Precalibration Evaluation Procedures for Mid-Infrared Milk Analyzers¹

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ABSTRACT

The purpose of this paper is to present a detailed account of the precalibration procedures developed and implemented by the USDA Federal Milk Market Administrators (FMMA) for evaluating mid-infrared (MIR) milk analyzers. Mid-infrared analyzers specifically designed for milk testing provide a rapid and cost-effective means for determining milk composition for payment and dairy herd improvement programs. These instruments determine the fat, protein, and lactose content of milk, and enable the calculation of total solids, solids-not-fat, and other solids. All MIR analyzers are secondary testing instruments that require calibration by chemical reference methods. Precalibration is the process of assuring that the instrument is in good working order (mechanically and electrically) and that the readings before calibration are stable and optimized. The main components of precalibration are evaluation of flow system integrity, homogenization efficiency, water repeatability, zero shift, linearity, primary slope, milk repeatability, purging efficiency, and establishment of intercorrection factors. These are described in detail and apply to both filter-based and Fourier transform infrared instruments operating using classical primary and reference wavelengths. Under the USDA FMMA Precalibration Evaluation Program, the precalibration procedures were applied longitudinally over time using a wide variety of instruments and instrument models. Instruments in this program were maintained to pass the criteria for all precalibration procedures. All instruments used similar primary wavelengths to measure fat, protein, and lactose but there were differences in reference wavelength selection. Intercorrection factors were consistent over time within all instruments and similar among groups of instruments using similar primary and reference wave-

lengths. However, the magnitude and sign of the intercorrection factors were significantly affected by the choice of reference wavelengths.

Key words: evaluation, mid-infrared, milk, precalibration

INTRODUCTION

The first detailed description of a mid-infrared (MIR) analyzer specifically designed for milk testing was published in 1964 (Goulden, 1964). The instrumentation has evolved over the years, incorporating many improvements and modifications, and today most milk payment and dairy herd improvement testing is done by MIR. These instruments provide rapid, cost-effective determinations of the fat, protein, and lactose contents of milk, and enable the calculation of total solids, SNF, and other solids. Early instruments were entirely filter-based but more recent equipment employs Fourier transform infrared (FTIR) technology with the capacity to use more detailed spectral information.

In 1989, we described the history of milk analysis for payment testing as it evolved from chemical methods to the Milko-tester and finally MIR (Barbano and Clark, 1989). At that time, we outlined the challenges that MIR-based testing presented and gave a general overview of plans to develop procedures to assure accurate and uniform milk testing that could be implemented throughout the dairy industry. The 3 major components in achieving this goal were 1) establishing and documenting the performance of chemical reference methods for calibration of the instruments, 2) developing uniform and meaningful procedures for assessing instrument performance and integrity prior to calibration, and 3) developing and validating instrument calibration procedures. This work was initiated and supported by the Test Procedures Committee of the USDA Federal Milk Market Administrators (FMMA), in collaboration with the authors at Cornell University.

All MIR analyzers are secondary testing instruments that require calibration by chemical reference methods. Thus, the first task was to identify appropriate reference methods for milk payment testing. Once methods were identified, they were reviewed, modified to improve performance, and clearly described. Interlabora-

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tory collaborative studies were conducted according to the guidelines of AOAC and method performance was documented. Using this approach, reference methods for milk true protein (Kjeldahl protein N; methods 991.22, 991.23), fat (modified Mojonnier ether extraction; method 989.05), total solids (oven dry; methods 990.20, 990.19), and SNF (by difference; method 990.21) were adopted as AOAC final action methods (AOAC, 2000). A collaborative study for determination of milk anhydrous lactose using an enzymatic spectrophotometric procedure is currently underway.

The second component of achieving accurate tests with MIR involves ensuring proper instrument performance before calibration by means of what is commonly called precalibration evaluation. Many of the basic principles of precalibration are described both in the literature (Barbano and Clark, 1989; AOAC, 2000; IDF, 2000; Wehr and Frank, 2004) and in the instrument manufacturers' manuals. The purpose of the present paper is to present a detailed account and summary of the results of the procedures developed and implemented by the USDA FMMA. Work on the final component in achieving optimum testing performance of MIR instruments, calibration and validation, is in progress.

PRINCIPLE OF INFRARED ANALYSIS

The principle behind infrared analysis is the absorbance of infrared energy by specific chemical groups. Most MIR analyzers use 4 primary wavelengths to measure fat, protein, and lactose. On filter-based instruments, these wavelengths correspond to the fat A filter (carbonyl groups, 5.723 μm), fat B filter (carbon-hydrogen groups, 3.48 μm), protein filter (amide groups, 6.465 μm), and lactose filter (hydroxyl groups, 9.610 μm). In reality, each filter selects a range (bandwidth) of wavelengths centered around the specified wavelength. The majority of filter-based instruments also use 4 separate reference filters with wavelengths of approximately 5.6, 3.6, 6.7, and 7.7 μm , corresponding to the fat A, fat B, protein, and lactose filters, respectively (Smith et al., 1993a). Some early instruments used no reference filters and others used just 1 or 2. The purpose of reference filters is to account for the effects of water absorption and light scattering (Biggs et al., 1987). Water absorption is very strong within the 2 to 10 μm wavelength region and the absorption bands of the other components are superimposed on this background. Because water is the major constituent of milk, it makes a large contribution to the infrared absorption (Goulden, 1964; Biggs et al., 1987; Barbano and Clark, 1989). The readings from MIR instruments represent a ratioing of each primary component with its respective reference wavelength, and are referred to as measure-

ments from the fat A, fat B, protein, or lactose channels. Detailed discussion of the principles of milk MIR analysis is given in Biggs et al. (1987), Biggs and McKenna (1989), and Sjaunja (1984).

More recent MIR instrumentation employs FTIR technology that uses an interferometer to produce full spectral information within the midinfrared region (Agnet, 1998). However, most FTIR instruments can be set up to simulate filter instruments by selecting the appropriate primary and reference wavelengths, and bandwidth (fixed wavelength approach). Currently, only the fixed wavelength approach to MIR milk analysis (using either fixed filter or FTIR instrumentation) has been approved as an official method (AOAC, 2000) for use in payment testing in the United States.

PRECALIBRATION, CALIBRATION, AND VALIDATION

Precalibration is the process of assuring that the instrument is in good working order (mechanically and electrically) and that the readings before conducting a calibration are stable and optimized. The readings used during precalibration are the uncorrected signals from each of the component channels. The main components of precalibration are listed in Figure 1. At the end of precalibration, intercorrection factors are established.

Intercorrection factors (called, variously, correction factors, cross-correction factors, interaction factors, interference factors, and correction coefficients) are necessary because of water displacement by variation in concentration of the nonwater components and because each of the milk solids components absorb not only at their primary wavelength, but also to a lesser extent at the wavelengths where the other components are measured. So, for instance, the protein in milk contributes to most of the solids absorbance at 6.435 μm , but there will be some contribution of fat and lactose as well. Additionally, when the concentration of any of the milk solids component changes, the concentration of water also changes, which in turn affects the infrared signal at all wavelengths to a varying degree.

Applying intercorrection factors to the uncorrected signal produces an intercorrected signal. Calibration involves using linear regression to establish a secondary slope and intercept to convert the intercorrected signal to a corrected signal (Figure 1). This is done using a set of milk-based calibration samples (typically 10 to 14) whose composition has been established using chemical reference tests. Finally, calibration is verified by testing an independent set of validation milk samples and comparing the corrected signals to the chemical reference tests of these same milks (Figure 1).

Figure 1. Precalibration, calibration and validation flow chart. Precalibration steps 3 to 9 and adjustment of secondary slope and intercept during calibration are done for each component channel.

The general equations for the application of intercorrections, secondary slope, and intercept are presented in Table 1. The scheme presented here is referred to as the fixed intercorrection calibration approach, because the intercorrection factors, once established, are, for all practical purposes, constant for an instrument (if the wavelengths are not altered and the primary slope of the uncorrected signal is kept constant). Calibration in

this case consists solely of adjusting the final (secondary) slope and intercept. Note that the uncorrected signal is defined as the MIR reading before the application of intercorrection factors and the secondary slope and intercept. The uncorrected signal is essentially the linearized, gain-adjusted (described later on) instrument signal with the intercorrection factors set to 0 and the secondary slope and intercept set to 1 and 0, respectively.

An alternative calibration procedure is to use multiple linear regression in which intercorrections are determined as part of the final calibration (Barbano and Clark, 1989). We are not in favor of this approach for raw milk testing for a number of reasons. First, when the linearity and gain level at each measurement wavelength are controlled during precalibration, intercorrection factors are fixed characteristics of the filter (or wavelength) used and are not expected to vary unless something fundamental changes in the instrument (e.g., the filter is replaced). Secondly, the statistical basis for the multiple linear regression approach is very dependent on the number, characteristics (i.e., range and correlation of component concentrations), and quality of the milks used for calibration, and this can result

Table 1. Equations to convert uncorrected signals to corrected signals

Intercorrected signal ¹	Corrected signal ²
$FA_i = FA_u + (P_u)(p_{fa}) + (L_u)(l_{fa})$	$FA_c = (FA_i)(s_{fa}) + a_{fa}$
$FB_i = FB_u + (P_u)(p_{fb}) + (L_u)(l_{fb})$	$FB_c = (FB_i)(s_{fb}) + a_{fb}$
$P_i = P_u + (F_u)(f_p) + (L_u)(l_p)$	$P_c = (P_i)(s_p) + a_p$
$L_i = L_u + (F_u)(f_l) + (P_u)(p_l)$	$L_c = (L_i)(s_l) + a_l$

¹FA = fat A; FB = fat B; P = protein; L = lactose; FA_i, FB_i, P_i, L_i = intercorrected signals; FA_u, FB_u, P_u, L_u = uncorrected signals; F_u = fat A or fat B uncorrected signal [for all instruments participating in the USDA Federal Milk Market Administrator program, F_u = FB_u (fat B uncorrected)]; p_{fa}, l_{fa} = intercorrection factors for protein and lactose on fat A; p_{fb}, l_{fb} = intercorrection factors for protein and lactose on fat B; f_p, l_p = intercorrection factors for fat and lactose on protein; and f_l, p_l = intercorrection factors for fat and protein on lactose.

²FA_c, FB_c, P_c, L_c = corrected signals (predicted chemistry); s_{fa}, s_{fb}, s_p, s_l = secondary slopes; and a_{fa}, a_{fb}, a_p, a_l = intercepts (bias).

in intercorrection factors that are susceptible to wide and unrealistic fluctuations from one set of calibration samples to the next.

PRECALIBRATION PROCEDURES

Routine precalibration evaluations serve a number of purposes. First, they provide ongoing documentation of satisfactory instrument performance and a record of when adjustments have been made. Second, if the instrument fails one or more of the tests, they aid the operator and manufacturer's service representative in diagnosing the cause of the problem. A variety of precalibration procedures are discussed in *Official Methods of Analysis* (AOAC, 2000), International Dairy Federation Standard 141C:2000 (IDF, 2000) and Standard Methods (Wehr and Frank, 2004). The AOAC procedures were last updated in 1989 to reflect the approval of the fat B filter and give guidance on computer-based control of instruments in use at the time (Biggs and McKenna, 1989). The purpose of the following is to present a systematic and detailed approach to precalibration that has been tested for ruggedness, and applied longitudinally over time using a wide variety of instrument models.

The components of precalibration listed in Figure 1 are intended to be conducted in the order given and on a regular (e.g., monthly) basis. Although all the procedures can technically be applied to all models of instruments, the operator needs access to the uncorrected signal (with the ability to adjust linearity and gain separately) and intercorrection factors. We suggest that instrument manufacturers make these easily available to the operator of an MIR milk analyzer. The criteria used in the USDA FMMA for passing or failing a particular test are based on the manufacturer's guidelines, the recommendations of the various standardization organizations (AOAC, 2000; IDF, 2000; Wehr and Frank, 2004), and our experience working with different instruments and laboratories.

For each of the precalibration procedures, it is critical that all the zeroing solutions and milk samples to be tested be maintained at the appropriate temperature. We recommend $41 \pm 1^\circ\text{C}$, which is 1°C higher than current standards (AOAC, 2000; IDF, 2000). The reason for this is 2-fold. First, the efficiency of the instrument homogenizer is dependent on sample temperature and, within a reasonable range, higher temperatures result in better efficiency (Walstra and Jenness, 1984). However, this shortens the time that the milk can remain in a tempering water bath because increasing the temperature will accelerate milk deterioration and oiling off of fat. A temperature of 41°C was chosen as a reasonable compromise between the amount of time a good

quality milk can stay in the tempering water bath (approximately 15 min after reaching 41°C) and the increase in performance gained by using higher temperatures. The second reason for choosing 41°C is based on the realities of high-volume testing where automatic conveyer belts are used to feed racks of samples to the instrument. Once the samples in the racks are removed from the tempering water bath and placed on the conveyer, they start to cool. This can result in a significant decrease in sample temperature especially for the last samples in the rack that are exposed to the ambient temperature the longest. In addition to maintaining proper sample temperature, the milk (formulated or not) used in all the precalibration procedures must be of good quality and thoroughly mixed before being presented to the instrument. Although seemingly trivial, these requirements cannot be overemphasized. Testing irregularities are often improperly attributed to instrument problems when in reality they are caused by temperature deviations, poor quality samples, or improper sample handling.

Throughout the following discussion, milk and zeroing solution are described as being presented to the instruments in vials. These are standard 47-mL volume plastic vials with lids, filled approximately three-fourths full (e.g., Capitol Vial, Fulton, NY). The zeroing solution recommended by the instrument manufacturer (typically distilled water containing 0.01% Triton X-100) is used for all zero setting and checks. All evaluations are done solely using the uncorrected readings (signals) from the appropriate component channel or channels under evaluation.

A final comment is that, in routine operation, some instruments can be set up to apply a purge correction to the signal. All the precalibration procedures require that this option be disabled when performing the evaluations.

Procedure 1: Flow System Checks

Flow system checks involve evaluating the flow system and homogenizer temperature. The specific procedures and pass/fail criteria for evaluating the flow system are instrument-dependent and the operator needs to refer to the instrument manual.

The block of the homogenizer has small empty holes that can be filled with thermal heat-sink compound. The temperature of the homogenizer is measured by inserting a temperature probe in one of the holes. The temperature should be between $40 \pm 2^\circ\text{C}$. If not within this range, the temperature should be adjusted according to the instrument manufacturer's instructions.

Procedure 2: Homogenization Efficiency

The purpose of checking instrument homogenization efficiency is to determine if the fat globules are being broken down into small enough sizes to give accurate test results and minimize light scattering. Large milk fat globules cause spectral effects that result in analytical error and poor repeatability (Smith et al., 1994, 1995). Homogenizers wear over time and need to be replaced or rebuilt periodically. The homogenization test allows the operator to monitor wear and schedule replacement before test results are adversely affected.

The “recycle” test is the most common method for evaluating homogenizer performance (AOAC, 2000; IDF, 2000; Wehr and Frank, 2004). This test consists of running a nonhomogenized milk through the instrument, recording the instrument readings from the fat channels, and collecting the milk as it exits the machine. The milk collected upon exit (first-pass milk) represents milk that has been homogenized by the instrument homogenizer. This first-pass milk is then run through the instrument a second time, and again the readings are recorded. Homogenization is considered adequate if the difference in readings between the first and second passes of the milk is $<0.05\%$. The limitations of the recycle test have been previously discussed (Smith et al., 1993a, 1995). The recycle test is not used in the USDA FMMA program.

A more informative method is direct determination of the milk fat globule size distribution after whole milk is homogenized through the instrument. This is done using a laser light-scattering particle size analyzer capable of determining particle size distribution between 1 and 80 μm , as described by Smith et al. (1995). Fat globule size needs to be measured using forward light scattering (Smith et al., 1995), not side scattering. This is to minimize the contribution of casein micelles to light scattering, which would result in an overestimation of homogenization efficiency. The milk to be evaluated should be between 3 to 3.5% fat, unhomogenized, pasteurized (to inactivate milk lipase), preserved, and of good quality. Two vials of this milk are prepared and warmed to $41 \pm 1^\circ\text{C}$. Between 3 to 4 instrument measurement uptake cycles are run using the first vial to flush out the flow system and fill it completely with the milk of interest. This also helps with temperature stabilization of the system by heating the uptake tubing before the homogenizer. The second vial is presented to the instrument and the milk from 3 measurement cycles (total volume ~ 5 to 15 mL) is collected from the cuvette by-pass line. Only the cuvette by-pass line should be used because the milk from the bleeder line is not homogenized and there is too little milk exiting the cuvette line. The instrument-homogenized milk is

immediately cooled in ice and refrigerated before particle size analysis.

Results from the particle size analysis are evaluated by looking at the actual scan (from 1 to 80 μm) and the $d(0.9)$ value (Figure 2). The actual scan of the instrument-homogenized milk should be unimodal and resemble a normal distribution curve. If additional peaks (other than trace) appear on the high side outside the main distribution (e.g., $>10 \mu\text{m}$), this usually indicates fat globule clustering or coalescence of free fat and may be symptomatic of poor homogenization or sample quality problems. The $d(0.9)$, calculated on a volume basis, is the mean globule diameter below which 90% of the fat volume is contained. It is a more rugged parameter to evaluate than the volume mean diameter, $d(4, 3)$, because it is not influenced by the occasional appearance of a large air bubble in the distribution. Homogenizers with a $d(0.9) >1.70 \mu\text{m}$ are wearing out and should be replaced; homogenizers with a $d(0.9)$ of $>2 \mu\text{m}$ are unacceptable (Smith et al., 1995).

Procedure 3: Water Repeatability

Water (more correctly, the zeroing solution) repeatability is a check of the electronics and mechanics of the instrument. If repeatability is poor using the zeroing solution then the instrument results are fundamentally unreliable. After zeroing the instrument, water repeatability is determined by calculating the range of the readings from each channel of 18 or more consecutive measurements of $41 \pm 1^\circ\text{C}$ zeroing solution. The range of the readings for each channel should be ≤ 0.04 .

Procedure 4: Initial Zeros/Zero Shift

The purpose of the zero shift test is to determine if the windows of the instrument's cuvette are deteriorating. Regular commercially homogenized milk is used for this test, so as not to confound the interpretation with issues unique to raw milk. Four vials of homogenized milk and 4 or 5 of zeroing solution are warmed to $41 \pm 1^\circ\text{C}$. The flow system is cleaned according the manufacturer's instructions immediately before the test and the instrument is zeroed. After setting the zero, 3 measurements of zeroing solution from a new vial are taken and recorded for each channel (initial zeros). This is followed by 12 sample uptake cycles (do not use purge) of the homogenized milk (3 from each vial), in which the actual readings are unimportant. Three sample uptake cycles of zeroing solution from a single vial are then run to flush out the flow system to negate any influence of purging efficiency. Finally, another 3 measurements of zeroing solution from a new vial are made and recorded (final zeros).

Figure 2. Example of particle size analyses of A) raw milk, and B) raw milk after homogenization by a midinfrared homogenizer, where $d(4,3)$ = volume mean diameter and $d(0.9)$ = mean globule diameter below which 90% of the fat volume is contained.

The average of the 3 initial zero readings should be 0.00 ± 0.02 (initial zero check) for each channel. The difference between the average of the 3 initial zero readings and the average of the 3 final zero readings should be $\leq \pm 0.02\%$ on all channels (zero shift evaluation). Problems with cuvette wear often become evident on the protein or lactose channels before they appear on the fat channels and manifest themselves as a shift in

readings due to rapid accumulation of milk solids on the windows of a freshly cleaned cuvette. Regardless, a consistent failure on any channel means the cuvette should be replaced. The evaluation of initial zeros and zero shift is formally conducted early on in the prec calibration evaluation sequence, but the basic procedure is an integral part of most of the subsequent tests to assure that the zero is stable and maintained.

Procedure 5: Linearity

The purpose of linearity evaluation is to determine if linear changes in component concentration (wt/wt) are reflected in linear changes in the uncorrected signals. Linearity adjustment is necessary because the Beer-Lambert law is not always valid for low-resolution spectroscopy (Biggs et al., 1987). Nonlinearity has a negative impact on the standard deviation of the difference between instrument and chemical test results and can cause errors in the determination of the intercorrection factors (Biggs, 1979).

Smith et al. (1993b) described the limitations of the AOAC (2000) and IDF (1990, 2000) procedures for linearity evaluation and proposed an alternative designed to overcome them. Linearity is assessed using 2 sets of solutions: one for the fat channels and one for both the protein and lactose channels. The concentration ranges chosen for the linearity solutions are designed to encompass the range over which the final calibration will be used.

The fat linearity solutions are made using a 6% fat (approximate) stock material prepared by diluting commercial homogenized, pasteurized half-and-half (10 to 18% milk fat; Code of Federal Regulations, 2004) with pasteurized skim milk ($\leq 0.2\%$ milk fat). The stock material is diluted (wt/wt) with distilled water to achieve a series of 5 fat concentrations: 2, 3, 4, 5, and 6% fat. The fat chemistry for each solution is calculated from the weights used during preparation. Determination of the percentage fat in the stock solution by chemical analysis is not a requirement, although it is advised as a quality control tool.

For the protein/lactose linearity solutions, 6 solutions are prepared by diluting a can of evaporated skim milk with distilled water. In the absence of directly measuring true protein and lactose, the evaporated skim milk can be assumed to contain approximately 7% true protein and 11% anhydrous lactose. For true protein, the solutions range from approximately 2.8 to 3.9%. The corresponding range for anhydrous lactose is 4.3 to 6.0%. The chemistry for each solution is calculated from the weights obtained during preparation.

For each set of linearity solutions (either fat or protein/lactose), the instrument is zeroed and then 3 measurements of $41 \pm 1^\circ\text{C}$ zeroing solution are recorded (initial zeros). The linearity solutions ($41 \pm 1^\circ\text{C}$) are presented to the instrument, going from low to high concentration and taking 3 measurements per vial from the channel(s) under evaluation. Three measurement cycles of zeroing solution from a single vial are then run to flush out the flow system, followed by 3 recorded measurements of zeroing solution (final zeros).

Figure 3. Example of a residual nonlinearity plot (Fat B channel). The dashed lines represent the upper and lower limits of the linearity acceptance criteria.

The initial zeros and zero shift are calculated and evaluated as previous described. Failure to pass these tests invalidates the linearity evaluation. For each linearity solution, the first reading is not used to avoid possible distortions due to poor purging efficiency. The second and third readings are evaluated for repeatability and only accepted if the difference between the readings is $\geq 0.04\%$. Acceptable readings are averaged. A nonzero forcing linear regression is conducted with actual chemistry (calculated from the dilutions of the stock solutions) as the dependent variable and the uncorrected signal as the independent variable. Mid-infrared predicted chemical values are calculated using the derived regression equation. Residual nonlinearity values, calculated by subtracting actual chemistry from MIR-predicted chemistry, are plotted against actual chemistry (Figure 3). Residuals of $\leq \pm 0.02\%$ within the calibration range are deemed acceptable. For examples of residual plot calculations and additional details on making up the linearity evaluation solutions for fat and protein/lactose, see Smith et al. (1993b). Residual nonlinearity will influence the setting of primary slope and intercorrection factors (Biggs, 1979) and will weaken the agreement between instrument predicted and actual chemistry values.

Procedure 6: Primary Slope

Primary slope determines the responsiveness of changes in the uncorrected signal to changes in component concentration; the ideal primary slope is 1.00. Too low a primary slope will cause insensitivity and too high a primary slope will result in noise. When the primary slope deviates from 1 or is allowed to vary, the

burden of correcting for this falls to the intercorrection factors, and secondary slope and intercept. This is undesirable, as the purpose of intercorrection factors is to compensate for the absorbance of components other than the one being measured. Assuming that all other precalibration specifications are met, controlling primary slope and keeping the response on each channel in a one-to-one relationship with differences in chemistry is the single most important factor in maintaining stable instrument calibration and agreement with chemistry. In older model instruments, the primary slope is adjusted using knobs whereas in new instruments adjustments are made by changing “gain” factors in computer software or memory chips. The importance of maintaining primary slope is mentioned in IDF (2000), and both AOAC (2000) and Standard Methods (Wehr and Frank, 2004) suggest that the desired coefficient is 1.

The procedure described below uses milk-based ingredients to formulate pairs of milks differing only in the concentration of the component of interest. The difference between the 2 uncorrected readings from the primary component channel divided by difference in actual chemistry is the primary slope. An important characteristic within each pair of milks is a similar background chemistry. This removes the influence of variation in the coabsorbance of secondary components on the primary signal.

Separate sets of primary slope solutions are prepared for fat, protein, and lactose, each consisting of a “low” and “high” solution. The solutions are formulated using homogenized (pasteurized) whole milk, pasteurized skim milk, low-heat nonfat dry milk powder (NDM), lactose monohydrate, and a 6% fat stock solution as ingredients. The 6% fat stock solution is prepared by diluting commercial homogenized, pasteurized half-and-half with pasteurized skim milk. Chemical reference tests are run on each of the ingredients (except lactose monohydrate) to determine the true protein, fat and total solids contents. Lactose is determined either by difference or by spectrophotometric enzymatic analysis. Lactose by difference is calculated as % anhydrous lactose = % total solids – (% fat + % true protein + 0.19 + % ash), where 0.19 is the NPN factor to convert true protein to CP and % ash is either directly measured (method 945.46; AOAC, 2000) or indirectly calculated using an updated version of the equation described by Lynch et al. (1990) where % ash = (% true protein × 0.0596) + 0.5379. The spectrophotometric enzymatic analysis of lactose is based on AOAC method 984.15 (AOAC, 2000), modified by weighing all volume additions. The ingredients used to formulate the solutions and their typical composition are presented in Table 2.

Table 2. Example of the typical composition of ingredients used to formulate primary slope and intercorrection evaluation solutions

Ingredient	Typical composition ¹		
	Fat	True protein	Anhydrous lactose
	(g/100 g)		
Half-and-half ¹	10.0	2.77	4.30
6 % fat stock ²	6.00	2.93	4.41
Skim milk ³	0.10	3.17	4.56
NDM ⁴	0.80	35.0	51.0
Whole milk ⁵	3.25	3.01	4.50
Lactose monohydrate	0	0	95

¹Homogenized, pasteurized mixture of milk and cream (10 to 18% milk fat; Code of Federal Regulations, 2004).

²Prepared by diluting the half-and-half with the skim milk.

³Pasteurized skim milk (≤0.2 % fat).

⁴NDM = Commercial low-heat nonfat dry milk powder.

⁵Pasteurized, homogenized whole milk (≥3.25% fat).

The primary slope solutions are prepared by weight based on the chemical composition of the starting ingredients and the desired target values. Target values for the low and high solutions are approximately 2 and 6% for the fat set (4% range), 3 and 4% for the true protein set (1% range), and 4.5 and 5.5% for the anhydrous lactose set (1% range), respectively, while keeping the background chemistry similar within a set of solutions. It is critical for the target values of the solutions to be within the linear range of measurement determined in the linearity evaluation. Table 3 gives an example of the formulations and final component concentrations, using the composition of the ingredients listed in Table 2. Note that only the low and high solutions in Table 3 are used for evaluation of primary slope.

Each set of primary slope solutions are run on the instrument as described for linearity (i.e., solutions tempered to 41 ± 1°C, 3 readings per vial for the component channel under evaluation, initial zeros and zero shift evaluation between each set of solutions). The average of the second and third readings, evaluated for repeatability, is calculated and the difference between the low and high averages for each primary component is divided by difference in actual chemistry. Acceptable primary slopes are between 0.95 and 1.05. These same primary slope solutions can be used not only to evaluate primary slope, but also to adjust it.

Primary slope is dependent on cuvette path length, the level of light intensity striking the detector, and the linearity of the uncorrected signal. Generally, as a cuvette is used over time, there is erosion of the windows and the path length of the cuvette increases. This will cause the primary slope to increase gradually for all component channels. Current practice in the USDA FMMA laboratories is to adjust primary slope back to

Table 3. Example of the formulation and composition of primary slope and intercorrection evaluation solutions

Component	Level	Ingredient					Evaluation solution composition ¹		
		6% fat stock	Skim milk	NDM ²	Whole milk	Lactose monohydrate	Fat	True protein	Anhydrous lactose
		(g/100 g of formulation)					(g/100 g)		
Fat	Low	32.18	67.82				2.00	3.09	4.51
	Medium	66.03	33.77	0.20			4.00	3.08	4.55
	High	99.62		0.38			5.98	3.05	4.58
Protein	Low				98.21	1.79	3.19	2.96	6.12
	Medium			1.71	97.41	0.88	3.18	3.53	6.09
	High			3.38	96.62		3.17	4.09	6.07
Lactose	Low				100.00		3.25	3.01	4.50
	Medium				99.44	0.56	3.23	2.99	5.01
	High				98.86	1.14	3.21	2.98	5.53

¹Composition calculated using component values in Table 2. Numbers in bold show concentration changes for the target components.

²NDM = Commercial low-heat nonfat dry milk powder.

1.00 if it is ≤ 0.95 or ≥ 1.05 on any channel. However, large sudden changes in primary slope are typically caused by a change in the light source, detector, or cuvette. In these cases, the primary slope must be checked immediately after instrument repair and adjusted to 1.00 for all channels. This allows the optimized intercorrection factors already established for that instrument to continue to function properly.

The importance of maintaining primary slope is illustrated in the following example. Assume that the fat B uncorrected signal is 4.58 at a primary slope of 1.00. If the slope is changed to the upper limit of acceptability (1.05), then the signal increases to 4.81, and if it is changed to the lower limit (0.95) the signal becomes 4.35. Thus, a deviation in primary slope from 1.00 even within the narrow range of acceptable primary slope settings can change the uncorrected reading by as much as 0.23. If this were the only impact of variation in primary slope on the result, a compensating change in secondary slope would take care of it. However, adjusting secondary slope would not address the issue of the proper function of the intercorrection factors

Procedure 7: Milk Repeatability

Although seemingly counterintuitive, the milk repeatability test is conducted after linearity and primary slope have been evaluated and adjusted if necessary. The reason is that nonoptimized primary slope settings can either mask or cause poor repeatability. On a practical basis, an instrument that is not repeatable inherently gives unreliable results.

The milk repeatability test consists of 2 separate evaluations, the first using raw whole milk and the second using pasteurized, homogenized whole milk. If

the instrument passes the raw milk evaluation, there is no need to run the homogenized milk. However, if the instrument fails the raw milk evaluation, running the homogenized milk helps the operator determine the cause of the failure. If the instrument fails the raw milk evaluation but passes the evaluation of the homogenized milk, then poor repeatability is most likely caused by improper sample mixing or splitting, homogenizer failure, or sample quality. If the instrument fails both the raw and homogenized milk evaluation, then the problem is more serious and it is likely that the instrument was close to the failure limit on the previously performed water repeatability test.

For each test, 6 vials of milk (raw or homogenized, depending on the test) and at least 4 of zeroing solution (for setting the zero and determining zero shift) are warmed to $41 \pm 1^\circ\text{C}$. The instrument is zeroed, 3 measurements of zeroing solution are taken (initial zeros), followed by 18 measurement cycles of the milk (3 from each vial). Three measurement cycles of zeroing solution from a single vial are then run to flush out the flow system and another 3 measurements of zeroing solution from a new vial are made and recorded (final zeros).

The initial zeros and zero shift are evaluated as previously described and the range of the readings is calculated for each channel from the 2 through 18 measurements of the milk. The first measurement is not used to avoid possible distortions due to poor purging efficiency. The range of milk readings for each channel should be ≤ 0.04 .

Procedure 8: Purging Efficiency

Evaluation of purging efficiency is a check on the amount of carryover from one sample vial to the next.

The evaluation should initially be carried out using the normal mode of instrument operation: either manual or automatic. Automatic mixing and sampling poses additional carryover risk because there can be carryover from the mechanical stirrer used to mix the sample before measurement. Liquid adhering to the stirrer will be transferred from one sample to another. Because most instruments used for payment testing or herd evaluations make just one measurement per sample vial, the impact of carryover on actual test results can be significant. The influence of purging efficiency is made clear when going from zeroing solution (referred to as water) to milk (lowering the milk reading) or milk to zeroing solution (increasing the zero). Thus, the evaluation looks at these 2 situations.

To perform the test, 12 vials (of the same volume used for routine testing) are filled with zeroing solution and 10 vials are filled with pasteurized, homogenized whole milk. The solutions are warmed to $41 \pm 1^\circ\text{C}$, the instrument is zeroed, and the initial zeros are recorded. The 22 vials are then presented to the instrument in the order of alternating pairs: water (W_1), water (W_2), milk (M_1), milk (M_2), water (W_1), water (W_2), etc. A single measurement is made for each vial and the readings from each channel are recorded. At the conclusion, 3 measurement cycles of zeroing solution from a single vial are made to obtain the final zeros.

As usual, the initial zeros and zero shift are evaluated. Purging efficiency is calculated as follows

$$\text{Water to Milk} = \frac{(\Sigma M_1 - \Sigma W_2) \times 100}{(\Sigma M_2 - \Sigma W_2)}$$

$$\text{Milk to Water} = \frac{(\Sigma M_2 - \Sigma W_1) \times 100}{(\Sigma M_2 - \Sigma W_2)}.$$

Purging efficiency should be $\geq 99.0\%$ for all channels for both water to milk and milk to water. If an instrument fails the purging efficiency test in automatic mode then the test should be run again in manual mode. If the instrument fails in automatic mode but passes in manual mode, then the most likely explanation is carryover from the stirrer (which should be repaired, replaced, or a different shape should be utilized).

The procedures of IDF (2000) and AOAC (2000) evaluate purging efficiency in a similar manner, but only consider the efficiency of water to milk, and for AOAC (2000), the evaluation is limited to the fat channels. Both the water to milk and milk to water evaluations are described in the 17th edition of Standard Methods (Wehr and Frank, 2004). The pass/fail criteria for all the standards organizations are at the 99% level.

Procedure 9: Intercorrection Factors

As discussed previously, intercorrection factors are necessary to compensate for the influence of water displacement and absorbance of secondary components at the wavelength of the component of interest. Intercorrection factors for each channel are a function of linearity, primary slope, and the center wavelength and bandwidth of both the primary and reference wavelengths. Once established, intercorrection factors should be relatively constant unless there is a major change in the instrument (e.g., filter or filter angle for filter-based instruments; central wavelength and bandwidth for FTIR instruments) or changes are made to primary slope or linearity. From this discussion, it should be apparent that it is critical to establish linearity and primary slope of the uncorrected signal before setting or evaluating intercorrection factors.

For each primary component channel, separate intercorrections must be established for each of the secondary components. For instance, when true protein is measured at its primary wavelength ($6.465 \mu\text{m}$), intercorrections are applied for the effects of lactose on protein and fat on protein. Thus, for instruments using the full complement of channels (fat A, fat B, protein, and lactose), there will be 2 intercorrection factors for each channel (8 total). The issue arises as to whether the fat A or fat B signal should be used to establish intercorrections for the effect of fat on protein and lactose. Historically, the fat A signal was used because that was the only fat filter available in early instrumentation. Most modern filter instruments, however, are equipped with both fat A and B filters and, of course, FTIR gives access to both wavelengths. All the instruments in the FMMA program use the fat B wavelength to establish the fat intercorrections for protein and lactose. We consider it preferable to fat A because the magnitude of the fat B signal is greater and thus has the potential to provide greater sensitivity.

Intercorrection factors are both set and evaluated by determining them experimentally and comparing them to previously established values. The solutions used for the evaluation are identical to those used for primary slope except that a "medium" solution is added to each set (Table 3). Note that the solutions (e.g., low, medium, and high fat) are used to determine the effect of the secondary component (e.g., fat) on the primary signals of the other 2 components (e.g., lactose and protein). The ingredients used to formulate the solutions and their typical composition is presented in Table 2, and an example of the formulations and final component concentrations is given in Table 3. Formulations are done by weight, background component composition is similar within each set, and actual composition is deter-

Table 4. Example of calculating intercorrection factors: effect of fat on protein

Intercorrection evaluation solution	Chemical analysis		IR protein signal (uncorrected signal) –
	Fat	True protein	
	(g/100 g)		
Low fat	2.0365	3.0021	2.301
Medium fat	4.0969	2.9843	2.130
Difference	–2.0604	0.0178	0.171
Intercorrection of Fat B on protein = $\frac{\text{Difference protein} - \text{Difference IR protein}}{\text{Difference fat}}$			
	$= \frac{0.0178 - 0.171}{-2.0604}$		
	$= 0.074$		

mined by chemical analysis, as was described for primary slope. Each set of intercorrection solutions are run on the instrument as previously described (i.e., solutions tempered to $41 \pm 1^\circ\text{C}$, 3 readings per vial, initial zeros and zero shift evaluation between each set of solutions) and the average of the second and third readings, evaluated for repeatability, are used to calculate the intercorrection factors.

The intercorrection factor for the secondary component on the primary component determined using the low and high solutions (named after the secondary component) is calculated as follows:

$$\text{Intercorrection factor} = \frac{(\text{PriChem}_{\text{low}} - \text{PriChem}_{\text{high}}) - (\text{PriIR}_{\text{low}} - \text{PriIR}_{\text{high}})}{\text{SecChem}_{\text{low}} - \text{SecChem}_{\text{high}}}$$

where PriChem = g/100 g of primary component determined by chemical analysis, PriIR = uncorrected signal of primary component, SecChem = g/100 g of secondary component determined by chemical analysis, _{low} = low solution, and _{high} = high solution. An example of the calculations for the effect of fat on protein is given in Table 4.

The values for the medium solutions are substituted in the equation in place of those for the high solution to provide a second estimate of the intercorrection factor. This serves as a check, because the intercorrection factors established using the low/medium and low/high solutions should be similar (i.e., differ by <0.04). If the difference is larger, the solutions should be checked and the test should be repeated. The average of these 2 intercorrection estimates is used to derive the final intercorrection factor. Our experience has been that they are very constant within instruments and instrument models. This is discussed in detail in the next section.

USDA FMMA PRECALIBRATION EVALUATION PROGRAM

In 1995, the Test Procedures Committee of the USDA FMMA established a formal MIR Precalibration Evaluation Program based on the procedures described. Most participants are laboratories operated by, or under contract to, individual federal milk marketing orders. The characteristics of the instruments that have been part of the program are given in Table 5. Of the 20 instruments listed, 14 are filter-based and 6 are FTIR-based set up to simulate traditional filter instruments. All the instruments operate using fat A, protein, lactose, and fat B wavelengths and most use the 4 traditional reference filter wavelengths. The Dairy Lab 2 uses the protein reference wavelength for all channels and the Bentley 2000 uses the fat B reference wavelength for the fat B channel and the fat A reference wavelength for the other channels. Milk-o-Scan 6000 instruments (Foss Electric, Hillerød, Denmark) in the FMMA system that operate in the traditional fixed filter calibration mode have not yet been included in the precalibration evaluation program because the operator does not have access to uncorrected signals, linearity adjustments, and intercorrection factors.

Evaluations are conducted monthly for flow system checks, homogenization, water repeatability, zero shift, primary slope, milk repeatability, and purging. Linearity and intercorrection evaluations are conducted at 6-mo intervals and whenever there is an adjustment of primary slope. In addition, all precalibration evaluations are conducted in the event of instrument acquisition or major repair (e.g., replacement of detector or light source). Solutions for homogenization efficiency, primary slope, linearity, and intercorrection evaluations are prepared at a central location and sent out to each participating laboratory. A computer program, IR-

Table 5. Characteristics of the instruments that have participated in the USDA Federal Milk Market Administrators Precalibration Evaluation Program

Instrument model	Abbreviation	Currently manufactured	No. of filters/wavelengths		No. of instruments ¹
			Primary	Reference	
Milkoscan 134 ²	MS-134	No	4 filter	4 filter	2
Milkoscan 255 ²	MS-255	No	4 filter	4 filter	3
Multispec Dairy Lab 2 ²	DL-2	No	4 filter	1 filter	1
Milkoscan 300 (303) ²	MS-300	No	4 filter	4 filter	3
Milkoscan 605 ²	MS-605	No	4 filter	4 filter	3
Milkoscan 4000 ²	MS-4000	No	4 filter	4 filter	1
Bentley 2000 ³	BTY	Yes	4 filter	2 filter	1
FT120 ²	FT-120	Yes	FTIR ⁴ (4-filter simulation)	FTIR (4-filter simulation)	1
Delta Lactoscope FTIR ⁵	DELTA-FT	Yes	FTIR (4-filter simulation)	FTIR (4-filter simulation)	5

¹Number of instruments that have participated in the evaluation.

²Foss Electric, Hillerød, Denmark.

³Bentley Instruments, Chaska, MN.

⁴Fourier transform infrared.

⁵Delta Instruments, Drachten, The Netherlands.

QC, was developed (by D. Barbano, Cornell University, Ithaca, NY; M. Schweisthal, USDA Agricultural Marketing Service, Carrollton, TX; G. Houghton, Kestral Software Consulting, Caroline, NY) to guide the operator through the procedures, collect data directly from the instrument, calculate the results, and keep a database record. When changes in linearity, primary slope or intercorrections are made, the IR-QC program ensures that appropriate calibration adjustments are made prior to resumption of routine sample testing. The precalibration data are sent to a central location where they are evaluated, and summary reports are sent back to the laboratories.

Under this program, the MIR instruments in the FMMA system have routinely met the passing criteria for all precalibration procedures. Failure of any one procedure results in action to correct the problem. Determining the cause of failure is facilitated by the association of specific tests with identifiable causes. This has shortened downtime and reduced maintenance service costs. Especially valuable are the homogenization checks because they not only allow the user to preemptively replace or rebuild a homogenizer, but they also save the user from unnecessarily incurring the cost of replacing a correctly functioning homogenizer.

Of special interest are the intercorrection factors. When the program was initiated in 1995, no criteria were set for evaluating intercorrection factors. At that time, most of the instruments in the FMMA program were filter based and thus, the intercorrection factors were, in large part, dependent on the fixed characteristics of the individual instruments (e.g., dependent on filter wavelength and bandwidth). What was under operator's control, however, was linearity and primary slope, and it was not known at the time what effect

controlling these key determinants would have on the variability of intercorrection factors within and between instruments.

As previously mentioned, intercorrection factors are routinely evaluated every 6 mo by determining them experimentally and comparing them to the previous values. In the absence of criteria for evaluation, the decision was made to change them by default each time they were checked. What we found was that intercorrection factors remained very constant within an instrument (when linearity and primary slope were maintained within tolerances) and there was little effect of most instrument repairs on intercorrection factors. Furthermore, intercorrection factors tended to be very similar among instruments with the same wavelength characteristics.

Table 6 presents the most recent intercorrection factors for the instruments that are or have participated in the precalibration evaluation program, grouped by filter/wavelength. The standard deviation (SD) is indicative of the variation among instruments. For the categories in which information is available for a reasonable number of instruments (i.e., the 12 filter- and 6 FTIR-based instruments using 4 primary and 4 reference filters/wavelengths), intercorrection factors are similar among instruments with the same characteristics (SD ranging from 0.008 to 0.029 and 0.002 to 0.022 for the filter- and FTIR-based instruments, respectively) and, with the exception of the effects of lactose and protein on fat B, the differences between the corresponding intercorrection factors for the 12 filter- and 6 FTIR-based instruments are also very small (average difference ranging from 0.002 to 0.034).

The intercorrection factors for lactose and protein on fat B for the 12 filter and 6 FTIR instruments are nota-

Table 6. Intercorrection factors established for the instruments that are or have participated in the USDA Federal Milk Market Administrators Precalibration Evaluation Program

Component signal		Filter mid-infrared (MIR): 4 primary, ² 4 reference ³		FTIR ¹ : 4 primary, ² 4 reference ³		Filter MIR/FTIR: 4 primary, ² 1 reference ⁴		Filter MIR: 4 primary, ² 2 reference ⁵
Primary	Secondary	Factor ⁶	SD ⁷	Factor	SD	Factor	SD ⁸	Factor
Fat B	Lactose	-0.253	0.029	-0.165	0.007	-0.450	0.075	-0.356
Fat B	Protein	-0.140	0.019	-0.068	0.012	0.045	0.018	-0.196
Lactose	Fat B	0.035	0.010	0.041	0.011	-0.073	0.004	0.020
Lactose	Protein	0.027	0.022	0.025	0.017	0.082	0.038	-0.192
Protein	Fat B	0.075	0.008	0.067	0.009	0.060	0.009	0.156
Protein	Lactose	0.076	0.024	0.095	0.011	0.087	0.002	0.013
Fat A	Lactose	0.045	0.012	0.079	0.002	0.086	0.033	0.003
Fat A	Protein	0.016	0.012	0.038	0.022	0.423	0.001	0.001
Number of instruments		12		6		2		1
Models ⁹		MS-134, MS-255, MS-300, MS-605, MS-4000		DELTA-FT, FT-120 ¹⁰		DL-2, FT-120 ¹⁰		BTY

¹Fourier transform infrared.²Primary filter or wavelength selections correspond to ~5.723, 3.48, 6.465, and 9.610 μm for fat A, fat B, protein, and lactose, respectively.³Reference filter or wavelength selections correspond to ~5.6, 3.6, 6.7, and 7.7 μm for fat A, fat B, protein, and lactose, respectively.⁴The reference filter or wavelength selection for all channels is ~6.7 μm .⁵The reference filter is ~5.6 μm for fat A, protein, and lactose. The reference filter is ~3.6 μm for fat B.⁶Intercorrection factor.⁷SD = Standard deviation.⁸The range of the intercorrection factors between the 2 instruments with 1 reference filter is $\text{SD} \times \sqrt{2}$.⁹Instrument model abbreviations explained in Table 5.¹⁰This is the same FT-120 instrument using either 1 or 4 reference wavelengths.

ble because they are relatively large and negative. The sign of the intercorrection factor depends on the strength of the absorption of both the secondary components and water. At most of the primary component wavelengths, the absorption of water is stronger than the absorption of the secondary components. The result is a net decrease in signal (i.e., positive intercorrection factors) because the loss of absorption due to the displacement of water is greater than the increase due to the secondary components. At the fat B wavelength (3.48 μm), however, water absorption is not as strong as that of the secondary components and the net effect is a negative coefficient. (Biggs and McKenna, 1989). Although still negative, the intercorrections of lactose (-0.165) and protein (-0.068) on fat B for the 6 FTIR instruments are smaller than those for the 12 filter-based instruments (lactose: -0.253; protein: -0.140). We suspect this is because the FTIR allows the operator to directly set center wavelength and bandwidth for each "virtual" filter, and the benefit of this is most apparent at wavelengths where the effect of the secondary components is greatest. In fact, for FTIR instruments in which the user has the ability to specify and control center wavelength and bandwidth, it may be possible in the future to further optimize these parameters to improve analytical performance.

The effect of reference filter wavelength on the intercorrection factors is also illustrated in Table 6. Although the intercorrections for both the filter and FTIR instruments using the same 4 reference wavelengths were similar, the instruments using just 1 or 2 reference filters were not. When a single reference wavelength of 6.7 μm (protein reference wavelength) was used, significant changes in the magnitude and sign were observed for most of the intercorrections except the effects of fat B and lactose on protein (Table 6). When 2 reference wavelengths were used (5.6 μm for fat A, protein, and lactose; 3.6 μm for fat B), the effects were even more variable. The main message here is that, assuming common primary wavelengths, any differences between instrument type (filter or FTIR) or between models appear small when compared with the effect of the selection of reference wavelengths (and bandwidth). A clear definition of both primary and reference wavelengths is needed in official methods to ensure consistency. The most desirable situation for analytical testing is to have the intercorrections as small as possible.

CONCLUSIONS

Assuring that MIR instruments are in good working order and that the readings are stable and optimized

before calibration is an important component of assuring accurate and precise milk testing. The procedures utilized in the USDA FMMA Precalibration Evaluation Program provide a systematic and meaningful way of evaluating instrument performance before calibration. Under this program, properly maintained MIR instruments in the FMMA system have routinely met the passing criteria for all precalibration procedures. When linearity and primary slope of the uncorrected signal meet specifications, intercorrection factors are consistent over time within instruments and similar among instruments using similar primary and reference wavelengths. However, the magnitude and sign of the intercorrection factors are notably different for instruments using similar primary but different reference wavelengths.

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