Near-Infrared Analysis of Fat, Protein, and Casein in Cow's Milk

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Fat, crude protein, true protein, and casein were determined in cow milks by near-infrared transmission spectroscopy (NIR). Partial and overall PLS calibrations were performed on two sets of samples: partial calibration included 76 unhomogenized samples, whereas overall calibration used 96 homogenized and unhomogenized samples. Standard errors of calibration were 0.12% for fat, 0.06% for crude protein, 0.04% for true protein, and 0.05% for casein in the overall calibration. Validation of the overall calibration with an independent set of samples gave standard errors of prediction of 0.07% for fat, 0.06% for crude protein and casein, and 0.05% for true protein. Except for fat, all of the statistical parameters were better with overall than with partial calibrations, which indicates that homogenization has an effect on NIR fat determination. Despite the relatively small number of samples included in the calibration model, NIR transmission was found to be a reliable method for the determination of fat and nitrogenous constituents in milk.

Keywords: *Near-infrared spectroscopy; milk; PLS; fat; protein; casein*

INTRODUCTION

The determination of the principal milk constituents is a key issue for the different products in the dairy industry, but reference analyses for fat, protein, and casein are expensive and time-consuming. Although there are good infrared indirect methods for fat and protein analysis (Grapin and Lefier, 1995), there is still no near-infrared (NIR) method for real time analysis of casein in cow's milk. In recent years, two major changes have occurred: first, the industry has become more interested not just in protein but in true protein and casein determinations; second, there have been changes in infrared technology and related software that now allow a simple and rapid determination of these constituents.

NIR is used for analyzing many agricultural products (Osborne et al., 1993; Davies and Grant, 1987; Williams and Norris, 1987). In the dairy industry, NIR has been widely used for analyzing the major components in milk (Sato et al., 1987; Hall and Chan, 1993; Chen et al., 1994; Laporte and Paquin, 1998), skim milk (Ereifej and Markakis, 1983; Baer et al., 1983a; Frankhuizen and van der Veen, 1985), and fermented milk products (Rodriguez-Otero and Harmida, 1996) and for whey characterization (Baer et al., 1983b; Pouliot et al., 1997). The cheese industry already uses NIR for moisture, fat, protein, and lactose determination (Lee et al.., 1997; Rodriguez-Otero et al., 1995; Pierce and Wehling, 1994; Frank and Birth, 1982). Furthermore, NIR is a promising tool for monitoring cheese coagulation (Saputra et al., 1994; Payne et al., 1993; Laporte et al., 1998).

NIR has also been used for measuring casein in oil/water emulsion systems (Kamishikiryo-Yamashita et al., 1994). Even though good calibrations were obtained for fat, protein, casein, and casein fractions in goat's milk (Diaz-Carillo et al., 1993), few studies have been devoted to the NIR determination of casein in cow's milk.

NIR spectroscopy presents several advantages such as rapidity, precision, no need of sample preparation, and nondestructive aspect. Furthermore, NIR technology is an efficient tool for real-time control of production lines.

The objective of this study was to evaluate the feasibility of NIR for determining fat, crude protein, true protein, and casein in cow's milk. The effects of homogenization on NIR determinations of milk fat were also studied.

MATERIALS AND METHODS

Samples. Three sets of prepared unhomogenized and pasteurized (72°, 16 s) milk samples were produced during summer 1996 (June−August), which gave 37 samples. Fifteen unpasteurized and unhomogenized raw milks collected in August 1996 were also added to the samples. To account for seasonal variability, an additional 24 prepared unhomogenized and pasteurized samples were produced in January 1997. Finally, to briefly study the effects of homogenization on NIR determinations of milk fat, 20 homogenized and pasteurized samples were also prepared in May 1997, giving a total of 96 samples for NIR determinations of the major milk components. The calibration performed with these 96 samples was referred to the overall calibration because it included not only the prepared (homogenized and unhomogenized) samples but also the 15 unpasteurized and unhomogenized raw milks. A second calibration, referred to partial calibration, was also performed on the 76 unhomogenized samples.

Milk samples (except for the 15 raw milks) were produced from a single batch of pasteurized milk from a local dairy plant (Natrel, PQ, Canada) following the methods from the International Dairy Federation (IDF, 1996, appendix C). Samples were adjusted with controlled content of fat and protein.

The fractionation was carried out using the procedure shown in Figure 1: five milk fractions (whole milk, skim milk, cream,

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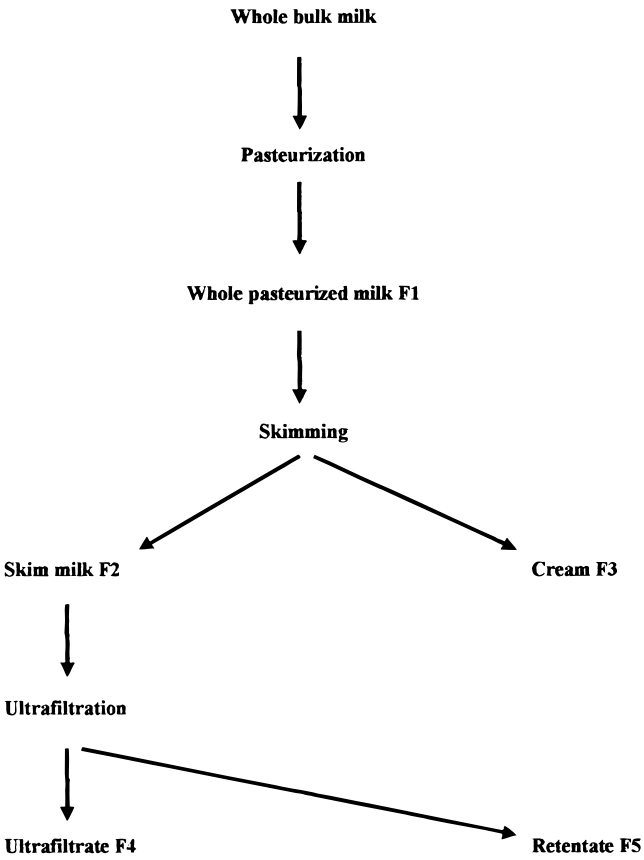

Figure 1. Preparation of calibration milk samples [according to IDF (1996), 141 B, appendix C].

retentate, ultrafiltrate), necessary for the sample preparation, were obtained.

Before recombination, fat and protein contents were measured in each fraction by Milko-Scan (133b A/S N. Foss Electric, type 10900, Denmark). The quantities required of each fraction (maximum of three in a given sample) were then combined to obtain the final composition of the aim target. Finally, 20 samples were homogenized on an Avestin C-50 (55 °C, 4000 psi).

Chemical Analysis. Fat content was measured according to the Mojonnier method (IDF, 1987, 1C). Crude protein (CP) (total nitrogen × 6.38), non-protein nitrogen (NPN), and non-casein nitrogen (NCN) were determinate by Kjeldahl. NPN was determined by trichloroacetic precipitation, and CP methodology was referred to standard IDF 20B (1993), whereas NCN was measured according to standard IDF 29 (1964) with the following modifications: milk is weighed at 38 °C and casein precipitation is performed by direct addition of acetic acid until pH 4.6 indicated by an electrode. True protein (TP) was calculated by subtracting NPN from crude protein: TP = CP − NPN. Casein was calculated by subtracting NCN (nitrogen content in filtrate) from crude protein: casein = CP − NCN. Standard deviations (SD) for duplicate assays were <0.03% for fat and <0.02% (nitrogen basis) for nitrogen compounds.

NIR Spectroscopy. Samples were heated at 40 °C in a water bath, then gently shaken, and an aliquot was transferred to a 0.5 mm cuvette quartz cell for spectroscopic analysis. Transmittance spectra were collected from 1100 to 2500 nm at 2 nm intervals using a NIRSystems model 6500 scanning spectrophotometer (Foss-NIRSystems) equipped with a temperature control module at 40 °C. The NIR spectrophotometer was interfaced to a personal computer running under DOS the Near Infrared Spectral Analysis software (NSAS version 3.26; NIRSystems Inc.).

Each spectra is a result of the average of 32 completed scans (1100−2500 nm). To minimize sampling error, triplicate samples were analyzed for all 96 samples. The average spectra was used for NIR analysis.

Table 1. Chemical Composition of Milk Samples for Partial and Overall Calibrations

	partial calibration, 68 samples (unhomogenized milks)				overall calibration, 91 samples (unhomogenized + homogenized)			
component	mean	min	max	SD	mean	min	max	SD
fat	2.63	0.12	6.22	1.69	2.73	0.12	6.84	1.68
CP	2.91	1.90	4.14	0.45	3.03	1.90	4.97	0.65
TP	2.75	1.75	3.94	0.44	2.87	1.74	4.79	0.64
casein	2.33	1.42	3.30	0.38	2.43	1.42	4.06	0.54
casein/CP (%)	80.00	74.74	84.66	2.02	79.94	74.49	84.66	1.96

Statistical Analysis. Mahalanobis distance (*H* statistic) was calculated from principal component analysis scores. The results indicate how different a sample spectrum is from the average sample of the set (Williams, 1987). A sample presenting an *H* statistic >3.0 standardized units from the mean spectrum was defined as a global *H* outlier and was then eliminated from the calibration set. Using this procedure, eight outliers were eliminated from the partial calibration, and five were deleted from the overall set. On the basis of the *H* statistic distribution, samples were classified from the most to the least similar compared with the average spectrum. To obtain a validation set as spectrally similar as possible to the calibration set, in the case of the partial calibration (unhomogenized samples), one sample was selected for every five samples (20%) from the *H* statistic distribution. The remaining samples, which represented 80% of the samples, were then used for the calibration. For the overall calibration (homogenized and unhomogenized milks), the validation set, which also represented 20% of the calibration set, was randomly selected.

All statistical analyses were performed using the ISI 3.1 software (NIRS 3.1 for network management, Near Infrared software (C), Infrasoft International, 1993, Foss-NIRSystems Inc.).

Calibrations were performed by modified partial least-squares regression (MPLS). To optimize the calibration accuracy, several scattering corrections and mathematical treatments were tested. The scattering correction standard normal variate (SNV) gave the best results and was used for the development of the calibration model. Four variables were considered in all of the mathematical treatments. For example, in the mathematical treatment 2621, the first number corresponded to the derivative order, the second to the subtraction gap, and the third and fourth numbers indicated, respectively, the number of data points in the first and second smoothings.

According to these explorative scattering and mathematical treatments, 512 calibration equations were generated. The best one was selected for each constituent on the basis of the highest R^2 and the lowest standard error of calibration and cross-validation (SEC and SECV, respectively). Samples from the validation set were then analyzed with these equations, which gave a standard error of prediction (SEP) for each constituent.

RESULTS AND DISCUSSION

Milk Samples Composition and Spectral Information. The chemical compositions of the milk samples used for partial and overall calibrations (including validation samples) are shown in Table 1. The use of milk samples prepared with target composition is an interesting alternative because it allows for the possibility of a calibration set that accounts for the entire seasonal variation, avoiding systematic sampling over the year. As presented in Table 1, for every component studied, the range covered in both milk sets is greater than that associated with seasonal variability in bulk milks (Paquin and Lacroix, 1992; Banks et al., 1984)

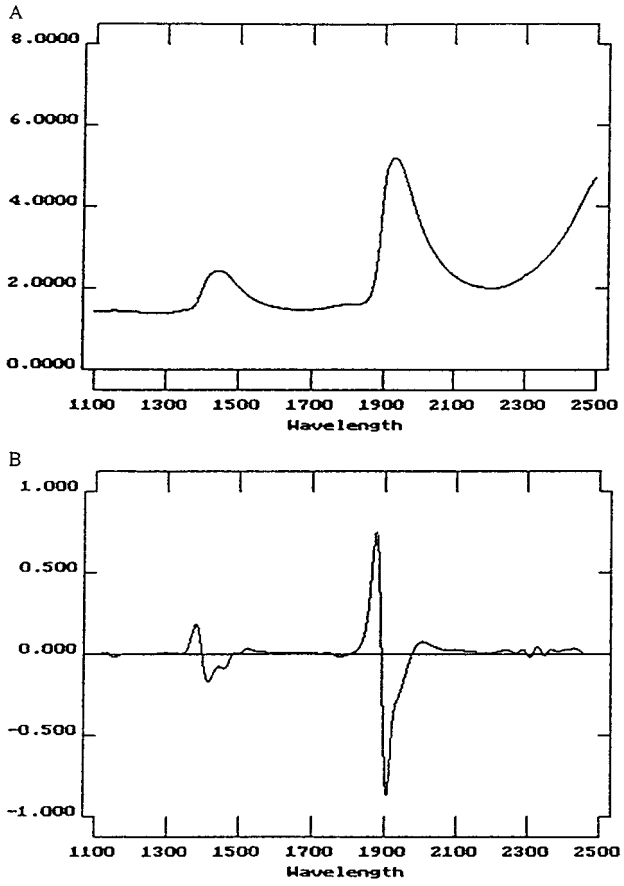

Figure 2. Average spectra of milks in the calibration set: (a) underivative; (b) second derivative.

and with variability resulting from the animal in individual cow's milk (Politis and Ng-Kwai-hang, 1988).

The average spectra (nonderivative and second derivative) of milks in the overall calibration are shown in Figure 2. Because of the strength of the overtones (1450 nm) and combination (1940 nm) water bands in the NIR region, spectral bands related to the other milk components are difficult to isolate on the nonderivative average spectra (Figure 2A). Furthermore, milk spectra result from the sum of each milk component and their specific interactions. However, the derivative mathematical treatment is an alternative approach for the problem of overlapping peaks (Hruschka, 1987). In the second-derivative average milk spectra (Figure 2B), the characteristic absorption peaks are more clearly separated. The lipid C−H combination and second overtone can be seen at 2320 and 2350 nm (Giangiacomo and Nzabonimpa, 1994). NH structures related to protein are located at approximately 2060 and 2170 nm (Diaz-Carillo at al., 1993).

NIR Determination of Fat and Nitrogenous Components in Unhomogenized Samples. The partial calibration was first performed on unhomogenized milk samples. After calculation of the Mahalanobis distance (*H* statistic) on the 76 milks, 8 samples were tagged outliers and were removed from this part of the study. The chemical reference ranges for the unhomogenized milk sample calibration set ($N = 57$) were 0.12−5.84% (2.50 ± 1.70) for fat, 1.90−4.14% (2.91 ± 0.46) for CP, 1.75−3.94% (2.75 ± 0.45) for TP, and 1.42−3.30% (2.33 ± 0.39) for casein.

The reference ranges for the validation samples ($N = 11$) were 0.03−6.22% (3.29 ± 1.49%) for fat, 2.03−3.32% (2.89 ± 0.40) for CP, 1.88−3.16% (2.76 ± 0.41%) for TP, and 1.56−2.69% (2.33 ± 0.34%) for casein. The statistic descriptors for the calibration equations of each component are shown in Table 2. These equations were used for the validation that gave the statistical descriptors of validation as shown in Table 3.

Table 2. Calibration Statistical Descriptors for the NIR Determination of Fat, Crude Protein, True Protein, and Casein in Unhomogenized Milk Samples

component	math	SEC	RSQ	SECV	factors (*n*)[a]
fat	2621	0.08	1.00	0.12	4
CP	2881	0.07	0.98	0.11	7
TP	2881	0.05	0.99	0.08	7
casein	2881	0.06	0.98	0.09	7

[a] Factors (*n*), number of factors.

Table 3. Validation Statistical Descriptors for NIR Determination of Fat, Crude Protein, True Protein, and Casein in Unhomogenized Milk Samples

component	math	SEP	bias	slope	RSQ
fat	2621	0.05	−0.01	0.98	1.00
CP	2881	0.09	−0.01	1.01	0.95
TP	2881	0.12	−0.02	0.99	0.91
casein	2881	0.07	0.01	1.00	0.96

Four PLS factors were required for the fat calibration, whereas seven factors were necessary for the calibrations of CP, TP, and casein (Table 2). The best calibration equations were obtained with mathematical treatments 2621 for fat and 2881 for CP, TP, and casein. For all components, better results were observed when the scattering option SNV was applied. SEC is the standard error of calibration or standard error of difference between references and NIR analysis values. The SEC indicates how well the NIR calibration model fits to data used for its determination. R^2 indicates the percentage of total variability explained by the PLS model. The standard error of cross validation (SECV) was obtained by partitioning the calibration set into several groups. For example, samples were predicted one-fourth at a time and the remaining three-fourths were used in the calibration until every sample had been predicted once. The predicted values gave validation errors, which were combined into SECV. This operation was done twice, and samples with large residuals ($T > 2.5$) were eliminated from the model. Although SECV is a good estimate of equation accuracy, validation of the model on an independent set of samples is still necessary to obtain real equation accuracy (Table 3). The statistical data for the calibration of each constituent show that the calibration models fit the reference data well. R^2 of 0.98−1.00 and SEC of 0.07, 0.05, and 0.06% for CP, TP, and casein, respectively, are similar or better values that those reported in previous studies (Diaz-Carillo et al., 1993; Hall and Chan, 1993). For fat calibration, SEC of 0.08 is better than that (0.178) reported in goat's milk by Diaz-Carillo et al. (1993) but not as good as the SEC reported in cow's milk (SEC = 0.04) by Hall and Chan (1993) and Chen et al. (1994). The validation results presented in Table 3 and, particularly, the SEP reported (0.05−0.07%) show that the calibrations developed are adequate for fat, protein, and casein determination. This is also supported by the low bias (−0.02−0.01), very good (0.98−1.01) slopes, and high R^2 values (0.91−0.99%) obtained for all milk components studied. Considering the relatively small number of samples used for the calibration, results demonstrate that NIR has the potential to become an alternative method for fat,

Table 4. Calibration Statistical Descriptors for NIR Determination of Fat, Crude Protein, True Protein, and Casein in Homogenized and Unhomogenized Milk Samples

component	math	SEC	RSQ	SECV	factors (n)[a]
fat	2621	0.12	1.00	0.16	4
CP	2881	0.06	0.99	0.08	8
TP	2881	0.04	1.00	0.07	8
casein	2881	0.05	0.99	0.08	8

[a] Factors (n), number of factors.

Table 5. Validation Statistical Descriptors for NIR Determination of Fat, Crude Protein, True Protein, and Casein in Homogenized and Unhomogenized Milk Samples

component	math	SEP	bias	slope	RSQ
fat	262	0.07	−0.01	0.98	1.00
CP	2881	0.06	−0.01	0.99	0.99
TP	2881	0.05	−0.02	0.99	0.99
casein	2881	0.06	−0.00	0.96	0.98

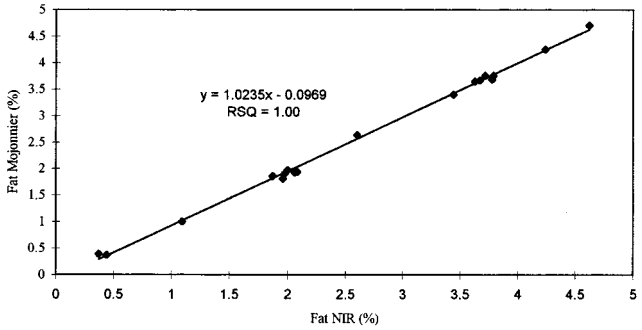

Figure 3. Correlation between determinations of fat content in homogenized and unhomogenized validation milk samples by reference (Mojonnier) and NIR transmission spectroscopy.

casein, and protein determinations in unhomogenized cow's milk.

Homogenization Effect on NIR Determination of Milk Major Components. To look at the effect of homogenization on the calibration, 20 homogenized prepared samples were added to the calibration set. For the 96 milk samples tested (76 unhomogenized and 20 homogenized), 5 samples had Mahanalobis distance >3.0 and were therefore eliminated from the study. For this second calibration, the reference ranges for the calibration set ($N = 73$) were 0.12−6.84% (2.76 ± 1.77%) for fat, 1.90−4.97% (3.01 ± 0.67%) for CP, 1.90−4.97% (3.00 ± 0.67) for TP, and 1.74−4.79% (2.85 ± 0.67%) for casein. The reference ranges for the validation set ($N = 18$) were 0.37−4.71% (2.60 1.31%) for fat, 2.10−4.47% (3.10 ± 0.56%) for CP, 1.96−4.29 (2.94 ± 0.55) for TP, and 1.64−3.59% (2.49 ± 0.0.46%) for casein.

Statistical descriptors for calibration and validation of components studied are shown in Tables 4 and 5, whereas Figures 3−6 illustrate the correlation between references (on validation samples) and NIR analysis for the four components.

According to Table 4, four PLS factors were required again for the calibration of fat, whereas eight PLS factors were necessary for the calibration of CP, TP, and casein. One more factor was needed for the overall calibration compared with the calibration performed on unhomogenized samples only (Table 2). This increase in PLS term number could be due to greater noise (Thomas and Haland, 1990), greater spectral variability (Shenk and Westerhaus, 1991), or simply the greater number of samples. As with the partial calibration, the

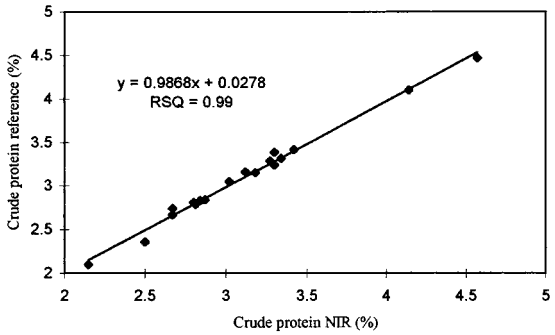

Figure 4. Correlation between determinations of CP content in homogenized and unhomogenized validation milk samples by reference (Kjeldahl) and NIR transmission spectroscopy.

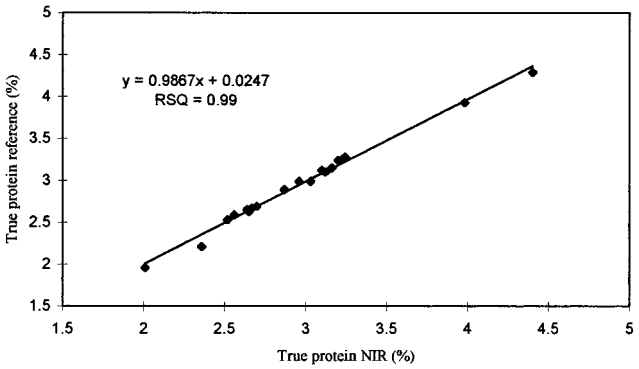

Figure 5. Correlation between determinations of TP content in homogenized and unhomogenized validation milk samples by reference (Kjeldahl) and NIR transmission spectroscopy.

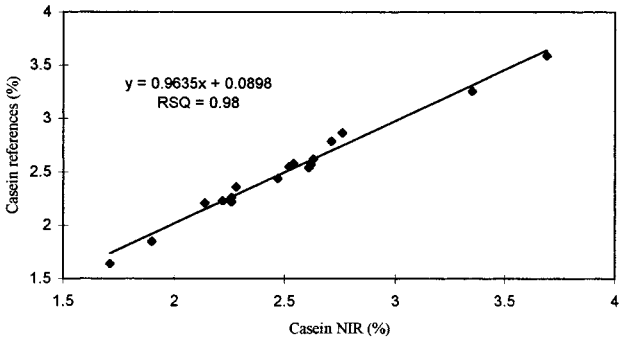

Figure 6. Correlation between determinations of casein content in homogenized and unhomogenized validation milk samples by reference (Kjeldahl) and NIR transmission spectroscopy.

best equations for components were obtained using the SNV option for scattering correction and mathematical treatments 2621 for fat and 2881 for nitrogenous components. For these latter components, the overall calibration (Table 4) gave a slight improvement in the SEC and SEVC values compared with the partial calibration (Table 2). This might be explained by the additional samples in the overall calibration which (1) allowed the use of more terms for PLS calibration, (2) accounted more efficiently for the large spectral variation of the nitrogenous components, and (3) improved the robustness of the calibration. These observations also applied to the statistical descriptors of validation (Table 5), particulary SEP and RSQ. In contrast, a decreased accuracy was observed for the NIR fat determination when homogenized samples were added to the calibration set, as shown by a decrease in statistical descriptors (Table 4). In fact, the addition of homogenized samples to the calibration led to a decrease in

both statistical descriptors of calibration and validation. The SEC and SECV for fat determination were, respectively, 0.08 and 0.12% in the partial calibration (unhomogenized milks) but increased to 0.12 and 0.16% in the overall calibration (homogenized and unhomogenized samples). This might be explained by the addition of homogenized samples, which caused a considerable increase in spectral variability. The mean size of fat globules is 3.5 μm in unhomogenized milk (Walstra, 1975) but only 0.75−0.85 μm in homogenized samples (IDF, 1990). There is also a greater heterogeneity among fat globules in unhomogenized compared with homogenized milks. In fact, unhomogenized milk samples are more susceptible to scattering effects (Smith et al., 1994). In general, results showed that NIR transmission technology is a promising method for the rapid analysis of major milk components. Furthermore, except for fat, the SEP for each component studied in the overall calibration met the required upper limit IDF standard of 0.06% (IDF, 1996).

Results also demonstrate that, as reported by Windham et al. (1989), many samples (≥150) are necessary to develop an accurate and robust NIR calibration, particulary for a complex product such as milk and for an overall calibration that includes homogenized and unhomogenized samples.

Conclusion. The NIR equations developed on a calibration set as small as 57 samples are applicable for the rapid determinations of fat, protein, and casein contents in cow's milk. However, an increase in the number of samples led to an improvement in both calibration and validation results for nitrogenous components. Results also showed an effect of homogenization on NIR fat determination. In fact, partial calibration including unhomogenized or homogenized samples only would be better for accurate NIR fat determination. As for a mid-infrared milk dedicated analyzer, a system for homogenization could also be integrated into the NIR apparatus. Considering the variability of milk and its components, it appears that larger calibration sets (≥150 samples) are necessary to achieve accurate and robust calibrations for NIR determinations of milk fat, protein, and casein. The low standard errors of prediction and validation obtained in this study as well as the numerous advantages of the technology make NIR transmittance a very promising tool for quality control in dairy plants.

ABBREVIATIONS USED

Math, mathematical treatment; NIR, near-infrared; PLS, partial least-squares regression; R^2, correlation coefficient; SD, standard deviation; SEC, standard error of calibration; SECV, standard error of cross-validation; SEP, standard error of prediction; SNV, standard normal variate.

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