Quantification of bovine milk protein composition and coagulation properties using infrared spectroscopy and chemometrics: A result of collinearity among reference variables

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ABSTRACT

Predicting protein fractions and coagulation properties in bovine milk using Fourier transform infrared (FT-IR) measurements is desirable. However, such predictions may rely on correlations with total protein content. The aim of this study was to show how correlations between total protein content, protein fractions, and coagulation properties are responsible for the successful prediction of protein fractions and rennet-induced coagulation properties in milk samples. This study comprised 832 bovine milk samples from 2 breeds (426 Holstein and 406 Jersey). Holstein samples were collected from 20 Danish dairy herds from October to December 2009; Jersey samples were collected from 22 Danish dairy herds from February to April 2010. All samples were from conventional herds and taken while cows were housed. The results showed that κ-CN, αS1-CN, αS1-CN with 8 phosphorylated groups attached (αS1-CN 8P), and curd firming rate could be predicted from FT-IR measurements of the milk samples (with coefficients of determination between 0.66 and 0.71). However, the success of these FT-IR–based predictions was based on indirect relationships with total protein content. Hence, the FT-IR predictions relied on covariance structures with total protein content rather than absorption bands directly associated with the protein fractions and coagulation properties. If covariance structures between the protein fractions, coagulation properties, and total protein content used to calibrate partial least squares models for β-CN. The 2 breeds had different covariance structures between β-CN and total protein content, and the validation samples yielded incorrect predictions. This finding may limit the usefulness of FT-IR–based predictions of protein fractions in milk recording, because indirect covariance structures in the calibration set must be valid for future samples, or future samples will show incorrect predictions.

Key words: bovine, coagulation properties, infrared spectroscopy, milk, protein composition

INTRODUCTION

In recent years, Fourier transform infrared (FT-IR) spectroscopy has been suggested as a method that could outcompete chromatography for detailed milk analyses (Soyeurt et al., 2006; Rutten et al., 2011; De Marchi et al., 2014). In particular, the high-throughput measurements of FT-IR analyses are an advantage over the more time-consuming chromatography-based analyses. If FT-IR–based predictions do not compromise the quality of the detailed milk composition estimates, FT-IR could be used as a phenotyping tool for breeding programs, for example (Bovenhuis et al., 2013). It has been proposed that the major fatty acids can be routinely estimated from FT-IR measurements (Soyeurt et al., 2006; Rutten et al., 2009), but protein fractions have been predicted with less accuracy (Bonfatti et al., 2011; Rutten et al., 2011).

Combined, the 4 caseins (αS1-CN, αS2-CN, β-CN, and κ-CN) and 2 whey proteins (α-LA and β-LG) comprise approximately 90% of the bovine protein fraction (wt/wt; Walstra, 1999). Heterogeneity in the major milk proteins is related to underlying genetic polymorphism (Heck et al., 2009), and it is also affected by DIM and parity (Poulsen et al., 2016). Posttranslational modification of the proteins also adds to their isoform complexity and has been shown to affect the technological properties of milk, including milk coagulation.
(Frederiksen et al., 2011; Jensen et al., 2012). In particular, the relative fraction of κ-CN to total protein (TP) is important for milk coagulation, together with the level of glycosylated κ-CN (Jensen et al., 2012; Bonfatti et al., 2014). Furthermore, bovine milk proteins are sold as ingredients based on their nutritional or functional properties, so reliable high-throughput estimation of protein content is warranted.

Several studies have attempted to use FT-IR measurements to predict protein fractions or the coagulation properties of bovine milk samples (Dal Zotto et al., 2008; Bonfatti et al., 2011; Rutten et al., 2011). Infrared radiation is absorbed by exciting the fundamental vibrations of molecular bonds, expressing a change in the dipole moment. For proteins in a milk matrix, the most pronounced absorption signal is related to the amide II band (primarily N-H bending vibration), located at approximately 1,540 cm\(^{-1}\) (Luinge et al., 1993). This band originates from the peptide backbone. Specific milk proteins are differentiated by the composition of their amino acid residues. The different amino acids are present in more or less all the protein fractions, although in varying relative contents, but the FT-IR active groups in the amino acids are more or less the same (Walstra et al., 2006). Therefore, it might be difficult to obtain unique FT-IR signals for specific protein fractions in milk.

Eskildsen et al. (2014) have argued that successful prediction of individual fatty acids in milk by FT-IR measurement is due to covariance between individual fatty acids and total fat content. Bonfatti et al. (2015) have speculated that similar covariance between protein fractions and TP content is responsible for the successful prediction of protein fractions. Total protein can be easily predicted from FT-IR measurements using the amide II band (Luinge et al., 1993), and good correlations between TP and individual protein fractions might enable indirect predictions of protein fractions using the amide II band. However, the indirect relationships between TP and protein fractions may change with factors such as breed or feed; indirect relationships for calibrating partial least squares (PLS) regression models may compromise calibration robustness. If an indirect relationship is used to calibrate a regression model, the model will not be valid for future samples unless the indirect relationship is conserved in the new samples. If such an indirect relationship is not conserved, the model will provide incorrect predictions, as shown by Eskildsen et al. (2014).

McDermott et al. (2016) have argued that when predicting protein fractions from FT-IR measurements, TP-related information is being used for prediction but FT-IR spectra also provide additional information. If TP is part of the information involved in predicting a given protein fraction, the prediction will be indirect and a change in TP will affect the prediction of the protein fraction. Furthermore, McDermott et al. (2016) have not identified this additional information; it could be related to indirect relationships with other major milk components or be a consequence of overfitting.

This study investigated whether FT-IR can be used to obtain reliable prediction models for the major milk proteins and coagulation properties, and how PLS models for predicting protein fractions, curd firming rate (CFR), and rennet coagulation time (RCT) depend on correlations between these traits and TP in raw milk samples from 2 Danish dairy breeds.

**MATERIALS AND METHODS**

**Experimental Design**

Morning milk samples from 406 Jersey cows, collected from 22 Danish dairy herds between February and April 2010, and samples from 426 Holstein cows, collected from 20 Danish dairy herds between October and December 2009, were included in this study. All samples were from conventional herds and taken while cows were housed. The sample set used in this study was a subset of that used in Eskildsen et al. (2014) to predict individual fatty acid content. Compared with Eskildsen et al. (2014), some samples were excluded from the present study, because they were not analyzed for protein fraction content. As also pointed out by Eskildsen et al. (2014), the sampling strategy was aimed at minimizing environmental variation but maximizing genetic variation in the sample population (Poulsen et al., 2012). The milk samples were placed on ice during transport to the laboratory and analyzed the same day using MilkoScan FT2 (Foss Analytical A/S, Hillerød, Denmark) and a ReoRox4 rheometer (MediRox AB, Nyköping, Sweden). The FT-IR measurements were performed on the raw milk samples, but the rheological analysis was performed on skim milk samples (Jensen et al., 2012). A part of each skim milk sample was stored at −20°C for quantification of protein fractions.

Protein fractions were quantified using electrospray mass spectrometry coupled to liquid chromatography as described by Jensen et al. (2012). Relative reference values obtained were multiplied by TP to convert protein fractions into quantities per unit milk (grams of specific protein per 100 g of milk), because quantities of protein fractions in units of TP are not comparable with FT-IR spectra obtained from liquid milk. We determined TP content using MilkoScan FT2 as outlined by Eskildsen et al. (2014).
Rennet-induced coagulation of skim milk samples was determined using a ReoRox4 rheometer, as outlined in Poulsen et al. (2013) and adding chymosin (ChyMax; Chr. Hansen, Hørsholm, Denmark). Milk coagulation properties for individual samples were described as RCT and CFR. We defined RCT as the amount of time from chymosin addition to when the phase angle reached 45° (θ = 45°). We calculated CFR from consecutive points of the linear (lin) part of the gelation profile, defining changes in the storage modulus ($G'_c$) over time $t$, $[\Delta G'_c/\Delta t]_{lin}$. Descriptive statistics (in quantities per unit milk) are presented in Table 1. Further descriptive statistics (in quantities per unit protein), including breed differences, for protein composition and milk coagulation properties of the samples have been described by Poulsen et al. (2013) and Poulsen et al. (2016).

As described by Eskildsen et al. (2014), FT-IR full spectra were recorded on all samples using MilkoScan FT2. Each FT-IR measurement was ratioed against an FT-IR water spectrum (as background). Spectra were obtained from 5,008 cm$^{-1}$ to 925 cm$^{-1}$, with a total of 1,060 data points for each sample. The FT-IR spectra were preprocessed by Savitzky-Golay first derivative (window size of 9 points and second-order polynomial) to remove offset differences between samples and then mean centered before PLS modeling. Both Holstein and Jersey samples were included (simultaneous) in fitting the PLS models. All PLS models were built using a mean centered univariate response variable.

We used Venetian blinds with 10 data splits (starting at samples 1 through 10, each validation set was determined by selecting every tenth sample in the data set) to validate PLS models calibrated on both Jersey and Holstein samples. Model parameters [cross-validated coefficient of determination ($R^2_{CV}$) and root mean squared error of cross-validation] were reported and used to choose the number of latent variables.

To investigate whether indirect relationships were responsible for successful prediction of protein fractions, we calculated and compared 3 parameters. The first parameter was the $R^2_{CV}$ from the PLS models (measured vs. predicted), which showed how well the individual milk proteins (or coagulation properties) were predicted. The second was the coefficient of determination ($R^2$) between the measured values of the individual proteins (or coagulation properties) and predicted TP. In cases where the protein fractions were predicted only by correlation with TP, this second parameter would equal the model performance (the first parameter). The third parameter was the $R^2$ between predicted values of the individual proteins (or coagulation properties) and predicted TP. If the protein fractions were modeled only by correlation with TP, then the protein fractions and TP would be modeled by the same wavenumbers on the FT-IR spectra (i.e., the same linear combination of wavenumbers). This would cause the correlation

Data Analysis

We used MATLAB version R2014a (8.3.0.532; MathWorks Inc., Natick, MA) and PLS_Toolbox version 7.5 (Eigenvector Research Inc., Manson, WA) for data analysis.

The FT-IR spectra were obtained in transmittance and transformed into absorbance to obey Beer’s law. Spectra were obtained from 5,008 cm$^{-1}$ to 925 cm$^{-1}$,
between predicted values and predicted TP to be 1. Hence, if the third parameter was 1, then the protein fractions were modeled only by correlation with TP.

To highlight the consequences of indirect models, we calibrated 1 additional PLS model predicting β-CN on a subset of the Holstein samples that included 16 Holstein herds (338 samples). The remaining 4 Holstein herds (88 samples) were used as 1 test set, and all Jersey samples were used as another test set. The 4 Holstein herds were randomly selected. Data were preprocessed similarly to the multi-breed PLS models. We compared the root mean squared error of calibration for the Holstein calibration samples with the root mean squared error of prediction for the Holstein test set samples and Jersey samples, respectively.

RESULTS AND DISCUSSION

The FT-IR absorption spectra of the milk samples are presented in Figure 1a. The spectra are colored by TP. The region from 2,968 cm⁻¹ to 5,008 cm⁻¹ and from 1,692 cm⁻¹ to 1,604 cm⁻¹ was excluded from modeling because of low signal-to-noise ratio. We found no peaks in the region from 1,773 cm⁻¹ to 2,802 cm⁻¹, so this region was also excluded from modeling. Finally, due to possible saturation of the single beam spectra, a narrow region from 2,915 cm⁻¹ to 2,930 cm⁻¹ was excluded from modeling. Figure 1b shows the spectral parts included for modeling. The spectra in Figure 1b are also colored by TP.

Offset differences between spectra may affect PLS models. To remove offset differences, spectra were preprocessed by Savitzky-Golay first-derivative prior modeling, also done in Eskildsen et al. (2014).

Spectral data were modeled by principal component analysis (results not shown). The spectral sample set used in this study differed only slightly from the set used by Eskildsen et al. (2014), so the results from the principal component analysis were also very similar. Eskildsen et al. (2014) found that Holstein and Jersey samples were separated to a large extent by spectral variation associated with total fat and TP, with the Jersey samples having higher content of both.

To explore how individual fatty acids related to the FT-IR spectra, Eskildsen et al. (2014) calculated $R^2$ values between the absorption intensities of each wavenumber and concentrations of individual fatty acids. Eskildsen et al. (2014) found that fatty acids showed a correlation pattern similar to total fat content with FT-IR absorption intensities. The fatty acids correlated primarily with the spectral regions associated with symmetric and antisymmetric stretching vibrations of methylene groups and the stretching vibration of carbonyl groups. Similarly, $R^2$ values were calculated between absorption intensities of each wavenumber of the preprocessed (Savitzky-Golay first derivative) FT-IR spectra and the measured values of the individual protein fractions, CFR, and RCT (Figure 2). Descriptive statistics for the protein fractions and coagulation traits are presented in Table 1. Figure 2 shows a trend for TP and protein fractions similar to what Eskildsen et al. (2014) found for total fat content and fatty acids. The individual protein fractions (such as κ-CN and αS1-CN 8P) and CFR had more or less the same correlation pattern as TP, which was a high correlation with the expected area between 1,600 cm⁻¹ and 1,500 cm⁻¹ related to the amide II band. This could very well indicate that individual proteins and coagulation properties do not give rise to specific absorption bands in FT-IR, and are predicted mainly by the amide II band.

Table 2 shows the results of the PLS models for protein fractions, CFR, and RCT. We obtained acceptable predictions for κ-CN, αS1-CN, αS1-CN 8P, and CFR, which all yielded $R^2$CV values around 0.7. The results for αS1-CN and κ-CN were in agreement with reports from De Marchi et al. (2009b) and Bonfatti et al. (2011). However, we obtained poor predictions for glycosylated κ-CN, αS2-CN, αS2-CN 11P, β-CN, α-LA, β-LG, and RCT, which all had $R^2$CV values from 0.06 to 0.47. The results for αS2-CN and β-CN were in agree-
ment with De Marchi et al. (2009b) and Bonfatti et al. (2011), but our predictions for glycosylated κ-CN, β-CN, α-LA, and β-LG were worse than previous reports (De Marchi et al., 2009b; Bonfatti et al., 2011; Rutten et al., 2011). Moreover, in the present study, the prediction of RCT was remarkably worse than in other studies (Dal Zotto et al., 2008; De Marchi et al., 2009a, 2013). It is worth noting that some protein fractions and coagulation properties were well predicted in some studies and poorly predicted in others. As highlighted by De Marchi et al. (2014), different reference methods have been used across studies. When calibration models are evaluated and compared based on R²CV values, differences in uncertainties among the reference methods affect those values (DiFoggio, 1995; Faber and Kowalski, 1997; De Marchi et al., 2014). Furthermore, in the present study, milk coagulation properties were determined using skim milk, but this was not the case in other studies (Dal Zotto et al., 2008; De Marchi et al., 2009a, 2013), making it difficult to compare calibration models directly. Finally, if protein fractions and coagulation properties are predicted by indirect relationships, then those relationships will also affect evaluation of the calibration models. In some studies, certain indirect relationships may have enabled good predictions for specific traits, but those relationships may not have been present in other studies because of differences such as sampling strategies, leading to poor predictions for those traits.

As already mentioned in the Materials and Methods, the protein fractions per unit protein (obtained using electrospray mass spectrometry coupled to liquid chromatography) were multiplied by TP to obtain the protein fractions per unit milk. This multiplication imposed higher correlations between the protein fractions and TP (and therefore also between the FT-IR spectral parts associated with TP and the protein fractions). Nevertheless, if the protein fractions have unique signals in the FT-IR spectra, then it is still possible to fit a regression vector (for each individual protein fraction) that is orthogonal to the TP signal (Sanchez and Kowalski, 1988). If the regression vector for that protein fraction is orthogonal to the TP signal, predictions of that protein fraction will not be influenced by variation in TP (i.e., the predictions will be independent of TP). However, if the regression vector
for a given protein fraction is not orthogonal to the TP signal, then the predictions of that protein fraction will be influenced by variation in TP (i.e., the predictions will be dependent on TP). The protein fraction and TP will be (partially) fitted by the same FT-IR spectral wavenumbers. If this is the case, the correlations between the predicted TP and predicted protein fraction will be higher than the correlation between predicted TP and measured protein fraction, because the 2 traits are predicted (partially) by the same linear combination of the spectral wavenumbers.

To investigate these indirect relationships, we calculated and compared the 3 parameters: $R^2_{CV}$ (model performance), $R^2$ (measured traits vs. predicted TP), and $R^2$ (predicted traits vs. predicted TP). Model performance is plotted on the x-axis in Figure 3. The further to the right the protein fractions are located in Figure 3, the better the protein fractions were predicted. On the y-axis (Figure 3), the $R^2$ between the measured traits and predicted TP is plotted with an open square, and the $R^2$ between predicted traits and predicted TP is plotted with a filled triangle.

Figure 3 reveals that the protein fractions $\alpha_S$-CN 8P, $\kappa$-CN, and $\alpha_{S1}$-CN, as well as CFR, which were all modeled well, were very dependent on correlation with TP ($R^2$-values for $\alpha_{S1}$-CN coincided with $R^2$-values for CFR, and $R^2$-values for $\alpha_{S1}$-CN 8P coincided with $R^2$-values for $\kappa$-CN in Figure 3). Total fat and lactose were also modeled very well (Figure 3). However, these models were not dependent on correlation with TP, because the $R^2$ between the measured values and TP and the $R^2$ between the predicted values and TP were almost the same. Hence, total fat and lactose must have been predicted by parts of the FT-IR spectra, which were different from the parts used for predicting TP as also shown by Luinge et al. (1993). In other words, the regression vectors for total fat and lactose must have been orthogonal to the TP FT-IR signal.

Figure 4 illustrates the problems in modeling individual proteins by the indirect relationship with TP. In Figure 4a, $\beta$-CN is plotted against TP for the 2 breeds (Holstein and Jersey). From Figure 4a, it is clear that

![Figure 3. Relationship between cross-validated coefficient of determination ($R^2_{CV}$) from cross-validated partial least squares models (x-axis) and coefficient of determination ($R^2$) between measured and predicted values of individual proteins, coagulation properties, lactose, total fat, and predicted total protein (TP; y-axis). CFR = curd firming rate; RCT = rennet coagulation time.](image)

### Table 2. Results from partial least squares models

<table>
<thead>
<tr>
<th>Protein fraction or coagulation trait</th>
<th>Range</th>
<th>Mean</th>
<th>SD</th>
<th>CV</th>
<th>LV</th>
<th>$R^2_{CV}$</th>
<th>RMSECV</th>
<th>Relative error</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\kappa$-CN</td>
<td>0.15-0.40</td>
<td>0.25</td>
<td>0.05</td>
<td>0.21</td>
<td>2</td>
<td>0.71</td>
<td>0.03</td>
<td>0.13</td>
</tr>
<tr>
<td>Glycosylated $\kappa$-CN</td>
<td>0.03-0.08</td>
<td>0.05</td>
<td>0.01</td>
<td>0.16</td>
<td>4</td>
<td>0.20</td>
<td>0.02</td>
<td>0.30</td>
</tr>
<tr>
<td>$\alpha_{S1}$-CN</td>
<td>0.75-1.51</td>
<td>1.05</td>
<td>0.15</td>
<td>0.14</td>
<td>2</td>
<td>0.66</td>
<td>0.11</td>
<td>0.10</td>
</tr>
<tr>
<td>$\alpha_{S1}$-CN 8P</td>
<td>0.53-1.21</td>
<td>0.80</td>
<td>0.13</td>
<td>0.17</td>
<td>2</td>
<td>0.71</td>
<td>0.09</td>
<td>0.11</td>
</tr>
<tr>
<td>$\alpha_{S2}$-CN</td>
<td>0.13-0.31</td>
<td>0.20</td>
<td>0.04</td>
<td>0.18</td>
<td>2</td>
<td>0.36</td>
<td>0.05</td>
<td>0.24</td>
</tr>
<tr>
<td>$\alpha_{S2}$-CN 11P</td>
<td>0.07-0.22</td>
<td>0.13</td>
<td>0.03</td>
<td>0.23</td>
<td>2</td>
<td>0.47</td>
<td>0.03</td>
<td>0.25</td>
</tr>
<tr>
<td>$\beta$-CN</td>
<td>0.82-1.61</td>
<td>1.25</td>
<td>0.09</td>
<td>0.07</td>
<td>7</td>
<td>0.25</td>
<td>0.14</td>
<td>0.11</td>
</tr>
<tr>
<td>$\alpha$-LA</td>
<td>0.06-0.13</td>
<td>0.11</td>
<td>0.01</td>
<td>0.06</td>
<td>4</td>
<td>0.06</td>
<td>0.02</td>
<td>0.21</td>
</tr>
<tr>
<td>$\beta$-LG</td>
<td>0.13-0.42</td>
<td>0.27</td>
<td>0.04</td>
<td>0.14</td>
<td>9</td>
<td>0.34</td>
<td>0.05</td>
<td>0.19</td>
</tr>
<tr>
<td>Curd firming rate</td>
<td>1.48-34.94</td>
<td>14.80</td>
<td>6.86</td>
<td>0.46</td>
<td>2</td>
<td>0.66</td>
<td>4.90</td>
<td>0.33</td>
</tr>
<tr>
<td>Rennet coagulation time</td>
<td>10.37-17.38</td>
<td>13.29</td>
<td>0.86</td>
<td>0.06</td>
<td>4</td>
<td>0.12</td>
<td>2.19</td>
<td>0.17</td>
</tr>
</tbody>
</table>

1LV = number of latent variables; $R^2_{CV}$ = cross-validated coefficient of determination; RMSECV = root mean squared error of cross-validation; relative error = RMSECV/mean. Range, mean, SD, and RMSECV are in units of g/100 g of milk for protein fractions, units of Pa/min for curd firming rate, and units of min for rennet coagulation time. In $\alpha_{S1}$-CN 8P and $\alpha_{S2}$-CN 11P, P denotes the number of phosphorylated groups attached.
the relationship between β-CN and TP was different for the 2 breeds. We observed moderately good correlation between β-CN and TP for the Holstein samples, but poor correlation for the Jersey samples. Samples of the 4 randomly selected Holstein herds are highlighted with triangular symbols in Figure 4a. These 4 herds were kept aside for testing, together with the Jersey samples, and we calibrated a PLS model on the remaining Holstein samples from 16 herds. Figure 4b shows the measured versus predicted β-CN values for the calibration set and the 2 test sets. The root mean squared error of calibration for the Holstein calibration set was 0.09 g of β-CN per 100 g of milk. The root mean squared error of prediction for the Holstein test set was also 0.09 g of β-CN per 100 g of milk, but the root mean squared error of prediction for the Jersey samples was 0.29 g of β-CN per 100 g of milk, remarkably higher than the error for the Holstein test set. Hence, Figure 4 highlights the problem with using models based on indirect correlations. The indirect relationship used to calibrate the PLS model on Holstein samples was not valid for Jersey samples. For a model based on indirect relationships to be valid, the indirect relationships used for calibrating the model must be conserved in the new data set.

Building universal models (for example, using a high number of samples from multiple breeds in the calibration set) will not necessarily solve the problem of indirect models. Including a high amount of variation in the calibration set (such as different breeds, feeding systems, or lactation stages) will most likely break the indirect relationship between protein fractions and TP. The consequence will presumably be a poor global model, because the links between the FT-IR spectra and the protein fractions are lost. In Figure 4b, the PLS model was calibrated on the Holstein samples only. We observed a good relationship between β-CN and TP for the Holstein samples, and the Holstein

![Figure 4](https://example.com/figure4.png)

**Figure 4.** (a) Relationship between measured β-CN and total protein (TP) in milk samples from individual Jersey and Holstein cows; (b) measured versus predicted values of β-CN; β-CN was predicted by partial least squares regression applied to Fourier transform infrared measurements. The partial least squares model is calibrated on a subset of the Holstein sample and tested with the remaining Holstein samples and the Jersey samples. LV = latent variables, RMSEC = root mean squared error of calibration, RMSEP = root mean squared error of prediction. Color version available online.
samples were predicted well (Figure 4b). The PLS models presented in Table 2 were calibrated on both Jersey and Holstein samples. Figure 4a reveals that the overall correlation between TP and β-CN was poor when taking both breeds into account simultaneously. Consequently, the model for β-CN was poor when both breeds were included in the calibration set (Table 2). An option would be to construct only indirect local models on single breeds. In this way, the correlations between protein fractions and TP would be expected to be strong, and the link between the FT-IR spectra and the protein fractions established. Nevertheless, such indirect relationships need to be validated. Furthermore, the indirect prediction of protein fractions are of limited use as they (in principle) do not provide additional information (or very limited additional information) compared with a model predicting TP.

CONCLUSIONS

Concentrations of protein fractions, as well as coagulation properties, were predicted by applying PLS to FT-IR measurements of milk samples. This paper has illustrated that predictions of protein fractions and coagulation properties for the samples available in this study relied on indirect relationships with TP. This fact compromised the robustness of the calibration models for protein fractions and coagulation properties. The calibration models are no longer valid if these indirect relationships change. Hence, calibration models may not be valid for samples of a different nature (e.g., different breeds or lactation stages). Therefore, recommendations on applying FT-IR based estimates of protein fractions and coagulation properties for breeding purposes must account for these indirect relationships. It is simply not sufficient to evaluate model performance parameters such as $R^2_{CV}$ and root mean squared error of cross-validation as has been done in previous studies. The indirect relationships must be fully understood and controlled for. Furthermore, we need to be aware that indirect models provide very limited additional information compared with models of TP.

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