Prediction of immunoglobulin G content in bovine colostrum by near-infrared spectroscopy

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ABSTRACT

The objective of this work was to assess the potential of near infrared spectroscopy to predict the immunoglobulin G (IgG) content in bovine colostrum. Liquid colostrum samples ($n = 157$) were collected from Holstein cows from 2 dairy farms in southern Chile. Samples were obtained within 1 h of parturition and scanned in folded transmission (transflectance) in the visible-near infrared range. Multivariate regression models (modified partial least squares) were developed with spectral data against IgG content measured by radial immunodiffusion. The best calibration included a mathematical treatment of the spectra by a second derivative plus standard normal variate and detrending. The best equation explained a high proportion of the variation in IgG content (R^2 of 0.95 in calibration and 0.94 in cross-validation). Average (91.5 g/L), standard deviation (37.6 g/L), and range, as highest minus lowest values (171.9 g/L) of reference values were 10.1, 4.2, and 19 times the value of the root mean square error of cross-validation (9.03 g/L) respectively. Near-infrared spectroscopy, scanned in folded transmission, is an effective tool to predict the IgG content in liquid colostrum.

Key words: colostrum quality, immunoglobulin G, near-infrared spectroscopy, transflectance

INTRODUCTION

Adequate passive transfer of immunoglobulins is critical to the survival and health of young mammal (Chigerwe et al., 2008) either via the placenta, in those species where placental transfer is effective, or postnatally via colostrum in those where it is not, such as in ruminants (Stelwagen et al., 2009). Colostrum contains a high concentration of immunoglobulins that provide protection against microbial infection (Wheeler et al., 2007). Although bovine colostrum contains 3 major

classes of immunoglobulin: IgG, IgM, and IgA (Korhonen et al., 2000), IgG (particularly IgG₁) is the most abundant in bovine colostrum (Stelwagen et al., 2009), comprising around 80 to 90% of the immunoglobulins in bovine colostrum whey (Korhonen et al., 2000; El-Loly, 2007).

Factors associated with successful immunity transfer in the newborn calf are related to colostrum management, including concentration and volume of colostrum fed, time of feeding after birth, and minimal bacterial contamination of colostrum (Beam et al., 2009). An accurate method to measure IgG content is essential to assess colostrum quality. Several techniques have been developed. Radial immunodiffusion (**RID**), developed by Mancini et al. (1965), is considered the gold standard (Tyler et al., 1999; Biemann et al., 2010). In RID, samples containing IgG are applied to small circular wells cut in an agarose gel that contains an antiserum to the immunoglobulin being evaluated. Measurement is based on the formation of a visible ring of antigen-antibody precipitin (Gapper et al., 2007). Other techniques to estimate colostrum IgG are the use of hydrometer (Chigerwe et al., 2008; Rudovsky et al., 2008), weight of the first milking (Chigerwe et al., 2008), colostrum color (Argüello et al., 2005), refractometry (Chavatte et al., 1999; Biemann et al., 2010), size-exclusion HPLC (Dolman and Thorpe, 2002), electrophoresis (Page and Thorpe, 2002), and protein affinity liquid chromatography (Abernethy and Otter, 2010). For an extensive review of the topic, the reader is referred to Gapper et al. (2007). However, like RID, most of these methods are impractical for field situations, because time to results can exceed 24 h. Digital and optical refractometers have been tested as practical management tools and found capable of differentiating between good and poor quality colostrum, but variations in total solids, casein micelles, and fat globules can affect accuracy of the readings (Biemann et al., 2010).

Near-infrared spectroscopy (**NIRS**) is a widely used method for evaluating food and feedstuffs. It has many advantages over other techniques: it is fast, accurate,

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and easily performed, and it provides objective data (Givens et al., 1997; Dračková et al., 2008). The near-infrared (NIR) spectrum originates from overtones and combinations (weaker signals) from the fundamental vibrations that occur in the infrared region, to the H-containing groups in biological macromolecules, with overlapping absorption bands. The method requires appropriate calibrations to extract relevant information to develop a useful model to predict the analyte of interest or a particular quality of the samples (Osborne et al., 1993; Miller, 2001; Bosco, 2010). A critical aspect to consider is that prediction errors can stem from several factors associated with the samples, the instrument, the operators, and the reference techniques, as NIRS is a secondary technique, relying on the model developed from the spectral data and reference values (Williams and Norris, 2001). This method has been used extensively to evaluate food quality in general (Cen and He, 2007), including milk composition (Laporte and Paquin, 1999; Kawamura et al., 2007; Wu et al., 2008) and SCC (Tsenkova et al., 2001). However, despite the ample body of literature on NIRS applications to predict composition and quality traits of different foods and agricultural products, including prediction of main components of bovine colostrum (Navrátilová et al., 2006), no studies have reported the analysis of IgG content in colostrum by this technique. Therefore, the objective of this work was to evaluate the potential of NIRS technology to predict the IgG content in liquid colostrum from dairy cows.

MATERIALS AND METHODS

Colostrum Samples

The study was performed using 157 samples of colostrum collected from Holstein-Friesian cows of different parities, with average production of 7,000 L, from 2 commercial dairy farms with spring and autumn calving, respectively, located in the province of Valdivia, Región de Los Ríos, southern Chile. Cows were fed on permanent pastures and supplemented with concentrate. Samples were collected from the colostrum obtained from the 4 quarters of the udders in the first hour after calving, thoroughly mixed, and divided into 2 subsamples, one for spectra collection and the other for reference analysis. All subsamples were stored at -20°C until spectra collection and reference analysis were performed.

Spectra Collection

Prior to NIRS analysis, samples were thawed at 2 to 5°C for 24 h, left for 10 min at room temperature

($18\text{--}20^{\circ}\text{C}$), and then agitated for 1 min with a vortex mixer at maximum speed to ensure a uniform dispersion of colostrum components. Samples were scanned in the visible and near infrared (VIS-NIR) region (400–2,500 nm, at 2-nm intervals) in a NIRSystems 6500 monochromator (Silver Springs, MD). The software WINISI II (Infrasoft International, Port Matilda, PA) was used for scanning and calibration. All samples were scanned in duplicate by transmittance (folded transmission). Each duplicate (500 mg) was placed immediately after agitation in a cam-lock cell for liquid products with an aluminum reflector and 0.1-mm pathlength (part number IH-0345-1; NIRSystems). The transmittance cell was inserted in a spinning module (part number NR-6506; NIRSystems) for the readings. Each scan was composed of 32 readings of the sample plus 32 of a white ceramic tile provided in the instrument as a reference. The resulting spectra were recorded and stored in suitable files as $\log(1/R)$, where R is the reflectance (in this case, transmittance) energy recovered by detectors in the VIS (silicon detectors) and NIR (lead sulfide detectors) range, positioned at 45° of the sample.

RID Assay

Aliquots of colostrum samples reserved for reference analysis were thawed at 2 to 5°C for 24 h and then analyzed by RID at the Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan (Canada). The RID assay was performed using 1.0% agar gel containing 2.5% rabbit anti-bovine IgG (Jackson Laboratories Inc., West Grove, PA). Dilution series of test colostrum and reference colostrum were dispensed (5 replicates of 4 μL) in wells (2 mm diameter) cut into the agar on the plate. Plates were incubated at 20 to 25°C for 18 to 24 h in a humid chamber, and ring diameters of precipitin surrounding the wells were measured using a RID plate reader (The Binding Site, Birmingham, UK). The content of IgG was determined using a regression line generated with 2 replicates of known standards (Bovine IgG Serum Calibrator, Midlands BioProducts Corp., Boone, IA) diluted serially 1/4 to 1/32 and incubated on each plate.

Spectra Analysis

A principal component analysis (PCA) was applied to raw spectral data [$\log(1/R)$] to highlight relevant absorption patterns. This technique is widely used in NIRS analysis as it is able to transform all the absorbance data in a few “synthetic” variables—linear combinations of the original data—called principal components (PC), which are uncorrelated and preserve

the main variation of the spectra (Robert et al., 1996). The first PC reveal the main information in the data, whereas the last ones contains mostly noise. In this way, patterns of variability ("loadings") can be related to relevant absorption bands.

Calibrations

Modified partial least squares (**MPLS**) regression was used to obtain prediction equations, testing different mathematical treatments of the spectral data. Treatments consisted of gap-segment derivative, also referred to as subtraction order, or Norris derivative (Hopkins, 2001). No derivate (raw spectra), first and second derivatives were tested. Derivatives were applied across different data point intervals (gap) and with different smoothing segments of the spectra to resolve overlapping peaks and remove linear baselines (Osborne et al., 1993; Hruschka, 2001). Standard normal variate (**SNV**) and detrending (**DT**) treatment were applied for scatter correction (Barnes et al., 1989). The SNV was applied to scale each spectrum by its own standard deviation and correct shifts in the y-axis, and DT removes baseline curvature caused by interactions of moisture and particular effects across the NIR spectral range (Barnes et al., 1993). The option of no scatter correction was also tested. Equations were evaluated by one-out full cross-validation. This technique separates the calibration set into groups, omitting one for prediction and calibrating with the remaining groups. Four, 5, or 6 cross-validation groups were tested. If 4 groups are used, the first group is created from samples in position 1, 5, 9, and so on, and the second group, with samples in position 2, 6, 10, and so on. Consecutively, all groups were predicted and an error term; that is, standard error of cross-validation (Shenk and Westerhaus, 1996), also referred to as root mean square error of cross-validation (**RMSECV**), was computed. A coefficient of determination of cross-validation (**R²CV**) was also calculated. Two passes of elimination of outliers were applied to remove aberrant predictions ("T" outliers) or abnormal spectra ("H" outliers). A critical value of 2.5 was set for T outliers (residuals of predicted vs. reference values) and of 10 for H outliers (distance between each spectrum and average spectra). Best equations were those with a lowest **RMSECV**, which is considered a good estimator of prediction accuracy (Infrasoft International). As a complementary criteria to select the best equation, the residual predictive deviation (**RPD**); that is, the relation between standard deviation of RID measurements (reference data) and the **RMSECV**, and the range error ratio (**RER**); that is, the relation between the range

of RID values and the **RMSECV**, were used (Williams and Sobering, 1996; Williams 2001).

RESULTS

Spectra

Raw ($\log 1/R$) transreflectance spectra of all fresh colostrum samples are shown in Figure 1a. Low, combined bands are present in the VIS range, albeit with high variability. Strong absorption bands are shown at 1,440 to 1,460 and 1,932 to 1,960 nm, due to the high water content of samples. Standard deviation of the spectra (Figure 1b) reveals a strong variability along the VIS and NIR range, with 3 double variation peaks around 1,700, 1,900, and 2,300 nm, suggesting a combination of absorption bands in the average spectrum.

The PCA restructured the spectral data into 11 synthetic, uncorrelated variables (PC) that comprised 99.97% of total variation. The first 4 PC explained 98.1% of total variability (PC1: 70.5%, PC2: 21.6%, PC3: 4.4%, and PC4: 1.6%). The patterns of spectral variation, or loadings, explained by the first 4 PC (Figure 2) show several absorption bands: PC1 presented a flat trend and a few negative bands in the VIS and NIR sectors, whereas PC2 showed a positive signal in the early VIS (416–464 nm), a couple of negative bands at around 1,450 nm and 1,950 nm, a weak positive signal at 1,720, and mixed bands from around 2,180 to 2,350 nm. The loading values for PC3 reveal highly negative bands from 400 to 500 nm (VIS) and at 1,940 nm (NIR). It also depicted a broad positive signal at around 1,450, a peak at 1,884, and combined smaller bands thereafter (2,010 to 2,100 nm). Principal component 4 presented a high peak at around 440 and around 500 and 600 nm (VIS range). In the NIR range, a broad signal is present from 1,380 to 1,500 nm, a well-defined band at 1,892 nm, another at 1,980, and a mixed absorption at 2,060 to 2,076 nm.

RID Assay

The IgG content of colostrum samples obtained by RID ranged from 1.7 to 185.4 g/L, with an average of 93.3 g/L and a standard deviation of 37.9 g/L.

Calibration

Calibration statistics for the best equation developed are presented in Table 1. This calibration was performed with the full VIS and NIR segments of the electromagnetic range considering absorption values recorded every 8 data points (16 nm), which resulted in

Figure 1. Visible and near infrared transfectance spectra of colostrum samples. (a) Complete calibration set; (b) average spectrum and standard deviation. R = reflectance.

computation of a total of 252 variables (wavelengths). A second derivative order was performed on the spectral values across a subtraction gap of 16 nm (8 data points) and a smoothing segment of 8 nm (4 data points). The SNV and DT algorithms were also applied. Eight samples with unusual prediction residuals and 1 with an abnormal spectrum were excluded from the calibration in 2 passes of elimination of outliers, resulting in a final inclusion of 148 samples. The prediction equation included 7 partial least squares terms. The selected equation performed slightly better than those obtained without derivatives or with a first derivative treatment.

DISCUSSION

This work evaluated the use of NIRS to assess colostrum quality of Holstein cows by the prediction of IgG content in liquid samples, by the use of folded transmission (transfectance).

The complex nature of the NIR spectra resulted from the multitude of overtone and combination bands produced by fundamental vibrations, in the mid infrared region, of several chemical groups, such as OH, CH, NH, and CO, which can present different configurations in a large group of molecules (Miller, 2001). The strong water absorptions observed at 980 nm and within the ranges of 1,440 to 1,460 and 1,932 to 1,960 nm, were related to different order OH stretch overtones (Osborne et al., 1993; Miller, 2001). Wide variation can be observed in water absorption signals, which is probably due to different solid contents, among them lipids, which are known to cause important baseline shifts in the milk spectrum (Tsenkova et al., 2000) and, by extension, in colostrum. This is suggested in the present study by the standard deviation spectrum (Figure 1b), which shows a high diversity in other absorption bands that are concealed in the raw spectra by the water bands. That seems also to be the case in the range of 1,720 to 1,770 nm and at 2,308 nm, where CH

Table 1. Statistics of reference data and for the best equation obtained¹

Mathematical treatment ²	PLS terms (no.)	Mean (g/L)	Range (g/L)	SD (g/L)	R ²	RMSECV (g/L)	R ² CV	RPD	RER
2-8-4 SNV+DT	7	91.5	171.9	37.6	0.95	9.03	0.94	4.2	19.0

¹PLS = partial least squares; range = difference (highest minus lowest); SD = standard deviation of reference data; RMSECV = root mean square error of cross-validation; R²CV = coefficient of determination of cross-validation; residual predictive deviation (RPD) = SD divided by RMSECV; RER = range error ratio: range divided by RMSECV.
²2-8-4 SNV+DT = derivative order (first digit)-subtraction gap (second digit)-smoothing segment (third digit), plus standard normal variate (SNV) and detrending (DT).

groups associated with lipids absorb, and at around 2,180 nm, where bands related to NH, CH, and CO groups (associated with proteins) are present (Osborne et al., 1993). Colostrum is expected to present complex absorption patterns, because it contains several components, including water, proteins, carbohydrates, lipids, vitamins and minerals of different types and in variable amounts (Kehoe et al., 2007). In addition, important spectral variations are caused by other factors, such as scatter due to fat particles (Norris, 2001), especially if fat content is high. Although no reference data for colostrum fat or solid content were available in this study, samples showed a considerable heterogeneity in appearance, from thin and milky, to thick and creamy, the latter more typical of colostrum. Variability in colostrum composition has been reported in a survey of

55 Holstein dairy herds in Pennsylvania (Kehoe et al., 2007) with wide ranges for solids (18 to 43%), fat (2 to 26%), lactose (1.2 to 5.2%), protein (7 to 22%), and IgG (14 to 94 mg/mL). Loadings obtained from PCA of raw spectra [log (1/R)] show the patterns of variation recovered by each PC (Figure 2). By definition, PC are orthogonal (independent) of each other and because successive components are calculated in the analysis, each accounts for a maximum residual variance in the data, in this case, the spectra (Mark, 2008). Although explaining most of the spectral variation, the first PC is highly affected by scattering effects, and is typically of little use in studying the chemical composition of the product (Robert et al., 1996). In this work, more than 70% of the spectral variability was explained by the first PC

Figure 2. Patterns of variation (loadings) as explained by the principal component (PC) analysis of the visible-near infrared reflectance spectra. Lines show loadings for PC1 (—), PC2 (---), PC3 (— · —), and PC4 (····).

and the slight absorption signals involved were located in bands related to oil (1,412 nm), water (1,420, 1,940 nm), and protein (1,924 nm). This is coherent with the high light scattering in milk and colostrum explained by variations in water content, fat globules, and protein micelles (Šašić and Ozaki, 2001). The second PC loadings show clear bands associated negatively with OH in water (1,420 to 1,460 nm, 1,940 nm) and positively with oil (1,725 nm and 2,300 to 2,340 nm). Loadings of PC3 indicated positive lipid (1,412 to 1,440) and protein (1,460 nm) bands and a negative band for water (1,940 nm). A positive peak at 1,884 nm was also present, which could be attributed to a carboxyl group or to a disulfide bond (Miller, 2001). The same peak was present in the loadings of PC4, along with several other bands related to proteins. This finding is consistent with the fact that antibodies in general, and bovine IgG in particular, are made up of 4 protein chains (2 heavier and 2 lighter) hinged by disulfide bonds (Farrell et al., 2004).

With respect to the VIS range, positive and negative bands were present in loadings of the first 4 PC, and particularly in PC4. These were probably related to different types and content of carotenoids that influence the color of bovine colostrum (Calderón et al., 2007).

Mathematical treatments were applied to spectral data to decrease scatter, enhance bands, reduce band-shifts, and resolve mixed or overlapped absorptions. Figure 3 shows colostrum spectra after a second subtraction (derivative) order plus SNV and DT. Although higher variability can be seen in the VIS compared with the NIR segment, important information appeared in the latter region. As an example, in the encircled portion of Figure 3, a small segment was enhanced between 2,040 and 2,070 nm, where combination bands of N-H bonds associated with proteins are present. An important variation can be observed, representing different protein content in the samples. With a second derivative, absorption bands are shown as a minimum (negative values) at the peak value of the original spectrum (Hruschka, 2001). Therefore, samples with higher protein content should have higher magnitude negative peaks in this sector. Thus, these samples represent those with higher IgG concentrations, as globulins are the main protein fraction in colostrum. Plotting this spectral segment for the 5 samples with the highest and the 5 with the lowest IgG content (Figure 4) demonstrates the relevant differences in absorption; that is, immunoglobulin content.

The IgG content of the colostrum samples was highly variable and some samples had very low values. All samples were taken within 1 h after calving and before the newborn calf could nurse the dam. The very low IgG content of a few samples suggests that some cows

may have experienced leaking of colostrum from the udder before calving. The variability in IgG content of the colostrum samples was desirable for this study, because a good calibration set should have a wide and evenly distributed composition (Murray, 1986).

The statistics of the selected equation showed a high coefficient of determination for the calibration (R^2) with a minor but expected reduction in R^2_{CV} . A slight increase of error from calibration (standard error of calibration = 8.31 g/L) to cross validation (RMSECV = 9.03 g/L) was also expected and did not diminish the usefulness of the equation. Although RMSECV reached slightly more than 0.1 of the average IgG content of colostrum samples, the RPD and RER values allow routine operation of this equation with acceptable confidence. With a standard deviation equal to 4 times and a range of reference data almost 20 times the prediction error (Williams and Sobering, 1996; Williams, 2001), this equation could be used to predict IgG content of colostrum.

The encouraging results found in this study could be attributed in part to the ample variation of the reference data. However, the final equation was obtained without the inclusion of the sample with the highest IgG content (185.4 g/L) because that sample was excluded as a T outlier in the calibration routine. The samples with abnormally low values (IgG content <10 g/kg) were not eliminated and several were well predicted. A new calibration was developed without these samples (data not shown) to explore how relevant these samples were for the quality of the equation obtained. The equation obtained presented RPD and RER values of 3.5 and 17.5, respectively, rendering it useful for prediction purposes. Either equation could be useful in programs to select animals as colostrum donors for pooling purposes or to obtain high quality colostrum.

Fourier-transform NIRS was successfully used by Navrátilová et al. (2006) to develop prediction models for the main components of bovine colostrum, including total solids, fat, and protein contents. They also used transreflectance, which is a fast and easy method to obtain the readings in liquid samples or slurries. The use of a spinning module in a vertical plane for the cells, such as in the present work, is advantageous because rotation prevents fat globules from accumulating in the upper level of the cell. In fixed systems with transmittance or transreflectance analysis of milk, movement of fat globules may cause a significant particle size effect that can affect the readings (Williams, 2001).

If reference values are plotted against NIRS predictions of IgG content obtained with the selected equation (Figure 5), a positive and close relationship can be observed, as values are distributed along and near the equal response ($y = x$) line. This was to be expected,

Figure 3. Second derivative of visible-near infrared reflectance spectra; the segment from 2,028 to 2,081 nm is enhanced.

Figure 4. Second derivative of near infrared reflectance spectral segment (2,028 to 2,081 nm) of 5 colostrum samples with high IgG content and 5 with low IgG content.

Figure 5. Near infrared reflectance spectroscopy (NIRS) predicted versus reference (radial immunodiffusion) values for IgG content (g/L) in bovine colostrum.

given the high proportion of the variation explained (R^2CV) by the MPLS model. It is also apparent that the samples presented a wide and even distribution along the axis, although the model could probably be improved if more samples were included in the range of 40 to 60 g/L. Some samples in the lowest range of IgG content were slightly overestimated by the model, but, from a practical point of view, those colostrum samples would still be predicted as poor quality by the model.

CONCLUSIONS

Folded transmission (transflectance) is a fast and suitable technique to scan liquid colostrum samples. The VIS-NIR spectra can be used to develop regression models capable of obtaining reliable predictions of the content of IgG, and therefore to assess the passive immunity value of bovine colostrum. Modified partial least squares regression, along with a suitable mathematical treatment of the spectra, can be used to obtain such prediction models.

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