Automatic determination of protein fractions in manchega ewe's milk by near infrared reflectance spectroscopy

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Introduction

Ewes are milked in large numbers in Europe and in the Mediterranean region to produce milk which is largely used for manufacturing cheese and other dairy products. The fat and protein content of ewe's milk is of interest because it affects both the characteristics and quality of dairy products. Factors such as season, breed, stage of lactation, climate and feeding practices can influence the composition of ewe's milk.[1]

In many countries, the ewe's milk payment system is still based on milk weight and only in a few cases on fat content. As the importance of milk protein continues to increase, it will be necessary to change to a multiple-component and bacteriological quality pricing system. This system should include payments that reflect differences in the protein content of milk.

The reference method for milk protein determination is the Kjeldahl method. For many years, the dairy industry has measured the total nitrogen (TN) content in milk which includes both protein nitrogen and non-protein nitrogen (NPN). The determination of protein nitrogen and casein nitrogen eliminates the question of inaccurancies caused by NPN variability.[2]

The Kjeldahl method is time-consuming and requires considerable skill, without resulting in useful routine quality control. Because of this, a large number of comercial automatic instruments for the determination of milk nitrogen fractions have been developed. Also, the International Dairy Federation introduced the IDF 20B[3] standard to unify criteria in the utilization of this equipment.

In recent years, analysis based on physical measurements for the simultaneous determination of several constituents has replaced the chemical methods. Near infrared (NIR) reflectance spectroscopy is a widely used method in various fields, especially in the food industry, because it is rapid, chemically non-destructive and sample preparation is very easy.[4] This technique needs a calibration step for each constituent with samples tested by the chemical reference methods.

Many calibrations and checking systems have been described for the cow's milk,[5–7] but only a few references have been found for the application of NIR in the analysis of ewe's milk, especially for true protein and casein.

The objetive of this study was to identify wavelengths in the NIR region of the spectrum related to the chemical composition of ewe's milk. To achieve this, a NIR calibration for nitrogen fractions (protein and casein) using IDF 20B standard as a reference method has been developed and evaluated.

Materials and methods

Sample collection

A total of 150 milk samples were taken from the experimental Manchega ewe's flock at Polytechnic University of Valencia, Spain. Samples came from single animals at different days and milking phases to obtain a wide range of values for each protein fraction.

Calibration of crude and true protein was carried out using 110 samples and caseins with 86 samples. For the validation step, 40 samples were chosen from the total sample collection. Samples in the validation set were not used in the calibration set or *vice versa*.

Chemical analysis

TN and NPN fractions were determinated in duplicate by the Kjeldahl method according to IDF 20B:1993 standard (sections 3 and 4) and non-casein nitrogen (NCN) fraction by the Kjeldahl procedure described in AOAC.[8] A semi-automatic analyzer (Tecator) equipped with a digestion unit (Digestion System 1015 Digester) and a distillation unit (Kjeltec Auto 1030 Analyzer) was used.

The nitrogen components were: total or crude nitrogen = TN; true protein nitrogen = TN – NPN, and casein nitrogen = TN – NCN. Protein equivalents were calculated by multipling the nitrogen content by the factor 6.38.

NIR reflectance measurement

The NIR analysis was carried out on a Technicon InfraAlyzer 400D. The analysis is based on the measurement of the light reflected by the sample, in the near infrared region, at 19 wavelenghts ranging between 1000 and 2700 nm.

Samples, after being heated to 40ºC, were put into the InfraAlyzer 400D in order to read the logarithms of reflectances [log(1/*R*)] at 19 wavelengths. These logarithms are used as empirical regression coefficients (F-values). All determinations were done in duplicate.

Regression analysis

The InfraAlyzer 400D was calibrated for crude protein, true protein and casein in ewe's milk taking true N values for the contents obtained by the reference method.

The scanning, mathematical processing and statistical analysis was performed by a IACAL P01 program (Bran+Luebbe) with the help of a IBM PC (model 80286).

The best equation for each constituent was chosen by the optimal combination of the statistics from equation development: high *R* (multiple-correlation coefficient), low standard error of calibration (*SEC*) and high F-values in the calibration set. Each equation selected for a given protein fraction was subsequently used to predict the composition of validation set samples. Finally, predicted values were correlated to laboratory analysis data by simple linear regression.

Results and discussion

Sample composition

Table 1 shows the characteristics of the sample sets used in the study determined by the reference methods. All protein fractions varied over a wide range in order to establish robust calibration equations.

The protein contents of samples had mean values similar to those reported by Juarez *et al.*[9] and Molina[10] for Manchega ewe's milk. The range and mean values of the samples for calibration and validation were very close.

Table 1. Contents of protein fractions in the ewe's milk samples set obtained by the reference method.

Component (g/100g)	Calibration			Validation		
	Range	Mean	*SD*[a]	Range	Mean	*SD*[a]
Crude protein	3.47–6.02	5.07	0.51	3.61–6.01	5.08	0.59
True protein	3.34–5.85	4.76	0.52	3.52–5.80	4.74	0.57
Casein	2.65–4.72	3.75	0.43	2.68–4.66	3.77	0.52

[a]Standard deviation.

NIR calibration and validation

Wavelength selection

Table 2 contains the NIR results for calibration, together with the selected wavelengths. Wavelengths around 1700–1900 nm are used frequently for moisture determination of agrofood products, probably due to the strong relationship between water and other chemical components in living systems, while the 2100–2300 nm region is used frequently for protein determinations (Figure 1).

As can be seen, the statistics of the chosen equation for crude protein were satisfactory (R = 0.986 and SEC = 0.089). De Vilder and Bossuyt[11] obtained worse values (R = 0.96 and SEC = 0.18) in cow's milk using the same NIR equipment; this could be due to the lower number of samples used (n = 37). In spite of the scarce reports about true protein and casein calibrations in ewe's milk, the statistics values obtained can be considered very acceptable from a statistical point of view for these components.

Table 3 illustrates mean values and repeatibility obtained by Kjeldahl and NIR methods. Mean values are very similar in both, whereas repeatibility is better using NIR, which means that in this method the experimental error is lower.[12]

Prediction of chemical composition

Validation statistics from linear regression analysis comparing the results of chemical analysis with those predicted from NIR analysis are shown in Table 4. The correlation coefficients obtained for crude protein, true protein and casein were 0.982, 0.980 and 0.965 respectively, showing a good relationship between the values obtained by both methods.

Table 2. Calibration statistics and wavelengths used to predict the protein fractions content in ewe's milk.

Component	No. of samples	R[a]	SEC[b]	Selected wavelengths (nm)
Crude protein	110	0.986	0.089	1759, 1982, 2100, 2180
True protein	110	0.984	0.094	1734, 1759, 2139, 2180, 2336
Casein	86	0.968	0.111	1445, 1734, 1778, 2230, 2336

[a]Coefficient of multiple correlation.
[b]Standard error of calibration.

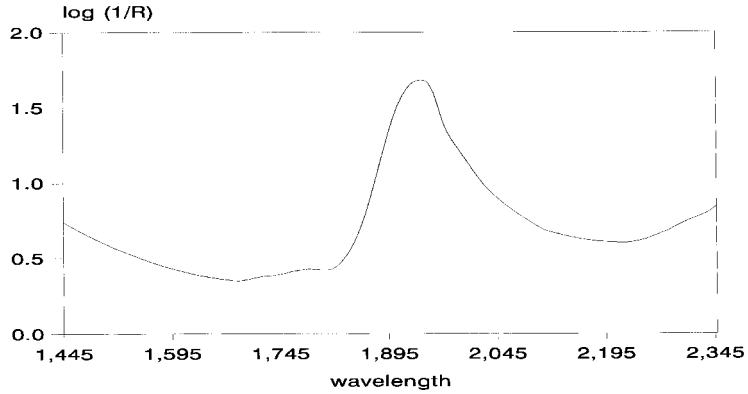

Figure 1. NIR spectrum of ewe's milk at protein wavelengths.

Table 3. Mean values and repeatibility of reference and NIR analysis for protein fractions in ewe's milk.

	Kjeldahl (%)			NIR (%)		
	Crude protein	True protein	Casein	Crude protein	True protein	Casein
Mean	5.071	4.761	3.752	5.069	4.757	3.754
Repeatibility[a]	0.070	0.042	0.087	0.029	0.030	0.036

[a]Repeatibility is expressed as the standard deviation of the difference between duplicates.

For all parameters, the standard error of prediction (*SEP*) was higher than the standard error of calibration (*SEC*). This may be explained by the fact that *SEP* includes the error associated with the Kjeldahl analysis and the error associated with the NIR equipment.[13] However, *SEP* did not exceed *SEC* by 33% (Table 2), criterion suggested by Shenk *et al.*[14]

The standard error, slope (1/skew) and mean bias values of selected equations indicated that ewe's milk samples were estimated acceptably, since the relationships between chemically determined and NIR predicted values are practically linear (Figure 2).

Conclusions

Based on the results, it can be indicated that NIR is effective for the prediction of protein fraction contents in ewe's milk. The results obtained with the prediction equations show a good relationship with the reference values when they are applied to unknown samples. Furthermore, in the automatic method, NIR accuracy and repeatibility are higher than those showed by reference methods.

NIR allows a rapid, simple and simultaneous determination of different components of great economic importance in ewe's milk such as protein (crude protein, true protein and casein), fat, lactose etc. Also, NIR has the advantage that no chemicals are used and no pretreatment of samples is needed.

Table 4. Statistical parameters obtained in the validation of the calibration.

Component	R^a	SEP^b	Bias[c]	Skew[d]
Crude protein	0.982	0.112	0.039	0.995
True protein	0.980	0.115	–0.009	0.992
Casein	0.965	0.133	0.076	0.964

[a]Coeffficient of multiple correlation.
[b]Standard error of prediction.
[c]Difference between reference and NIR values.
[d]Slope inverse.

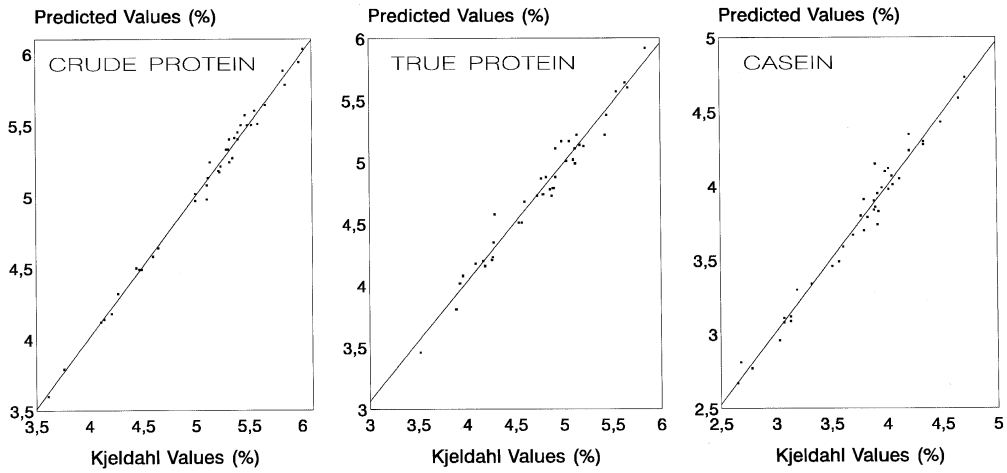

Figure 2. Relationship between protein fractions content and NIR predicted values.

References

1. P. Molina and L. Gallego, "Composición de la Leche: Factores de Variación", in *Ganado Ovino, R. Manchega.* Mundi-Prensa, Madrid, Spain, pp. 191–208 (1994).
2. D.B. Emmons and A.F. Kertz, *J. Dairy Sci.* **75,** 3191 (1992).
3. International Dairy Federation, IDF 20B:1993 standard. *Determination de la Teneur en Azote.* Secrétariat Général FIL, Bruxelles (1993).
4. B.G. Osborne and T. Fearn, *Near Infrared Spectroscopy in Food Analysis*. Longman Scientific & Technical, Harlow, UK, pp. 117–161 (1986).
5. T. Sato, M. Yoshimo, S. Furukama, Y. Someya, N. Yano, J. Vozumi and M. Iwamoto, *Japan J. of Zootech. Sci.* **58,** 698 (1987).
6. P. Robert, D. Bertrand and M.F. Devaux, *Anal. Chem.* **59,** 2187 (1987).
7. H. Kamishikiryo-Yamashita, Y. Oritani, H. Takamura and T. Matoba, *J. Food Sci.* **59,** 313 (1994).

8. AOAC,*Official Methods of Analysis*, 15th Edn. Association of Official Analytical Chemists, Arlington, VA (1990).
9. M. Juarez, M. Ramos, A. Goicoechea and S. Jimenez-Perez, *Chem. Mikrobiol. Technol. Lebeusm.* **8,** 143 (1984).
10. P. Molina, *Composicion y Factores de Variación de la Leche de Oveja de Raza Manchega.* Tesis doctoral (Ph.D.). Polytechnic University, Valencia, Spain (1987).
11. J. De Vilder and R. Bossuyt, *Milchwissenchaft* **38,** 65 (1983).
12. D.M. Barbano and J.M. Lynch, *J. Dairy Sci.* **75,** 3210 (1992).
13. J.L. Holechek, J.S. Shenk, M. Vavra and D. Arthum, *J. Anim. Sci.* **55,** 971 (1982).
14. J.S. Shenk, I. Landa, M.R. Hoover and M.O. Westerhaus, *Crop. Sci.* **21,** 355 (1981).