Rapid determination of main components in rabbit's milk by near infrared spectroscopy

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Introduction

In the hyperprolific rabbit lines developed in the last years, pups show low viability during their first days of life due to their deficient thermoregulation or to an insufficient energy intake.[1] Throughout this period, milk yield of does determines the viability and growth of pups, since they drink almost only maternal milk during their first 20 days. In the last two decades, the main studies of rabbit doe nutrition have been devoted to improving the value or the yield of rabbit's milk by the use of high-energy diets. In fact, all authors have showed that an increase in dietary energy (especially through fat addition) clearly stimulates the energy milk yield of reproductive rabbit does, inducing a higher litter weight gain and survival.[1–4] In connection with these facts, the determination of rabbit milk composition, in addition to the assessment of milk output, is necessary to study the specific effect of nutrition on the performance of lactating rabbit does.

However, to determine the chemical components of rabbit milk by using current analytical methods requires an amount of milk (40 ml, approximately) not always available in this specie, especially during the first and last days of lactation. Besides that, they are laborious and time-consuming.

Near infrared (NIR) reflectance spectroscopy is a widely used method in various fields, especially in the food industry, because it is rapid, accurate, chemically non-destructive and sample preparation is very easy.[5] This technique needs a calibration step for each constituent, with samples tested by the chemical reference methods.

The development of automated infrared instruments for the rapid determination of milk components has madeit possible to analyse milk samples from a large number of animals. Many calibrations and checking systems have been described elsewhere for the milk of species like cows[6–8] and ewes,[9] but no reference for the application of NIR in the analysis of rabbit's milk has been found.

An homogeniser, designed to decrease the milk fat globule diameter, is usually supplied with the commercial equipment, but the amount of sample required (40–50 ml for a simple analysis), can hardly ever be obtained from any doe. Recently, a new aluminium cup (British cup) has been developed which allows a simple and fast prediction of main components of a viscous fluid with a small amount of sample (200–300 µl).

The object of the present work was to identify wavelengths in the NIR region of the spectrum related to the chemical composition of rabbit milk. To achieve this, an NIR calibration for the main components of rabbit milk (dry matter, ash, protein, fat and energy), has been developed and evaluated, using, as a reference, the official analytical methods.

Material and methods

Sample collection

Twenty-eight New-Zealand × Californian rabbit does, giving a total of 62 lactations, were used to obtain 184 samples on different days of the lactation period. Litters were standardised to eight pups at birth and they were kept constant throughout lactation. Pups were separated from their mother to prevent suckling for a period of 24 h before sample collection in the morning. Each does was injected intravenously with 5 IU oxytocin to enhance maximum contraction of myoepithelial cells and milk was collected manually by gently massaging the mammary gland. The milk sample (30 to 40 ml per doe) were obtained from all mammary glands. Samples were taken on 7th, 21st and 28th days of lactation in order to obtain a wide range of values.

All does were kept under the same managerial conditions and were presented to the male 14 days after parturition. They were housed in individual cages, provided with feeders, automatic nipple drinkers and nest boxes.

A cross-validation method was used to validate the regression models. This method splits the total sample collection (184) into two sets: (a) a calibration set, that was used to develop the multilinear model (133 samples of milk were used for this set); and (b) a validation set, that was used to validate the model (51 samples). Samples in the validation set were not used in the calibration set or *vice versa.*

Chemical analysis

Milk samples were analysed for dry matter, ash, protein, fat and energy. Dry matter and ash contents of milk were obtained using the methods of the AOAC.[10] Milk protein content was calculated by the Kjeldahl method according to the FIL Standard: 20B.[11] Milk fat content was determined using the Gerber method according to the British Standards Institution.[12] An adiabatic bomb calorimetry was used to determine the gross energy content of the milk, which was previously freeze-dried.

NIR reflectance measurement

The NIR analysis was carried out in 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 wavelengths ranging between 1000 and 2700 nm.

Samples were heated to 40ºC and 200–300 µl of each homogenised sample was placed on the aluminium cup (British cup). Afterwards, samples were allocated into the InfraAlyzer 400D in order to read the logarithms of reflectance [$\log(1/R)$] at 19 wavelengths. These logarithms were used as empirical regression coefficients (*F*-values). All determinations were done in duplicate.

Regression analysis

The InfraAlyzer 400D was calibrated for dry matter, ash, protein, fat and energy in rabbit's milk with the values obtained from the reference analytical methods. The scanning, mathematical processing and statistical analysis was performed by a SESAME v. 3.01 program[13] (Bran+Luebbe) with the help of a personal computer (Pentium/233 MHz IBM-compatible). Calibrations for the main constituents of rabbit milk were carried out by stepwise multiple linear regression (MLR) analysis with the chemical data and NIR data.

The best equation for each constituent was chosen by the optimal combination of the statistical parameters from the equation development: high R^2 (multiple correlation coefficient), low standard error of calibration (*SEC*), high *t*-student values for the coefficients (*t*-values) and high *F*-values in the calibration set. Each equation for a given milk rabbit constituent was subsequently used to predict the composition of validation set samples and standard error of prediction (*SEP*) was also used to choose

Table 1. Means, standard deviations, range and coefficients of variation for rabbit milk measurements.

	N	Mean	SD	Minimum	Maximum	*CV*
Calibration set						
Dry matter (%)	133	34.97	4.8588	26.80	49.75	13.89
Ash (%)	133	2.061	0.3909	0.93	3.49	18.97
Fat (%)	133	18.19	3.5418	11.80	27.80	19.47
Protein (%)	133	12.87	1.6114	9.62	18.45	12.52
Energy (MJ kg^{-1})	133	10.02	1.7239	6.66	14.84	17.20
Validation set						
Dry matter (%)	51	36.56	5.2967	27.0	48.38	14.49
Ash (%)	51	2.192	0.4264	1.44	3.40	19.45
Fat (%)	51	19.13	3.3143	13.2	27.0	17.33
Protein (%)	51	13.33	2.0881	9.84	18.15	15.66
Energy (MJ kg^{-1})	51	10.59	1.7342	7.46	14.25	16.38

SD: standard deviation,
CV: coefficient of variation.

Table 2. Wavelengths, t-student values, determination coefficients and standard errors of calibration and prediction for the different multilinear regression methods developed for the determination of protein, fat and energy contents in the milk of rabbit does (*n* = 103).

	NF	*1734*	*1759*	*1778*	*1818*	*2100*	*2139*	*2180*	*2230*	*2270*	*2310*	*2336*	R^2	*SEC*	*SEP*
Protein	4	38.9	–38.5	—	—	—	—	—	—	30.7	–31.5	—	0.959	0.471	0.528
	5	12.8	—	—	–16.3	—	–4.4	—	—	22.5	–32.7	—	0.960	0.462	0.522
	6	—	—	—	—	4.3	–5.3	13.8	–11.4	10.6	–8.4	—	0.962	0.454	0.500
Fat	4	—	—	—	—	–20.3	—	13.17	—	–9.29	8.12	—	0.880	1.515	1.674
	5	–8.35	—	10.09	–10.5	—	–14.1	17.4	—	—	—	—	0.890	1.428	1.995
	6	—	—	10.7	–10.3	—	–10.6	10.9	—	–7.2	—	6.3	0.903	1.376	1.829
Energy	4	—	—	—	—	—	–24.5	17.7	—	–9.1	8.8	—	0.923	0.638	0.729
	5	—	—	—	—	—	–12.4	18.1	3.0	–7.4	8.4	—	0.926	0.626	0.719
	6	—	8.7	—	–8.2	—	–13.4	14.0	—	–8.9	—	7.7	0.932	0.602	0.649

NF: number of filters used in the method (4, 5 or 6)
R: multiple correlation coefficient
SEC: standard error of calibration
SEP: standard error of prediction

the best combination of wavelengths for each constituent. Finally, predicted values were correlated to laboratory analysis data by a simple linear regression.

Results and discussion

Sample composition

The main descriptive characteristics of the sample set used in the present work are listed in Table 1. These data show that all constituents varied over a wide range in order to establish robust calibration equations. As expressed by the coefficient of variation (*CV*) values in this table, there was a large variation in the milk fat content (*CV* = 19.5%) in the calibration set. The range obtained for the milk constituents so far analysed seems to include the values found in the literature for milk of rabbit does during all lactation.

Mean values obtained for each constituent were similar to those reported by Fraga *et al.*[1] and Pascual *et al.*[4] for New Zealand × Californian rabbit milk. Rabbit milk is characterised by a high fat content (18–19%) that has been related by some authors[14] to the daily interval between two consecutive suckling periods. The range, *CV* and mean values of the samples for calibration and validation were very close.

NIR calibration and validation

Data in Table 2 show the results obtained for the MLR analyses of the main constituents (protein and fat) and energy of rabbit milk. Wavelengths selected (4, 5 or 6 filters) , *t*-student values of the coefficients, R^2, *SEC* and *SEP* were used to determine the best method for prediction of the protein, fat and energy contents in the milk of rabbit does.

The statistical parameters found for the determination of milk protein and fat contents with only four wavelengths were not significantly improved, even *SEP* for milk fat prediction worsened, when one or two other wavelengths were included in the method. Furthermore, these equations with four filters use the typical wavelengths for the protein and fat determinations (1734, 1759, 2310 nm). Wavelengths around 1700–1900 nm are used frequently for moisture determination of agricultural products, probably due to the close relationship between water and other chemical constituents in living systems,[15] while the 2100–2300 nm region is frequently used for protein and fat determinations (Figure 1). On the other hand, the best equation for the milk energy prediction seems to be the six-filter equation when R^2, *SEC* and *SEP* were considered. The energy content of rabbit milk depends on many factors like protein, fat and lactose content, which could be the reason of the higher number of filters needed for its accurate prediction.

Table 3 contains the total NIR results for calibration and validation, together with the selected wavelengths. The multiple regression coefficients of the chosen equation for milk protein, dry matter, energy and fat were satisfactory (0.96, 0.95, 0.93 and 0.89, respectively), but for milk ash was lower (0.76). The standard errors showed that the calibration sets were far higher (0.230–1.582) than those found for other species[15,16] (cow and sheep) using this same NIR equipment. However, they were comparable to the standard error of laboratory (*SEL*) obtained in the present work for the chemical analysis of the milk constituents. These high *SEL* seem to be related to problems on fat homogeneity, that

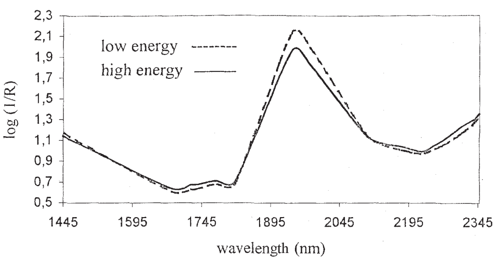

Figure 1. NIR log 1/*R* spectra of two rabbit's milk samples with different milk energy content.

Table 3. Calibration (n = 133) and validation (n = 51) statistics and wavelengths selected to predict the chemical composition of rabbit's milk.

Component	R^2	*SEL*	*SEC*	*SEP*	Selected wavelegths (nm)
Dry matter	0.947	1.105	1.582	1.743	1778, 1818, 2139, 2180, 2270, 2336
Ash	0.763	0.137	0.230	0.303	1680, 1722, 1759, 1778, 2139, 2190
Protein	0.959	0.188	0.471	0.528	1734, 1759, 2270, 2310
Fat	0.889	1.027	1.376	1.829	2100, 2180, 2270, 2310
Energy	0.932	0.260	0.602	0.649	1759, 1818, 2139, 2180, 2270, 2336

R: multiple correlation coefficient
SEL: standard error of laboratory
SEC: standard error of calibration
SEP: standard error of prediction

could be due to the high fat content of rabbit milk samples. There are no reports about NIR calibrations in rabbit milk, but taking into account that the main object of the present work was obtain a rapid method for determining the milk energy content for the evaluation of rabbit diets, the statistical values obtained could be considered acceptable from a statistical point of view.

Figure 2 shows the relationship between NIR predicted and values determined in the laboratory for the validation set. The simple correlation coefficients obtained for fat, protein, dry matter and energy were 0.84, 0.95, 0.91 and 0.91 respectively, showing a high relationship between the values obtained by both methods. The *SEP* was higher than the *SEC* for all components, but *SEP* did not exceed *SEC* by

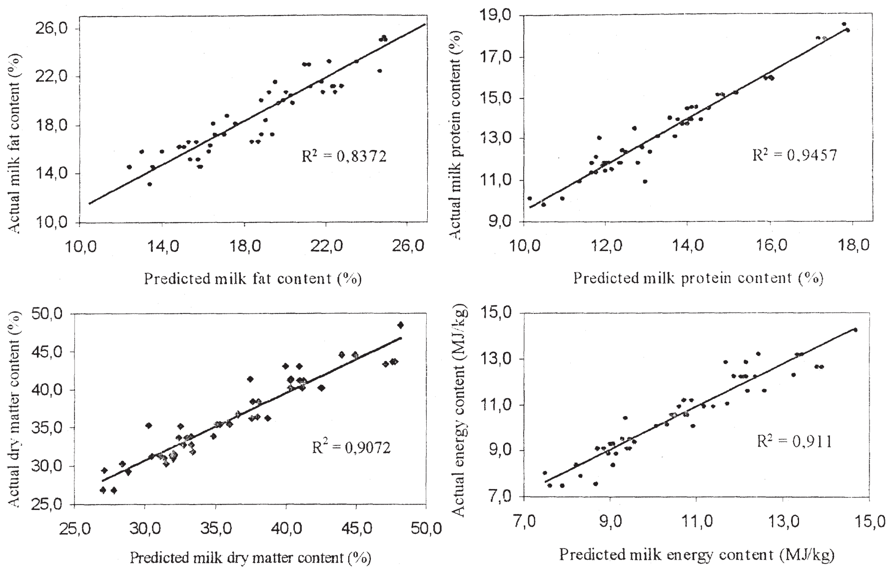

Figure 2. Relationship between NIR predicted and laboratory determined values of milk fat, protein, dry matter and energy content by IA-400D.

33% (only for ash prediction), a criterion suggested by Shenk *et al.*[17] The fact that *SEP* includes the error associated with the chemical analysis and the error associated to the NIR equipment,[18] could explain their higher values.

Based on these results, it could be concluded that NIR is an effective method for the prediction of dry matter, protein, fat and energy contents in rabbit milk, that could mean a substantial improvement in the methodology used in experimental research on lactating rabbit does. The prediction equations are robust and the results obtained from them show an adequate correspondence to the reference values when they are applied to unknown samples. In conclusion, NIR seems to allow a rapid, simple and accurate prediction of the different constituents of rabbit milk, from quite a small amount of sample (200 µl). However, further research is needed on developing a suitable method for predicting the chemical composition of rabbit milk.

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