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USE OF FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY TO PREDICT VFA AND AMMONIA FROM *IN VITRO* RUMEN FERMENTATION

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SUMMARY

The aim of the present study was to develop a FTIR method to quantify amounts and proportions of volatile fatty acids (VFA) and ammonia nitrogen (N-NH_3) in fermentation fluids collected in vitro using innovative Bayesian models as chemometric technique. A set of 170 fluids, collected before and after 4 in vitro incubations of 8 diets in 5 replication plus 5 blanks, were analysed for VFA, N-NH_3 and scanned using the MilcoScan FT2 (Foss Electric, Hillerød, Denmark) in the spectral range between 5000 and 900 cm^{-1}. A Bayes B model was used to calibrate equations for each fermentative trait. The calibration equation predicts well VFA and N-NH_3 amounts in calibration and also in validation (R^2_{VAL} ranged from 0.93 to 0.83 for iso-valeric and n-butyric acid, respectively). However, the prediction of VFA expressed as proportions of total amount was much less accurate (R^2_{VAL} ranged from 0.81 to 0.52 for iso-valeric and n-butyric acid, respectively). In conclusion, FTIR and Bayesian models can be used as tools to accurately predict VFA amounts in vitro.

Key-words: mid infrared spectroscopy; in vitro rumen fermentation; Bayesian regression model

INTRODUCTION

Volatile fatty acids (VFAs) and ammonia nitrogen (N-NH_3) are the mean products of microbial fermentation and their production reflect the diet degradation in the rumen (Tagliapietra et al., 2011). The Fourier Transform Infrared (FTIR) spectroscopy has been applied in many different fields because it is simple, rapid, economic and doesn't require sample pre-treatments. For these reasons, FTIR can be a useful tool to evaluate fermentative parameters of samples collected both in *in vitro* and *in vivo* (Bhagwat et al., 2012). In our knowledge, few attempts have been done to predict VFAs expressed as proportions of total VFA amount with FTIR (Udén and Sjaunja, 2009). Different statistical approaches were used to calibrate FTIR equipment like the Partial Least Square Regression (PLS) being a popular multivariate calibration technique used to analyse spectra data. Recently, Ferragina et al. (2015) compared the traditional PLS approach with diverse Bayesian regression models, commonly used for genomic selection, founding the "Bayes B" model as a powerful predictor of milk properties. Therefore, the present work aims to develop a FTIR method to quantity amounts and proportions of VFA and N-NH_3 in fermentation fluids collected *in vitro* using the Bayes B regression model.

MATERIAL AND METHODS

Data of 4 *in vitro* incubations were used to calibrate the FTIR equipment. Two incubations were stopped at 24 h, whereas the other two lasted for 48 h. In each incubation 8 diets were tested in 5 replication plus 5 blanks, where the rumen fluid (RF) was incubated without any substrate. At the beginning of the incubation 3 samples of RF, of buffer and of their mix were also collected. A total number of 54 samples per incubation were collected and stored for chemical and FTIR analysis. The tested diets were formulated for lactating cows and differed for fibre, crude protein, lipids and starch

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content to be representative of Italian intensive dairy system (Dal Maso et al., 2009). Of each test diet, 1 g of sample was incubated with 100 mL of buffer (Menke and Steingass, 1988) and 50 mL of RF collected from 3 dry cows as described by Tagliapietra et al. (2012). The fermentations were monitored using a fully automatic gas production system described by Tagliapietra et al. (2010). RF, buffer and buffered RF at the beginning of incubation and fermentation fluid at the end of incubation were sampled for the immediate infrared (IR) analysis and others two aliquots were stored at −20°C with metaphosphoric acid (25%, w/v). The N-NH3 content was measured using a FIAstar 5000 analyzer FOSS (FOSS Analytical, Hilleroed, Denmark) following the internal procedure (Method Cassette Ammonium). The VFA profile was determined by GC with flame ionization detection (7820A GC system, Agilent Technologies, Milan, Italy) using a 30-m stainless steel column (J&W DB-FFAP, Agilent Technologies, Milan, Italy) and H_2 as a carrier gas (flow rate: 30 mL/min; isothermal oven temperature: 150°C). The Fourier transform equipment, designed for milk analysis (MilcoScan FT2, Foss Electric A/S, Hillerod, Denmark), was used for scanning the fresh samples within 3 h from collection over the spectral range of 5000 to 900 waves $\times$ cm^{-1}. Two spectral acquisitions were carried out for each sample and the results were averaged before data analysis. For technical reasons, for 1 incubation, 3 of 5 sample replications and also the samples collected at the beginning of incubation were not scanned. The Mahalanobis distances were used for the detection of spectra outliers, and those showing a distance higher than three times the standard deviation were discarded. Data editing was done in R environment (R Core Team, 2013). Finally 170 spectra were available for the study. A Bayesian model (Bayes B), implemented in R-software BGLR (Pérez and De Los Campos, 2014), was used to calibrate equations for each fermentative traits as recently described by Ferragina et al. (2015). The calibration was performed on a random dataset of 80% of data values and the remained 20% of values were used in validation. This guarantees that calibration and validation sets were independent. The procedure was repeated 20 times for each trait. As index of prediction accuracy of calibration was used the determination coefficient was calculated as square of the correlation between observed and predicted values in the calibration dataset (R^2_{CAL}). Similarly, the R^2 of validation was computed as square of the correlation between the observed and predicted values in validation dataset (R^2_{VAL}).

RESULTS AND DISCUSSION

The mean values and the standard deviation of the fermentative parameters used in the study are given in Table 1. On the average the concentration of total VFA in fermentation fluid was about 3.06 g/L with a large variability (SD $\pm$1.06 g/L) reflecting the different degradability of diets incubated. Also the inclusion in the calibration set of samples were collected at the beginning of incubation: RF (on the average 3.77 g/L of total VFA), buffer (without VFA) and buffered RF (1.39 g/L of total VFA). The variability of VFA parameters expressed as a proportion of total VFA was much lower. The SD of VFA proportions expressed as percentage of mean values ranged from 6.2% to 22.3% respectively for acetic acid and N-valeric acid. Also the $N\text{-}NH_3$ concentration in the calibration set showed a large variability both for different CP content of fermented diets and the inclusion in the data set of RF (74 mg/L), buffer (172 mg/L) and buffered RF (132 mg/L). Finally, the pH was on the average close to 6.8 and rather stable among samples for the high concentration of bicarbonate in the medium and for the ammonia produced throughout the fermentations. Therefore, except for pH, the variability of measurements was comparable to the reported by previous studies (Udén and Sjaunja, 2009) and allows the development of robust calibrations. The sample spectra were homogeneous but some outliers were identified probably for the presence of small particles in suspension that could interfere with the instrument sensors designed for milk analysis.

The coefficients of determination between the measured and predicted values obtained in calibration (R^2_{CAL}) and validation (R^2_{VAL}) datasets are given in Table 1. The amounts of VFAs were accurately predicted as shown by the R^2 that on the average ranged between 0.97 to 0.93 of respectively acetic acid and n-butyric acid. Moreover, the individual calibration, repeated 20 times, always exceeded 0.90 R^2_{CAL}. In validation the performance of prediction remain high with mean R^2_{VAL} values always greater than 0.90 with the only exception of n-butyric acid (R^2=0.84). Also the values of $RMSE_{VAL}$, suggest an expected error analysing an external sample of about 0.15, 0.08 and 0.06 g/L for acetic acid, propionic acid and n-butyric acid respectively. Udén and Sjaunja (2009) reported comparable performance of calibration working with semi artificial rumen fluids, obtained removing the VFA naturally present in the samples by acidification and adding defined amounts of acetic, propionic, n-butyric acid and bicarbonate.

Table 1. Statistics of samples used for the calibration and prediction R-squared in calibration (R^2_{CAL}) and validation (R^2_{VAL}) and root mean square error in validation ($RMSE_{VAL}$)

	Mean	SD	R^2_{CAL}			R^2_{VAL}			$RMSE_{VAL}$
			Mean	Max	Min	Mean	Max	Min	Mean
VFA amounts, g/L									
- Acetic acid	1.76	0.60	0.97	0.98	0.95	0.92	0.97	0.83	0.15
- Propionic acid	0.71	0.26	0.96	0.96	0.95	0.90	0.96	0.76	0.08
- Iso-butyric acid	0.04	0.01	0.96	0.97	0.95	0.91	0.97	0.83	0.00
- N-butyric acid	0.43	0.17	0.93	0.94	0.91	0.84	0.91	0.65	0.06
- Iso-valeric acid	0.07	0.03	0.97	0.98	0.97	0.93	0.98	0.87	0.01
- N-valeric acid	0.06	0.02	0.96	0.97	0.95	0.91	0.96	0.81	0.01
Total VFA	3.06	1.06	0.97	0.98	0.97	0.93	0.97	0.85	0.26
VFA proportion, g/100 g									
- Acetic acid	58.1	3.60	0.87	0.91	0.85	0.64	0.80	0.43	2.1
- Propionic acid	22.9	2.27	0.81	0.87	0.76	0.55	0.82	0.36	1.5
- Iso-butyric acid	1.2	0.19	0.87	0.89	0.84	0.72	0.84	0.55	0.1
- N-butyric acid	13.7	1.92	0.69	0.75	0.65	0.52	0.75	0.36	1.4
- Iso-valeric acid	2.4	0.51	0.92	0.93	0.90	0.81	0.92	0.70	0.2
- N-valeric acid	1.8	0.39	0.92	0.95	0.90	0.77	0.89	0.55	0.2
Ammonia nitrogen, mg/L	176	47.3	0.86	0.89	0.84	0.73	0.86	0.50	23
pH	6.85	0.16	0.54	0.59	0.47	0.39	0.50	0.30	0.12

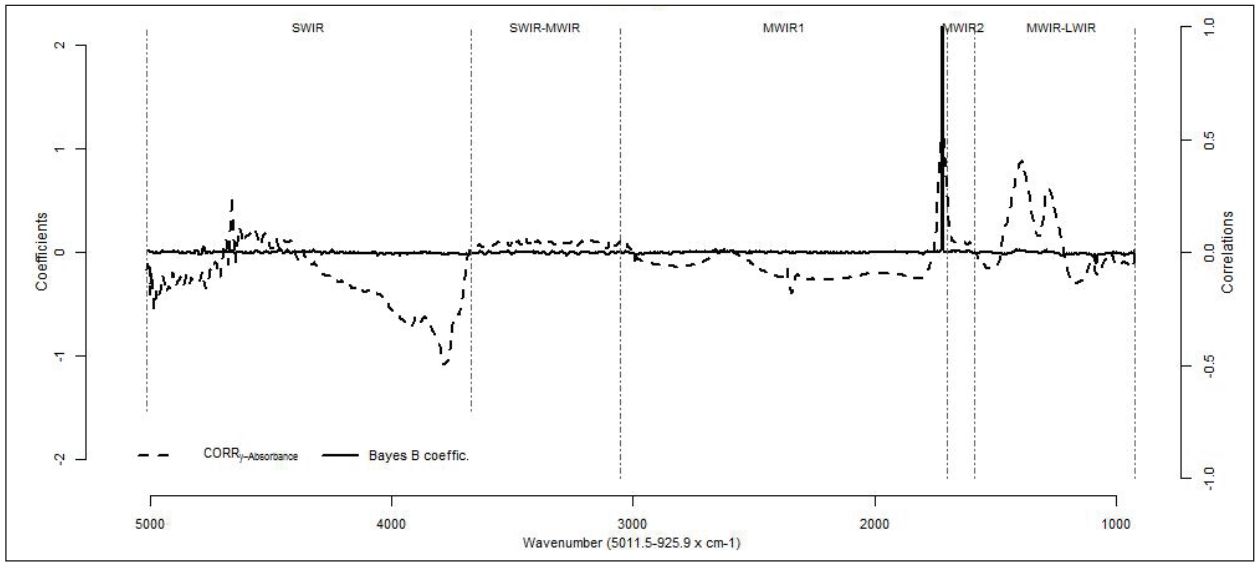
R^2_{CAL}= coefficient of determination calculated as the square of the correlation between observed and predicted values in calibration (80% of entire data set); Mean= mean of the R^2 of 20 replicas; Min= minimum value of R^2 obtained in 20 replicas; Max= maximum value of R^2 obtained in 20 replicas; R^2_{VAL}= coefficient of determination calculated as the square of the correlation between observed and predicted values in validation (20% of entire data set); Mean= mean of the R^2 of 20 replicas; Min= minimum value of R^2 obtained in 20 replicas; Max= maximum value of R^2 obtained in 20 replicas; $RMSE_{VAL}$= mean of the root mean square errors in validation of 20 replicas

The prediction accuracy of VFA values, expressed as proportion of total amounts, was acceptable in calibration with R^2 that ranged between 0.92 to 0.69 for valeric acid and n-butyric acid respectively. However, the correlation between measured and predicted values decreased in validation but with large differences among VFAs. The ability of the model to predict the $N\text{-}NH_3$ in fermentation fluids was slightly lower compared to VFA amounts as evidenced by the lower R^2_{VAL} and the higher $RMSE_{VAL}$.

In Figure 1 the correlation coefficients between the trait and each wavelength absorbance, and the estimated coefficients of the Bayes B equations for the prediction of acetic acid are shown. For the prediction of the amount of acetic acid (Figure 1a), it could be seen that the absorbance recorded at several wavelength is correlated with the measured VFA, but only in two cases the correlation coefficient approached 0.50.

In both cases, the spectral areas more correlated with VFA measurements were the spectral regions characterized by the large variability of absorbance due to water bonds: SWIR-MWIR and MWIR2 regions as classified by Bittante and Cecchinato (2013). The SWIR region and the area between MWIR1 and MWIR2 regions were negatively and positively correlated with VFA data, respectively. The Bayesian method B selected one wavelength in this last area (1721 cm^{-1}) as the most important one for predicting acetate quantity. This specific wavelength corresponds to the absorption peak of C=O bond of the carboxylic group (Bittante and Cecchinato, 2013). This result clearly evidences a direct relation between the prediction model and the chemical-physical properties of acetic acid. A similar condition was observed for the others VFAs and for $N\text{-}NH_3$. A different behaviour has been observed when VFA were expressed as proportion of their total amount. The pattern of correlations between absorbance at individual wavelengths and the acetate proportion is similar in shape, lower in extension and often opposite in sign than when acetate is expressed in g/L (Figure 1b). To predict the acetic acid proportion, the Bayes B model attributed a high coefficient to the wavelengths 4356, 3989 and especially 1644 waves $\times$ cm^{-1} with a negative, positive and negative sign respectively. These wavelengths are not directly related to the absorbance properties of acetic acid and the prediction depend on the correlation between acetic acid proportion and other chemical compounds in the fermentation fluid. *In vitro* experiments aimed to evaluate the effects of different feed combination or additives (Cattani et al, 2012) would take benefits from contemporary measurements of FTIR predicting changes in VFA composition.

A) Acetic acid, g/L



B) Acetic acid, g/100 g VFA

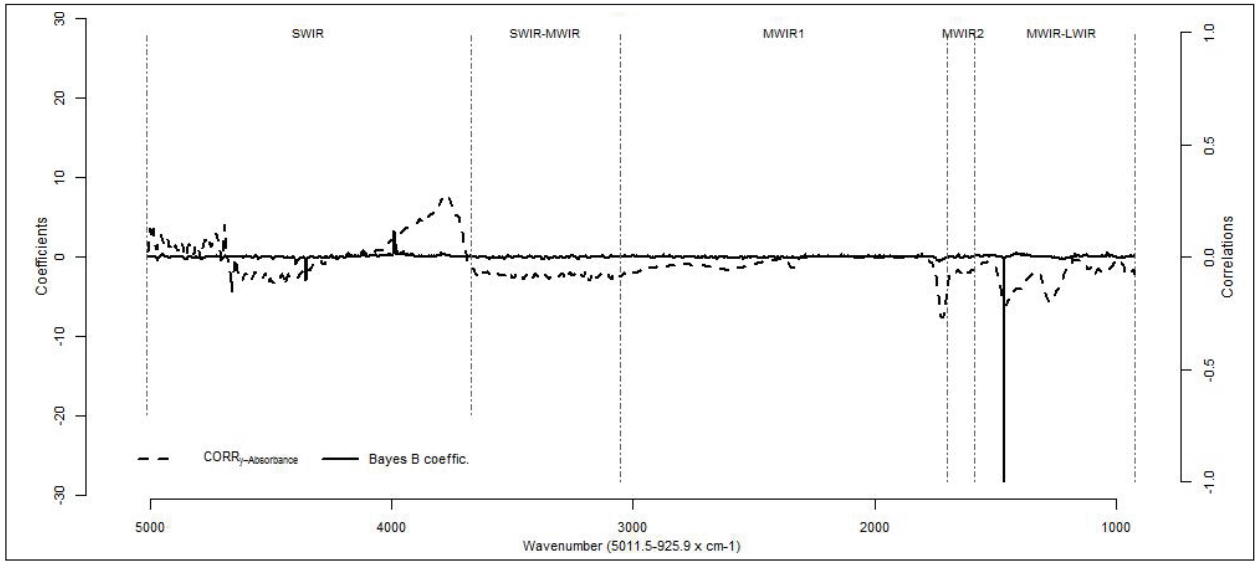


Figure 1. Graphs of correlation coefficients (r) between *in vitro* chemical compounds (A: acetic acid mg/L; B: acetic acid, % VFA) and NIR spectrum wavenumber absorbance (dot line), and prediction equation coefficients of each spectrum wavenumber (solid line) between 5000 and 900 waves × cm^{-1}

CONCLUSION

The FTIR technique, calibrated using a Bayesian regression approach, was able to predict accurately the VFA and ammonia amounts in fermentation fluids. However, the prediction of VFA expressed as proportions of their total amount was much less accurate. This is due to the fact that FTIR absorbances are mainly related to the concentration of specific chemical bonds in the fluid and not to their proportions. VFA proportions seem to be predicted only in an indirect way on the bases of correlations with the amount of some compounds present in the sample. For these parameters a greater number of samples in calibration set could be needed for a good prediction accuracy.

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