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Total antioxidant activity of bovine milk: Phenotypic variation and predictive ability of mid-infrared spectroscopy

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ABSTRACT

The phenotypic variation of milk total antioxidant activity (TAA) and the ability of mid-infrared spectroscopy to predict this novel trait was investigated. Total antioxidant activity was measured through the reference spectrophotometric method on 1249 individual milk samples of Holstein Friesian cows. Sources of variation of milk TAA were investigated using a mixed model, which included the fixed effects of days in milk, parity and calving season, and the random effects of herd-test-date and error. Mid-infrared spectroscopy prediction models were developed using partial least squares regression approach. The average level of milk TAA was 6.93 mmoL L^{-1} of Trolox equivalents; this exhibited a coefficient of variation of 15% and showed weak phenotypic correlations with milk quality traits. Values of TAA were lower in early lactation than in late lactation. Mid-infrared spectroscopy prediction models reached a coefficient of determination in external validation of 0.41, suggesting that they are not adequate for analytical purposes.

1. Introduction

Milk contains a variety of antioxidant compounds, such as tocopherols (Chauveau-Duriot, Doreau, Nozière, & Graulet, 2010), retinol and carotenoids (Nozière et al., 2006), ascorbate (Nielsen, Hald, Kjeldsen, Andersen, & Østdal, 2001), phenols (Velázquez Vázquez et al., 2015) and low molecular weight thiols (Niero, De Marchi, Masi, Penasa, & Cassandro, 2015). Also, peptides derived from casein (CN) digestion and lactoferrin have been studied for their antioxidant properties (Pihlanto, 2006; Suetsuna, Ukeda, & Ochi, 2000).

Milk antioxidants play a central role in the maintenance of milk oxidative stability, especially during milk technological processing, cheese manufacturing and ripening. Firstly, milk antioxidants are involved in the prevention of proteins oxidative alteration, that may lead to the loss of their biological and nutritional properties, as a consequence of protein hydrolysis and amino acids release (McDermott et al., 2016). Secondly, lipid peroxidation in milk and cheese is significantly limited in presence of antioxidants, resulting in a delay of oxidative rancidity and oxidised flavour appearance (Richardson & Korycka-Dahl, 1983). Thirdly, severe heat treatment of cream improves the oxidative stability of butter, due to the exposure of antioxidant sulphydryl groups (Fox, Uniacke-Lowe, McSweeney, & O'Mahony, 1998). Fourthly, total antioxidant capacity is determined by cheese-making process itself as well as by ripening time, in relation to the rate of formation of soluble antioxidant peptides as a consequence of proteolysis development (Gupta, Mann, Kumar, & Sangwan, 2009; Lucas et al., 2006; Perna, Intaglietta, Simonetti, & Gambacorta, 2015). Finally, Niero et al. (2018) documented that skimming procedures and ultra-high temperature treatments significantly decreased milk vitamin E in terms of α- and γ-tocopherols.

Food antioxidants play an important role in human health because they are responsible for neutralisation and deactivation of free radicals. Indeed, oxidative stress and free radicals cause cytological stress, mainly associated with lipid peroxidation, oxidative alteration of proteins and DNA cleavage (Robbins & Cotran, 2010). This damage at a biochemical level has been associated with clinical diseases such as cancer, atherosclerosis, rheumatoid arthritis, neurodegeneration and diabetes (Gilbert, 2000).

Despite the great variety of antioxidants in milk, the majority of these molecules are present in traces or at low concentration. Moreover, the characterisation of these compounds is expensive, time-consuming and highly demanding in terms of milk sample preparation and analytical procedures (Niero et al., 2017). On the

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other hand, total antioxidant activity (TAA) of milk, defined as the sum of antioxidant contribution related to tocopherols, retinol, carotenoids, ascorbate, phenols, low molecular weight thiols, CN derived peptides and lactoferrin (Chen, Lindmark-Mansson, Gorton, & Åkesson, 2003), can be easily and cheaply measured using near infrared spectrophotometric method and might have broader impact on human nutrition and health, compared with that of each single antioxidant molecule (Niero et al., 2017).

Gaining knowledge on phenotypic variation of TAA across different dairy species and breeds, days in milk (DIM), parities and feeding and management systems and on the relationships of this novel phenotype with milk yield and quality traits, is of relevant interest for several reasons: (i) from a physiological point of view, it would allow a better understanding on how, and to what extent, milk antioxidants are transferred from feed to animal and from the animal into the milk (Havemose, Weisbjerg, Bredie, Poulsen, & Nielsen, 2006); (ii) from a technological point of view, it would allow examination of the correlations between milk quality traits and TAA and analysis of the impact of different management conditions on milk TAA (Niero, Penasa, Gottardo, Cassandro, & De Marchi, 2016a); (iii) with regard to human nutrition and health, it would provide added value to milk composition and milk nutraceutical properties (Haug, Høstmark, & Harstad, 2007); (iv) the assessment of phenotypic variation for a new trait such as TAA is a prerequisite to develop mid-infrared spectroscopy (MIRS) prediction models to record phenotypes at population level (De Marchi, Toffanin, Cassandro, & Penasa, 2014).

Therefore, the objectives of the present study were to assess the associations between milk TAA and traditional milk quality traits, to investigate sources of variation of milk TAA, and to evaluate the feasibility of MIRS to predict TAA in milk.

2. Materials and methods

2.1. Milk sample collection and chemical composition

Individual raw milk samples (n = 1249) of Holstein Friesian cows from parity 1 to 9 and from 6 to 536 DIM were collected in 17 herds between September 2016 and February 2017. Only one milk sample per cow was collected. Herds selected for the present study were commercial farms located in the lowlands of Veneto region and were characterised by comparable size, feeding system, and management. Animals were reared in indoor barns with access to exercise paddock. Diet was based on corn silage and concentrates (no grazing pasture), distributed through total mixed ration.

Immediately after sampling, 200 μL of preservative (Bronopol; 2-bromo-2-nitropropan-1,3-diol) were added to 40 mL of milk and transferred at 4 °C to the laboratory of the Breeders Association of Veneto Region (ARAV, Padova, Italy). Before chemical analyses, milk samples were warmed at room temperature and gently mixed by inversion for 10 times to promote fat and solids homogenisation. Milk samples were analysed within 12 h for fat, protein, CN and lactose content using a MilkoScan FT6000 (Foss Electric A/S, Hillerød, Denmark). Spectra information of analysed samples was stored and used to develop prediction model for milk TAA. Finally, somatic cell count (SCC; cells μL^{-1}) was determined using a Fossomatic (Foss Electric A/S) and values of SCC were transformed to somatic cell score (SCS) to achieve normality and homogeneity of variances using the formula of Wiggans and Shook (1987): $SCS = \log_2(SCC/100) + 3$.

2.2. Analysis of milk TAA

An aliquot of each individual milk sample was transferred to the laboratory of the Department of Agronomy, Food, Natural Resources, Animals and Environment of the University of Padova (Legnaro, Italy) for the determination of milk TAA according to the method developed and validated by Niero et al. (2017). Briefly, before milk TAA assay, 14 mM 2,20-azino-bis [3-ethilbenzotiazolina-6 sulphonic acid] diammonium salt (ABTS; aqueous) and 4.9 mM $K_2S_2O_8$ (1:1) were added. The resulting mixture was stored in the dark for 12 h at room temperature to activate the ABTS radical. Acetone (80%, w/w) was added with activated ABTS radical solution until an absorbance of 1.10 ± 0.05 $OD_{730\ nm}$ was reached and used as milk antioxidants extraction solvent. Subsequently, 1 mL of extraction solvent was added with 0.1 mL of milk diluted in water (1:20). Immediately after milk addition, samples were vortexed to promote antioxidant extraction, incubated at room temperature for 10 min and centrifuged at 18,000×*g* for 5 min to promote milk protein precipitation. Finally, the absorbance of supernatant was read at 730 nm and subtracted from the absorbance of the blank. This difference, directly proportional to milk TAA, was expressed in mmol L^{-1} of Trolox equivalents (TE). Full details about method development, repeatability, reproducibility, linearity, limit of blank, limit of detection and range of calibration are reported in Niero et al. (2017). The potential interfering effects of Bronopol on milk TAA assays were excluded by the same authors.

2.3. Statistical analysis

Shapiro–Wilk's test and visual inspection of data distribution highlighted that milk TAA was normally distributed. Pearson correlations of TAA with traditional milk quality features were assessed through the CORR procedure of the SAS software (ver. 9.4; SAS Institute Inc., Cary, NC) and sources of variation of TAA were investigated using the MIXED procedure of the same software, according to the following linear mixed model:

$$y_{ijkl} = \mu + DIM_i + P_j + C_k + HTD_l + \varepsilon_{ijkl},$$

where y_{ijkl} is milk TAA; μ is the overall intercept of the model; DIM_i is the fixed effect of the *i*th class of stage of lactation of the cow ($i = 1$ to 9, with the first being a class from 6 to 40 d, followed by 7 classes of 40 d each and the last being an open class > 320 d); P_j is the fixed effect of the *j*th parity of the cow (j = first, second, third and fourth and later parities); C_k is the fixed effect of the *k*th calving season (k = spring, summer, autumn and winter); HTD_l is the random effect of the *l*th herd-test-date ($l = 1$ to 17) ~ $N(0, \sigma^2_{HTD})$; and ε is the random residual ~ $N(0, \sigma^2_{\varepsilon})$. In the model, the herd and test-day effects were confounded because cows in each herd were sampled only once, all on the same test-day. Multiple comparisons of means were performed for DIM, parity and calving season effects. Significance was set at $P < 0.05$.

2.4. Development of MIRS prediction model for TAA

A prediction model for TAA was developed using the SAS software. Spectral data were converted to absorbance by taking the $\log_{10}$ of the reciprocal of the transmittance. Spectral regions between 1580 and 1710 cm^{-1} and 2990 and 3690 cm^{-1} were discarded as characterised by low signal-to-noise ratio due to water absorption. Principal component analysis was carried out on edited spectral matrix to identify similarities and differences between milk spectra. The 98.96% of the total spectral variability was represented by the first 5 principal components. In descending order, the first 5 principal components explained 70.51, 12.36, 10.73, 3.98 and 1.38% of the total variation. Based on the Mahalanobis distance, a total of 17 milk samples were identified as outliers and discarded from the dataset.

The prediction model was developed using partial least squares regression analysis, including the vector of milk TAA as dependent

variable and the matrix of spectral wavenumbers as predictor. The dataset was firstly sorted by milk TAA and subsequently divided in two different subsets, namely a calibration set (75% of the total observations) and a validation set (the remaining 25% of the total observations). The calibration set was used to develop the prediction model and the validation set was used to assess the predictive ability of the developed model, as the samples included in this last dataset were not used to generate the MIRS model. This process was repeated four times; in the first round the first observation every four was excluded from the calibration set, in the second round the second observation every four was excluded and similarly for the third and fourth round. For each iteration, one-at-a-time cross validation was performed on the calibration set. Mathematical pre-treatments (Savitzky–Golay first and second derivatives) were applied to the raw spectra but no improvement on the prediction model accuracy was detected; therefore, the untreated spectra were used to generate the models. The optimal number of model terms was the least number of extracted factors whose residuals were not significantly greater than those of the model with minimum error (van der Voet, 1994). Variable importance in the projection (VIP) scores were calculated accordingly to Wold (1994).

Goodness-of-fit statistics considered in the present study were the coefficients of determination in cross- and external validation (R^2_{CV} and R^2_V, respectively) and the standard errors of prediction in cross- and external validation (SE_{CV} and SE_V, respectively). The ratio of prediction to deviation (RPD) was calculated as the SD of the trait divided by the SE_V. Bias was calculated as the mean difference between the reference TAA and the respective MIRS-predicted TAA and a *t*-test was carried out to check if the bias was statistically different from zero. The predicted values were also linearly regressed on the respective reference values and a *t*-test was performed to verify if the coefficient of regression (slope) differed statistically from one.

3. Results and discussion

3.1. Mean and variation of milk TAA

Milk TAA averaged 6.93 mmol L^{-1} TE, with values ranging from 3.71 to 10.18 mmol L^{-1} TE (Table 1). The coefficient of variation (CV) of milk TAA (15%) suggested that exploitable phenotypic variability exists for this trait, being similar to that of protein and CN percentages and not far from that of fat percentage. Average milk TAA of the present study was slightly lower than the values obtained by Niero et al. (2017) for several types of commercial milk (from 7.11 to 7.52 mmol L^{-1} TE for whole UHT milk and partially skimmed pasteurised milk, respectively), but considerably greater than that (2.24 mmol L^{-1} TE) reported by Chen, Lindmark-Månsson, Gorton, and Åkesson (2003), probably due to differences in milk antioxidant extraction procedures. Comparisons with other studies (e.g., Zulueta et al., 2009) is difficult due to different units of measure to express TAA and different assays involved in the quantification of antioxidant capacity. In terms of variability, the CV reported in our study (15%) was much greater than those (2.18–3.52% for raw milk and whole UHT milk, respectively) obtained by Niero et al. (2017). Two reasons can be argued to explain this difference: first, milk analysed by Niero et al. (2017) was purchased in commercial stores as bulk milk, whereas the present study analysed individual milk, which accounts for more variability of the trait of interest; second, the sample size of Niero et al. (2017) was smaller than that of the present work.

Table 1
Descriptive statistics of milk total antioxidant activity, production related traits and milk quality traits.[a]

Trait	n	Mean	Minimum	Maximum	CV (%)
TAA (mmol L^{-1} TE)	1239	6.93	3.71	10.18	15
Production related traits					
Milk yield (kg d^{-1})	1245	29.8	4.0	59.9	32
DIM (d)	1249	174	6	536	69
Parity (n)	1249	2.14	1.00	9.00	60
Milk quality traits					
Fat (%)	1240	3.95	1.30	6.80	22
Protein (%)	1240	3.36	2.40	4.69	12
Casein (%)	1239	2.63	1.80	3.70	13
Lactose (%)	1226	4.86	4.10	5.57	4
SCS (units)	1249	3.43	−1.64	9.73	60

[a] Abbreviations are: TAA, total antioxidant activity, expressed as mmol L^{-1} of Trolox equivalents (TE); DIM, days in milk; SCS, somatic cell score; CV, coefficient of variation.

3.2. Relationships of milk TAA with milk yield and quality traits

Phenotypic correlations of milk TAA with milk yield and quality traits were weak (Table 2). Overall, the complex pattern and variety of milk antioxidants, their low concentration distributed among different milk constituents (including fat, protein and CN), and thus their small individual contribution to milk TAA, are possible reasons to explain the weak relationships between TAA and traditional milk quality traits observed in the present study. Milk TAA was unfavourably correlated with milk yield (−0.22; $P < 0.001$), meaning that high-producing cows yielded milk with lower TAA compared with low-producing animals; a dilution effect can be hypothesised, similarly to what happens for other milk constituents such as protein and fat (Ng-Kwai-Hang, Hayes, Moxley, & Monardes, 1982; Niero et al., 2016b).

Among composition traits, CN and protein percentages had the most favourable correlation with TAA (0.15 and 0.18, respectively; $P < 0.001$); this was somewhat expected and in agreement with findings from other studies. Indeed, Zulueta et al. (2009) reported that CN is one of the main compounds contributing to milk TAA,

Table 2
Pearson correlations of milk total antioxidant activity with milk yield and quality traits.[a]

Trait	Total antioxidant activity
Milk yield	−0.22***
Fat content	0.13***
Protein content	0.18***
Casein content	0.15***
Lactose content	−0.11***
Somatic cell score	0.13***

[a] Abbreviations are: SCS, somatic cell score; ***P < 0.001.

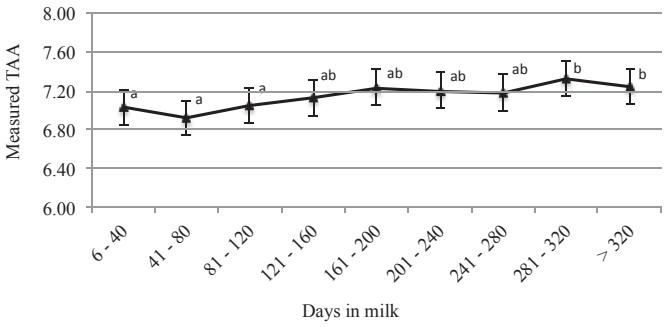

Fig. 1. Least squares means (with standard error) of total antioxidant activity (TAA) of milk, expressed as mmol L^{-1} of Trolox equivalents, across lactation; least squares means with different letters differ significantly ($P < 0.05$).

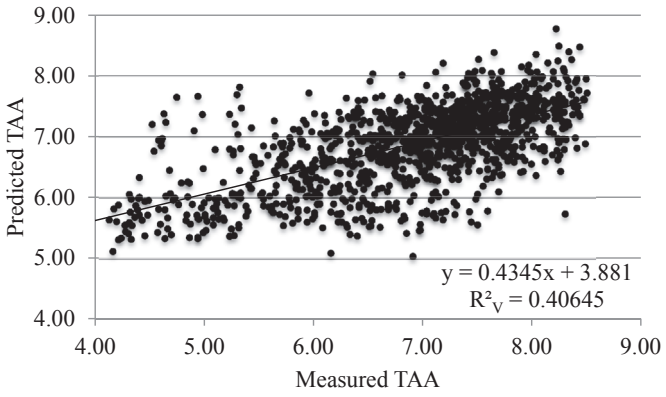

Fig. 2. Scatter plot of predicted total antioxidant activity (TAA) (y-axis) versus measured TAA (x-axis) of milk, expressed as mmol L^{-1} of Trolox equivalents.

due to its high content of potentially antioxidant amino acids (Rival, Boeriu, & Wichers, 2001); the latter are expected to be present in milk as free molecules in response to protein hydrolysis (McDermott et al., 2016). Furthermore, in a review of Lindmark-Månsson and Åkesson (2000) on antioxidant factors in milk, it has been reported that CN is in complex with glutathione peroxidase enzyme, responsible of glutathione antioxidant capacity.

Regarding protein percentage, the favourable association with TAA is due to the previously discussed CN antioxidant properties and to the whey protein contribution to milk TAA. Whey proteins have been studied for their antioxidant properties, in particular lactoferrin which is able to bind iron and to block its pro-oxidant action (Cichosz, Czeczot, Ambroziak, & Bielecka, 2017). Also, β-lactoglobulin and derivative peptides have antioxidant effects, firstly preserving retinol and α-tocopherol from oxidation along the digestive trait (Liang, Tremblay-Hèber, & Subirade, 2011), and secondly deactivating free radicals through Trp, Tyr and Met amino acid residues (Cichosz et al., 2017).

A favourable but weak correlation (0.13; $P < 0.001$) was observed between milk TAA and fat percentage (Table 2), similar to the findings of Chen et al. (2003). Milk fat contains several liposoluble vitamins and antioxidant compounds, which contribute to milk TAA. Small amounts of retinol, α- and γ-tocopherol and β-carotene in milk fat have been observed by Calderón et al. (2007), Chauveau-Duriot et al. (2010) and Ramalho, Santos, Casal, Alves, and Oliveira (2012). The correlations of TAA with fat and protein

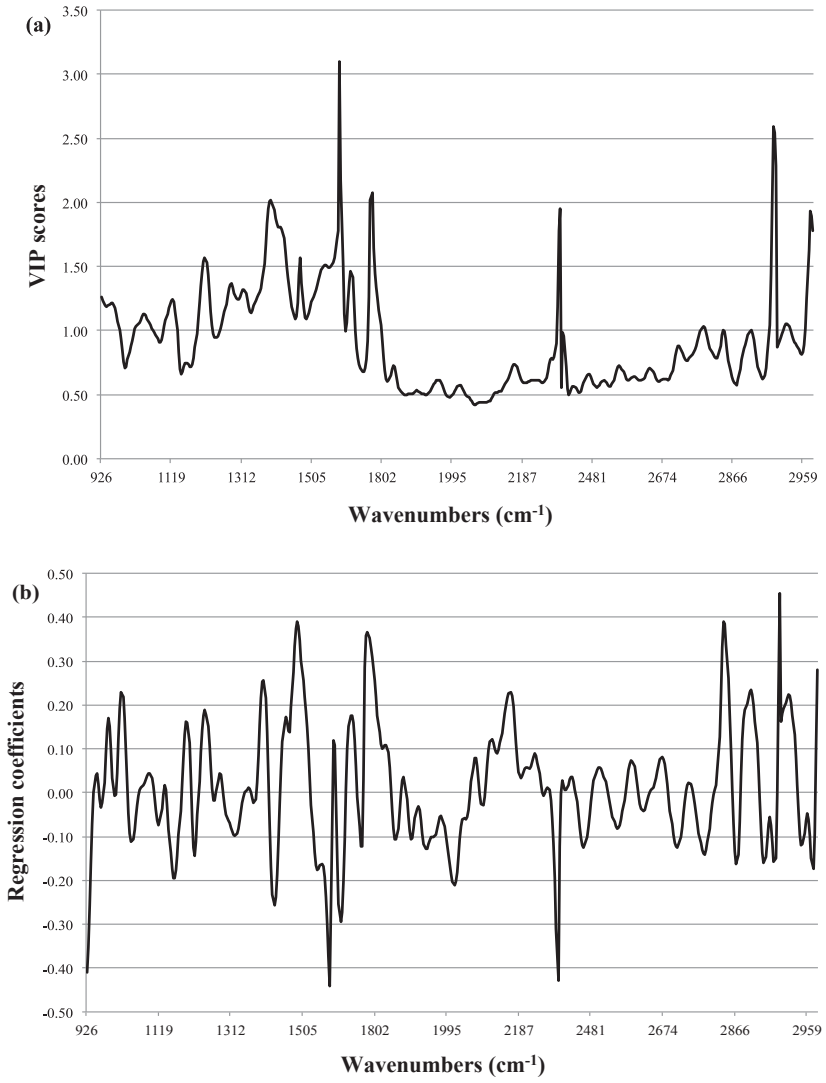

Fig. 3. Variable importance in the projection (VIP) scores (a) and coefficient of regression (b).

of the present work were higher than those reported by Ramalho et al. (2012).

High milk SCS is one of the main indicators of mastitis, defined as inflammation of udder tissues as a consequence of pathogens infection (Pyörälä, 2003). This altered physiological status is associated with high free radical development (Atakisi et al., 2010), thus resulting in amplified antioxidant response (Conner & Grisham, 1996) and explaining the favourable relationship between TAA and SCS of the present study. Additionally, the negative correlation between TAA and lactose (Table 2) confirms this hypothesis, since milk lactose content decreases in presence of high SCS and mastitis (Miglior et al., 2007).

3.3. Effects of lactation stage, parity and calving season on milk TAA

The fixed effect of classes of DIM was significant in explaining the variation of milk TAA ($P < 0.01$). Total antioxidant activity decreased slightly from 6 to 40 DIM and increased thereafter (Fig. 1), exhibiting a trend that was opposite to that of milk yield (results not shown). This is in agreement with the previously discussed unfavourable correlation between milk TAA and milk yield (Table 2). Fat, protein and casein percentages resembled the trend of milk TAA, i.e. they were lower in the first part of the lactation and increased thereafter, corroborating the positive correlations assessed between milk TAA and the aforementioned quality traits (Table 2).

Parity and calving season did not significantly contribute to explain the variation of milk TAA. Overall, TAA was almost stable across classes of parity and calving season. This equilibrium may be due to the complex biological systems designed to maintain cellular redox status, that is crucial not only to maintain normal cellular function, but also to prevent damage mediated by oxidative stresses (Ray, Huang, & Tsuji, 2012).

3.4. Accuracy of prediction model for milk TAA

Fig. 2 depicts the scatter plot between milk TAA measured by spectrophotometric reference method and milk TAA predicted by MIRS. Mid-infrared spectroscopy prediction model was developed using 17 factors and it showed R^2_{CV} and R^2_V of 0.46 and 0.41, respectively, and both SE_{CV} and SE_V of 0.72 mmol L^{-1} TE. The results indicated that prediction models are not adequate for analytical purposes.

To our knowledge no studies have attempted to investigate the potential of MIRS to predict milk TAA, and thus the comparison of our results with the scientific literature can be made only with regard to other innovative and trace milk quality traits. For example, R^2_V observed in the present study is close to that reported by Visentin, Penasa, Gottardo, Cassandro, and De Marchi (2016) on MIRS-predicted milk coagulation properties, but lower than R^2_V reported by Niero et al. (2016a) on detailed milk protein composition and Visentin et al. (2015) on major milk mineral composition. The RPD value in external validation was 1.30 and the mean bias of prediction (i.e., the average of the difference between the gold standard and predicted values for each individual sample) was not significantly different from zero in external validation. The slope of the linear regression of the reference on the predicted values was 0.44 (SE = 0.02). Again, due to the low RPD, prediction model cannot be considered adequate for analytical purposes because a prediction model can be considered useful for analytical purposes when RPD is greater than 2 (Williams, 2007).

According to VIP scores and regression coefficients, the prediction of milk TAA showed specific absorption peaks, as previously reported by De Marchi et al. (2009): 1550–1570 cm^{-1} related to protein absorption and 2800–2959 cm^{-1} related to lipid absorption (Fig. 3). This result corroborates the previously discussed positive correlations of milk TAA with protein and fat content.

4. Conclusions

The present study is the first contribution to the phenotypic characterisation of TAA of bovine milk. This new phenotype exhibited an interesting and exploitable variability (CV of 15%), similar to that of traditional quality traits. Positive but weak phenotypic correlations of TAA with fat, protein and CN percentages were observed, as well as with SCS. Total antioxidant activity of milk increased across lactation. So far, the MIRS prediction models developed to predict milk TAA were not enough accurate for analytical purposes. Feeding and genetic aspects need to be investigated to account for other possible sources of variation influencing this phenotype.

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