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**PHENOTYPIC AND GENETIC ASPECTS
OF BOVINE MILK TECHNOLOGICAL TRAITS
PREDICTED BY MID-INFRARED SPECTROSCOPY**

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RIASSUNTO

Tra i Paesi dell’Europa-28, l’Italia è il primo produttore di formaggi a Denominazione di Origine Protetta (DOP); di conseguenza, l’industria casearia riveste un ruolo centrale nei settori agricolo e alimentare italiani. Circa il 70% del latte disponibile in Italia viene utilizzato per la produzione di formaggio e il 35% è trasformato in prodotto DOP.

Negli ultimi anni, vi è stata una crescita dell’interesse per il latte e i suoi derivati, specialmente per le loro caratteristiche tecnologiche e nutrizionali. Le proprietà coagulative del latte, la sua acidità titolabile e il suo contenuto minerale sono al momento gli argomenti maggiormente discussi e studiati a livello d’industria casearia e di ricerca. L’attitudine casearia, che comprende il tempo di coagulazione (RCT, min), il tempo di rassodamento (k_{20} , min) e la consistenza del coagulo (a_{30} , mm), influenza l’efficienza dell’intero processo produttivo. L’acidità titolabile gioca un ruolo fondamentale sulla velocità di aggregazione delle micelle para-caseiniche e sineresi e sulla reattività del caglio. Infine, la composizione minerale del latte riveste importanza sia a livello tecnologico, influenzando il processo di produzione casearia, sia sulla salute umana.

L’obiettivo generale della presente tesi di dottorato è stato quello di studiare gli aspetti fenotipici e genetici delle proprietà coagulative del latte, della sua acidità titolabile e del suo contenuto minerale, predicendo questi parametri attraverso la spettroscopia del medio infrarosso (MIRS).

Nel primo capitolo si è analizzata la bibliografia inerente alla tecnologia MIRS applicata all’analisi del latte ed è stata riscontrata la capacità del MIRS nel predire, con differenze a livello di accuratezza, gli acidi grassi del latte, la sua composizione proteica e minerale, le MCP, la sua acidità, i corpi chetonici e lo stato energetico della vacca e le emissioni di metano. In generale, gli articoli analizzati hanno evidenziato come la variabilità dei dati, i metodi di riferimento e le unità di misura influenzino notevolmente la robustezza dei modelli di predizione. Un punto cruciale a favore dell’applicazione del MIRS è il possibile scambio di dati tra diversi Paesi al fine di sviluppare equazioni che tengano conto della variabilità biologica dei parametri in base alle differenti condizioni. Data la grande variabilità dei metodi di analisi di riferimento utilizzati per le calibrazioni MIRS, sembra essenziale una standardizzazione di questi tra i diversi Paesi.

Nel secondo capitolo si è studiata la capacità del MIRS di predire i latti che non coagulano e i risultati ottenuti hanno dimostrato che a livello spettrale non vi è una specifica informazione che permetta una distinzione a priori tra questo tipo di campioni e quelli che

coagulano. Il modello di predizione più accurato si è ottenuto per il tempo di coagulazione del latte, seguito da quelli per il tempo di rassodamento e coesistenza del coagulo a 30 minuti.

Nel terzo capitolo si sono sviluppati i modelli di predizione MIRS per il contenuto di calcio (Ca) e fosforo (P) del latte e per la sua acidità titolabile (TA), ottenendo risultati soddisfacenti: per tutti e tre i parametri è stato ottenuto un coefficiente di correlazione in cross-validation maggiore di 0.73. Inoltre, sono state rilevate delle correlazioni favorevoli tra questi caratteri e le proprietà coagulative del latte.

Infine, nel quarto e ultimo contributo, è stata dimostrata l'esistenza di una varianza genetica additiva per Ca, P e TA predetti col MIRS, per i quali si sono stimate ereditabilità pari a 0.10, 0.12 e 0.26, rispettivamente. Inoltre, tra i tre suddetti caratteri sono state stimate correlazioni fenotipiche da moderate a forti, mentre da deboli a moderate tra essi e la composizione qualitativa e quantitativa del latte.

La presente tesi di dottorato ha dimostrato come la tecnologia MIRS possa essere un utile strumento per predire le proprietà coagulative, l'acidità titolabile e il contenuto minerale del latte. La possibilità di sfruttare il MIRS su vasta scala e l'esistenza di una base genetica per i caratteri precedentemente menzionati, rappresentano un punto di partenza per definire specifici programmi genetici per il miglioramento delle caratteristiche tecnologiche e nutrizionali del latte.

ABSTRACT

Italy is the first producer of Protected Designation of Origin (PDO) cheeses in Europe-28, and thus the dairy industry plays a central role in the agriculture and food sectors. About 70% of available milk in Italy is used for cheese production and 35% is transformed into PDO products.

In the last few years, the interest for milk and dairy products has grown, especially in relation to their technological and healthy features. Coagulation properties, titratable acidity (TA) and mineral content of milk are among the main topics discussed at research and dairy industry level. Milk coagulation properties (MCP), namely rennet coagulation time (RCT), curd firming time (k_{20}) and curd firmness (a_{30}), influence the efficiency of cheese-making process. TA plays a fundamental role in the aggregation rate of para-casein micelles, reactivity of rennet, and rate of syneresis. Finally, mineral composition of milk has an impact on human bone health and on milk technological properties, which affects cheese-making process.

The overall aim of the present work was to study the phenotypic and genetic aspects of coagulation traits, mineral content and acidity of bovine milk predicted by mid-infrared spectroscopy (MIRS).

The analysis of the literature (Chapter 1) demonstrated the feasibility of MIRS to predict milk fatty acid, protein and mineral composition, MCP, acidity, ketone bodies, body energy status, and methane emissions, with different accuracies and improvement of prediction equations. In general, the reviewed papers underlined the influence of data variability, reference method, and unit of measurement on the development of robust models. A crucial point in favour of the application of MIRS is to stimulate the exchange of data among countries to develop equations that take into account the biological variability of the traits under different conditions. Due to the large variability of reference methods used for MIRS calibration, it is essential to standardize the methods within and between countries.

Chapter 2 investigated the ability of MIRS to predict not coagulating (NC) from coagulating milks. Results showed that there is no specific spectral information that distinguishes NC from coagulating samples. The most accurate prediction model was developed for RCT followed by k_{20} and a_{30} .

Mid-infrared models to predict calcium (Ca), phosphorus (P) and TA were developed in Chapter 3 and results were satisfactory: coefficients of correlation of cross-validation for CA, P and TA were greater than 0.73. Moreover, favourable relationships of these traits with MCP were estimated.

Exploitable additive genetic variation for predicted Ca, P, and TA exists (Chapter 4) and heritability estimates were 0.10, 0.12, and 0.26 for Ca, P and TA, respectively. Positive moderate to strong phenotypic correlations were estimated between Ca, P and TA, while phenotypic weak to moderate correlations were assessed between these traits with both milk quality and yield. Moderate to strong genetic correlations existed between Ca, P, and TA, and between these predicted traits with both milk fat and protein percentage.

The present thesis demonstrated that MIRS might be used as phenotyping tool to predict MCP, mineral content and acidity of milk. The feasibility of using MIRS on a large scale coupled with the existence of a genetic basis for the aforementioned predicted features, represent the starting point to set up specific genetic programs to improve these nutritional and technological characteristics of milk.

GENERAL INTRODUCTION

Milk production is growing worldwide and the most productive countries are the European Union (EU-28), United States of America (USA), New Zealand and Australia, which have increased their production of about 5% during the last year (Ismea, 2014). Within the EU-28, a global trend of increase (2%) was registered between April 2013 and February 2014, with differences across European countries: the highest increase was shown in Belgium and the Netherlands, while Sweden and France exhibited the lowest (Ismea, 2014). Moreover, the major producers of European milk remain Germany, France, United Kingdom, Poland, the Netherlands and Italy (Eurostat, 2014).

The EU-28 is the main exporter of cheese worldwide, with about 790 tonnes in 2013 (Ismea, 2014). Within EU-28, the Italian dairy industry is very important; about 70% of available milk is used for cheese production and 35% is transformed into Protected Designation of Origin (PDO) products (Pieri, 2011). Italy is the first producer of PDO cheeses in EU-28 (Ismea, 2014). Therefore, in the last years, milk and dairy products have been widely studied for their technological and healthy features.

Milk technological traits

The technological ability of milk for cheese production can be established by milk coagulation properties (MCP) measurements. Milk coagulation properties are defined as rennet coagulation time (RCT), curd-firming time (k_{20}) and curd firmness (a_{30}) (Summer et al., 2002). The RCT measures the time in minutes between the rennet addition to milk and the occurrence of the first coagulum, the k_{20} the time in minutes to achieve 20 millimetres of curd and a_{30} the thickness millimetres of curd obtained 30 minutes after the rennet addition. These characteristics are the result of interactions between different milk features as acidity, mineral contents, protein polymorphisms and breed, stage of lactation and parity of cows (Summer et al., 2002; Malacarne et al., 2013; De Marchi et al., 2007). Moreover, the MCP are also influenced by laboratory conditions like instrument setup, type, reproducibility and repeatability and pre-treatments of milk samples (Pretto et al. 2011). In several studies a heritable additive genetic variance of MCP was reported (Ikonen et al., 1997, 1999; Cassandro et al., 2008), therefore an improving of cheese yield seems to be possible through the genetic selection of cows which show better MCP (Ikonen et al., 2000).

As previous explained the cheese-making process is also highly influenced by the acidity of milk: pH and titratable acidity (TA) affect the starting enzymatic phases and the

following aggregation rate of para-casein micelles, reactivity of rennet, and rate of syneresis (Formaggioni et al., 2001). In literature, different authors investigated the effect of acidity changes on milk rheological properties of milk, reporting strong relationships between milk acidity and optimal or late-non-coagulating samples of milk. Malacarne and colleagues (2013) declared that non-coagulating samples are characterised by high values of pH and low of TA, while the opposite behaviour of acidity traits represent good coagulating milks. Moreover, Ikonen et al. (2004) and Cassandro et al. (2008) reported positive correlations between pH and RCT. In this studies moderate heritability estimates for pH and TA of milk were reported, suggesting an achievable genetic improvement of them.

Another technological aspect of milk is represented by its mineral composition. Fossa et al. (1994) reported that the milk ability to coagulate and the final consistency of the coagulum are strongly influenced by calcium (Ca) and phosphorus (P) content. In particular, the colloidal Ca phosphate affects the paracaseinate particles' aggregation speed and the casein reticulum's properties (Mariani et al., 1996). Milk minerals play a key role on the human health. Calcium provides rigidity to the skeleton by virtue of its phosphate salts and takes part in neuromuscular function, enzyme-mediated processes and blood clotting. Moreover, during skeletal growth the increase of bone mineral density is affected by an adequate Ca intake, while during old age this element prevents bone loss and osteoporotic fractures. The principal disease referred to a Ca (and vitamin D) deficiency is osteoporosis (Caroli et al., 2011), which has been defined by the Consensus Development Conference (1993) as "a systemic skeletal disease characterized by low bone mass and micro-architectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture". Cashman et al. (2006) summarized results on the effects of phosphorus on human health: in particular, the Ca to P ratio is very important as a high concentration of P combined to a low amount of Ca seems to be responsible of a stimulation of parathyroid hormone (PTH) with an eventual reduction of bone mass and density, especially in young people.

Mid-infrared spectroscopy

Over the years, the need of cheap, fast and high-throughput chemical analysis methods widely increased in dairy sector, therefore a powerful technique as mid-infrared spectroscopy (MIRS) has become more and more relevant. Several studies were conducted on the application of MIRS to milk analyses (De Marchi et al., 2014), investigating this technology as alternative method of milk analysis and attaining promising and satisfactory results. Mid-infrared spectroscopy is an indirect method of analysis given its functioning: it's based on the

study of the interaction between matter and electromagnetic waves in mid-infrared region (2500 - 25000 nm), and it includes statistical and mathematical treatments for each specific traits calibration and validation procedure. Recently, mid-infrared spectroscopy (MIRS) has been proposed as a valid tool for the prediction of innovative milk phenotypes as MCP, TA (Dal Zotto et al., 2008; De Marchi et al., 2009) and minerals (Soyeurt et al., 2009). The MIRS technology is used in routine milk recording programs of different countries for determining traditional milk quality traits such as milk fat, protein, casein and lactose content. Therefore, the developments of robust MIRS prediction models for innovative milk phenotypes could permit their implementation on MIRS instruments present in milk laboratories, allowing the rapid and simultaneous recording of large amount of different data. The use of MIRS to predict milk phenotypes has relevant genetic implications. Berry et al. (2012) reported that a phenotype should be easily, routinely and cheaply measured, and they endorsed MIRS technique given its possible optimal accuracy of prediction, exhibition of genetic variation and high genetic correlations with traits of interest. The genetic implications of applying MIRS at population level were also inferred by Cecchinato et al. (2009) who concluded that for their studied traits (RCT and a_{30}) the selection using MIRS predictions as indicator traits can ensure a genetic response equal to or slightly lower than to those achievable through actual analysis of the traits.

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AIMS OF THE THESIS

The possibility to reduce time and cost of milk analyses is increasingly required by the dairy industry and MIRS seems to reply to this need. The general aim of the present thesis was to investigate the phenotypic and genetic aspects of coagulation traits, mineral content and acidity of bovine milk predicting these features by MIRS. The four chapters aimed to:

1. review papers which have investigated the use of MIRS to predict milk quality, nutritional and technological traits, and other features of economic relevance;
2. investigate the capability of MIRS to predict coagulating and not-coagulating milk in dairy cows;
3. investigate the effectiveness of MIRS in predicting milk Ca, P and TA using different statistical approaches and to study variation of Ca, P, and TA in individual milk of Italian Holstein-Friesian cows and their relationship with milk quality and coagulation properties;
4. estimate genetic parameters for milk Ca, P and TA predicted by MIRS and genetic correlations of these traits with milk production and quality in Italian Holstein-Friesian cows.

CHAPTER 1

Invited review: Mid-infrared spectroscopy as phenotyping tool for milk traits

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ABSTRACT

Interest in methods that routinely and accurately measure and predict animal characteristics is growing in importance, both for quality characterization of livestock products and for genetic purposes. Mid-infrared spectroscopy (MIRS) is a rapid and cost-effective tool for recording phenotypes at the population level. Mid-infrared spectroscopy is based on crossing matter by electromagnetic radiation and on the subsequent measure of energy absorption, and it is commonly used to determine traditional milk quality traits in official milk laboratories. The aim of this review was to focus on the use of MIRS to predict new milk phenotypes of economic relevance such as fatty acid and protein composition, coagulation properties, acidity, mineral composition, ketone bodies, body energy status, and methane emissions. Analysis of the literature demonstrated the feasibility of MIRS to predict these traits, with different accuracies and with margins of improvement of prediction equations. In general, the reviewed papers underlined the influence of data variability, reference method, and unit of measurement on the development of robust models. A crucial point in favour of the application of MIRS is to stimulate the exchange of data among countries to develop equations that take into account the biological variability of the studied traits under different conditions. Due to the large variability of reference methods used for MIRS calibration, it is essential to standardize the methods used within and across countries.

Key words: mid-infrared spectroscopy, phenotyping, quality trait, dairy cattle.

INTRODUCTION

In the genomic era, phenomics is becoming a compulsory research field. This new science is concerned with the acquisition of phenotypic data on a large scale (Houle et al., 2010) and the phenotype can be described as the outcome of the interacting development between the genotype of an individual and its specific environment throughout life (Bowman, 1974). The interest in methods that routinely and accurately measure and predict animal characteristics (i.e., phenotypes) is rapidly growing. Accurate phenotypes and efficient phenotyping tools are the key ingredients, especially for genomic selection of livestock animals, which is expected to increase the genetic gain of the selected traits (Pryce et al., 2010; Lillehammer et al., 2011; Mc Hugh et al., 2011).

Furthermore, in recent years, consumers and the dairy industry have shifted the concept of quality in relation to market requirements: for example, milk coagulation traits have been studied to improve cheese production and FA composition to enrich the nutritional

value of milk for human health. Breeding goals have followed these changes: if phenotypes are accurately and cheaply measured at the population level and integrated in the national milk recording systems, it may be possible to enhance the traits using genetic or genomic tools.

The need for fast, cheap, and high-throughput methods of chemical analysis has also led to the application of infrared spectroscopy in both the livestock and food sectors. The spectroscopic technique is based on the study of the interaction between matter and electromagnetic waves. Electromagnetic radiation comprises different regions according to the following wavelengths: the x-ray region (0.5–10 nm), UV region (10–350 nm), visible region (350–800 nm), near-infrared region (800–2,500 nm), mid-infrared region (2,500–25,000 nm), microwave region (100 μm–1 cm), and radio frequency region (1 cm–1 m).

One of the most important historical events for the spectroscopic technique was the development of the Fourier transform in the 1700s; in later years, this mathematical transform was improved with the use of the interferometer. In 1969, Digilab Inc. (Marlborough, MA) put the first Fourier-transform infrared spectro-photometer with a dedicated minicomputer on the market, which was later refined in 1983 by the same company (Spectra-Tech Inc., Oak Ridge, TN). From the 1980s, Fourier-transform infrared spectrophotometers were combined with personal computers and this method of analysis became widely used due to its versatility and cost effectiveness. Since then, many studies have investigated the relationships between spectra wavelengths and several quality traits through the explanation of chemical bond variations.

Mid-infrared spectroscopy (**MIRS**) has been evaluated as a potential tool to collect data at the population level for phenotypic and genetic purposes, and it is becoming one of the major topics in dairy science. In the mid-infrared region, when matter is crossed by electromagnetic radiation, the bonds of the molecules make movements (e.g., vibration and rotation), which involve a more or less marked absorption of the provided energy. On the basis of supplied energy and the amount absorbed by the irradiated sample, and using spectra mathematical pretreatments, it is possible to determine the sample's chemical composition and correlated compounds (Figure 1).

The present review summarizes papers that have investigated the use of MIRS to predict milk quality traits. Furthermore, studies that adopted this technique to predict nutritional, technological, and other traits of economic relevance are also reviewed.

PHENOTYPING OF MILK BY MIRS

Studies that aimed at investigating the effectiveness of MIRS to predict phenotypes for dairy industry applications or for genetic purposes have markedly increased over the years. Figure 2 depicts the trend in the number of papers published from 2001 to 2013 on the application of MIRS to milk, highlighting a growing interest for the topic, particularly in the last 3 yr. This is confirmed also by large international research projects that aimed at predicting new traits in the dairy industry (e.g., OptiMIR, 2012; RobustMilk, 2012). In animal science, phenomics is mainly related to the study of phenotypes of an individual. A phenotype should be routinely, cheaply, and easily measurable, should show good to optimal accuracy of prediction, depending on its use, and should exhibit genetic variation or, if it is a predictor of the real phenotype of interest, high genetic correlation with the trait of interest (Berry et al., 2012).

Currently, MIRS is used to determine quality traits in bulk and individual milk samples. In particular, most countries use MIRS in official milk-recording schemes to predict protein, casein, fat, lactose, and urea contents. Besides these traditional traits, MIRS has been used to predict other milk characteristics: FA composition (Soyeur et al., 2006, 2008, 2011; Rutten et al., 2009; De Marchi et al., 2011; Ferrand et al., 2011; Maurice-Van Eijndhoven et al., 2013), milk protein composition (Luginbuhl, 2002; Sorensen et al., 2003; Etzion et al., 2004; De Marchi et al., 2009a; Bonfatti et al., 2011; Rutten et al., 2011), milk coagulation properties (**MCP**; Dal Zotto et al., 2008; De Marchi et al., 2009b, 2013), milk acidity (De Marchi et al., 2009b), mineral composition (Soyeur et al., 2009), melamine content (Balabin and Smirnov, 2011), ketone bodies (Heuer et al., 2001; de Roos et al., 2007; van Knegsel et al., 2010; van der Drift et al., 2012), body energy status (McParland et al., 2011), and methane emissions (Dehareng et al., 2012).

In addition, several laboratories involved in routine milk-recording systems have been storing spectral data to predict *a posteriori* several phenotypes; this approach is very interesting for genetic purposes.

SPECTRA PRETREATMENTS

Pretreatments of MIRS spectral data are very common and often of great importance to obtain robust prediction models (Rinnan et al., 2009). The main goal of using statistical procedures for preprocessing spectral data is to improve the linear relationship between the spectra and the gold reference. As reported by Rinnan et al. (2009), several phenomena can

cause a deviation from the aforementioned linear relation: scatter from particulates, molecular interactions, and changes in sample size. The most common preprocessing techniques for MIRS data are those that directly use available reference values, such as scatter correction and derivation methods. The scatter correction methods include multiplicative scatter correction, standard normal variate, and normalization. Regarding the derivation methods, the most commonly used is the Savitzky-Golay derivative.

Briefly, multiplicative scatter correction aims at removing nonlinearities in the spectral data caused by scatter from particulates in the samples (Martens et al., 1983) and this is very similar in result to standard normal variate (Rinnan et al., 2009). Figure 1 depicts the variation of spectra peaks moving from untreated data to first and second derivatives. The main benefits of the use of derivative spectra pretreatments are to improve the resolution of the spectra (first derivative) and to give a negative peak for each band and shoulder (second derivative); the most common derivatives used for these purposes were developed by Savitzky and Golay (1964). Generally, the sharp bands are enhanced at the expense of broad ones (Figure 1) and this may allow for the selection of suitable peaks (Stuart, 2004).

Despite mathematical pretreatments being widely used in the building of MIRS prediction models, the authors usually report only the best model with the related mathematical method and, hence, the comparison among different mathematical pretreatments is not possible. Only Soyeurt et al. (2011) and De Marchi et al. (2011) investigated the variation in the accuracy of MIRS prediction models for FA composition using different mathematical pretreatments; they found quite similar results, showing better accuracies using derivative pretreatment than untreated spectral data. In a similar manner, De Marchi et al. (2009b) investigated the effect of different spectra pretreatments for the prediction of MCP and they found better accuracies using untreated spectral data; this has been recently confirmed by De Marchi et al. (2013).

FITTING STATISTICS OF CHEMOMETRIC ANALYSIS

Partial least squares (**PLS**) modelling is a powerful multivariate statistical tool that has been widely applied to the quantitative spectral analyses of MIRS and near-infrared data (De Marchi et al., 2012; Riovanto et al., 2012). Because several software packages using the PLS technique are available, it is important to understand how chemometric results can be compared. Several statistic parameters exist that can be used to assess the goodness of PLS models both in calibration and validation (Nas et al., 2002). Reference statistics are the

validation ones, which are calculated after the development of the calibration equation; they are essential in determining the ability of calibration models to predict unknown values. The most important parameters are the coefficient of determination and standard error of calibration and validation, the relative ability of prediction, ratio to performance deviation (**RPD**), range error ratio (**RER**), relative prediction error (**RPE**), and concordance correlation coefficient (**CCC**; Williams and Norris, 2001; Williams, 2003).

The coefficient of determination in calibration (R^2_C) and validation ($1 - VR$, where $VR = \text{variance ratio}$) are calculated as the square of the correlation coefficient between the reference values and their corresponding predicted values obtained using calibration and validation sets. The coefficient of determination in validation depends on the validation technique: (1) use of an external data respect to calibration set or (2) cross-validation on calibration data according to Stone (1974).

The standard error is essential to determine the ability of the calibration equation to predict unknown values as good calibration equations have small standard errors. Three types of standard error exist: (1) standard error of calibration, which is obtained by testing the calibration equation directly on calibration data and it is usually a highly over-optimistic estimate of the prediction ability; (2) standard error of external validation (**SEP**), which is based on splitting the data set into 2 subsets, one for calibration and the other for validation; and (3) standard error of cross-validation, based on the previously described cross-validation technique (Williams and Norris, 2001; Sivakesava and Irudayaraj, 2002; Williams, 2003). During the development of calibration models, the SEP is used for determining the optical number of components (#L); usually the SEP is large when #L is low and decreases as #L increases. Partial least squares models allow the selection of appropriate #L to model as much of the complexity of the system without overfitting the data (Haaland and Thomas, 1988; Nas et al., 2002). The relative ability of prediction is defined as a variant of the correlation coefficient and it aims at correcting the standard correlation for the variance of error in the calibration set (Hildrum et al., 1983).

The RPD, RER, RPE, and CCC provide information on the practical utility of prediction models and allow the comparison of models among different studies. The RER is calculated by dividing the range of a given parameter by the standard error in validation; models with RER values lower than 3 have little practical utility, values between 3 and 10 have limited to good practical utility, and values greater than 10 have high utility (Williams, 1987). The RPD is calculated by dividing the standard deviation and standard error in

validation of a given trait; values of RPD greater than 10 are considered equivalent to reference methods, whereas values larger than 2 are considered adequate for analytical purposes (Karoui et al., 2006). As reported by Fuentes-Pila et al. (1996) and Lopez-Villalobos et al. (2009), RPE values lower than 10% indicate satisfactory predictions, from 10 to 20% indicate relatively acceptable predictions, and larger than 20% indicate poor predictions. Regarding the CCC, values from 0.81 to 1.00 indicate perfect prediction ability, from 0.61 to 0.80 substantial prediction ability, from 0.41 to 0.60 moderate prediction ability, and from 0.21 to 0.40 fair prediction ability (Lin, 1989; Lopez-Villalobos et al., 2009).

FA COMPOSITION OF MILK

Milk fat and FA composition are important quality traits, as they influence the sensory attributes, technological properties, and nutritional value of milk, and are related to human health. Bovine milk contains 70% SFA, 25% MUFA, and 5% PUFA (Grummer, 1991). The daily intake of SFA from dairy products has great relevance for the consumer (Chilliard et al., 2001) and the effects of variation of SFA, MUFA, and PUFA content in the diet on human health have been widely described (Mensink and Katan, 1992; German et al., 2009), along with the role of conjugated linoleic acid. Furthermore, FA composition influences the technological traits of butter, such as the spreadability (MacGibbon and McLennan, 1987). Therefore, feeding and breeding strategies to favourably alter the FA composition of bovine milk could be very beneficial. Recently, Lopez-Villalobos (2012) reviewed the genetic basis of FA and reported moderate heritability for FA, suggesting that the improvement of these traits through selection is feasible.

In recent years, several authors have attempted to predict FA and groups of FA (**GFA**) using MIRS (Table 1). Studies were conducted using (1) different numbers of samples, (2) different spectra pretreatments, (3) different reference methods, and (4) different units of measures of FA and GFA. The number of milk samples used to build prediction models for FA and GFA ranged from 49 to 3,622 (Table 1) and influenced the PLS analysis, in particular the validation procedures (cross-validation vs. independent validation). The cross-validation procedure has been used for a limited number of samples, as reported by Soyeurt et al. (2006, 2008) and De Marchi et al. (2011); however, independent validation is more commonly used (Rutten et al., 2009; Ferrand et al., 2011; Soyeurt et al., 2011; Maurice-Van Eijndhoven et al., 2013). These studies were conducted on individual milk samples, mostly collected on different cow breeds; only De Marchi et al. (2011) used milk samples from a single breed and

reported difficulties in developing MIRS prediction models for several FA due to the limited range of variation of these compounds.

Concerning the methods of spectra preprocessing, most studies have been conducted on untreated spectral data or, to a less extent, using first-derivative spectra pretreatment. Soyeurt et al. (2011) and De Marchi et al. (2011) investigated the variation in the accuracy of MIRS prediction models using different mathematical pretreatments; they reported quite similar results, with better accuracies of prediction models using derivative pretreatment with respect to untreated spectral data.

The reference method for the assessment of FA composition is based on 2 major phases: fat extraction and gas chromatography analysis. The extraction procedure has been conducted using 3 methods and gas chromatography using very different columns and analytical conditions (Table 1). Because calibration models were carried out using different spectral data, statistical procedures, and gold standard methods, the effect of reference method on the accuracy of MIRS prediction models is not determinable.

The unit of measure of FA and GFA is another crucial point in the development of accurate MIRS prediction models. Most studies expressed FA and GFA as the quantity per unit of milk, and only Soyeurt et al. (2006) and Rutten et al. (2009) predicted FA composition for FA expressed as quantity of total fat (Tables 2, 3, and 4). Both studies reported that, on average, the accuracy of MIRS prediction models were better when FA and GFA were expressed as quantity per unit of milk. This was also confirmed by De Marchi et al. (2011), who reported that the prediction of FA using MIRS is the combined effect of predicting fat content and fat composition. The prediction of FA is performed on milk samples, whereas reference methods for FA determination are performed on fat extracted from milk, which means that their relationship is affected also by the variation in fat percentage. Therefore, we will hereafter focus on prediction models developed on FA and GFA expressed per unit of milk. The difficulty of MIRS to predict FA when they are expressed on a fat basis represents a limitation in the application of this technique; in fact, this information is essential when the interest is to change only the fat composition (e.g., through selection). This situation represents a critical point also in the case of protein, as described in the following section and as previously reported by Rutten et al. (2011).

The accuracies of prediction models for SFA from different studies are reported in Table 2. Overall, prediction models for C4:0, C6:0, C8:0, C10:0, C12:0, C14:0, and C16:0 showed high accuracy. Stearic acid (C18:0) had quite good predictability by MIRS, with 1 –

VR from 0.09 to 0.88. Only De Marchi et al. (2011) have attempted to predict C20:0, but with unsatisfactory results (1 – VR of 0.29). Prediction models achieved better results for SFA than unsaturated FA. Mid-infrared spectroscopy showed quite good potential to predict *cis*-9 C14:1 (1 – VR from 0.07 to 0.53), *cis*-9 C16:1 (1 – VR from 0.28 to 0.65), *trans*-11 C18:1 (1 – VR from 0.31 to 0.63), and *cis*-9,*cis*-12,*cis*-15 C18:3 (1 – VR from 0.14 to 0.60; Table 3). Very good results were obtained for C18:1 (1 – VR from 0.53 to 0.91) and *cis*-9 C18:1 (1 – VR from 0.53 to 0.95). Only 3 studies have attempted to predict linolenic acid (*cis*-9,*cis*-12,*cis*-15 C18:3), obtaining low to medium accuracy of prediction. Mid-infrared spectroscopy showed medium potential to predict the 2 isomers of conjugated linoleic acid (*cis*-9,*trans*-11 C18:2 and *cis*-9,*cis*-12 C18:2; Table 3), with 1 – VR from 0.07 to 0.71 and a wide variability of accuracy of prediction models.

Regarding the GFA, MIRS prediction models highlighted very good accuracies; SFA and MUFA achieved 1 – VR up to 0.85 (Soyeurt et al., 2006, 2011; Ferrand et al., 2011; Maurice-Van Eijndhoven et al., 2013), with the exception of De Marchi et al. (2011), who reported 1 – VR of 0.52 and 0.55 for SFA and MUFA, respectively. Polyunsaturated FA were not very well predicted by MIRS (1 – VR from 0.10 to 0.41); only Soyeurt et al. (2011) found high 1 – VR for PUFA (0.81). Regarding the short-chain, medium-chain, and long-chain FA, results were often very satisfactory, with 1 – VR up to 0.90 (Table 4). The notably better results obtained by Soyeurt et al. (2011) could be related to (1) huge biological variability of calibration data (milk samples were collected from different breeds, countries, and production systems) and (2) statistical procedures that used several different mathematical pretreatments of spectral data.

The accuracies of MIRS prediction models for major FA were better than for minor FA; the relationship between FA content in milk and accuracy of prediction was discussed by Soyeurt et al. (2006), Rutten et al. (2009), De Marchi et al. (2011), and Maurice-Van Eijndhoven et al. (2013). In particular, Rutten et al. (2009) and De Marchi et al. (2011) reported a strong relationship of FA concentration with 1 – VR and the RPD (which is the ratio of the standard deviation to standard error of validation of the trait; Williams, 2001).

The accuracy of prediction models can be affected by the characteristics of the data set; in particular, breed of cows, stage of lactation, and season of sampling are the main aspects to take into consideration during sample collection (Rutten et al., 2009; Maurice- Van Eijndhoven et al., 2013). Moreover, as reported by Soyeurt et al. (2011), the variation of MIRS prediction accuracy is not only related to FA concentration but also to spectra

variability, the maximization of which is an effective way to improve the accuracy of prediction.

In general, MIRS has widely demonstrated its potential to predict FA and GFA. Nevertheless, milk composition of calibration data set plays a fundamental role in building good prediction models. The development of MIRS prediction equations using milk selected from different breeds, countries, and seasons, and the use of the same reference method seem the best way to improve the accuracy and robustness of MIRS calibrations.

MILK COAGULATION PROPERTIES AND ACIDITY

The volume of milk destined for cheese manufacturing is growing worldwide and, thus, the characteristics of milk related to cheese making, and cheese yield and quality are relevant for the dairy industry. Milk coagulation properties, acidity, and protein composition are important actors in cheese production (Aleandri et al., 1989; Wedholm et al., 2006; De Marchi et al., 2009a; Pretto et al., 2013).

Milk clotting characteristics affect the efficiency of the cheese-making process. Milk with good aptitude to coagulate after rennet addition and to form a firm curd has been associated with increased cheese yield compared with milk that poorly reacts to the presence of the enzyme (Bynum and Olson, 1982; Riddell-Lawrence and Hicks, 1989). Common measures of MCP are rennet coagulation time (**RCT**; min), curd-firming time (k_{20} ; min), and curd firmness (a_{30} ; mm). Several studies reported across-breed (Auldist et al., 2002, 2004; De Marchi et al., 2007, 2008) and genetic variation of MCP, measured by reference mechanical methods (Ikonen et al., 2004; Tyrisevä et al., 2004; Cassandro et al., 2008) and predicted by MIRS (Cecchinato et al., 2009).

Prediction of MCP by MIRS has been investigated by Dal Zotto et al. (2008) and De Marchi et al. (2009b, 2013; Table 5). Dal Zotto et al. (2008) and De Marchi et al. (2009b) analysed individual cow milk samples using the computerized renneting meter (Polo Trade, Monselice, Italy) as reference method, and they developed MIRS prediction models for RCT and a_{30} , and De Marchi et al. (2013) developed MIRS prediction models for MCP (including k_{20}) of samples that coagulated beyond 30 min from rennet addition, using the Formagraph (Foss Electric A/S, Hillerod, Denmark) as a reference instrument. The best calibration models were developed by De Marchi et al. (2013); according to Table 5, 1 – VR were 0.76, 0.72, and 0.76; the root mean square error of cross-validation (**RMSEcv**) were 7.05 min, 3.54 min, and 7.68 mm; and the RPD were 2.03, 1.86, and 1.80 for RCT, k_{20} , and a_{30} , respectively.

Results of De Marchi et al. (2013) on an extended testing time of analysis (60 min instead of the typical 30 min) showed notably better prediction models for RCT and a_{30} than those reported by Dal Zotto et al. (2008) and De Marchi et al. (2009b; Table 5). Moreover, De Marchi et al. (2013) highlighted the potential of MIRS to predict k_{20} , which is considered a trait of great practical importance in the dairy industry, as it suggests the optimal time at which curd-cutting should commence and, thus, it is related to product yield and quality (Bynum and Olson, 1982). Nevertheless, the comparison of papers in the literature is difficult because of different reference methods and the different types and concentrations of rennet used. The role of different methodologies in the assessment of MCP was investigated by Pretto et al. (2011), who proposed a method for the transformation of MCP obtained from various methodologies, and by Cipolat-Gotet et al. (2012), who compared MCP determined by Formagraph and by an optical instrument.

The prediction model for curd firmness 60 min after rennet addition was not completely satisfactory, as 1 – VR, RMSEcv, and RPD for this trait were 0.40, 7.26 mm, and 1.26, respectively (De Marchi et al., 2013). The low accuracy of prediction might be related to low accuracy of reference analysis for late-coagulating milk samples. This was previously reported by Cipolat-Gotet et al. (2012), who found large variability for curd firmness measured 45 min after rennet addition.

Finally, in most studies MIRS prediction models were developed using untreated spectral data. This consisted with the results reported by De Marchi et al. (2009b) who investigated the effect of different spectra pretreatments for the prediction of MCP, showing better accuracies using untreated spectral data with respect to first- or second-derivative pretreatments.

Concerning milk acidity, 2 main measures could be identified: pH and titratable acidity (**TA**). Both traits are very important in cheese production. Titratable acidity is related to the aggregation rate of paracaseinate micelles, the reactivity of rennet, and the rate of syneresis; usually, milk with low values of TA (hypoacid milk) is considered unsuitable for cheese making (Formaggioni et al., 2001). The pH of milk affects enzymatic and aggregation reactions. De Marchi et al. (2009b) evaluated the potential of MIRS to predict TA and pH (Table 5); results were quite satisfactory, as 1 – VR were 0.59 and 0.66, and RMSEcv were 0.07 and 0.25 Soxhlet-Henkel degrees ($^{\circ}\text{SH}$)/50 mL for pH and TA, respectively. Quite similar results were found by Colinet et al. (2010), who predicted TA with 1 – VR greater than 0.90 and RPD of 3.13.

Overall, MIRS is a valid tool for predicting MCP and milk acidity of bovine milk and, thus, it might be adopted in payment systems to reward or penalize producers of milk, according to its clotting characteristics, as well as for breeding purposes (Tiezzi et al., 2013; Penasa et al., 2014).

MILK PROTEIN COMPOSITION

Milk proteins have gained interest in dairy organizations worldwide mainly for their role in cheese production. Several studies have dealt with the effects of milk protein polymorphisms, in particular those of casein, on MCP (Comin et al., 2008; Heck et al., 2009; Penasa et al., 2010) and cheese yield (Wedholm et al., 2006; Bonfatti et al., 2011). Furthermore, milk proteins are relevant for some aspects related to human nutrition, such as the release of peptides with biological function (Caroli et al., 2009). Lactoferrin is one of these proteins and it can be found in most biological fluids (e.g., colostrum, milk, and blood). Lactoferrin is important for immune system maintenance, as it has antibacterial, antifungal, and antiviral properties (Farnaud and Evans, 2003; Baker and Baker, 2005).

Heritability estimates of individual milk proteins are moderate to high, suggesting wide opportunity to alter the composition in cow milk using breeding if individual measurements of milk protein fractions are available on a large scale (Lopez-Villalobos, 2012).

Mid-infrared prediction models for protein, casein, and casein fractions are shown in Table 6. Only De Marchi et al. (2009a), Bonfatti et al. (2011), and Rutten et al. (2011) developed prediction models for casein fractions, whereas Luginbuhl (2002), Sorensen et al. (2003), and Etzion et al. (2004) reported predictions only for casein and protein contents. As in the case of FA composition, reference methods play a basic role in the development of prediction models for milk protein composition. Studies that dealt with the use of MIRS to predict protein and casein contents referred to the Kjeldahl analytical method (Sorensen et al., 2003), and they reported excellent 1 – VR and RMSEcv of 0.94 to 0.97, and 0.08 to 0.05 g/100 g of milk, respectively (Table 6). On the other hand, MIRS models for casein and whey fractions measured using capillary zone electrophoresis or reversed-phase HPLC as reference methods showed moderate predictive ability (De Marchi et al., 2009a; Bonfatti et al., 2011; Rutten et al., 2011). On average, casein fractions were better predicted when the reference method was HPLC than capillary zone electrophoresis. We can hypothesize that the accuracies of gold standard methods play a key role in the development of infrared prediction

models. Moreover, in this specific case and as reviewed by Recio et al. (1997), HPLC methods showed better accuracies with respect to capillary zone electrophoresis in the determination of protein composition.

The best prediction models were described by Bonfatti et al. (2011), with 1 – VR of 0.66, 0.49, 0.53, 0.63, and 0.40 for α S1-CN, α S2-CN, β -CN, κ -CN, and γ -CN, respectively, whereas no satisfactory results were obtained by Rutten et al. (2011), who reported 1 – VR of 0.18 and 0.28 for α S1-CN and κ -CN, respectively. De Marchi et al. (2009a) showed slightly lower accuracies compared with Bonfatti et al. (2011), analysing similar data and using the same reference methods. Bonfatti et al. (2011) tried to predict casein fractions changing the unit of measurement of the traits (percentage of protein or casein); results were not satisfactory compared with those obtained from traits expressed per unit of milk and this confirms previous findings for FA.

Regarding the total whey protein and whey protein fractions, very similar results were obtained by De Marchi et al. (2009a), Bonfatti et al. (2011), and Rutten et al. (2011), with 1 – VR, on average, of 0.55, 0.35, and 0.55 for total whey protein, α -LA, and β -LG, respectively (Table 7). As with casein fractions, MIRS predictions of whey fractions were better when traits were expressed per unit of milk. Concerning the spectra pretreatments, the scientific literature reported contradictory results; in fact, De Marchi et al. (2009a) and Rutten et al. (2011) used untreated spectral data, whereas Bonfatti et al. (2011) used several preprocessed spectra methods.

According to Table 7, 1 – VR values for predicted lactoferrin would be 0.66, 0.73, and 0.75 in Lopez-Villalobos et al. (2009), Soyeurt et al. (2012), and Soyeurt et al. (2007), respectively. The prediction models were developed using 2 gold standard methods: (1) a commercial ELISA kit in the case of Soyeurt et al. (2007, 2012) and (2) HPLC in the case of Lopez-Villalobos et al. (2009). The large number ($n = 2,499$) and the origin (3 countries) of milk samples, combined with first derivative pretreatments, were probably responsible for the better prediction of lactoferrin from Soyeurt et al. (2012) compared with the other studies.

In general, results of the reviewed studies indicate that MIRS cannot predict milk protein composition with high accuracy and, hence, the prediction models are not currently suitable for the dairy industry (e.g., milk payment system).

COW HEALTH AND ENERGY STATUS

Mid-infrared spectroscopy has been studied as potential tool to predict several milk traits related to cow health and robustness, which are closely related to cow fertility and production. This is the case for ketosis, a metabolic disorder that affects high-producing cows and causes a loss of production and infertility. Ketosis is related to the abnormal increase of acetone and BHBA in blood and milk, and the potential to predict the ketone bodies using MIRS has been tested by several authors. Hansen (1999) obtained 1 – VR and RMSEcv of 0.81 and 0.27 mM, respectively, for acetone content of bovine milk; these results were useful for screening purposes (healthy vs. potential ketotic cows). Heuer et al. (2001) studied the ability of MIRS to predict acetone content and found greater 1 – VR compared with Hansen (1999), a specific relationship between 1,370 and 1,239 cm⁻¹ wavelengths and acetone prediction, and an advantage in the accuracy of prediction related to second-derivative spectra pretreatment. The results reported by Hansen (1999) and Heuer et al. (2001), which allowed the screening of cows with subclinical ketosis, were confirmed by the calibration models developed by de Roos et al. (2007). de Roos et al. (2007) investigated the potential of MIRS to predict acetone and BHBA concentrations on 1,080 bovine samples and reported RMSEcv of 0.184 and 0.064 mM for acetone and BHBA, respectively. The ability of MIRS to predict acetone and BHBA concentrations was also demonstrated by van Knegsel et al. (2010), who reported that cow hyperketonemia could be better predicted using models developed for acetone and BHBA contents than using fat-to-protein ratio.

The negative energy balance, typical of dairy cows, especially in early lactation (Berry et al., 2006, 2009), is known to be related to animal health and fertility (Beam and Butler, 1999). Several studies estimated genetic association between negative energy balance and animal health (Collard et al., 2000; Veerkamp et al., 2000), and proposed energy balance predictors such as change in BCS, milk fat-to-protein ratio, and FA composition of milk (de Roos et al., 2007; Roche et al., 2009; Stoop et al., 2009).

McParland et al. (2011) investigated the feasibility of using the MIRS spectrum as an indicator of body energy status in Holstein cows; the authors found quite satisfactory accuracies for direct energy balance, with 1 – VR from 0.50 to 0.56. However, the prediction models were developed using data from a single herd and 2 diets that differed only for the level of concentrates offered, and this might have affected the variability of data. McParland et al. (2012) went on to evaluate the ability of MIRS to predict body energy status across the United Kingdom and Ireland in both confinement and grazing systems. The accuracies of

prediction models were quite satisfactory, with square root of the coefficients of multiple determination from 0.47 to 0.69, 0.51 to 0.56, and 0.76 to 0.80 for direct energy balance, body energy content, and energy intake, respectively. Moreover, in the same study, McParland et al. (2012) highlighted the importance of limiting the error on the reference measurements to obtain a satisfactory equation. In conclusion, MIRS prediction models developed by McParland et al. (2011, 2012) provided useful information on the energy status of cows to dairy farmers; in fact, through routine recording of these traits, changes in the energy status of cows could be related to herd management practices or cow individual characteristics (e.g., genetic merit).

MINERAL COMPOSITION OF MILK AND OTHER TRAITS

Minerals in milk play a key role for human health and for some technological aspects (e.g., coagulation process). Recently, Caroli et al. (2011) reviewed the relationships between the intake of several milk and dairy products and bone health (e.g., osteoporosis), with particular emphasis to calcium and other macro- and micronutrients. Calcium associated with phosphorus influenced the ability of milk to coagulate and affected the final consistency of coagulum (Fossa et al., 1994; Mariani et al., 1996).

As for the previously reviewed traits, the opportunity to predict mineral composition using MIRS is desirable, as it allows rapid and large-scale data recording. Only Soyeurt et al. (2009) investigated the potential of MIRS to predict the calcium, phosphorus, magnesium, sodium, and potassium content of cow milk based on the inductively coupled plasma atomic emission spectrometry gold method. Results showed the ability of MIRS to predict calcium and phosphorus (1 – VR of 0.87 and 0.85, respectively), reasonable accuracies for magnesium and sodium (coefficient of determination in cross-validation of 0.65), and unsatisfactory results for potassium. Moreover, Soyeurt et al. (2009) reported that inductively coupled plasma atomic emission spectrometry without mineralization was an inappropriate method to determine the sodium concentration in milk.

Recently, Toffanin and De Marchi (2013) investigated the effectiveness of MIRS to predict calcium and phosphorus, and the relations of these traits with MCP. Milk samples of about 200 Holstein-Friesian cows were collected and MIRS spectra and reference values obtained by the inductively coupled plasma atomic emission spectroscopy method were recorded. Statistical analysis using external and cross-validation procedures showed quite

satisfactory results, with $1 - VR$ ranging from 0.50 to 0.60 and from 0.67 to 0.80 for calcium and phosphorus, respectively.

Mid-infrared spectroscopy has also been used to predict hydrochloride tetracycline concentration in milk (Sivakesava and Irudayaraj, 2002); those authors reported very high accuracy of prediction using a limited range of tetracycline concentration (4 to 2,000, 520 to 2,000, and 4 to 520 ppb) and first-derivative spectra pretreatment. More recently, MIRS has been used to predict methane emission of dairy cows (Dehareng et al., 2012) on a limited number of animals fed 2 diets to induce large variation in methane emissions, measured using the sulphur hexafluoride method. Prediction models were developed using the average daily milk spectrum that was collected at 5 different times over 5 d from each diet to find the best relationship between methane emissions and spectra information. The best results were obtained for spectra collected for 1.5 d after methane determination, with $1 - VR$ of 0.79. Moreover, an interesting relationship between FA and methane emissions was confirmed. Dehareng et al. (2012) suggested the use of MIRS models for screening purposes; however, the application of MIRS to predict methane emission needs to be further studied.

CONCLUSIONS AND PERSPECTIVES

Mid-infrared spectroscopy is a fast, large-scale, and low-cost methodology for collecting phenotypes. Its potential to predict milk quality traits (e.g., FA composition, MCP, and mineral content) and other milk characteristics related to cow health and energy status has been demonstrated. In the near future, MIRS could be used for the prediction of other milk traits: (1) potassium, magnesium, and zinc content, which are important for transmitting nerve impulses, for mineral structure of bones, for wound healing, and healthy immune systems; (2) phospholipids and acidic glycolipids, which are important for infant development; (3) vitamins A and B, which are important for healthy eyes and skin; (4) sensory features, which are important for the characterization of milk taste, beyond its nutritional value; (5) cheese yield; and (6) whey components, such as glutathione, α -tocopherol, and vitamin C.

Several studies on the effect of spectra pretreatment and type of software are needed. From this point of view, the effect of the use of mathematical pretreatments is not completely known, especially when prediction models are developed for a new trait for which the success of calibration models is related to several spectra peaks. Moreover, the potential of different statistical software packages should be investigated; in fact, no studies have been conducted to compare the performance of chemometric with general statistics software.

Efforts should be made to transfer prediction models for new phenotypes to MIRS instruments available in milk laboratories to allow routine data recording at the population level. To facilitate the implementation of MIRS models in field conditions, close collaboration with companies producing MIRS instruments is recommended.

Another crucial point for improving the use of MIRS is to favor the exchange of spectra databases among countries to develop across-country MIRS prediction models that take into account the biological variability of the studied traits in different environmental conditions. To do this, standardized reference methods within and across country are needed. As reviewed in this paper, large variability of reference methods used for MIRS calibration exists, and this does not facilitate the exchange and comparison of predicted phenotypes from different countries.

Finally, MIRS allows the recording of many new phenotypes that can be used for breeding purposes. The application of MIRS predictions in breeding programs depends upon the genetic correlation between the predicted and measured values. If the correlation and the genetic variance of MIRS phenotypes are sizable with reasonable accuracies, practical utility exists in the MIRS models.

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Figure 1. Example of untreated, first derivative, and second derivative spectra.

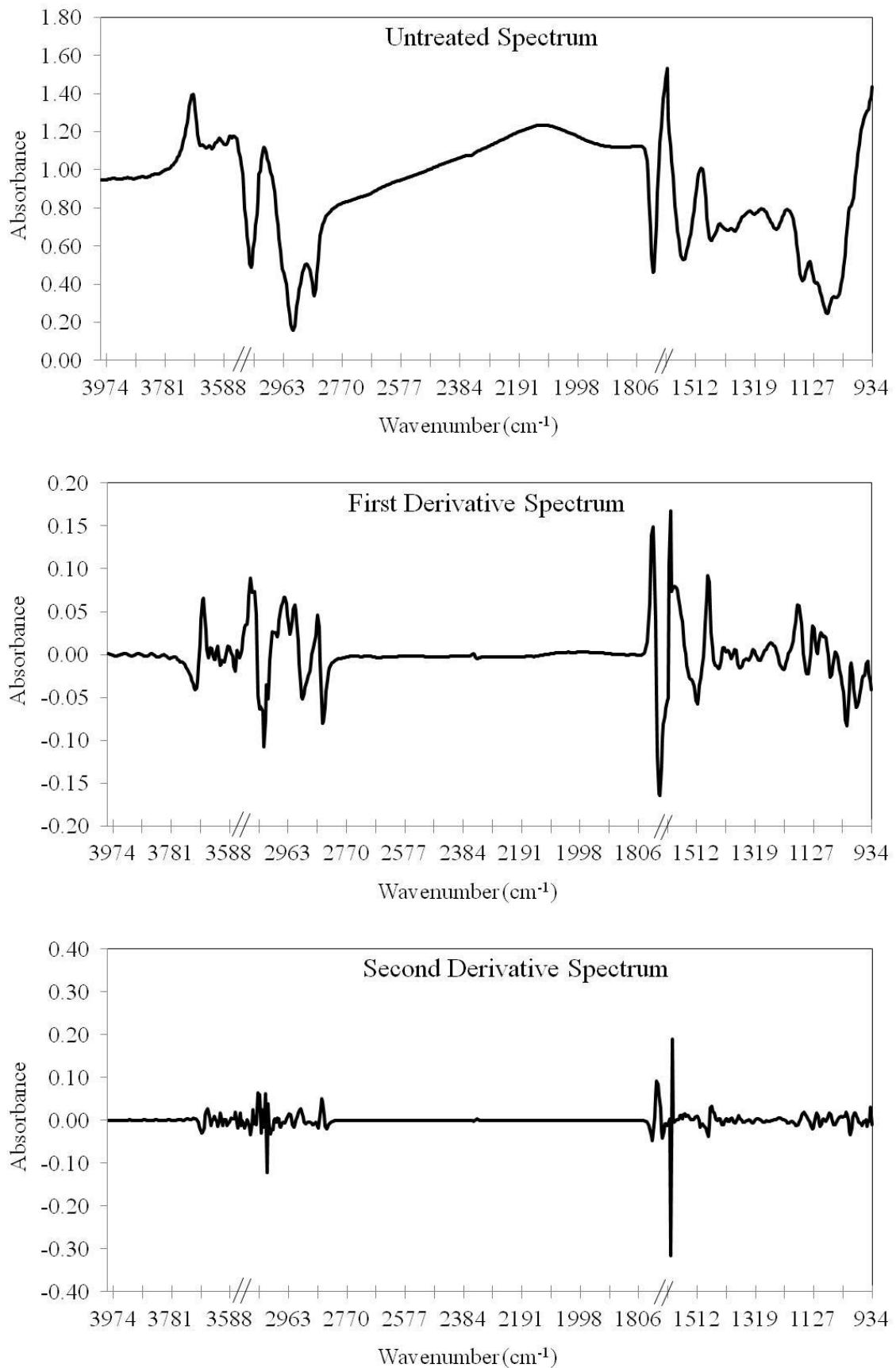


Figure 2. Published papers (retrieved from ISI Web Of Science; <http://thomsonreuters.com/web-of-science/>) on mid-infrared spectroscopy (MIRS) and milk. For 2013, papers published up to October are reported.

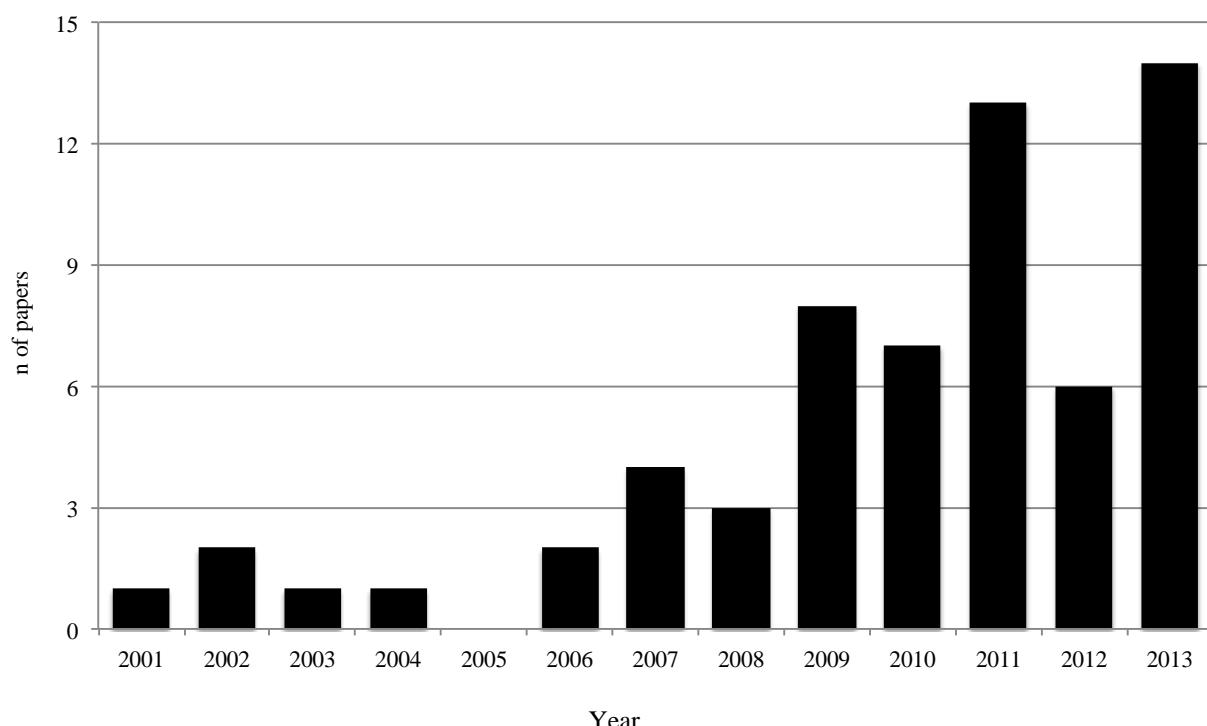


Table 1. Number of calibration samples, number of breeds, reference method, and validation procedures used in the studies aimed at developing mid-infrared spectroscopy (MIRS) prediction models for milk FA

Reference	n ¹	Dairy breed	Reference method	Spectra pre-treatments ²	Validation procedure
Soyeurt et al. (2006)	49	Multibreed	ISO standard 14156:2001. Column: length of 50 m, internal diameter of 0.25 mm, film thickness of 0.20 µm. Average velocity of 35cm/s, flame-ionization detector at 265°C, temperature program from 40 to 250°C. ISO standard 14156:2001.	Untreated	Cross-validation
Soyeurt et al. (2008)	78	Multibreed	Column: length of 100 m, internal diameter of 0.25 mm, film thickness of 0.20 µm. Average velocity of 19 cm/s, flame-ionization detector at 255°C, temperature program from 60 to 225°C. ISO standard 15884.	Untreated	Cross-validation
Rutten et al. (2009)	3,622	-	Column: length of 100 m, internal diameter of 0.25 mm. Temperature held at 225°C for 5 min. Mele et al. (2009).	VSS of Hoskuldsson	External
De Marchi et al. (2011)	267	Single breed	Column: length of 100 m, internal diameter of 0.25 mm, film thickness of 0.20 µm, injector temperature set at 270°C, detector temperature set at 300°C, temperature program from 60 to 230°C. ISO standard 14156, IDF172.	1D, MSC+1D	Cross-validation
Ferrand et al. (2011)	250	Multibreed	Column: length of 100 m. Splitless injector at 250°C, flame detector at 250°C, temperature program from 70 to 215 °C. ISO standard 14156:2001.	Untreated	External
Soyeurt et al. (2011)	517	Multibreed	Column: length of 50 m, internal diameter of 0.25 mm, film thickness of 0.20 µm, average velocity of 19 cm/s, cold on-column injector, flame-ionization detector at 255°C, temperature program from 60 to 225°C. ISO standard 15884.	1D, 2D	External
Maurice-Van Eijndhoven et al. (2013)	1,236	Multibreed	Column: length of 100 m, internal diameter of 0.25 mm.	1D	External

¹Number of samples used for calibration models.

²VSS = variable selection strategy; 1D = first derivative; 2D = second derivative; MSC = multiplicative scatter correction.

Table 2. Unit of measurement, coefficient of determination, and prediction error (in parentheses) of validation procedures for mid-infrared spectroscopy (MIRS) prediction models of selected SFA

Reference	Unit	SFA								
		C4:0	C6:0	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	C20:0
Soyeurt et al. (2006)	g/dL of milk	0.51 (0.08)	0.52 (0.04)	0.59 (0.02)	0.64 (0.04)	0.74 (0.02)	0.82 (0.05)	0.82 (0.17)	0.69 (0.13)	-
	g/100g of fat	0.39 (1.60)	0.41 (0.98)	0.46 (0.50)	0.53 (0.90)	0.64 (0.53)	0.67 (1.14)	0.50 (3.50)	0.09 (2.77)	-
Soyeurt et al. (2008)	g/dL of milk	-	-	-	-	-	-	0.90 (0.05)	0.84 (0.17)	0.85 (0.10)
Rutten et al. (2009) ¹	g/dL of milk	0.91 (0.1)	0.96 (0.2)	0.94 (0.5)	0.92 (0.1)	0.85 (0.3)	0.94 (0.03)	0.94 (0.1)	0.82 (0.7)	-
	g/100g of fat	0.55 (0.0)	0.73 (0.3)	0.73 (0.6)	0.75 (0.2)	0.68 (0.3)	0.73 (0.3)	0.71 (0.0)	0.51 (1.2)	-
De Marchi et al. (2011)	g/kg of milk	-	-	-	0.55 (0.07)	0.53 (0.19)	0.56 (0.25)	0.59 (0.60)	0.49 (1.59)	0.42 (0.75)
Ferrand et al. (2011)	g/100mL	0.90 (0.005)	0.96 (0.002)	0.96 (0.002)	0.91 (0.006)	0.91 (0.007)	0.93 (0.015)	0.90 (0.058)	0.77 (0.033)	-
Soyeurt et al. (2011)	g/dL of milk	0.89 (0.01)	0.95 (0.01)	0.93 (0.00)	0.92 (0.01)	0.92 (0.01)	0.95 (0.03)	0.93 (0.08)	0.88 (0.06)	-
Maurice-Van Eijndhoven et al. (2013)	g/dL of milk	0.92 (0.012)	0.93 (0.006)	0.92 (0.005)	0.93 (0.019)	0.85 (0.036)	0.95 (0.039)	0.93 (0.192)	0.72 (0.132)	-

¹Prediction bias within parentheses.

Table 3. Unit of measurement, coefficient of determination, and prediction error (in parentheses) of validation procedures for mid-infrared spectroscopy (MIRS) prediction models of selected unsaturated FA

Reference	Unit	Unsaturated FA							
		<i>cis</i> -9 C14:1	<i>cis</i> -9 C16:1	C18:1	<i>cis</i> -9 C18:1	<i>trans</i> -11 C18:1	<i>cis</i> -9 <i>trans</i> -11 C18:2	<i>cis</i> -9,12 C18:2	<i>cis</i> -9,12,15 C18:3
Soyeurt et al. (2006)	g/dL of milk	0.07 (0.01)	0.65 (0.02)	0.88 (0.18)	-	-	0.07 (0.02)	0.62 (0.02)	0.14 (0.01)
	g/100g of fat	0.23 (0.28)	0.37 (0.37)	0.53 (3.99)	-	-	0.34 (0.37)	0.44 (0.11)	0.20 (0.20)
Soyeurt et al. (2008)	g/dL of milk	0.53 (0.01)	0.28 (0.03)	-	-	-	-	-	-
Rutten et al. (2009) ¹	g/dL of milk	-	-	-	0.92 (0.3)	0.63 (0.4)	0.58 (1.0)	0.36 (0.9)	0.45 (3.3)
	g/100g of fat	-	-	-	0.84 (0.5)	0.57 (0.6)	0.56 (1.1)	0.28 (0.6)	0.38 (2.8)
De Marchi et al. (2011)	g/kg of milk	0.46 (0.08)	0.36 (0.11)	-	0.53 (1.13)	0.31 (0.09)	0.34 (0.04)	-	-
Ferrand et al. (2011)	g/100mL of milk	-	-	0.91 (0.037)	0.91 (0.036)	-	-	0.65 (0.004)	-
Soyeurt et al. (2011)	g/dL of milk	-	-	-	0.95 (0.06)	-	0.63 (0.01)	0.71 (0.01)	0.60 (0.01)
Maurice-Van Eijndhoven et al. (2013)	g/dL of milk	-	-	-	-	-	-	-	-

¹Prediction bias within parentheses.

Table 4. Unit of measurement, coefficient of determination, and prediction error (in parentheses) of validation procedures for mid-infrared spectroscopy (MIRS) prediction models of selected groups of FA

Reference	Unit	FA category ¹					
		SFA	UFA	MUFA	PUFA	SC	MC
Soyeurt et al. (2006)	g/dL of milk	0.94 (0.20)	0.66 (0.34)	0.85 (0.22)	0.39 (0.04)	-	-
	g/100g of fat	0.63 (3.75)	0.63 (3.75)	0.52 (4.10)	0.10 (0.74)	-	-
Rutten et al. (2009) ²	g/dL of milk	-	-	-	-	0.95 ¹ (0.0)	0.97 ² (0.0)
	g/100g of fat	-	-	-	-	0.82 (0.3)	0.77 (0.1)
De Marchi et al. (2011)	g/kg of milk	0.52 (2.97)	0.50 (1.57)	0.55 (1.39)	0.41 (0.22)	-	0.53 (2.66) (1.94)
Ferrand et al. (2011)	g/100mL of milk	0.98 (0.038)	0.91 (0.043)	0.92 (0.040)	0.38 (0.008)	0.97 ³ (0.008)	-
Soyeurt et al. (2011)	g/dL of milk	0.99 (0.08)	0.97 (0.07)	0.97 (0.06)	0.81 (0.02)	0.95 (0.02)	0.96 (0.12) (0.12)
Maurice-Van Eijndhoven et al. (2013)	g/dL of milk	0.99 (0.078)	-	-	-	0.95 (0.028)	0.96 (0.190)

¹UFA = unsaturated FA; SC = short-chain FA; MC = medium-chain FA; LC = long-chain FA.

²Prediction bias within parentheses.

Table 5. Number of calibration samples, dairy breed, reference method, range, spectra pre-treatments, coefficient of determination (1-VR, where VR = variance ratio), and SE of cross-validation (SE_{CV}) of validation procedures for mid-infrared prediction models of milk coagulation properties and milk acidity

Reference	Trait ¹	n	Dairy breed	Reference method ²	Range	Spectra pre-treatments ³	1-VR	SE_{CV}
Dal Zotto et al. (2008)	RCT, min	158	Holstein-Friesian	CRM	9.20-25.8	1D	0.73	1.80
	a_{30} , mm				11.00-55.00	1D	0.45	5.49
De Marchi et al. (2009b)	RCT, min	1,064	Brown Swiss	CRM	4.40-29.30	Untreated	0.62	2.36
	a_{30} , mm				6.00-64.00	Untreated	0.37	6.86
De Marchi et al. (2013)	pH	350	Holstein-Friesian	FOR	5.88-7.03	1D, 1D+N	0.59	0.07
	TA, °SH/50mL				1.19-4.77	1D	0.66	0.25
De Marchi et al. (2013)	RCT, min	350	Holstein-Friesian	FOR	7.75-59.00	Untreated	0.76	7.05
	k_{20} , min				2.00-28.45	Untreated	0.72	3.54
	a_{30} , mm				0.36-51.30	Untreated	0.70	7.68
	a_{60} , mm				0.76-40.96	Untreated	0.40	7.26

¹RCT = rennet coagulation time; a_{30} = curd firmness 30 min after rennet addition; TA = titratable acidity; °SH = Soxhlet-Henkel degree; k_{20} = curd-firming time; a_{60} = curd firmness 60 min after rennet addition;

²CRM = computerized renneting meter (Polo Trade, Monselice, Italy); FOR = Formagraph (Foss Electric A/S, Hillerød, Denmark).

³1D = first-derivative; 1D+N = first derivative and normalization.

Table 6. Unit of measurement, coefficient of determination, and prediction error (in parentheses) of validation procedures for mid-infrared spectroscopy (MIRS) prediction models of total protein, total casein, and protein fractions

Reference	Spectra pre-treatments ¹	Unit	Protein						
			Protein	Casein	α_{S1} -CN	α_{S2} -CN	β -CN	κ -CN	γ -CN
Luginbühl et al. (2002)	-	g/100g of milk	-	0.997 (0.047)	-	-	-	-	-
Sørensen et al. (2003)	-	%	-	0.97 (0.035)	-	-	-	-	-
Etzion et al. (2004)	-	%	0.94 (0.08)	-	-	-	-	-	-
De Marchi et al. (2009a)	Untreated	g/L of milk	0.58 (3.11)	0.58 (2.76)	0.50 (1.07)	0.35 (0.58)	0.33 (1.77)	0.44 (0.68)	-
		g/L of milk	0.78 (2.13)	0.77 (1.91)	0.66 (0.89)	0.49 (0.48)	0.53 (1.37)	0.63 (0.55)	0.40 (0.10)
Bonfatti et al. (2011)	SNV, De, MSC, 1D, 2D	% protein % casein	- -	- -	0.23 (1.95) 0.20 (2.34)	0.17 (1.08) 0.19 (1.25)	0.13 (2.42) 0.16 (2.63)	0.36 (1.44) 0.36 (1.62)	0.08 (1.00) 0.09 (1.14)
Rutten et al. (2011)	Untreated	g/100g of milk	-	0.25 (1.50)	0.18 (1.52)	0.26 (1.20)	0.19 (1.42)	0.28 (0.49)	-

¹SNV = standard normal variate; De = detrend; MSC = multiplicative scatter correction; 1D = first derivative; 2D = second derivative.

Table 7. Unit of measurement, coefficient of determination, and prediction error (in parentheses) of validation procedures for mid-infrared spectroscopy (MIRS) prediction models of whey protein and selected whey protein fractions

Reference	Spectra pre-treatments ¹		Unit	Protein			
				Whey protein	α-LA	β-LG	Lactoferrin
Soyeurt et al. (2007)	Untreated		mg/L of milk	-	-	-	0.75 (103.93)
De Marchi et al. (2009a)	Untreated		g/L of milk	0.53 (0.51)	0.29 (0.19)	0.55 (0.43)	-
Lopez-Villalobos et al. (2009)	Untreated		mg/L of milk	-	-	-	0.81 ²
			g/L of milk	0.61 (0.45)	0.31 (0.18)	0.64 (0.37)	-
Bonfatti et al. (2011)	SNV, De, MSC,		% protein	-	0.31 (0.42)	0.42 (0.74)	-
	1D, 2D		% whey protein	-	-	0.36 (3.02)	-
Rutten et al. (2011)	Untreated		g/100g of milk	0.53 (0.84)	0.20 (0.29)	0.56 (0.79)	-
Soyeurt et al. (2012)	Untreated, rep, 1D, 1D+rep, 2D, 2D+rep		mg/L of milk	-	-	-	0.73 (50.55)

¹SNV = standard normal variate; De = detrend; MSC = multiplicative scatter correction; 1D = first derivative; 2D = second derivative; rep = repeatability file.

²Concordance correlation coefficient calculated according to Lin (1989).

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CHAPTER 2

Prediction of coagulating and noncoagulating milk samples using mid-infrared spectroscopy

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ABSTRACT

Over the last years, a general deterioration of milk coagulation properties (MCP) has been observed in Italy, and thus the prediction of noncoagulating (NC) milk, defined as milk not forming a curd within 30 min from rennet addition, is of immediate interest in the Italian cheese industry. The present study investigated the ability of mid-infrared (MIR) spectroscopy to predict NC milk using individual and bulk samples from Holstein cows. Samples were selected according to MIR analysis to cover the range of coagulation time between 5 and 60 min. Milks were then analysed for MCP through the reference instrument (Formagraph) over an extended testing-period of 60 min to identify coagulating and NC samples. Measured traits were rennet coagulation time, curd-firming time, and curd firmness 30 and 60 min after rennet addition. Results showed that there is no specific spectral information that distinguishes NC from coagulating samples. The most accurate prediction model was developed for rennet coagulation time followed by curd-firming time and curd firmness 30 min after rennet addition, whereas curd firmness 60 min after enzyme addition could not be accurately predicted. Based on these findings, MIR spectroscopy might be proposed in payment systems to reward or penalize milk according to MCP. Moreover, the ability of MIR spectroscopy to predict MCP of samples that form a curd beyond 30 min from enzyme addition may be of interest for genetic improvement of coagulation traits in dairy breeds, because until now most studies have excluded NC information from genetic analysis, leading to possible biases in the estimation of genetic parameters and in the prediction of sire's merit for MCP.

Key words: mid-infrared spectroscopy, milk coagulation property, Holstein Friesian, phenotyping

INTRODUCTION

The Italian dairy industry relies heavily on milk coagulation properties (**MCP**) as they influence the efficiency of cheese-making process (Pretto et al., 2013). Milk with good aptitude to coagulate after rennet addition and to form a firm curd has been associated with increased cheese yield compared with milk that poorly react to the presence of the enzyme (Bynum and Olson, 1982; Riddell-Lawrence and Hicks, 1989; De Marchi et al., 2008). During the last decades, MCP have been deteriorating in Italy (Sandri et al., 2001), with an elongation of the rennet coagulation time (**RCT**, min) and an increase of the percentage of noncoagulating (**NC**) milk, which has been conventionally defined as milk not forming a curd within the testing time of 30 min (e.g., Ikonen et al., 1999; Tyrisevä et al., 2003) or 31 min

(e.g., Ikonen et al., 2004; Cassandro et al., 2008; Penasa et al., 2010) from rennet addition. As the proportion of cows producing NC milk is increasing, a negative impact on profitability and efficiency of the dairy sector is expected. Noncoagulating milk is detrimental for the cheese industry and thus it is economically penalized in certain dairy chains, such as in Parmigiano-Reggiano and Trentingrana cheese production, where coagulation traits are determined by the Formagraph and are considered in milk quality payment system. Also, blending well-coagulating with NC (Frederiksen et al., 2011) or poorly coagulating (Okigbo et al., 1985b) milk compromises MCP of the resulting mix.

Several studies on MCP faced the problem of NC samples and investigated causes underlying their occurrence. Although neither environmental nor genetic factors can thoroughly explain the phenomenon, associations of NC milk with pH (Okigbo et al., 1985c; Tyrisevä et al., 2003), SCC and mastitis (Okigbo et al., 1985a), k-CN concentration and genotypes (Wedholm et al., 2006; Hallén et al., 2010; Frederiksen et al., 2011), and stage of lactation (Okigbo et al., 1985c; Tyrisevä et al., 2003; Ikonen et al., 2004) have been reported. Moreover, Harzia et al. (2012) found that the metabolic profile of NC milk was significantly different from that of well-coagulating milk, and thus a relationship between metabolic profile and MCP exists. The same authors compared NC and well-coagulating milk, and highlighted significant differences in terms of pH, SCS, and fat, protein, and urea contents.

Besides environmental factors, also breed and additive genetic effects seem to influence the occurrence of NC milk. There are marked differences in the proportion of NC samples among cattle breeds: for example, it was 8 to 13.2% in Finnish Ayrshire (Ikonen et al., 1999 and 2004; Tyrisevä et al., 2003 and 2004), 1.3 to 9.7% in Holstein-Friesian (Tyrisevä et al., 2004; Cassandro et al., 2008), and 0.3% in Estonian Holstein (Vallas et al., 2010). However, the comparison of the occurrence of NC samples from different breeds should be made with caution as the experimental conditions often differ among studies, particularly in terms of sampling procedures and analytical conditions, such as the use or not of preservative in the milk, the age of sample, the type of instrument (e.g., mechanical vs. optical), the type and activity of the coagulant, and the temperature of analysis (Pretto et al., 2011; Cipolat-Gotet et al., 2012). Evidence exist that additive genetic effects (Ikonen et al., 1999 and 2004; Tyrisevä et al., 2003 and 2004) and two potential candidate genes (Tyrisevä et al., 2008) are associated with the noncoagulation of milk and that there are large differences in the proportion of daughters with NC milk among sires. Although several studies discarded NC samples from the genetic analysis, the inclusion or not of NC milk information in statistical models aiming

at estimating covariance components for MCP has an influence on the assessment of genetic parameters for coagulation traits (Ikonen et al., 2004; Cecchinato et al., 2011).

Few studies have dealt with measures of MCP beyond the conventional time of 30-31 min from coagulant addition (Mariani et al., 1997; Auldist et al., 2002 and 2004; O'Brien et al., 2002; Frederiksen et al., 2011; Cipolat-Gotet et al., 2012). Extending the coagulation analysis beyond this threshold may notably reduce the proportion of NC milks as longer time is allowed for the sample to coagulate (Cipolat-Gotet et al., 2012). However, the use of traditional instruments (e.g., the Formagraph) to determine MCP is applicable only to small-scale experiments and not at population level.

Mid-infrared (**MIR**) spectroscopy has been recently evaluated as potential tool to collect MCP data at population level for phenotypic and genetic purposes (Dal Zotto et al., 2008; Cecchinato et al., 2009; De Marchi et al., 2009). De Marchi et al. (2009) discussed the prediction of RCT and curd firmness 30 min after rennet addition (a_{30} , mm) through MIR spectroscopy using coagulated samples of Brown Swiss cows, and found that models were most applicable for RCT. However, MIR spectroscopy predictions of individual samples may play an important role as indicator traits in selective breeding to enhance MCP. No studies are currently available in the literature that attempted to predict NC samples. Therefore, the aim of this work is to investigate the capability of MIR spectroscopy to predict coagulating and NC milk in dairy cows.

MATERIALS AND METHODS

Milk Samples Collection and Reference Analysis

In September 2011, prediction models for MCP were installed on Milko-Scan FT6000 (Foss Electric A/S, Hillerød, Denmark) in the laboratory of the Breeders Association of Veneto region (ARAV, Padova, Italy) and were used for routine analysis of MCP on samples collected during monthly test-day milk recording. Mid-infrared spectroscopy calibration models were developed by De Marchi et al. (2009, 2012) and exhibited coefficients of determination in cross-validation of 0.76 and 0.70 for RCT and a_{30} , respectively (De Marchi et al., 2012). The use of MIR spectroscopy models allowed the prediction of MCP only for samples that coagulated within 30 min from rennet addition.

To assess the capability of MIR technology to predict also NC samples, individual and bulk milks from Holstein Friesian cows were collected in the Veneto region from November 2011 to February 2012. Immediately after sampling, milks were treated with preservative

(Bronopol, Knoll Pharmaceuticals, Nottingham, UK), transferred to the Regional Laboratory of Breeder Association (ARAV, Padova, Italy), and selected according to MIR spectroscopy analysis. The aim of the selection process was to store samples (one time per week) that covered a wide range of RCT, from very early coagulation (i.e., within 10 min from enzyme addition) to very late coagulation (i.e., beyond 30 min from enzyme addition). As a result, spectral information from 200 individual milks from 5 dairy herds and 135 bulk milks were extracted from the MIR database and analysed for MCP within 2 to 5 d from collection through the reference method (Formagraph; Foss Electric, Hillerød, Denmark) over an extended testing-time of 60 min to allow the determination of coagulation characteristics of NC milks (Ikonen et al., 1999; Cassandro et al., 2008; De Marchi et al., 2009). Samples (10 mL) were heated to 35°C and 200 µL of a rennet solution (Hansen Standard 160; Pacovis Amrein AG, Bern, Switzerland) diluted to 1.6% (w/v) in distilled water was added at the start of analysis. Besides RCT and a_{30} , measured traits were curd-firming time (k_{20} , min; the time interval from the addition of rennet to the time at which the width of the graph attains 20 mm) and curd firmness 60 min after rennet addition (a_{60} , mm). All samples coagulated within 60 min from enzyme addition.

MIR Spectra Acquisition and Multivariate Data Analysis

Mid-infrared spectra were selected from stored data and were initially collected from 0.25 mL of sample over the spectral range of 4,000 to 900 cm⁻¹ using a Milko-Scan FT6000 (Fourier transform infrared interferometer; Foss Electric A/S, Hillerød, Denmark) within 4 h from reference analysis.

Prediction models were obtained from The Unscrambler software (version 9.6; Camo A/S, Oslo, Norway). To optimize the accuracy of the calibration, the data underwent (1) detection of anomalous spectra in the calibration dataset, accomplished using the Mahalanobis distance (Global H statistics, GH) to the centre of the population (Williams and Norris, 2001); samples with GH > 3 were considered outliers and were then removed from the calibration dataset, so that the final data available for investigation accounted for 319, 147, 224, and 178 samples for RCT, k_{20} , a_{30} , and a_{60} , respectively. (2) Different combination of scattering corrections and derivative mathematical treatments; normalized and multiplicative scatter corrected, first derivative (Savitzky-Golay, 3 data points each side), and normalized plus first derivative according to Williams (2001) and De Marchi et al. (2009). However, no significant improvement was found compared with the use of untreated spectra, as reported by De Marchi et al. (2009).

Statistical analysis was carried out using principal component analysis (**PCA**) and partial least squares (**PLS**) regression analysis. Spectral data subjected to PCA and PLS produces a new and smaller set of variables called principal components (**PC**) and loadings (#**L**), respectively. Principal component analysis provides graphical representations of similarities and differences across spectra, and was employed to investigate sample distribution (Martens and Naes, 1989), and to discriminate NC milks from samples that coagulated within 30 min from enzyme addition.

The optimal number of #**L** was calculated as the number of #**L** after which the standard error of cross-validation (**SEC_{CV}**) no longer decreased substantially. Prediction models were confirmed using cross-validation method by dividing the calibration dataset in 10 subgroups: 9 of them were used to construct the calibration model and 1 to check the results. This operation was repeated until all groups were treated both as calibration and prediction set. The effectiveness of calibration and validation models was assessed using the standard error of calibration, R^2 , **SEC_{CV}**, and the coefficient of determination of cross-validation (**1-VR**). According to Williams (2003) values of 1-VR between 0.50 and 0.65 indicate poor predictions; values between 0.66 and 0.81 indicate fairly accurate predictions; and values greater than 0.82 reveal good predictions.

The ratio performance deviation (**RPD**) and the range error ratio (**RER**) were calculated to provide indications on the practical utility of predictive models. The RPD is the ratio of the SD to **SEC_{CV}** of a given trait and RER is the ratio of the **SEC_{CV}** of the trait relative to the range of the reference data (Edney et al., 1994; Williams, 2001). High values of RPD are desirable; in particular RPD greater than 2 enables good predictions (Sinnaeve et al., 1994; De Marchi et al., 2011). The RER is a method for standardizing the **SEC_{CV}** by relating it to the range of the reference data. For example, RER values lower than 6 indicate very poor classification and are not recommended for any application; RER values between 7 and 20 classify the model as poor to fair and indicate that the model could be used for screening purposes; and RER values between 21 and 30 indicate a good classification suggesting that the model would be suitable for a role in a quality control application (Williams, 2001).

RESULTS AND DISCUSSION

MCP

Descriptive statistics of MCP are summarized in Table 1. Rennet coagulation time, k_{20} , a_{30} , and a_{60} averaged 30.55 min, 9.61 min, 26.97 mm, and 14.76 mm, respectively. The CV

ranged from 47 (RCT) to 68% (k_{20}), suggesting a wide degree of variability, which should facilitate the development of robust prediction models. The comparison with other studies is difficult because our results originated from selected NC samples. If milks with RCT longer than 30 min are removed from the dataset (data not shown), RCT and a_{30} averaged 16.51 min and 29.36 mm, respectively, which are similar to findings reported by Cassandro et al. (2008) in Italian Holstein cows. However, RCT of samples that coagulated within 30 min is longer than results from De Marchi et al. (2009) in Brown Swiss cows and Ikonen et al. (2004) in Finnish Ayrshire breed.

MIR Spectra and PCA Analysis of NC Milk Samples

Prediction models were obtained using modified spectra according to several authors (Pillonel et al., 2003; Dal Zotto et al., 2008; De Marchi et al., 2009 and 2011) who suggested omitting the spectral regions related to low signal-to-noise ratio. In the present study, the spectral regions 3,040 to 3,470 cm^{-1} and 1,600 to 1,700 cm^{-1} were found to have high noise levels and thus they were discarded prior to the analysis; these regions were related to the absorption of water as reported by Hewavitharana and Brakel (1997) and Jørgensen and Næs (2004). The analysed spectra showed specific peaks previously reported by De Marchi et al. (2009 and 2011): 1,550 to 1,570 cm^{-1} related to protein absorption (Curley et al., 1998; Etzion et al., 2004), 2,800 to 3,000 cm^{-1} , 2,855 to 2,928 cm^{-1} , and 1,805 to 1,736 cm^{-1} related to lipid absorption (Coates, 2000; Lefèvre and Subirade, 2000), and 900 to 1,500 cm^{-1} related to C-H bending (1,493 cm^{-1}) and C-O stretching (1,080 and 1,157 cm^{-1}).

Principal component analysis of the untreated spectra allowed the investigation of the influence plot and the identification of outliers. Milk samples did not show extreme values of leverage and residual variance, and thus none of them was classified as outlier. The score plot of first and second PC provided information on samples distribution; in particular, samples were classified in 2 categories, namely those with RCT longer than 30 min and those with RCT shorter than 30 min, with the purpose of investigating spectral information from well-coagulating and NC milks.

Principal components 1 and 2 explained 86 and 8% of the observed variation in the spectra, respectively (Figure 1). The plot clearly depicts the mixing of all samples, indicating that there is no specific spectral information related to well-coagulating and NC milks. This outcome was somewhat expected as the potential of MIR spectroscopy to predict MCP is related to specific protein and lipid peaks (De Marchi et al., 2009), and NC samples did not differ in chemical composition from well-coagulating milks. According to results from PCA,

a discriminant analysis (presence or absence of NC samples) is not feasible; the only way to rapidly predict NC samples is to develop MIR prediction models using milk with a wide range of RCT (from 30 to 60 min).

Prediction of Rennet Coagulation Time, Curd-firming Time, and Curd Firmness

Fitting statistics of prediction models for MCP using untreated spectra are in Table 2. Prediction models were developed using three MIR spectral regions: 900 to 1,600 cm^{-1} , 1,700 to 3,040 cm^{-1} , and 3,470 to 4,000 cm^{-1} . The number of modified PLS factors used in the calibration ranged from 12 (k_{20}) to 17 (a_{30}), which is slightly lower than that reported by De Marchi et al. (2009). The most accurate prediction model was developed for RCT (1-VR and SEC_{CV} of 0.76 and 7.05 min, respectively), followed by k_{20} (1-VR and SEC_{CV} of 0.72 and 3.54 min, respectively), and a_{30} (1-VR and SEC_{CV} of 0.70 and 7.68 mm, respectively). Models for the prediction of a_{60} were not satisfactory (1-VR of 0.40 and SEC_{CV} of 7.26 mm). Regarding the practical utility of the prediction models, RPD and RER ranged from 1.26 (a_{60}) to 2.03 (RCT) and from 5.53 (a_{60}) to 7.47 (k_{20}), respectively.

Only Dal Zotto et al. (2008) and De Marchi et al. (2009) assessed the ability of MIR spectroscopy to predict RCT and a_{30} , whereas no studies investigated the potential of MIR spectroscopy to predict MCP of samples that coagulate beyond 30 min from rennet addition. Prediction models for RCT and a_{30} were notably better than those reported by Dal Zotto et al. (2008) and De Marchi et al. (2009) in milk with preservative; the authors reported 1-VR for a_{30} that ranged from 0.55 to 0.73, and from 0.35 to 0.45, respectively. Nevertheless the reference method (Computerized Renneting Meter) used in these studies was different than that adopted in the present research (Formagraph). The reference method plays a key role in determining the accuracy of prediction models as reported by Rutten et al. (2009) and De Marchi et al. (2011) for milk fatty acids. Also, the rennet used by Dal Zotto et al. (2008) and De Marchi et al. (2009) was different than that adopted in the present work. The implementation of different methodologies to assess MCP was investigated by Pretto et al. (2011), who proposed a method for the transformation of values of MCP obtained from different methodologies, and by Cipolat-Gotet et al. (2012), who compared MCP determined with an optical instrument, the Optigraph, with MCP obtained from the Formagraph.

Results of the present study highlighted the potential of MIR spectroscopy to predict k_{20} . Curd-firming time is considered a trait of great practical importance in the dairy industry as it suggests the optimal time at which curd-cutting should commence, and thus it is related to product yield and quality (Bynum and Olson, 1982). However, k_{20} has not been much

studied in the past as it is available only for samples attaining 20 mm of curd firmness and thus not for NC milks. To our knowledge, this is the first study attempting to predict k_{20} for samples that coagulated beyond 30 min from enzyme addition using MIR spectroscopy. The worst prediction model developed in the present research was for a_{60} . The low accuracy of prediction might be related to low accuracy of reference analysis for NC samples. This was reported by Cipolat-Gotet et al. (2012) who found large variability for curd firmness measured 45 min after rennet addition.

The scatter plots of predicted vs. measured MCP are shown in Figure 2. A quite high degree of scatter was observed for a_{60} indicating that the current model is not enough accurate to be used in the dairy industry but demonstrates the potential of MIR spectroscopy to predict a_{60} .

To investigate the molecular basis of MCP, the loadings weight of the first, second, and third PLS components of prediction models were examined (Figure 3). There was considerable structure in the loading plots of all studied traits. In general, the pattern of loadings weight of RCT and k_{20} was specular to that of a_{30} and a_{60} . A number of peaks were observed in the fingerprint region from 900 to 1,500 cm⁻¹ (e.g., 992, 1,138, and 1,184 cm⁻¹) of the spectra and this might be attributable to C-O or C-C stretchings. Characteristic peaks were also observed from 1,543 to 1,593 cm⁻¹, which can be attributable to amide II. Peaks associated with lipids (from 2,828 to 2,970, and from 1,751 to 1,763 cm⁻¹) were also apparent in loadings 1, 2, and 3, as well as peaks from 1,238 and 1,265 cm⁻¹ and from 1,455 to 1,462 cm⁻¹, which may be attributable to O-C-H, C-C-H, or C-O-H bendings. The peaks associated with protein were found to be very dominant for RCT and k_{20} , whereas those associated with lipids seem to be more dominant for curd firmness traits. These results highlighted the role of protein and lipid regions of the MIR spectra to predict MCP, and coincided with findings of De Marchi et al. (2009) and of several authors who reported the effect of protein and fat content on milk coagulation (Bastian et al., 1991; Castillo et al., 2003).

CONCLUSIONS

Mid-infrared spectroscopy combined with PLS regressions has been proposed as potential tool to predict RCT, k_{20} , a_{30} , and a_{60} in bovine milk samples. Results indicated that the prediction performance of MIR technique was satisfactory for RCT measured up to 60 min from rennet addition, and for k_{20} and a_{30} , and thus it might be used in payment systems to reward or penalize milk according to MCP. The prediction of MCP in milk samples that

coagulate beyond 30 min from rennet addition (NC samples) may have a great importance in genetic evaluation of animals for MCP. In fact, until now MCP information from NC samples has been often discarded from genetic analysis, leading to possible biases in the prediction of sires merit for coagulation traits.

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Table 1. Descriptive statistics of milk coagulation properties

Trait ¹	n ²	Mean	SD	CV	Minimum	Maximum
RCT, min	319	30.55	14.30	0.47	7.75	59.00
k ₂₀ , min	147	9.61	6.58	0.68	2.00	28.45
a ₃₀ , mm	224	26.97	13.88	0.51	0.36	51.30
a ₆₀ , mm	178	14.76	9.18	0.62	0.76	40.96

¹RCT = rennet coagulation time of samples that coagulated within 60 min from enzyme addition; k₂₀ = curd-firming time of samples attaining 20 mm of firmness within 60 min from enzyme addition; a₃₀ = curd firmness 30 min after enzyme addition; a₆₀ = curd firmness 60 min after enzyme addition.

²n = number of samples used in the calibration after removing outliers.

Table 2. Fitting statistics¹ of prediction models for milk coagulation properties

Trait ²	#L	SEC	R ²	SEC _{CV}	1-VR	RPD	RER
RCT, min	15	6.14	0.82	7.05	0.76	2.03	7.28
k ₂₀ , min	12	2.94	0.80	3.54	0.72	1.86	7.47
a ₃₀ , mm	17	5.06	0.87	7.68	0.70	1.80	6.63
a ₆₀ , mm	12	6.20	0.56	7.26	0.40	1.26	5.53

¹#L = number of modified partial least squares factors used in the calibration; SEC = standard error of calibration; R² = coefficient of determination of calibration; SEC_{CV} = standard error of cross-validation; 1-VR = coefficient of determination of cross-validation; RPD = ratio performance deviation; RER = range error ratio.

²RCT = rennet coagulation time of samples that coagulated within 60 min from enzyme addition; k₂₀ = curd-firming time of samples attaining 20 mm of firmness within 60 min from enzyme addition; a₃₀ = curd firmness 30 min after enzyme addition; a₆₀ = curd firmness 60 min after enzyme addition.

Figure 1. Plot of first (PC-1) and second (PC-2) components from principal component analysis of spectral data. Triangles = samples with rennet coagulation time equal or longer than 30 min; Circles = samples with rennet coagulation time shorter than 30 min.

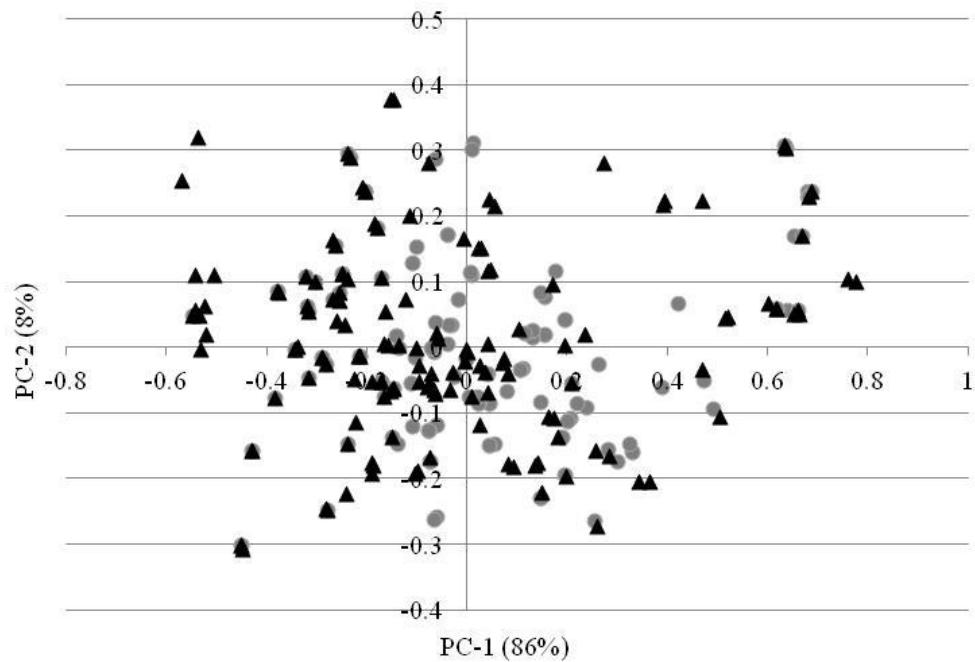
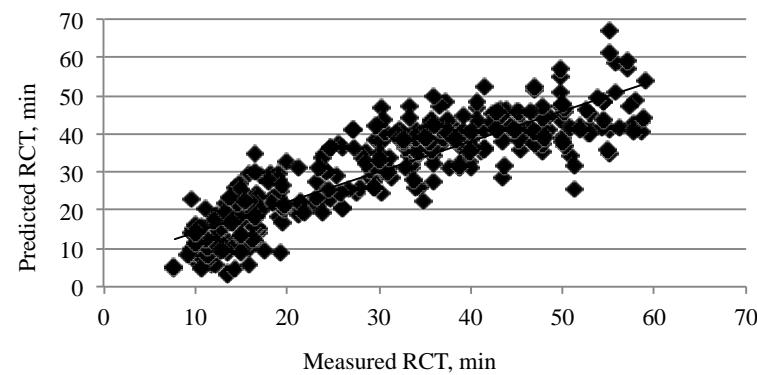


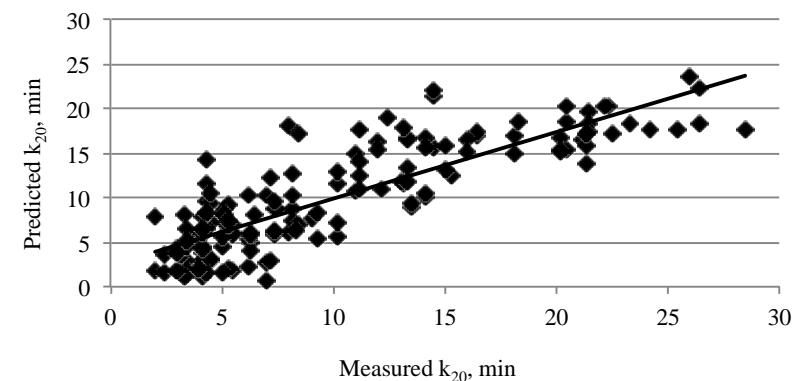
Figure 2. Scatter plots of predicted (y-axis) versus measured (x-axis) milk coagulation properties. (A) Rennet coagulation time of samples that coagulated within 60 min from enzyme addition (RCT); (B) curd-firming time of samples attaining 20 mm of firmness within 60 min from enzyme addition (k_{20}); (C) curd firmness 30 min after enzyme addition (a_{30}); and (D) curd firmness 60 min after enzyme addition (a_{60}).

L9

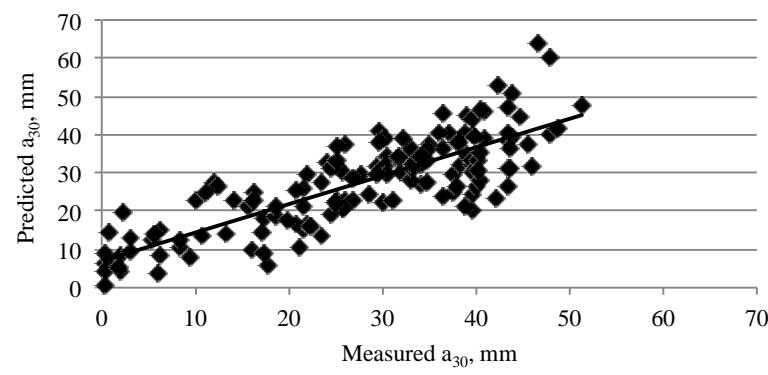
A



B



C



D

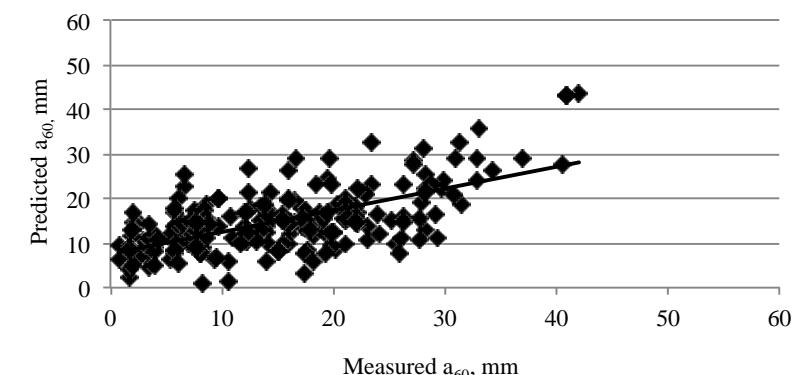
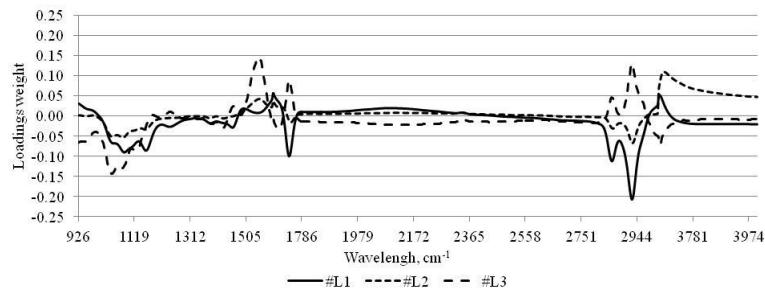
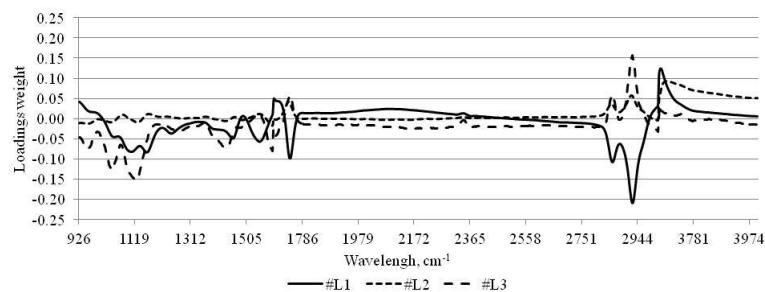


Figure 3. Loadings weight of the first (#L1), second (#L2), and third (#L3) partial least squares regression component for (A) rennet coagulation time of samples that coagulated within 60 min from enzyme addition; (B) curd-firming time of samples attaining 20 mm of firmness within 60 min from enzyme addition; (C) curd firmness 30 min after enzyme addition; and (D) curd firmness 60 min after enzyme addition.

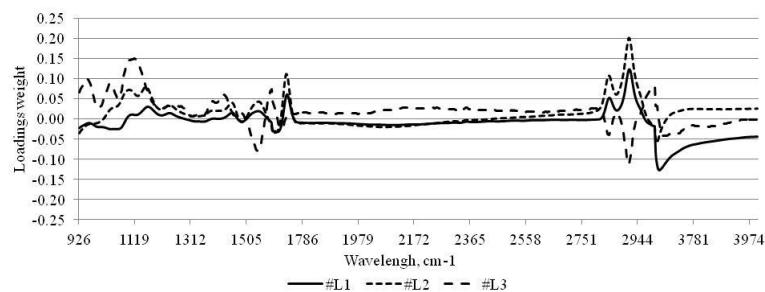
A



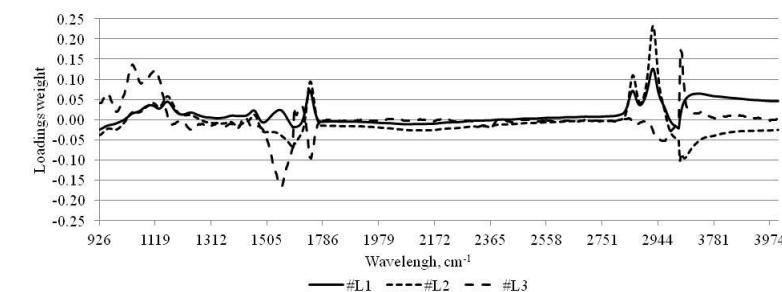
B



C



D



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CHAPTER 3

Effectiveness of mid-infrared spectroscopy for prediction of the contents of calcium and phosphorus, and titratable acidity of milk and their relationship with milk quality and coagulation properties

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ABSTRACT

Individual milk samples from Holstein Friesian cows were collected and analysed by inductively coupled plasma optical emission spectrometry (ICP-OES) and titration for the determination of Ca and P, and TA contents, respectively. Prediction models were obtained using partial least squares (PLS) regression analyses using two statistical packages. The average Ca, P and TA were 1156 mg kg^{-1} , 934 mg kg^{-1} and $3.42 \text{ SH}^{\circ} 50 \text{ mL}^{-1}$, respectively. Pearson's correlations between Ca and P and other milk traits were significant ($P < 0.05$) and ranged from 0.16 to 0.53 for chemical composition traits and from 0.17 to -0.35 for milk coagulation properties (MCP). Results from the two statistical packages were comparable. Prediction models using MIR spectroscopy were satisfactory for Ca, P and TA, with coefficients of correlation of cross-validation greater than 0.73. Moreover, the study highlighted favourable relationships of these traits with milk coagulation properties.

INTRODUCTION

Minerals from milk, and in particular calcium (Ca) and phosphorus (P), play a key role on human health. As reported by the FAO (2001), milk Ca provides rigidity to the skeleton by virtue of its phosphate salts and takes part in neuromuscular function, enzyme-mediated processes, and blood clotting. During skeletal growth, the increase in bone mineral density is affected by Ca intake, while during old age this element prevents bone loss and osteoporotic fractures (Caroli et al., 2011). The effect of P on human health is not completely understood (Cashman et al., 2006); the Ca to P ratio in milk has been studied intensively, and a high content of P combined with a low level of Ca seems to be responsible for the stimulation of a parathyroid hormone, with a reduction of bone mass and density, especially in young people.

Moreover, Ca and P are important in cheese-making process because they have a strong influence on the ability of milk to coagulate and on the final consistency of the coagulum (Fossa et al., 1994). In fact, colloidal calcium phosphate (CCP) plays a fundamental role in all stages of cheese-making, affecting the aggregation speed of paracaseinate particles and the properties of the casein curd (Mariani et al., 1996). Several other factors are related to milk coagulation (e.g., type and concentration of the coagulation enzyme, temperature, acidity, and protein content; O'Callaghan et al., 2002). Milk acidity, and in particular titratable acidity (TA), affects the aggregation rate of paracaseinate micelles, the reactivity of rennet, and the rate of syneresis (De Marchi et al., 2009); as reported by Formaggioni et al. (2001), milk with low acidity is generally considered unsuitable for cheese-making, because of negative effects

on the rheology of the rennet curd and on the textural properties of the cheese paste. Moreover, several studies have investigated the favourable relationships between TA and milk coagulation properties (MCP; Cassandro et al., 2008; De Marchi et al., 2007; De Marchi et al., 2009; Penasa et al., 2010; Harzia et al., 2012; Toffanin et al., 2012) and cheese yield (De Marchi et al., 2013).

The determination of milk minerals as well as TA is time-consuming and has high costs (Soyeurt et al., 2009; De Marchi et al., 2009); these disadvantages could be overcome by mid-infrared (MIR) spectroscopy, an innovative technique widely used in milk laboratories for the routine prediction of traditional milk components (De Marchi et al., 2014). The possibility of recording cow phenotypic information on a large scale would enable genetic selection since a genetic basis, as has been shown for Ca and P content (Davis et al., 2001, Soyeurt et al., 2009, Van Hulzen et al., 2009) and TA (Colinet et al., 2012).

Recently, De Marchi et al. (2014) reviewed the application of MIR spectroscopy as a phenotyping tool in the dairy industry, showing the potential of this technique for the prediction of several non-traditional milk quality traits, such as fatty acid composition (Coppa et al., 2010; De Marchi et al., 2011; Soyeurt et al., 2011), protein composition (De Marchi et al., 2009a; Rutten et al., 2011) and MCP (De Marchi et al., 2013). Soyeurt et al. (2009) investigated the effectiveness of MIR spectroscopy in predicting milk minerals, showing promising results for Ca, P, and Mg, and suggesting an improvement in the accuracy of the reference method by using a mineralization process before ICP-OES analysis. Therefore, the aims of this study were (i) to study variation in levels of Ca, P, and TA in bovine milk and their relationship with milk quality and coagulation properties and (ii) to investigate the effectiveness of MIR spectroscopy in predicting milk Ca, P and TA levels using different statistical approaches.

MATERIAL AND METHODS

Milk samples collection, reference analyses, and spectra collection

A total of 208 individual milk samples of Holstein-Friesian cows from parity 1 to 10 and between 10 and 620 days in milk were collected between January and March 2011 from 13 dairy herds in the North of Italy. Immediately after sampling, milk samples (100 mL) with preservative (Bronopol, Knoll Pharmaceuticals, Nottingham, UK) were transferred to the laboratory of the Breeders Association of the Veneto region (Padova, Italy) for milk analyses. Milk fat, protein and casein contents and pH were determined by Milko-Scan FT6000 (Foss

Electric A/S, Hillerød, Denmark), and somatic cell count (SCC) by Cell Fossomatic 250 (Foss Electric A/S).

Milk coagulation properties (MCP) were determined using a Formagraph (Foss Electric) as described by Pretto et al. (2011) and De Marchi et al. (2013). Briefly, samples (10 mL) were heated to 35°C and 200 µL of a rennet solution (Hansen Standard 160; Pacovis Amrein AG, Bern, Switzerland) diluted to 1.6% (w/v) in distilled water was added at the start of analysis. Rennet coagulation time (RCT, min), which corresponds to the time between the addition of the clotting enzyme to the milk and the beginning of coagulation, the curd-firming time (k_{20} , min), the time required to achieve 20 mm of firmness, and the curd firmness (a_{30} , mm), which is defined as the width, in millimetres, of the diagram 30 min after the addition of the rennet addition, were determined.

Calcium and P contents in milk were determined after mineralization with nitric acid in closed vessels by a microwave system using inductively coupled plasma optical emission spectrometry (ICP-OES), Ciros Vision EOP (SPECTRO Analytical Instruments GmbH, Kleve, Germany). ICP-OES was employed to determine Ca at 315.887 nm, and P at 178.287 nm. Instrument operating parameters were optimized for acid solution and calibration standards were matched with nitric acid 5% “suprapure” grade. The elements to be determined were added from single element solutions (Inorganic Ventures, Christiansburg, VA, USA). The concentration range of the calibration solutions was between 0 and 100 mg L⁻¹ for both elements. The accuracy and precision of this method were investigated analysing the certified reference material BCR® – 063R “Skim milk powder” (Institute for Reference Materials and Measurements (IRMM), Geel, Belgium) prepared as described above. The measured values and the certified values were in excellent agreement for all the elements.

Titratable acidity and pH were determined using the Crison Compact D (Crison Instruments SA, Alella, Spain). Titratable acidity was expressed in Soxhlet-Henkel degrees (°SH 50 mL⁻¹) as described by Anonymous (1963).

The MIR spectra were collected on milk samples over the spectral range of 4000-900 cm⁻¹ using a Milko-Scan FT6000 (Foss Electric A/S).

Statistical analysis

Prediction equations were obtained using partial least squares (PLS) regression analyses using two statistical software, The Unscrambler version 10.1 (PLS1; Camo A/S, Oslo, Norway) and the PLS procedure of SAS version 9.3 (PLS2; SAS Institute Inc., Cary, NC). Before PLS analyses, two spectra regions (3040 to 3470 cm⁻¹ and 1,600 to 1,700 cm⁻¹)

characterized by high noise levels were removed according to Hewavitharana and Brakel (1997), Jørgensen and Næs (2004), and De Marchi et al. (2013), and anomalous spectra and reference values were discarded according to the procedures reported by De Marchi et al. (2013). Models were developed using raw spectra data. The final data accounted for 203, 207, and 207 records for Ca, P, and TA, respectively.

To compare statistical software both prediction models (PLS1 and PLS2) were developed using a full cross-validation; this was achieved with a one-at-a-time cross-validation method in which Unscrambler and SAS used all observations but one for the calibrated model, which then was used to predict the left-out observation; this process was repeated for every observation to find the best prediction equation. The prediction equations chosen were those that minimized the predicted residual sum of squares with the minimum number of PLS factors.

Several criteria were used to determine the proficiency of predictive models based on MIR spectroscopy: the coefficient of correlation of cross-validation (r_{cv}), the root mean square error of cross-validation (RMSE_{cv}), optimum number of PLS factors (#L), and ratio performance deviation (RPD). The latter statistic is calculated as the ratio of the SD to RMSE_{cv} for a given trait (Edney, Morgan, Williams, & Campbell, 1994; Williams, 2001). High values of RPD are desirable; in particular, RPD values greater than 2 enable good predictions (Sinnaeve et al., 1994; De Marchi et al., 2011).

Finally, the relationships between protein, fat and casein contents, somatic cell count (SCC) and somatic cell score (SCS, calculated as the logarithm of SCC), RCT, k₂₀ and a₃₀, measured Ca, P and TA and predicted Ca (pCa), P (pP) and TA (pTA) were investigated through SAS procedures (SAS, 2008). Milk samples that did not coagulate within 30 min (about 13% of total records) were considered missing values with no MCP information.

RESULTS AND DISCUSSION

Descriptive statistics and Pearson correlations

Calcium and P levels were on average 1156.33 and 933.98 mg kg⁻¹, respectively (Table 1). These values are very similar to those reported by Soyeurt et al. (2009) and van Hulzen et al. (2009) for bovine milk with Ca and P values that ranged from 1051 to 1447 mg kg⁻¹ and from 919 to 1221 mg kg⁻¹, respectively. Moreover, values of the present study were consistent with findings of Cashman (2011) who reported average values of 1120 mg kg⁻¹ and 890 mg kg⁻¹ for measured Ca and P in bovine milk, respectively. The mean values of TA and

pH were 3.42°SH 50 mL^{-1} and 6.68, respectively (Table 1), which are very similar to those reported by De Marchi et al. (2008) and Cassandro et al. (2008) for Holstein Friesian bovine milk. Calcium, P and TA were normally distributed, allowing the development of robust prediction models. Milk chemical composition and SCS are typical of Italian Holstein Friesian cow milk and consistent with values reported by the National Herdbook of Italian Holstein Friesian (ANAFI, 2011) and by Penasa et al. (2014).

Rennet coagulation time, k_{20} , and a_{30} averaged 18.23 min, 5.70 min, and 24.58 mm, respectively (Table 1); the CV ranged from 25 (RCT) to 51% (a_{30}). Twenty-seven samples (12.9% of the total data) did not form a curd within 30 min from the rennet addition. This percentage of so-called non-coagulating (NC) milk samples is similar to that reported by Ikonen et al. (2004), Tyrisevä et al. (2004), and Cassandro et al. (2008), who reported percentages of NC samples between 7.5 and 13.2% for Finnish Ayrshire and Holstein Friesian cows. Milk coagulation properties of the present study indicated a type of milk not very suitable for cheese-making and a worsening of these traits across years (Zannoni and Annibaldi, 1981). Nevertheless, values of MCP are similar to those reported by Penasa et al. (2014); they reported RCT and a_{30} values of 21 min and 20.8 mm, respectively, for milk from multi-breed dairy herds.

Overall, phenotypic correlations between measured Ca, P and TA and milk quality traits (Table 2) were very similar to those between pCa, pP and pTA and quality traits (Table 3). This was expected because of the high accuracy of MIR spectroscopy prediction models. Calcium and P were positively correlated with milk chemical composition and varied from 0.27 (Ca and fat content, and P and Fat content) to 0.53 (Ca and protein content, and P and protein content). Calcium and P were positively correlated with TA, which is consistent with results reported by Mariani (1982), Mariani and Artoni (1983), Mariani et al. (1989), and Formaggioni et al. (2001). Those authors found values of correlation close to 0.72 between P and TA using both individual (Mariani et al., 1983) and bulk (Formaggioni et al., 2001) milk samples. Moreover, Ca showed a weak positive correlation with pH. As expected, a high correlation (0.68; $P<0.001$) was calculated between Ca and P, highlighting their relationship and reflecting the importance of the Ca/P ratio in milk (Cashman et al., 2006); similar results were reported by Soyeurt et al. (2009), with a correlation of 0.73. A weak negative correlation (-0.16; $P<0.05$) was found between P and SCS; this result was consistent with those reported by Summer et al. (2010), who studied milk salt equilibria and reported a decrease in total P with increasing SCC, and so confirmed the opposite relationship between the two traits.

Moreover, Summer et al. (2010) found no significant relationship between Ca and SCC, consistent with the results of the present study.

A favourable relationship was estimated between milk minerals and MCP; an increase in Ca and P is correlated with an increase of a_{30} , while an increase in Ca reduces RCT. These relations were found also by Malacarne et al. (2013), who found optimal milk for cheese-making when these minerals were present at high concentrations.

Milk chemical composition was positively related with TA, with values ranging from 0.22 ($P<0.05$, TA and fat content) to 0.57 ($P<0.001$, TA and casein content); these estimates are comparable to those between pTA and fat content (0.23), and pTA and casein content (0.64). Cassandro et al. (2008) and Colinet et al. (2010) reported similar correlations in their studies.

As expected, TA was negatively correlated with RCT and k_{20} and positively with a_{30} ; this agrees with results of Cassandro et al. (2008) and Toffanin et al. (2012) for Italian Holstein Friesians, and of De Marchi et al. (2007) in mixed Brown Swiss and Holstein Friesian herds. Moreover, considering the strong and negative correlation between TA and pH -0.53 ($P<0.001$), the correlations between pH and RCT and a_{30} were 0.42 ($P<0.001$) and -0.47 ($P<0.001$), respectively.

MIRS prediction models and PLS methods

Fitting statistics of PLS analysis are shown in Table 4. On average, the accuracy of the prediction models developed by cross-validation and through different statistical packages was very similar within each trait; moreover, the satisfactory results of the prediction models suggested that there is a relation between MIR spectra and the traits studied.

Generally, a robust prediction model should have high r_{CV} , low RMSE_{CV} and RPD value greater than 2. Results of prediction models for Ca and P were satisfactory, with r_{CV} of about 0.75 and 0.85, respectively, and this confirmed the effectiveness of MIR spectroscopy in predicting Ca and P contents of milk. The RPD and #L were 1.47 and 4, and 1.84 and 6 for Ca and P, respectively.

Very limited information has been reported in the literature about the application of MIR spectroscopy for the prediction of mineral composition of milk. Results of the present study are quite similar than those reported by Soyeurt et al. (2009); they found values of 95 mg kg⁻¹ (SE_{CV}), 0.93 (r_{CV}), and 2.74 (RPD) for Ca, and 50 mg kg⁻¹ (SE_{CV}), 0.92 (r_{CV}), and 2.54 (RPD) for P.

However, the present study used a different reference method respect to Soyeurt et al. (2009); in the present study, the mineral contents were assessed by ICP-OES after mineralization, while Soyeurt et al. (2009) used ICP-AES without mineralization. Moreover, in the study of Soyeurt et al. (2009), 6 different cow breeds were included which provided great biological variability, as confirmed by the coefficients of variation of the studied traits. Recently, Wu et al. (2012) investigated the potential of near-infrared and MIR spectroscopy techniques for predicting the calcium content of powdered milk. The predicted results of PLS models gave very good results, with a coefficient of determination of 0.93 and 5.22% for relative error of prediction.

The prediction models for TA gave satisfactory results, with correlation coefficients of cross-validation of 0.86 (Table 4). The RPD values were close to 2, suggesting good predictive models (Sinnaeve et al., 1994) and #L in cross-validation procedure was 6. These results were slightly better than those reported by De Marchi et al. (2009), with RMSE_{CV} of 0.26 °SH 50 mL⁻¹ and r_{CV} of 0.80, and slightly worse than those reported by Colinet et al. (2010), with SE_{CV} of 0.64°D (Dornic Degree) and r_{CV} of 0.95. Nevertheless, Colinet et al. (2010) used a different reference method and unit of measurement for TA. Compared to statistical procedures used in the present study, predictive models proposed by De Marchi et al. (2009) were developed using untreated spectra data and the Unscrambler software, while prediction models developed by Colinet et al. (2010) used first derivative spectra pre-treatment and WinIisi software.

For each trait, the best regression models are represented in Figure 1; the plot of measured against predicted values showed a moderate degree of scatter in all cases. Moreover, the coefficients of determination of cross-validation (R^2_{CV}) were 0.56, 0.72, and 0.74 for Ca, P and TA, respectively. Considering these values, predictions of TA and P were found to be approximate, and the model for Ca could only discriminate between low and high values (Williams, 2003).

This is the first study that compares the performance of chemometric with two statistical packages using the same data and validation procedures. As expected, the results were very similar for both statistical packages. From a practical point of view, chemometric analysis by the Unscrambler is more complex and requires more time and steps; in contrast, SAS seems to be less suitable for spectra visualization and outlier identification. Moreover, the PLS procedure of SAS allows large data analysis (more than 50,000 samples). This considerations should be taken into account, because in the future the use of large MIR

spectroscopy data to predict new milk phenotypes will be an important tool for dairy industry (De Marchi et al., 2014).

CONCLUSIONS

Mid-infrared spectroscopy combined with different PLS regressions has been proposed for prediction of contents of Ca and P, and TA in bovine milk. Prediction equations were effective in predicting P and TA, and were effective in discriminating between low and high contents of Ca. Moreover, favourable relationships were found between these traits and MCP, which might be interesting in improving the suitability of milk for producing cheese. Results suggest the possibility of using MIR spectroscopy prediction models in large scale research programs aiming at improving the nutritional aspects of milk by genetic selection of dairy cattle.

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Table 1. Descriptive statistics of mineral content, acidity, chemical composition, and coagulation properties of milk.^a

Trait	n	Mean	SD	Skewness	Kurtosis
Ca, (mg kg ⁻¹)	208	1,156.33	130.46	0.31	-0.27
P, (mg kg ⁻¹)	208	933.98	109.71	0.18	-0.20
TA, (°SH 50 mL ⁻¹)	208	3.42	0.36	0.37	0.44
Fat, (%)	181	3.75	0.85	0.22	1.91
Protein, (%)	181	3.35	0.39	0.49	-0.25
Casein, (%)	154	2.55	0.33	0.79	0.38
SCS, (score)	181	4.40	1.26	0.46	-0.22
pH	202	6.68	0.07	-0.05	-0.10
RCT, (min)	181	18.23	4.56	0.15	-0.09
k ₂₀ , (min)	120	5.70	2.04	0.48	-0.13
a ₃₀ , (mm)	189	24.58	12.56	0.01	-0.44

^a Abbreviation are: Ca = calcium content; P = phosphorus content; TA = titratable acidity; SCS = somatic cell score; RCT = rennet coagulation time; k₂₀ = curd-firming time; a₃₀ = curd firmness.

Table 2. Phenotypic correlations between measured mineral levels, acidity, chemical composition, and coagulation properties of milk.^a

Trait	Ca, (mg kg ⁻¹)	P, (mg kg ⁻¹)	TA, (°SH 50 mL ⁻¹)	Fat, (%)	Protein, (%)	Casein, (%)	SCS, (score)	pH	RCT, (min)	k ₂₀ , (min)	a ₃₀ , (mm)
Ca, mg kg ⁻¹	0.68***	0.21**	0.27***	0.53***	0.47***	-0.07ns	0.13†	-0.11ns	-0.35***	0.29***	
P, mg kg ⁻¹		0.54***	0.27***	0.53***	0.47***	-0.16*	0.06ns	-0.01ns	-0.23*	0.17*	
TA, °SH 50mL ⁻¹			0.22*	0.48***	0.57***	-0.11ns	-0.53***	-0.25**	-0.22*	0.44***	
Fat, %				0.30***	0.27†	-0.01ns	0.07ns	-0.08ns	-0.22*	0.18*	
Protein, %					0.99***	0.11ns	-0.08ns	0.04ns	-0.44***	0.27***	
Casein, %						0.12ns	-0.31*	-0.10ns	-0.49*	0.43**	
SCS, score							0.03ns	0.08ns	-0.09ns	-0.07ns	
pH								0.42***	0.13ns	-0.47***	
RCT, min									0.39***	-0.79***	
k ₂₀ , min										-0.86***	

^a Abbreviation are: Ca = calcium content; P = phosphorus content; TA = titratable acidity; SCS = somatic cell score; RCT = rennet coagulation time; k₂₀ = curd-firming time; a₃₀ = curd firmness. † P<0.10; * P<0.05; ** P<0.01; *** P<0.001.

Table 3. Phenotypic correlations of predicted mineral levels and titratable acidity with chemical composition and coagulation properties of milk.^a

Trait	pCa, (mg kg ⁻¹)	pP, (mg kg ⁻¹)	pTA, (°SH 50 mL ⁻¹)	Fat, (%)	Protein, (%)	Casein, (%)	SCS, (score)	pH	RCT, (min)	k ₂₀ , (min)	a ₃₀ , (mm)
pCa, mg kg ⁻¹		0.60***	0.40***	0.35***	0.70***	0.77***	0.01 ^{ns}	0.08 ^{ns}	0.04 ^{ns}	-0.40***	0.23**
pP, mg kg ⁻¹			0.69***	0.26**	0.58***	0.55***	-0.12 ^{ns}	-0.07 ^{ns}	-0.08 ^{ns}	-0.23*	0.26***
pTA, °SH 50 mL ⁻¹				0.23**	0.55***	0.64***	-0.06 ^{ns}	-0.42***	-0.15*	-0.28**	0.35***

^a Abbreviations are: pCa = predicted calcium content; pP = predicted phosphorus content; pTA = predicted titratable acidity; SCS = somatic cell score; RCT = rennet coagulation time; k₂₀ = curd-firming time; a₃₀ = curd firmness.

† P<0.10; * P<0.05; ** P<0.01; *** P<0.001.

Table 4. Fitting statistics of prediction models for calcium, phosphorus and titratable acidity.

Trait ^a	Calcium,		Phosphorus,		Titratable acidity,	
	(mg kg ⁻¹)	PLS1 ^b	(mg kg ⁻¹)	PLS2 ^c	(°SH 50 mL ⁻¹)	PLS2 ^c
n	203		207		207	
Mean	1,155.56		933.04		3.42	
SD	125.04		103.78		0.34	
RMSE _{CV}	85.70	85.25	54.67	56.38	0.17	0.17
r _{CV}	0.75	0.73	0.85	0.84	0.86	0.86
RPD	1.46	1.47	1.89	1.84	1.98	2.01
#L	5	4	6	6	6	6

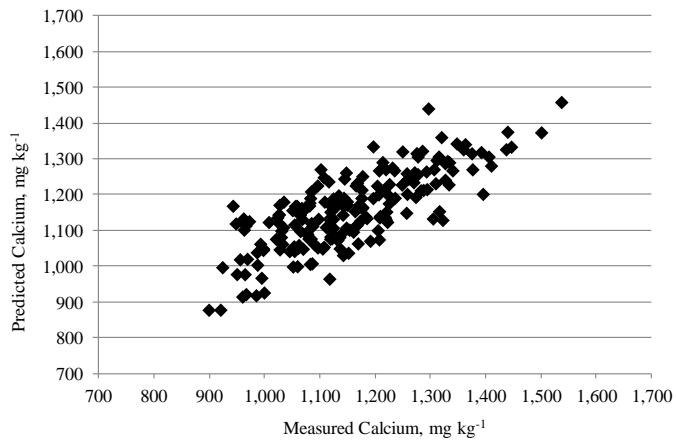
^a Abbreviations are: n = number of samples used in the calibration after removing outliers; SD = standard deviation; RMSE_{CV} = root mean square error of cross-validation; r_{CV} = coefficient of correlation of cross-validation; RPD = ratio performance deviation; #L = number of modified partial least squares factors used in the prediction models.

^b Developed using the Unscrambler software.

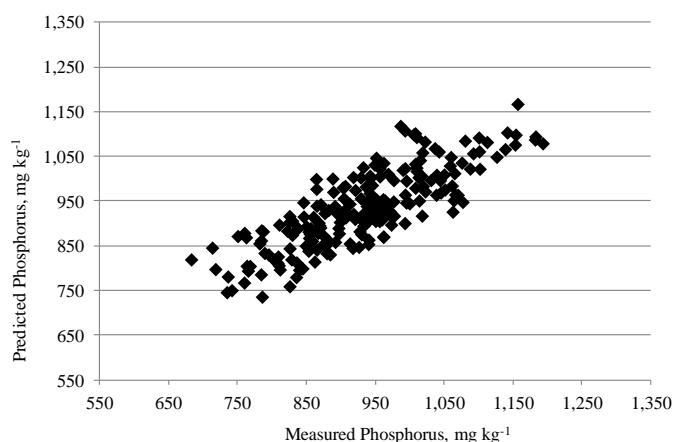
^c Developed using SAS software.

Figure 1. Scatter plots of predicted versus measured calcium (panel A), phosphorus (panel B), and titratable acidity (panel C) using PLS.

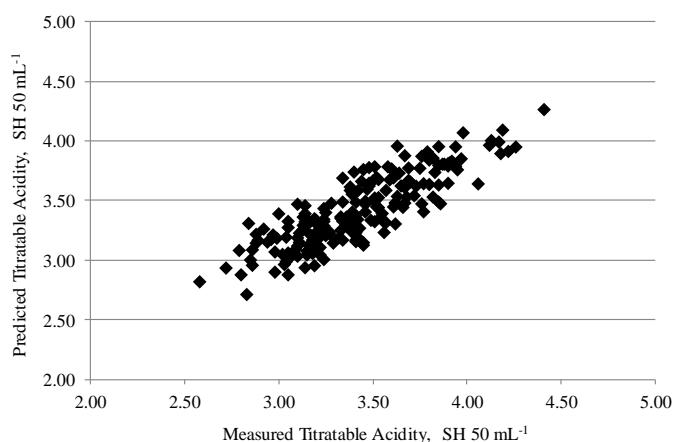
A



B



C



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CHAPTER 4

Genetic parameters for milk mineral content and acidity predicted by mid-infrared spectroscopy in Holstein-Friesian cows

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ABSTRACT

The aim of the present study was to estimate genetic parameters for calcium (Ca), phosphorus (P) and titratable acidity (TA) in bovine milk predicted by mid-infrared spectroscopy (MIRS). Data consisted of 2458 Italian Holstein-Friesian cows sampled once in 220 farms. Information per sample on protein and fat percentage, pH, and somatic cell count, as well as test-day milk yield, was also available. (Co)variance components were estimated using univariate and bivariate animal linear mixed models. Fixed effects considered in the analyses were herd of sampling, parity, lactation stage, and a two-way interaction between parity and lactation stage; an additive genetic and residual term were included in the models as random effects. Estimates of heritability for Ca, P, and TA were 0.10, 0.12, and 0.26, respectively. Positive moderate to strong phenotypic correlations (0.33 to 0.82) existed between Ca, P and TA, while phenotypic weak to moderate correlations (0.00 to 0.45) existed between these traits with both milk quality and yield. Moderate to strong genetic correlations (0.28 to 0.92) existed between Ca, P, and TA, and between these predicted traits with both fat and protein percentage (0.35 to 0.91). The existence of heritable genetic variation for Ca, P, and TA, coupled with the potential to predict these components for routine cow milk testing, imply that genetic gain in these traits is indeed possible.

Keywords: milk mineral, milk acidity, mid-infrared spectroscopy, Holstein-Friesian cow, genetic parameter

IMPLICATIONS

The present study quantified the phenotypic and genetic characteristics of cow milk calcium, phosphorus and acidity predicted by mid-infrared spectroscopy. These milk features are very important for both human health and the technological properties of milk. The existence of heritable genetic variation coupled with the potential to predict these components for routine cow milk testing, imply that genetic gain in these traits is indeed possible.

INTRODUCTION

Global demand for dairy products is increasing, especially milk powder and butter, and further expansion of trade in dairy products is expected. Moreover, in recent years, there is a growing interest in milk components with potential benefits for human health. Milk mineral profile, in particular calcium (Ca) and phosphorus (P) content, play a key role in human health (Caroli et al., 2011), especially for osteoporosis which is a progressive bone disease related to

deficiencies in Ca and vitamin D. Both Ca and P are also important in cheese-making as they influence the coagulation process and the final consistency of the curd (Fossa et al., 1994).

Another characteristic which affects the technological properties of milk is titratable acidity (TA) which has a key role in the aggregation rate of para-casein micelles, in the reactivity of rennet (De Marchi et al., 2009) and also on milk coagulation properties (De Marchi et al., 2007).

There is a paucity of studies which have estimated genetic variation for milk mineral content and acidity. Van Hulzen et al. (2009) reported heritability estimates of 0.57 and 0.62 for milk Ca and P content, respectively, in 1860 Dutch Friesian cows. Reported heritability estimates for milk TA range from 0.17 to 0.23 in Italian Holstein cows (Cassandro et al., 2008; Cecchinato et al., 2011). To our knowledge, only Soyeurt et al. (2008a) used 5502 multi-breed cows to estimate genetic parameters for mineral content of milk predicted by mid-infrared spectroscopy (MIRS) and they reported heritabilities of 0.42 and 0.47 for Ca and P, respectively.

The limited number of studies on the genetics of milk mineral composition and acidity is likely related to the high analytical costs associated with measuring these traits, thereby hindering the generation of large data sets (De Marchi et al., 2014). Mid-infrared spectroscopy has already been proposed to predict milk Ca and P (Soyeur et al., 2008a; Toffanin et al., 2015), thereby providing a low-cost approach to generate large datasets useful for genetic evaluations. The objective therefore of the present study was to estimate genetic parameters for milk Ca, P and TA predicted by MIRS as well as the genetic correlations between these traits with milk production and quality in Italian Holstein-Friesian cows.

MATERIALS AND METHODS

Data and MIRS prediction models

A total of 2458 Holstein-Friesian cows representing the offspring of 210 AI sires were sampled once in 220 Italian dairy herds between May 2010 and March 2011. Cows were from parity 1 to 10, and between 5 and 600 days in milk (DIM). Information on parity, DIM, and milk yield (MY, kg/day) at the time of milk sampling was provided by the Breeders Association of the Veneto region (Padova, Italy). Samples were preserved with Bronopol (Knoll Pharmaceuticals, Nottingham, UK), transported to the laboratory of the Breeders Association of the Veneto region (Padova, Italy), and analysed within 12 hours of collection. The routine prediction of milk fat and protein percentage as well as pH were conducted with a

Milko-Scan FT6000 (Foss Electric A/S, Hillerød, Denmark) and somatic cell count (SCC) was determined using a Cell Fossomatic 250 (Foss Electric A/S, Hillerød, Denmark). Somatic cell count was transformed to somatic cell score (SCS) by taking the natural logarithm of SCC. Spectral information was stored for all milk samples.

Predicted Ca and P content, and TA were obtained using MIRS models developed by Toffanin et al. (2015). Briefly, calibration models were built using 208 cow milk samples collected from 13 herds chosen from those analysed in the present study. The gold standard method for the quantification of Ca and P was based on inductively coupled plasma optical emission spectrometry (ICP-OES) with previous milk mineralization; for TA the gold standard was milk titration. Prediction models were developed through partial least squares regression analysis using the PLS procedure of SAS (SAS Institute Inc., Cary, NC). Coefficients of correlation of the models were 0.73, 0.84 and 0.86 for Ca, P and TA, respectively, and the ratio performance deviation was 1.47 for Ca, 1.84 for P and 2.01 for TA (Toffanin et al., 2015).

Statistical analyses

Factors associated with the traits of interest were determined using the following linear model in PROC GLM (SAS Institute Inc., Cary, NC, USA):

$$Y_{ijkl} = \mu + \text{Herd}_i + \text{parity}_j + \text{stage}_k + (\text{parity} \times \text{stage})_{jk} + e_{ijkl},$$

where Y_{ijkl} is the dependent variable (Ca, P, TA, pH, fat percentage, protein percentage, SCS or MY); μ is the overall mean; Herd_i is the fixed effect of the i^{th} herd of sampling ($i = 1$ to 220); parity_j is the fixed effect of the j^{th} parity of the cow ($j = 1, 2, 3, \geq 4$); stage_k is the fixed effect of the k^{th} class of DIM ($k = 5-35, 36-65, 66-95, \dots, 306-350, 351-450, >450$ days); $(\text{parity} \times \text{stage})_{jk}$ is the fixed interaction effect between parity and stage of lactation; and e_{ijkl} is the random residual $\sim N(0, \sigma_e^2)$. 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.

Variance and covariance components for the studied traits were estimated using univariate and bivariate animal models in ASREML (Gilmour et al., 2009). Fixed effects considered in the analyses were the same as described previously, and the random effects were the additive genetic effect and the residual term. The number of animals in the additive relationship matrix was 33 197 and included individuals with phenotypic records and their ancestors up to 23 generations back. Pedigree information was provided by the Italian Holstein Friesian Cattle Breeders Association (Cremona, Italy).

RESULTS

Descriptive statistics

Predicted Ca, P, TA, and pH averaged 1171 mg/kg, 935 mg/kg, 3.41 °SH/50mL and 6.63, respectively (Table 1). The coefficient of variation was 0.13 for both Ca and P, 0.08 for TA, and 0.01 for pH.

The effect of lactation stage on Ca and P content was similar (Figure 1); both components decreased rapidly within the first 2 months of lactation and increased thereafter. The trend for TA and pH in early to mid-lactation was opposite to each other (Figure 2); milk TA decreased markedly in the 1st month after calving, reached a plateau during the 2nd month, and increased thereafter, whereas milk pH increased until the 5th month after calving and decreased slightly thereafter (Figure 2).

Least squares means for the milk characteristics in each parity are in Table 2. Predicted mineral content and TA were inversely related to parity ($P < 0.05$); pH did not change among parities with the exception of parity one which exhibited the lowest pH value ($P < 0.05$). Significant parity effects were detected for MY, protein percentage and SCS, while no effects were detected for fat percentage.

Heritability

Estimates of heritability for predicted Ca, P, and TA were 0.10, 0.12, and 0.26, respectively (Table 1), and they ranged from 0.09 (SCS) to 0.25 (fat percentage) for milk quality and yield. The coefficients of genetic variation for the minerals and TA were lower than those of fat percentage, SCS and MY, but comparable to that of protein percentage. Practically no genetic variation in milk pH existed with a detected coefficient of genetic variation of only 0.004.

Phenotypic and genetic correlations

Predicted Ca was moderately phenotypically correlated with both P (0.33) and TA (0.40), while P was strongly correlated (0.82) with TA (Table 3). A moderate negative phenotypic correlation (-0.38) existed between TA and pH. Phenotypic correlations of the milk minerals and TA with milk chemical composition varied from 0.00 (TA and SCS) to 0.45 (TA and protein percentage). Milk yield was negatively phenotypically correlated with Ca, P and TA, albeit all correlations were weak (-0.23 to -0.11; Table 3).

Strong and significant ($P < 0.05$) genetic correlations existed between TA and P (0.92) and between Ca and P (0.67). Genetic correlations of Ca, P and TA with fat and protein

percentages ranged from 0.35 (TA and fat percentage; $P < 0.05$) to 0.91 (P and protein percentage; $P < 0.05$), and with SCS and MY the correlations were weak to moderate and not significantly different from zero ($P > 0.05$; Table 3).

DISCUSSION

Descriptive statistics

Mean milk Ca and P in the present study are within the ranges of values documented by van Hulzen et al. (2009), Cashman (2011) and Toffanin et al. (2015), who reported average values from 1120 to 1235 mg/kg and from 890 to 1027 mg/kg for measured Ca and P in bovine milk, respectively. Moreover, van Hulzen et al. (2009) and Toffanin et al. (2015) documented a phenotypic coefficient of variation between 9% and 12% for measured Ca and P in milk of Holstein-Friesian cows. Soyeurt et al. (2009) investigated the feasibility of MIRS to predict the content of milk minerals using the ICP-AES without previous mineralization as the reference method. Despite differences in pre-treatment for ICP-AES as well as the data originating from two separate populations of cows, similar means and coefficients of variation were observed between the present study and the aforementioned study of Soyeurt et al. (2009).

Parity least squares means for Ca and P (Table 2) followed a similar trend to that described by Kume et al. (1998) in Japanese Holstein cows, who reported a slight decrease in milk mineral concentration after first parity, even if not statistically significant. The decrease in milk Ca and P content within increasing parity is probably due to the reduced utilization of these minerals in the mammary gland (Kume and Tanabe, 1993). Indeed, serum Ca and P concentration is also inversely related to parity number (Shappell et al., 1987; Romo et al., 1991) justifying the hypothesis of a lower utilization in older cows. Van de Braak and Van't Klooster (1987) argued that osteoplastic activities are less intense in older than in younger cows, which could explain a decreased responsiveness for PTH-mediated Ca resorption and intestinal absorption in older parity cows (Romo et al., 1991). Moreover, the greater amount of milk produced by older cows during lactation could explain the lower mineral content because of the dilution.

The association between lactation stage and Ca in the present study (Figure 1) corroborates the observed increase in milk Ca content across lactation documented by van Hulzen et al. (2009) in 1860 Dutch Holstein-Friesian cows. Similar effects have been documented in other dairy species such as goat and sheep (Sahan et al., 2005; Kondyli et al.,

2007). Trends of minerals across DIM might be due to changes in feeding composition across lactation (Cashman and Flynn, 2003) or to a dilution effect during lactation; negative phenotypic correlations between MY with both Ca (-0.23) and P (-0.14) were observed in the present study after adjustment for terms in the statistical model (Table 3).

Mean milk TA across all samples in the present study is similar to documented values of TA in the milk of Holstein-Friesian (Cassandro et al., 2008) and Brown Swiss cows (De Marchi et al., 2007, 2008; Cecchinato et al., 2011; Penasa et al., 2014) and in herd bulk milk samples destined for Grana Padano cheese production (Pretto et al., 2013). Moreover, mean TA in the present study is similar to reported values of MIRS-predicted TA by De Marchi et al. (2009), Colinet et al. (2010) and Toffanin et al. (2015). The lower pH in first compared to later parity cows corroborates results of Ikonen et al. (2004) and could be the consequence, at least partly, of the higher values of P and TA in first lactation cows (Table 2), and the inverse relationships between these minerals and pH (Table 3). The interrelationships between milk acidity and mineral content have been previously investigated (Mariani et al., 1989). The similar patterns of TA and minerals across stages of lactation (Figures 1 and 2) could be explained by the nature of these traits; TA is moderately phenotypically correlated with both protein percentage and Ca, as well as being strongly correlated with P (Table 3). As a consequence, it is expected that TA follows the trend of minerals across lactation, which decreases markedly in early lactation, increasing thereafter.

Descriptive statistics of milk chemical composition and MY are in agreement with those documented in several studies on Italian Holstein-Friesian cows (Cassandro et al., 2008; Pretto et al., 2011; Tiezzi et al., 2013), with the exception of SCS, which exhibited a smaller phenotypic coefficient of variation in the present study. Parity mean milk composition and yield (Table 2) were generally in agreement with other studied international populations of dairy cows (Ikonen et al., 2004; Hansen et al., 2006; Bastin et al., 2013).

Heritabilities

Heritability estimates of Ca and P were markedly lower than those reported by Soyeurt et al. (2008b) who predicted Ca and P using MIRS with similar accuracy to the equations used in the present study. Differences in number and variability of data could explain different estimates between the present study and that of Soyeurt et al. (2008b). Using data on measured Ca and P, Van Hulzen et al. (2009) reported similar heritability estimates for Ca and P to those documented by Soyeurt et al. (2008b). The lower heritability estimates obtained in the present study could be also related to the MIRS prediction models performance: the less

than perfect predictions will likely increase the residual variance and thus reduce the heritability. Nevertheless, the presence of genetic variation in these traits, albeit with coefficients of genetic variation generally less than observed for other performance traits like milk production, suggests that genetic improvement is still nonetheless possible. This is especially true because milk MIRS information is routinely available on all milk samples taken during milk testing. Moreover, it has been reported that also in the case of quite low accuracy of prediction, the genetic correlations between predicted and true values are strong. This was demonstrated by Cecchinato et al. (2009) who compared genetic parameters from predicted (with accuracies similar to those obtained in the present study) and measured values of milk coagulation properties: those authors concluded that the genetic response in milk coagulation properties from selection using MIRS predictions as indicator traits is expected to be equal to or slightly lower than the response achievable through direct measurement of the traits (Cecchinato et al., 2009).

The accuracy of selection for both milk Ca and P based on information on 100 half-sib daughters (ignoring parental contribution) in a univariate genetic evaluation is 0.86. Assuming a genetic gain of 0.22 standard deviations annually, this equates to a potential genetic gain of 7.3 mg/kg and 6.9 mg/kg for Ca and P, respectively. The actual gain achievable however will be dependent on the traits in the breeding goal, their covariances with Ca and P and their relative weighting within the breeding goal, as well as other factors like intensity of selection, accuracy of selection and generation interval (Rendel and Robertson, 1950).

To our knowledge, this is the first study to estimate genetic parameters for TA predicted by MIRS. Heritability estimates for actual TA, determined using Soxhlet-Henkel degrees, has been reported to range between 0.17 to 0.23 in Holstein-Friesian (Cassandro et al., 2008) and Brown Swiss cows (Cecchinato et al., 2011). The heritability of pH in the present study (0.16) is within the range of 0.06 (Cecchinato et al., 2011) to 0.38 (Ikonen et al., 2004) reported in other populations. The lack of genetic variation in pH observed in the present study suggests limited potential for change through breeding; the small phenotypic coefficient of variation for pH, also suggests limited potential for alteration of milk pH in the dairy cow through non-breeding means.

Phenotypic and genetic correlations

Both the phenotypic and genetic correlations between Ca and P observed in the present study were consistent with correlations reported by Soyeurt et al. (2008b) from a large multi-breed population of dairy cows. The phenotypic correlation between measures of P and TA

has been reported to range from 0.54 to 0.81 (Mariani et al., 1989; Toffanin et al., 2015), depending on whether individual or bulk milk samples or whether single or multi-breed populations were considered. As expected, the phenotypic correlation between P and pH in the present study was negative, although weak, reflecting the link between P and TA (positive and strong) and between TA and pH (negative and moderate). The phenotypic correlation between TA and P was also confirmed by the effect of lactation stage on each trait being a mirror of the other (Figure 2). Similar correlations were reported by Mariani et al. (1989) who assessed a phenotypic correlation of -0.61 between pH and soluble P.

No study has previously estimated the genetic correlations between milk mineral content and other milk components. Predicted Ca and P were positively and moderately (phenotypic) to strongly (genetic) correlated with milk protein and fat percentage. After all, as reported by Malacarne et al. (2013), micellar P can be present as part of colloidal calcium phosphate or covalently bound to caseins as phosphate groups, given that Ca and P are basic components of the milk micelles. Milk yield was moderately and not significantly ($P > 0.05$) related to Ca, P and TA, suggesting independence of production from mineral content and acidity of milk. However, it is likely that the lack of significance in the present study was the consequence of the relatively small sample size which led to quite high standard errors for some estimates of genetic correlations. The use of MIRS models to predict milk mineral content and TA at population level will reduce the sampling variance.

CONCLUSIONS

Improvement in milk mineral and acidity profiles could be of benefit for the human nutritional and technological characteristics of milk. The existence of heritable genetic variation coupled with the ability to predict these milk characteristics through routinely available MIRS suggests that genetic selection for these components in milk is indeed possible. Moreover, because Ca, P, and TA are genetically correlated with milk protein and fat percentage, breeding goals that select for improved milk fat and protein concentration are likely to be indirectly selecting also for improved milk Ca, P and TA.

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Sciences, Estonia) and Lucia Ancilotto (University of Padova, Italy) for technical support, and “CPDA134308/13” project for financial support.

Table 1. Mean, coefficient of genetic variation (CV_g), estimated heritability (h^2 ; standard errors in parentheses) of milk mineral components, acidity, chemical composition and yield

Trait ¹	N	Mean	CV_g	h^2 (s.e.) ²
Mineral component				
Ca (mg/kg)	2428	1171	0.03	0.10 (0.04)
P (mg/kg)	2353	935	0.04	0.12 (0.05)
Acidity				
TA (°SH/50mL)	2019	3.41	0.03	0.26 (0.07)
pH	2440	6.63	0.004	0.16 (0.05)
Quality traits				
Fat (%)	2456	3.69	0.09	0.25 (0.06)
Protein (%)	2458	3.37	0.04	0.20 (0.05)
SCS (units)	2451	4.62	0.08	0.09 (0.04)
Milk yield (kg/day)	2285	30.19	0.07	0.12 (0.05)

¹Ca = calcium; P = phosphorus; TA = titratable acidity; SCS = somatic cell score [SCS = ln(SCC)], where SCC is somatic cell count.

²All estimates of heritability are significantly different from zero ($P < 0.05$).

Table 2. Least squares means and pooled standard errors (s.e.) for milk mineral components, acidity, chemical composition and yield across parities

Trait ¹	Parity				s.e.
	1	2	3	4 and later	
Mineral component					
Ca (mg/kg)	1185 ^a	1185 ^a	1162 ^b	1152 ^b	6
P (mg/kg)	965 ^a	942 ^b	929 ^b	931 ^b	5
Acidity					
TA (°SH/50mL)	3.49 ^a	3.36 ^b	3.30 ^c	3.27 ^c	0.01
pH	6.62 ^a	6.64 ^b	6.63 ^b	6.64 ^b	0.00
Quality traits					
Fat (%)	3.80 ^a	3.80 ^a	3.76 ^a	3.77 ^a	0.03
Protein (%)	3.44 ^a	3.48 ^b	3.43 ^a	3.40 ^a	0.01
SCS (units)	4.55 ^a	4.82 ^b	4.94 ^{bc}	5.14 ^c	0.06
Milk yield (kg/day)	26.8 ^a	28.6 ^b	30.5 ^c	29.7 ^{bc}	0.3

¹Ca = calcium; P = phosphorus; TA = titratable acidity; SCS = somatic cell score [SCS = ln(SCC)], where SCC is somatic cell count.

^{a-c}means with different superscripts within a row are significantly different ($P < 0.05$).

Table 3. Phenotypic (above diagonal, with standard errors in parentheses) and genetic (below diagonal, with standard errors in parentheses) correlations between milk mineral components, acidity, chemical composition and yield

Trait ¹	Ca	P	TA	pH	Fat	Protein	SCS	MY
Ca		0.33(0.02)	0.40(0.02)	0.01(0.02)	0.32(0.02)	0.44(0.02)	0.06(0.02)	-0.23(0.02)
P	0.67(0.24)*		0.82(0.01)	-0.10(0.02)	0.25(0.02)	0.39(0.02)	0.03(0.02)	-0.14(0.02)
TA	0.28(0.25)	0.92(0.05)*		-0.38(0.02)	0.24(0.02)	0.45(0.02)	0.00(0.02)	-0.11(0.03)
pH	0.19(0.29)	-0.06(0.25)	-0.30(0.19)		-0.16(0.02)	-0.09(0.02)	0.13(0.02)	0.01(0.02)
Fat	0.76(0.15)*	0.83(0.14)*	0.35(0.17)*	-0.29(0.19)		0.37(0.02)	0.08(0.02)	-0.27(0.02)
Protein	0.84(0.14)*	0.91(0.10)*	0.69(0.12)*	-0.02(0.22)	0.75(0.11)*		0.16(0.02)	-0.35(0.02)
SCS	-0.02(0.33)	0.20(0.30)	0.38(0.25)	-0.14(0.29)	0.38(0.24)	0.40(0.24)		-0.18(0.02)
MY	-0.26(0.30)	-0.31(0.28)	-0.46(0.24)	0.28(0.25)	-0.19(0.23)	-0.44(0.21)	-0.08(0.31)	

¹Ca = calcium; P = phosphorus; TA = titratable acidity; SCS = somatic cell score [SCS = ln(SCC)], where SCC is somatic cell count.

*Genetic correlations are significantly different from zero at $P < 0.05$.

Figure 1. Least squares means (with standard errors) of predicted calcium (Ca - continuous line) and phosphorus (P - dashed line) across days in milk.

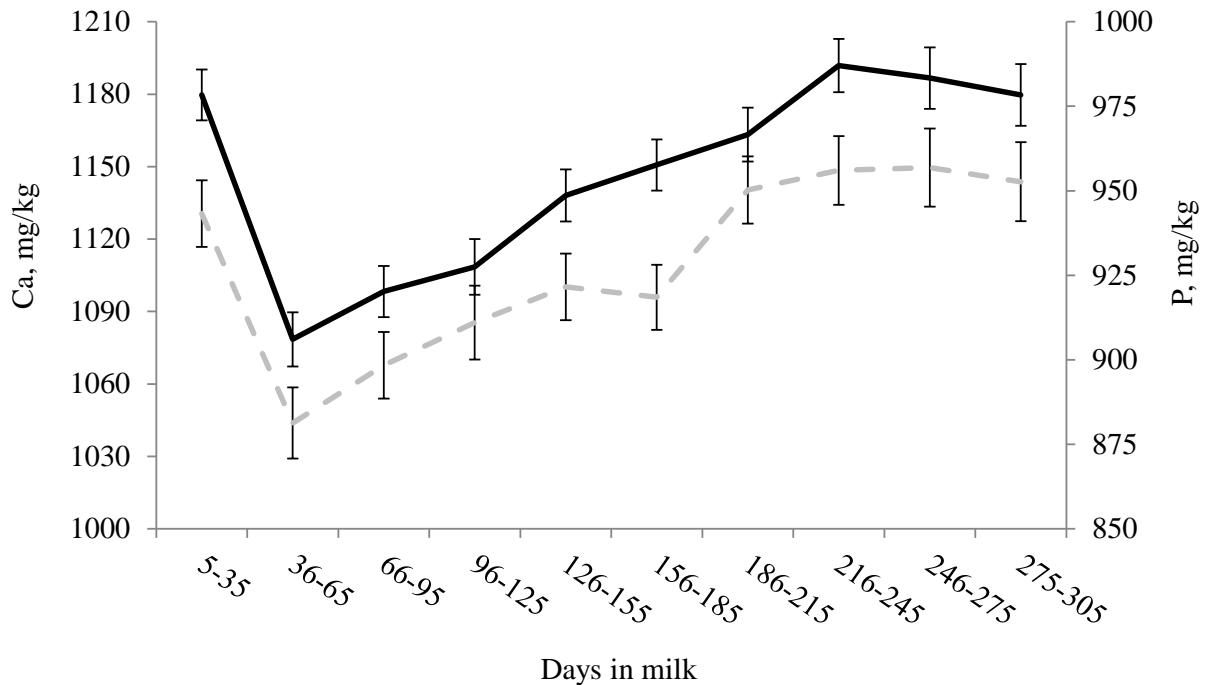
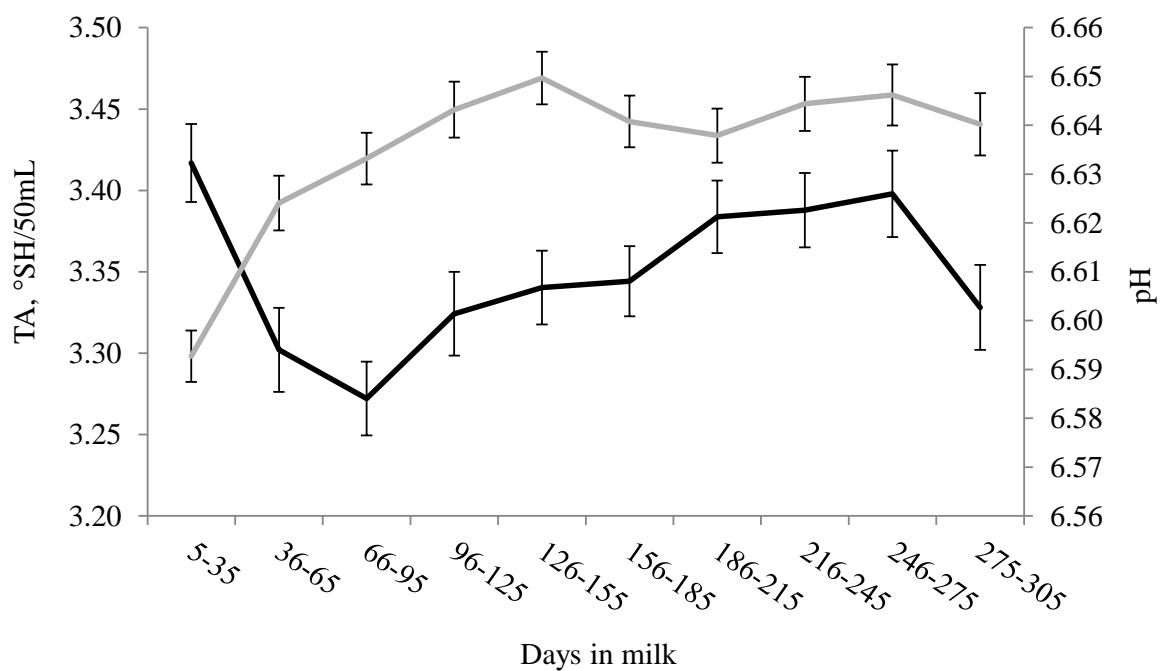


Figure 2. Least squares means (with standard errors) of predicted titratable acidity (TA - black line) and pH (grey line) across days in milk.



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GENERAL CONCLUSIONS AND PERSPECTIVES

The potentiality of MIRS as milk phenotyping tool has been documented in the literature. The accuracy of prediction models largely differs across milk traits, and the standardization of reference methods, the increase of biological variability of data through collaborations among different countries, the large scale data recording, and the study of the most suitable editing and spectra pre-treatments would lead to improvement of the robustness of prediction models.

In the present work the effectiveness of MIRS to predict milk MCP, Ca and P contents, and TA has been demonstrated. The prediction performance were satisfactory for RCT, k_{20} and a_{30} , given the improvement of prediction models based on a overextending of the traditional reference analysis time, while the accuracy of a_{60} prediction need to be enhanced. Milk Ca and P contents were predicted with good accuracy, even though Ca prediction model only permits to discriminate between low and high contents of Ca and need to be improved. Moreover, TA of milk was predicted with satisfactory accuracy.

The MCP, mineral contents and acidity of milk were moderately and favourably correlated to physical-chemical composition. Moreover, given the existence of heritable genetic variation of milk Ca, P and TA and high genetic correlations of Ca, P, and TA with milk protein and fat percentages, an indirect improvement of the former traits could be achieved through direct selection of the latter features.

The possibility to predict samples which coagulate after the traditional testing time of 30 minutes, should be considered for genetic purposes. In fact, these samples are often discarded from genetic analyses and considered as missing information, leading to possible biases in the prediction of sires merit for coagulation traits.

The aforementioned genetic purposes could be followed given the possibility to recorder large amount of milk phenotypes, with reduced costs and times thanks to MIRS technology. New milk phenotypes should be routinely available at population level if their prediction models are apply to MIRS instruments routinely used in milk laboratories for the milk-recording schemes to predict protein, casein, fat and lactose contents.

Concluding, the possibility to use mid infrared spectroscopy as phenotyping tool for milk MCP, mineral content and acidity was demonstrated and a genetic improvement of these nutritional and technological characteristics of milk could be facilitated by this technology.

LIST OF PUBLICATIONS

- Toffanin, V., M. De Marchi, N. Lopez-Villalobos and M. Cassandro. 2015. Effectiveness of mid-infrared spectroscopy for prediction of the contents of calcium and phosphorus, and titratable acidity of milk and their relationship with milk quality and coagulation properties. *International Dairy Journal* 41:68-73.
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