

Final OptiMIR Scientific and Expert Meeting: From milk analysis to advisory tools (Palais des Congrès, Namur, Belgium, 16-17 April 2015)

OPTIMIR - A PROJECT AIMING THE DEVELOPMENT OF NOVEL MID-INFRARED BASED MANAGEMENT TOOLS FOR DAIRY HERDS

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The dairy industry represents 13% of the turnover of the European food industry and north-west Europe produces 60% of the European milk. However, in 2014 the milk market has experienced a substantial fall in milk prices of some 50%. At these lower prices for milk the business of many milk producers is not sustainable. Due to recent advances, the mid-infrared (MIR) analysis of milk performed within milk recording (MR) promises more information than used traditionally. Beside the established MR parameters (protein, fat, lactose, and urea), the spectra could provide additional information on cows' status for a range of characteristics (*e.g.* fertility, health, energy balance, feeding and methane emission). Thus, the spectra routinely obtained from the MIR analysis of milk offer a possibility to develop novel, cost-effective tools which enable milk producers to improve the management of their dairy herds and in turn reduce the costs for milk production. The OptiMIR project aims to improve the sustainability of the dairy sector by developing and providing innovative, economical and standardized MIR-based tools for the management of dairy herds. Additionally, the project aims to create and promote a framework for the cross-border exchange of information and practices to enhance the service of milk recording organizations (MROs) in north-west Europe and to strengthen their competitiveness. In order to achieve these objectives, in 2011 the European OptiMIR project was officially launched for a 5-year-period. The INTERREG IV B funded OptiMIR project is a cooperation between three research centers, three universities, 11 MROs, and one laboratory from six north-western European countries. To accomplish the goals, the different processes from the identification of the priority areas to the development, validation, and implementation of the novel tools were outlined in three working packages comprising 10 actions. A common transnational database combines the phenotypic data of the cows and the MIR spectra from the European MR, which enables the detection of relevant phenotypic traits and their reflection in the MIR spectra. Additionally, a standardization has been installed among all instruments of the milk analyzing laboratories involved in the OptiMIR project to ensure a stable prediction over time and a correction of deviations. Due to the monthly standardization process the developed prediction equations can be used in routine on all instruments taking part in the standardization. Different models for the prediction of the pregnancy status, energy balance, the methane emission as well as the detection of ketosis and acidosis have been developed within OptiMIR. It is worth mentioning, that those models predict the status of the animal with different accuracies and that progress on development and implementation differs between the models. Some of the tools developed within OptiMIR are already used in the field and provide dairy farmers with useful information *e.g.* about the health status or methane emission of a certain cow. The OptiMIR project also resulted in a collaborative planning on an economic interest group formation of the participating MROs. The final OptiMIR scientific and expert meeting disseminates the results obtained through OptiMIR more detailed and also provides an overview of recent advances in the development of management tools for the dairy sector. Initial application of the MIR-based tools has shown their potential for providing dairy farmers with information to improve their herd management in a cost-effective way. However, further research and development is required to finish a greater extend of MIR-based tools so that north-western European stakeholders can acquire competitive advantage in the dairy sector.

Keywords. Dairy industry, livestock management, infrared spectrometry, research, Europe.

Session 1. Advances in infrared analysis of milk

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RECENT ADVANCES IN THE INFRARED ANALYSIS OF MILK

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Liquid milk is an excellent source of human nutrition that is widely consumed. In 2013 world milk production totalled 782 million t, more than 10 l per person globally. In the last 15 years milk production has increased annually at a rate of between 2-3%, and this is predicted to continue until 2020 (International Dairy Federation, 2014). Infrared spectroscopy is widely used in the analysis of liquid milk. Research and commercial applications go back to the 1970s. Both mid and near infrared (MIR and NIR) spectroscopic techniques have been used. Currently there are many thousands of infrared spectrometers routinely used for analyzing the chemical composition of milk. The last five years has seen a significant extension to the types of applications (Fagan et al., 2009; Holroyd, 2013). The use of chemometrics to develop quantitative predictive models has allowed the accurate measurement of the chemical constituents of milk (fat, protein, lactose). New chemometric approaches have shown that infrared spectroscopy has significant additional potential as a tool for milk quality assessment. Additionally, incidences of milk adulteration have further spurred the development of infrared spectroscopy in directions beyond those previously explored. Recently, three specific developments have had a major impact on the utility of MIR for analysis of milk:

- the ability to reliably create predictive models for minor components in milk;
- the development of lower cost and more robust instrument platforms;
- the use of targeted and untargeted calibration models for a wide range of potential adulterants.

NIR spectroscopy on liquid milk is challenging due to its turbidity and light scattering properties. Despite

this milk compositional analysis has been successfully implemented. Frequently this is in applications that leverage the advantages of NIR such as ease of application. In addition NIR has been applied to other areas such as the authentication of milk origin, differentiation of cow feeding regimes and measurement of milk microbiological content.

Keywords. Mid-infrared spectroscopy, near infrared spectroscopy, milk.

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QUANTIFICATION OF WHEY IN FLUID MILK USING CONFOCAL RAMAN MICROSCOPY AND ARTIFICIAL NEURAL NETWORK

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In this work, the use of confocal Raman microscopy and artificial neural network was assessed as a practical method to quantify adulteration of fluid milk by addition of whey. Confocal Raman microscopy uses an optical microscope to focus the laser in specific planes and spots along the sample. Raman technique is primarily advantageous because each material has a unique and unequivocal vibrational spectrum (fingerprint). Besides, no sample preparation is required, time analysis is brief and materials in different physical states and dimensions can be promptly analyzed. Milk samples containing whey percentage ranging 0 to 100% were prepared, simulating different levels of fraud. Even though the percentage of whey was up to 100%, the practical range focused was up to 30% of whey. All analyses were carried out by direct inspection of

the samples at the microscope after deposition of micro drops from each sample on microscope slide and dried under room temperature. Raman spectra were taken from 1,200 to 1,600 cm^{-1} , where two prominent peaks were observed at 1,430 and 1,540 cm^{-1} and considered as the most important regions to differentiate distinct levels of fraud. The excitation wavelength was 514.5 nm. No pre or post-treatment such as sample preparation or spectra correction were required in the analyses. Quantitative determination of the fraud was performed through a feed forward artificial neural network (ANN). Different ANN configurations were evaluated based on their values of R^2 and RMSE (Root Mean Square Error), which were the criteria for choosing and selecting the best predictor model. For the selected model, it was observed that both data from training and validation presented $R^2 > 99.99\%$, suggesting that the combination of confocal Raman microscopy and ANN consists on a swift, simple and efficient method to quantify adulterated milk by adding whey. Since sample preparation and post-processing of spectra were not required, the present method is interesting. It gives a linear model for the determination of percentage of whey added to milk. The method showed potential applications in sanitary vigilance and food quality monitoring.

Keywords. Raman spectroscopy, milk, artificial neural network.

DETECTION AND QUANTIFICATION OF WHEY IN RAW MILK BY NEAR INFRARED SPECTROSCOPY

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Brazil is one of the largest milk producer in the world and, until recently, milk used to be the major target of food frauds, such as adulteration with water, neutralizers to mask acidity, salt or sugar to mask extra water or high solid contents and cheese whey. The most frequent fraud consists of the addition of whey, which

is 90% cheaper than milk. Despite its noble uses, whey is often discarded by the dairy industry during cheese making. Whey has been used as an adulterant of both fluid and dry milk. It can be detected by analyzing its caseino macro peptides (CMP) or glycol macro peptides (GMP) contents by means of chromatography or electrophoresis measurements, which requires considerable time for preparation and analysis. In view of this, other techniques have been proposed for fast and reliable analysis of adulterated milk. Among the techniques, near infrared (NIR) absorption or reflectance has gained attention since it is fast and it did not require sample preparation. This work presents a method to quantify whey addition to raw milk by near infrared spectroscopy and comparing the results with the ones obtained by the determination of CMP by HPLC. Fluid samples with 0 (sample with no added whey), 1%, 4.25%, 7.5%, 10.75%, 14%, 17.25% and 20.5% were prepared in triplicate. For HPLC analysis of CMP a standard calibration curve was prepared using seven levels of CMP (Sigma) concentration: 0, 15, 30, 45, 60, 75, and 90 $\text{mg}\cdot\text{l}^{-1}$. An amount of 5 ml of trichloroacetic acid (24%) was slowly added to 10 ml of each standard solution, left to rest for 60 min at room temperature, followed by filtration in qualitative paper, discarding the first drops. The near infrared spectra were obtained by a FT-NIR Multi Purpose Analyzer (MPA model from BRUKER, Ettingen, Germany), in a range from 800 to 2,700 nm. As a result, the calibration model suitable for the detection of CMP in raw milk should be used with the PLS method. A linear correlation between CMP measurements and NIR was established with $R^2 = 0.9885$, for the calibration and 0.9543 for the validation data. It suggests that NIR measurements can be used to quantify whey addition in milk.

Keywords. Near infrared, whey, frauds.

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STANDARDIZATION OF MILK MIR SPECTRA, DEVELOPMENT OF COMMON MIR EQUATIONS

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This work is performed in the framework of the OptiMIR EU project that aims to use mid infrared (MIR) spectra of milk as indicator of the cow status. For this, milk spectra from different countries and laboratories are matched with physiological data in a common database to create calibrations predicting cow fertility, health, environmental and feeding indicators. Taking into account that the different spectrometers used to collect spectra produce different spectral responses, a step of spectral standardization is needed in order to merge all the spectra from the instruments into a common MIR database. Then, calibration models able to be used on all the instruments of the project can be built. The standardization method used is Piecewise Direct Standardisation (PDS), which match “slave” instruments spectra on those obtained with a “master” instrument, wavelength by wavelength, by measuring a set of common samples. The master is a fictional machine composed by an average of several instruments selected for their stability in time. The procedure is validated using different regression models as fat, poly-unsaturated fatty acids and methane produced by dairy cows. The RMSE (Root Mean Square Error) calculated between the master and the slave predictions, before and after standardization has been used as validation criterion. Using the fat model, the RMSE is reduced from 0.472 g fat·100 ml⁻¹ to 0.028 g fat·100 ml⁻¹ after standardization. The RMSE with the poly-unsaturated model is reduced from 0.061 g·100 ml⁻¹ to 0.009 g·100 ml⁻¹ and regarding the methane model the RMSE is lowered from 355.6 g CH₄ per day per cow to 30.2 g CH₄ per day per cow after standardization. These results have shown that the standardization step of MIR spectra of milk allows decreasing the inherent spectral variability between apparatus, and then permits the use of common equations on all instruments.

Keywords. Piecewise direct standardization, milk, MIR spectra.

IMPLEMENTATION OF CHEMOMETRIC TOOLS FOR MID-INFRARED CONTAMINANT SCREENING IN PRACTICE

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As a result of a number of crises relating to contamination of foods in the late 2000's focus on this subject has increased. Hence, solutions addressing the problem of intentional or unintentional contamination have been implemented in mid-infrared equipment for milk analysis. They are very efficient tools for screening for specific contaminants at levels down to less than 100 ppm, depending on the contaminant. It is also possible to screen for deviating milk by use of an untargeted approach. For the main constituents in milk (fat, protein, lactose, etc.) there are well-defined procedures for installing, monitoring, and adjusting prediction models, ensuring a good performance over time. This is somewhat different for these contamination screening models for two reasons: specific contaminants only appear rarely and the methods for screening for deviating milk produce results which do not follow standard distributions. Therefore, alternative methods for monitoring stability and triggering adjustments are required. Data from mid-infrared instruments being used for testing at milk receival in the field was collected before and after introducing contaminant screening. Furthermore, continuous data from instruments using contaminant screening was collected. Screening for deviating milk based on spectral features, *e.g.* based on Principal Component Analysis (PCA), will detect any spectral deviation, irrespective of its origin. This means that spectral deviations not caused by the milk will also be detected, *e.g.* instrument errors or spectral shifts. It is therefore very important to ensure that all instruments produce similar spectra prior to applying the same PCA model to all of them. This highlights the importance of using well maintained and standardized instruments for such untargeted screening methods. Results from the same instrument before and after introducing regular standardization procedures show this. A proposed test parameter for monitoring such untargeted screening models is the median of the distance measure produced by the model. Such a median may be calculated for a given period of time, *e.g.* one day. This parameter will be insensitive to the presence of a limited number of deviating samples and hence it will not be necessary to clean up the data prior to using it for monitoring. Prediction models targeting specific adulterants may be monitored by a similar approach. As there may be biases between results from different instruments there is a need to adjust for such biases. This may be done by assuming that the median of many samples should equal zero, *i.e.* that the median sample is not contaminated. Hence, it is an indication of a bias error if the median of predictions performed over a period of *e.g.* one day deviates from zero. This bias can be corrected for and monitored over time.

Keywords. Contaminant milk, chemometrics, monitoring.

Session 2 – Rapid analytical methods as phenotyping tools

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APPLICATION OF MID-INFRARED SPECTROSCOPY TO ENHANCE BOVINE MILK TECHNOLOGICAL TRAITS IN DAIRY INDUSTRY

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Worldwide demand for dairy products is increasing and further expansion is expected. In this scenario the improvement of the aptitude of milk to produce cheese is an attractive challenge for several European countries, especially those manufacturing high quality products (*e.g.* Protected Designation of Origin cheeses). One of the crucial steps of cheese making is the coagulation process that is related with yield and quality of the final product. Milk protein composition, milk coagulation properties (MCP), milk acidity (mainly titratable acidity, TA) and calcium (Ca) and phosphorus (P) contents are the most important traits involved in coagulation process. Traditionally, the determination of MCP, TA, and mineral content is time-consuming, expensive and not applicable at population level. The use of mid-infrared spectroscopy (MIRS) is a rapid and cost-effective tool for recording phenotypes at population level and it is commonly used to determine traditional milk quality traits in official milk laboratories. Lasting years the effectiveness of MIRS to predict phenotypes useful for dairy industry or for genetic purposes has been widely investigated, highlighting a growing interest for this technology. Several studies have demonstrated the potential of MIRS to predict MCP reporting quite satisfactory results (average coefficient of determination, 1-VR, of 0.70). Mid-infrared models have been implemented in several Italian laboratories to provide routine measures of MCP, and the information is mainly used by dairy factories to reward or penalize milk according to MCP. A recent study estimated (co) variance components for MCP using repeated data

($n = 63,470$) predicted during routine milk recording in Holstein-Friesian cows. Results indicated that genetic variation for MCP predicted by MIRS exists and thus selection at population level is feasible. The same study highlighted that several repeated measures per cow during a lactation are required to estimate reliable breeding values for MCP. Genetic correlations of MCP with production traits, milk composition, and somatic cell score were generally low. Besides MCP, MIRS has been indicated also as a tool to predict TA, and Ca and P contents, reporting 1-VR values of about 0.60 for Ca and greater than 0.70 for P and TA. Mid-infrared models were used to predict Ca and P contents, and TA in milk of about 2,500 Holstein-Friesian cows and to assess (co) variance components for these MIRS-predicted traits. Heritability estimates were 0.10, 0.12, and 0.26 for Ca content, P content, and TA, respectively; 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. Mid-infrared spectroscopy is a fast, large-scale, and low-cost tool for collecting phenotypes and its potential to predict MCP, milk acidity, and Ca and P contents has been demonstrated. All these new phenotypes could be used for breeding purposes to improve profitability of dairy chain.

Keywords. Dairy industry, mid-infrared spectroscopy, milk technological trait.

MID-INFRARED PREDICTION OF BETA-HYDROXYBUTYRATE, ACETONE, AND CITRATE CONTENTS IN MILK

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Mid-infrared (MIR) spectrometry is a rapid and cost-effective tool, which offers the opportunity to collect routinely a wide range of milk components useful for both management and genetics of dairy cattle. Among the milk biomarkers that could be potentially predicted by MIR spectrometry, citrate and ketone bodies are of particular interest in the monitoring of negative energy balance of dairy cows. Negative energy balance occurs in early lactation of dairy cows and might cause production diseases such as ketosis. Therefore, the objective of this study was to investigate the potential of MIR spectrometry to determine the contents in milk of acetone, beta-hydroxybutyrate (BHB) and citrate. A total of 566 milk samples were collected in commercial and experimental farms in Luxembourg, France and Germany. Acetone, BHB, and citrate contents were determined by flow injection analysis (Skalar, Breda, The Netherlands). Milk MIR spectra were recorded and standardized for all samples. Acetone content ranged from 20 to 3,355 $\mu\text{mol}\cdot\text{l}^{-1}$ with an average of 103 $\mu\text{mol}\cdot\text{l}^{-1}$; BHB content ranged from 21.3 to 1,595.6 $\mu\text{mol}\cdot\text{l}^{-1}$ with an average of 215.4 $\mu\text{mol}\cdot\text{l}^{-1}$; and citrate content ranged from 4.5 to 15.5 $\text{mmol}\cdot\text{l}^{-1}$ with an average of 8.9 $\text{mmol}\cdot\text{l}^{-1}$. Acetone and BHB content were log transformed to approach a normal distribution. Prediction equations were developed using partial least square regression after a first-derivative pre-treatment and the selection of informative wavelengths. The calibration coefficient of determination (R^2_c) was 0.73 for acetone, 0.75 for BHB and 0.90 for citrate with root mean square error (RMSE) of 87.7 $\mu\text{mol}\cdot\text{l}^{-1}$, 86.5 $\mu\text{mol}\cdot\text{l}^{-1}$ and 0.75 $\text{mmol}\cdot\text{l}^{-1}$ respectively. An external validation was performed and RMSE of validation was 45.2 $\mu\text{mol}\cdot\text{l}^{-1}$ for acetone, 65.33 $\mu\text{mol}\cdot\text{l}^{-1}$ for BHB and 0.80 $\text{mmol}\cdot\text{l}^{-1}$ for citrates. Although the practical usefulness of the equations developed in this study should be further verified with field data, results from this study demonstrated the potential of MIR spectrometry to predict citrate content with good accuracy and to provide indicative contents of BHB and acetone in milk for screening purposes.

Keywords. Mid-infrared spectrometry, ketone bodies, citrate.

BIODIVERSITY OF RAW MILK MICROBIOTA USING THE EXAMPLE OF TWO INDIVIDUAL FARMS

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Despite decades of research on the composition of raw milk (RM) microbiota, there is still very little known about them. Also, information on whether farm-specific floras exist and to what extent these are constant is still missing. In order to develop effective strategies to improve the quality of RM, more knowledge about the composition, variability, and stability of RM microbiota is needed because the microbiological quality of RM has a significant impact on the quality of the products made thereof. To get an idea of how the microbiota of RM is composed, the floras of two individual farms were analysed using an in depth culture-dependent sampling. Both farms were sampled nine times from December 2009 to February 2013, each time with a sample size of 50 isolates. The randomized isolated colonies were identified by Fourier-Transform Infrared (FTIR)-spectroscopy and *inter alia* the results were grouped into six higher taxonomic groups to determine the stability of the flora. In February 2010 the sample size was increased to 500 isolates. The identification was achieved using FTIR and 16S rDNA sequencing to describe the biodiversity and also the quantitative composition in more detail. Results of experiments with 50 and 500 colonies were compared to evaluate the influence of the sample size on the detected biodiversity. Based on a classification of microorganisms into six taxonomic groups, it could be shown that the composition of the two floras in the course of the study was very stable. Only at one time point in the period was the composition of one farm brought out of balance due to a limited coliform mastitis. For the 50 colonies approach the detected biodiversity was composed of 37 and 42 different genera in total on both farms, respectively. However, the composition of each sample at the genus or species level differed enormously. More than half of the detected species could only be found in a single sample at one time point, which is due to the high biodiversity, compared to the small sample size of only 50 colonies. With an increased sample size of 500 colonies the detected biodiversity was significantly higher, 66 and 117 species, respectively could be found. However, it was also found that only a few species are accounted for the majority of total isolates and that those could even be detected with small sample sizes. In addition, a significant number of new species and genera could be detected. In some cases the isolates of the same new species were isolated on both farms. Generally, this study has shown how large the diversity of RM microbiota is and that there is still a considerable need for research. Only when there is more knowledge of the biodiversity of RM and how the composition is

influenced, it is possible to improve the quality of RM and consequently the quality of the products.

Keywords. Raw milk flora, biodiversity, culture-dependent approach.

PREDICTING HARD TO RECORD BODY ENERGY TRAITS USING A MERGED PHENOTYPIC DATASET AND MID-INFRARED (MIR) SPECTRAL ANALYSIS OF MILK SAMPLES

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The high energetic cost of milk production for the dairy cow frequently outweighs that obtained through feeding and can lead to a state of negative energy balance (NEB), prolonged time in which can be detrimental to fitness and fertility. Body energy measures such as energy balance (EB), therefore, may be useful indices in genetic selection, however are notoriously difficult to measure. This study's objectives were to (1) merge data from different European experimental resource populations of Holstein-Friesian dairy cows creating a transnational database of phenotypic measurements, (2) use this merged dataset to estimate informative energy traits for all cows and (3) align such energy traits with available milk mid infra-red (MIR) spectra in order to predict these on an international scale using partial least squared (PLS) analysis. A total of 526,509 daily records from 962 cows were available from herds in France and the UK relating to milk, fat and protein yield, dry matter (DM) intake, live weight and body condition score. Use of random regression modeling to predict missing values gave 549,024 smoothed, daily records upon which body energy traits were calculated for each cow/day/lactation based on the metabolizable energy content of diet DM (MJ·kg⁻¹ DM). Effective energy intake (EEI, MJ·d⁻¹), the daily energy taken in by a cow through feeding, averaged 143.5 (± 0.06); Energy balance (MJ·d⁻¹), the daily balance of energy from feed

with that spent on milk production and maintenance, averaged -18.3 (± 0.05); Energy content (EC, MJ·d⁻¹), the absolute body energy of a cow on a particular day in milk, averaged 4,625 (± 1.21) and Cumulative effective energy (MJ·d⁻¹), total body energy throughout lactation, averaged 129.8 (± 1.74). Alignment of these energy estimates with their concurrent "standardized" spectral data created a reference "calibration" dataset of over 12,800 animal test dates, analyzed using PLS to predict the energy traits for independent "validation" spectra from 354 animal test dates collected from German cows, with an accuracy (r²) of 0.89. This demonstrates the feasibility of merging international data for developing beneficial prediction tools in livestock systems.

Keywords. Dairy cow, phenotypes, energy balance, mid-infrared spectrometry.

PHENOTYPING NEW TRAITS USING MID INFRARED SPECTROSCOPY IN DAIRY SMALL RUMINANTS: PROGRESS AND POSSIBILITIES IN FRANCE

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Using mid infrared (MIR) spectroscopy tends to develop to estimate fine component contents in milk for over a decade. In France PhénoFinlait was the first program to study the feasibility of estimating milk fatty acid (FA) and protein contents in goats and sheep using MIR spectroscopy. To develop FA equations, 332 milk samples of Alpine and Saanen goats were collected in five farms, and 200 milk samples of Lacaune, Manech Tête Rousse (MTR) and Basco-Bearnaise

ewes were collected in four farms to cover a large range of feeding systems. They were both analyzed by MIR spectroscopy and gas chromatography, which is the reference method for FA content measurement. To develop protein equations 287 milk samples from Alpine and Saanen goats and 159 milk samples from Lacaune and MTR ewes were collected and analyzed by both MIR and liquid chromatography coupled with mass spectrometry. For each equation, two thirds of the samples were used as calibration set and the remaining third was used as external validation set. In both goats and sheep the best results were obtained on saturated FA and monounsaturated FA with a chain length of 18 carbon atoms. The validation coefficient of determination (R^2v) exceeded 0.91 and the residual standard deviation (sy,x) was lower than 4% for 15 to 20 equations in both species. In sheep, whole casein, β -casein and $\alpha s1$ -casein are estimated with a good precision ($R^2v > 0.91$ and $sy,x < 5\%$) whereas $\alpha s2$ and κ casein are a little less precisely estimated. However the precision of the prediction is lower concerning whey proteins. In goats the R^2v for whole casein equation reached 0.71 and its sy,x was 7%. These results suggest that estimating milk fine composition in small ruminants using MIR spectroscopy is feasible. Other programs take advantage of this new skill in order to diagnose subclinical acidosis in dairy goat herds using milk FA profile (AcID) and to evaluate the infectious status of udders in dairy goats and sheep (MAMOVICAP). This approach might provide a cheap and fast solution to monitor these new traits for a large number of animals.

Keywords. Milk, mid-infrared (MIR) spectroscopy, small ruminants.

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USE OF THE MILK MIR SPECTRA WITH A LACTATION STAGE SPECIFIC MODEL TO PREDICT CH_4 EMITTED BY DAIRY COWS

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Methane (CH_4) is naturally produced by cattle during ruminal fermentation. However CH_4 is also the most important greenhouse gas produced by dairy cattle. In addition to environmental concerns, CH_4 production implies losses of gross energy ingested. Consequently, the mitigation of those emissions is relevant in environmental and economic perspective. For this, the main levers of action are the diet composition, the genetic selection and the management of the herd. To investigate the link between those parameters and the CH_4 emissions, an equation has been developed to predict easily individual CH_4 emissions from milk mid-infrared (MIR) spectra. Indeed, it has been established that CH_4 emissions can be predicted from MIR equations. However body tissue mobilization alters milk composition during time (*e.g.*, FA profile) and should be considered to predict CH_4 from MIR milk spectra. Therefore an innovative method was developed to reflect the expected changes in the relationship between CH_4 and milk composition due to the metabolic status during lactation. For this, the MIR based prediction equation includes now the days in milk (DIM) information by the use of lactation stage specific prediction coefficients. A total of 446 CH_4 reference data linked with the corresponding milk MIR spectra were obtained using the SF6 method on 146 Jersey, Holstein and Holstein-Jersey cows. Constant (P0), linear (P1) and quadratic (P2) modified Legendre polynomials were computed from DIM of cows the day of CH_4 measurements and a first derivative was applied to the MIR spectra. The calibration model was developed using as variables first derivative x P0, first derivative x P1, first derivative x P2 and a modified PLS regression. The CH_4 emission prediction ($g \cdot CH_4 \cdot d^{-1}$) showed a calibration coefficient of determination (R^2c) of 0.75, a cross-validation coefficient of determination (R^2cv) of 0.67 and the standard error of calibration (SEC) was 63 $g \cdot d^{-1}$. This equation has been applied on the milk MIR spectra database of the Walloon Region of Belgium ($\approx 2,000,000$ records) to check if the predictions match with the expected and biological meaningful behavior. The observed trend across lactation was similar to what was expected, *i.e.* an increase of averaged CH_4 up to DIM 83 and a slight decrease after. This pattern shows that considering the DIM into the calibration bring relevant information when compared to predictions from previous equations. Taking the metabolism of the cows into account could be a good strategy to improve the equation.

Keywords. Methane, milk, mid-infrared.

PREDICTION OF MILK TECHNOLOGICAL TRAITS FROM MID-INFRARED SPECTROSCOPY ANALYSIS OF MILK IN GRAZING DAIRY SYSTEM

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Rapid, cost-effective monitoring of milk technological traits is an important challenge for dairy industries specialized in cheese manufacturing. For example, moderate to high rennet susceptibility and long heat stability of milk could significantly concur on the profitability of dairy industries. Therefore, the present study aimed to investigate the ability of mid-infrared (MIR) spectroscopy to predict rennet coagulation time (RCT), curd firming time (k20), curd firmness at 30 (a30) and 60 (a60) min after rennet addition, heat coagulation time (HCT), casein micelle size (CMS), and pH in cow milk samples, and to quantify associations between these milk technological traits and milk composition. Samples were collected from 605 cows from multiple herds; the samples represented multiple breeds, stages of lactation, parities, and milking times. Reference analyses were undertaken in accordance with standardized methods, while MIR spectra were available for all samples. Prediction models were developed using partial least square regression, and prediction accuracy was based on both cross and external validation. Proportion of variance explained by prediction models in external validation was greatest for pH (71%), followed by RCT (55%), and HCT (46%). Prediction models for a60 and CMS were not satisfactory. On average, all prediction models tended to be unbiased ($P > 0.05$). Such a conclusion is quite important because significant bias could have implication on milk pricing, if a milk pricing was based on technological traits. Linear regression coefficient of reference values on predicted values varied from 0.17 (CMS regression model) to 0.83 (pH regression model) but were all different ($P < 0.05$) from one. This can potentially have implications for breeding programs, where the true variance of technological traits may be underestimated using predicted rather than

reference values. Milk composition and in particular nitrogen fraction affected milk technological traits. Protein concentration was negatively correlated with RCT (-0.46) and k20 (-0.54) and positively correlated with a30 (0.52). Urea concentration had the strongest correlation with HCT (0.48), whereas no correlation was calculated between CMS and milk composition. Results suggest that the developed prediction models for RCT, k20, a30, HCT, and pH can be used as a screening method for milk segregation at the industry level for different dairy productions. For example, milk with short MIR-predicted RCT, as well as with moderate to low MIR-predicted pH, can be used for cheese-making, whereas milk with high MIR-predicted HCT can be processed for milk powder. Moreover, further investigations are needed to quantify sources of variation of milk technological traits at the population level, and to estimate genetic parameters of these traits. **Keywords.** Milk coagulation properties, milk heat stability, casein micelle size.

Session 3 – Advances in data interpretation and integration in management indicators

Chair: HÉLÈNE SOYEURT (University of Liege, Belgium)

FREQUENTLY RECORDED SENSOR DATA MAY CORRECTLY PROVIDE HEALTH STATUS OF COWS IF DATA IS HANDLED CAREFULLY AND ERRORS ARE FILTERED AWAY

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The implementation of sensor based decision support in commercial dairy herds is highly dependent on having reliable systems. Problems with sensors give missing and noisy data hampering their use. Also, the presentation of results needs to be in a form, which is simple and useful. These issues were addressed using

a mastitis sensor and decision support as example. This study aims at providing and evaluating a modular system applicable to the pipeline from sensor to decision support. The case of mastitis was chosen as it is of economic importance and also affects welfare of cows, and because we have worked with a commercial sensor. The problems with sensors causing missing data and noise are described and a range of filtering and monitoring modules are shown to be important to make systems functional for herd management purposes. On top of this a solid method need to be used to interpret and present data to end users, in terms of easy to read categories. Filtering and pre-adjustments of raw data are important in making algorithms robust and reliable for daily use. Re-definition of traits is needed going from traditional few groups to continuous definitions, and then to new action oriented health classes. Also, for this case focusing on mastitis, assignment to “permanently sick” groups can be helpful in keeping focus on new acute cases. The combined used of filtering, fix-up routines and time series models leading into action oriented categories is needed to provide simple and robust decision support. The systems may be vastly improved by opening for transmission of data between user groups and to common databases – also with a few to use data in genetic selection.

Keywords. Mastitis, dairy cows, algorithms, model performance, data quality.

A full paper associated with this abstract, “Frequently recorded sensor data may correctly provide health status of cows if data is handled carefully and errors are filtered away”, is submitted to *Biotechnology, Agronomy, Society, Environment*.

DETECTION OF KETOSIS IN DAIRY CATTLE BY DETERMINING INFRARED MILK KETONE BODIES AMOUNT

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Ketosis is a disease caused by a negative energy balance affecting 50% of dairy farms (Fourichon et al., 2000). Ketosis induces a decrease in milk yield, in fertility performance and an increase in inter-current diseases (Leblanc, 2010). The objective of this paper is to propose a ketosis indicator integrating the components

of milk and zootechnical criteria. Milking samples were collected from 70 French commercial dairy farms (184 dairy cows). Ketone bodies were determined using both infrared spectroscopy and the Skalar chemistry method reference (Skalar, Breda, The Netherlands). The connections between the variables were studied by principal component analysis coupled with a decision tree (Software R 2.11.1) to determine the threshold values of variables best discriminating healthy from ketosis cows (milk ketone bodies thresholds of 0.1 mmol.l⁻¹ for Beta Hydroxy Butyrate [BHB] and 0.15 mmol.l⁻¹ for Acetone) (De Roos, 2007). In this study, we could identify healthy cows and cows affected by ketosis within 100 days of lactation. Our model had a sensitivity of 91% and a specificity of 88%. We could further extend the status of the animals into six classes from a healthy status (class 0) to doubtful or subclinical (with 2 levels of severity: classes 1 and 2) and clinical status (with 3 levels of severity: classes 3, 4 and 5). This final diagnosis tool is CetoDetect®. CetoDetect® was validated on a data set containing 2,500 dairy cows, from 8 to 100 days in milk. Blood samples were taken on 60 cows detected by CetoDetect® as affected by ketosis. Blood concentrations of BHB and glucose were determined using the Optium Xceed technology (Optium Xceed®, Abbott, Witney, UK); and the hepatic enzymes aspartate aminotransferase (AST) and gamma glutamyl transferase (gGT) were determined using the Vet Test (IDEXX laboratories, Westbrook, Maine, USA). These cows had higher BHB, AST, gGT, and lower glucose concentrations than healthy cows. So, blood analyses match with CetoDetect® diagnosis. Furthermore, CetoDetect® is correlated to production performances. A study conducted on 115,960 Prim’Holstein cows showed that cows from class 5 had 6.10 kg of milk·d⁻¹ loss as compared to cows from class 0 (healthy cows). The impact of ketosis on fertility was also studied. A total of 14,587 Prim’Holstein (PH) cows, 5,925 Normande (NO) cows are included in this study. The CetoDetect® class assigned to each cow is the maximum class given during the first three milk records. The interval from calving to 1st service was longer for cows in class 5 than those in class 0 (+ 17 days for PH and + 14 days for NO). CetoDetect® is an indicator for diagnosing ketosis to improve the health, production and reproduction of cows. CetoDetect® can be applied routinely at the herd level from milk samples collected during milk recording.

Keywords. Ketosis, infrared analysis of milk.

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DIRECT AND INDIRECT LOSSES IN MILK YIELD ASSOCIATED WITH SUBCLINICAL MAMMARY INFECTIONS IN DAIRY COWS UNDER FIELD CONDITIONS IN WALLONIA

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Milk losses associated with mastitis can be attributed to either effects of pathogens *per se* (*i.e.*, direct losses) or of the immune response triggered by the infection (indirect losses). Their respective magnitude may have short and long-term consequences on the epidemiology of the disease and the host-parasite coevolution, affect the choice of preventive and therapeutic targets, and determine selection objectives. To estimate both types of losses, it is necessary to have timely information on milk yield, level of immune response (*e.g.*, somatic cell counts, SCC) and bacterial load (*e.g.*, number of colony forming units, CFU). If the information is readily available on test-day milk and SCC, methods to count CFU are costly and time consuming, and they are not recorded routinely in field studies. An alternative is to perform a mediation analysis in which test-day SCC and milk are regressed towards CFU measured at a few selected times (sampling dates) and towards the time elapsed between test-day and sampling dates. The objective of the study was to apply this method

on 95 clinically healthy cows sampled three times at one week interval for bacteriological cultures and with test-day records on up to ten test-days before the date of the first bacteriological sample. Staphylococci (23.68% of the samples) and streptococci (9.16% of the samples) were the pathogens most frequently isolated. No changes were observed in cows with three consecutive bacteriological negative results. In cows with three consecutive bacteriological positive results, we observed an indirect loss in test-day milk yield (loss of 0.26 kg for each unit increase in log₂-transformed CFU) and high and stable test-day SCC (increase of 0.40 units for each unit increase in log₂-transformed CFU). In cows with one or two bacteriological positive results, we observed high and stable test-day SCC, no effect of CFU on SCC, and a decrease in milk of 0.83 kg for each unit increase in log₂-transformed SCC. The study highlights the importance of the milk loss associated with an increase in SCC in subclinically infected cows.

Keywords. Mastitis, losses.

MONITORING THE RISK OF KETOSIS BY USING MID INFRARED SPECTROMETRY

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Mid infrared (MIR) spectrometry is used in all milk analysis laboratories to estimate fat and protein contents. This method is based on the fact that molecules present in the milk absorb infrared radiation at different wavelengths. For over a decade MIR spectrometry has been used to estimate new milk components such as fatty acids, proteins, and minerals. Currently, the OptiMIR European program aims to use this method to predict physiological status of the cows directly from the MIR spectrum. The purpose of this study was to consider the possibility of preventing ketosis by using MIR spectrometry. Ketosis is a metabolic disease due to a trouble of energy metabolism that affects highly productive dairy cows in high negative energy balance

in early lactation. Beta-hydroxybutyrate and non-esterified fatty acids (NEFA) contents in blood are the gold-standard for ketosis control. Indeed blood BHB and blood NEFA are respectively the biomarkers of energy metabolism deviation and the use of fatty reserves. A data collection took place in four experimental farms in France and Germany on 214 Holstein, Montbeliarde and Abundance cows in early lactation. Once a week from calving to 50 days in milk one blood sample and one milk sample from each cow were collected. The blood sample was analyzed to measure BHB and NEFA contents and the milk sample was analyzed by MIR spectrometry. Blood BHB and NEFA contents were used to classify the samples according to the level of risk using thresholds defined on the basis of the literature. Among the samples, 820 were identified as “low risk” and 304 as “high risk”. The samples identified as “high risk” were then classified into three statuses according to the type of risk: “high ketone bodies content”, “high fat mobilization” and “ketosis”. An 81% sensitive and 69% specific prediction equation of the risk level was developed by logistic Partial Least Square regression. In order to distinguish between Ketosis type I and type II a second prediction equation was developed using canonical powered PLS regression and discriminant analysis. Applied on the samples predicted as “high risk” a well classified rate of 85% could be accomplished. Thanks to those models an alert can be given to the farmer concerning cows to monitor and cows to treat.

Keywords. Mid-infrared spectrometry, milk analysis, ketosis.

Authors thank OptiMIR partners, Interreg IV B financial support and the experimental farms for their help in data collection.

PHENOTYPING COWS – NEW TOOLS BY COMBINING MIR-SPECTRA AND DHI-DATA

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In Bavaria, Germany, more than 950,000 cows are under milk recording. Over 750,000 milk recording

samples are analyzed per month by Milchprüfing Bayern e.V., the raw milk testing lab for Bavaria. Milchprüfing Bayern e.V. has one lab equipped with one type of mid infrared (MIR) apparatus (FOSS Milkoscan FT6000). All milk recording data, animal data and the MIR spectra are managed in the database of the Landeskuratorium der Erzeugerringe für tierische Veredelung in Bayern e.V., the Dairy Herd Improvement Association covering all Bavaria. This unified database makes it easy to combine MIR-spectra with animal data. The MIR-spectrum of milk contains more information than is used to analyze the typical milk recording parameters like fat and protein content. Consequently, current research is trying to determine biological parameters from MIR-spectra. Pregnancy and metabolic health status of the cow are of particular interest. The objective is to create new additional management tools for farmers. This kind of research needs reference data for the biological status of the cow, which can be linked to the MIR-spectra. Such data are recorded by dairy herd improvement associations and milk recording organizations. The focus of the presented project was on the cow’s pregnancy status from 60 to 160 days in milk. The pregnancy status of the cow was determined from calving and insemination dates. To exclude breed effects only data from Fleckvieh (German Simmental) cows were used. All MIR-spectra were cut to a spectral range of 980 to 1,580, 1,728 to 1,800 and 2,810 to 2,980 cm^{-1} (222 Data points). As pregnancy should be detected as early as possible data collected 11 to 70 days after insemination were used. Models were build with 565,632 MIR-Spectra of milk samples collected in 2013. Performance of the models was estimated on 100,946 MIR-spectra collected in 2014 with the same general constraints as the training set. The following methods for preprocessing of the MIR-spectra were investigated: none, first derivative, partial least squares regression and correction for changes in MIR-spectra during lactation using linear models. Linear Discriminant Analysis and linear models were used for classification, *i.e.* to distinguish between pregnant and non-pregnant cows. Linear Discriminant Analysis and a linear model based on the raw MIR-spectra achieved a sensitivity and specificity for pregnancy of 60%. Preprocessing the MIR-spectra did not result in appreciable improvements. Incorporating animal fertility data in the models improved sensitivity and specificity by 6 to 10%. The results show that there are fundamental differences in MIR-spectra of pregnant and non-pregnant cows. However, these differences alone are not sufficient for practical use. To improve prediction models for pregnancy status further research on the combination of MIR-spectra with information about the fertility status of the cow is necessary.

Keywords. MIR spectroscopy, biometrics, farm management tools.

LINK BETWEEN MILK FATTY ACIDS AND NON-ESTERIFIED FATTY ACIDS IN THE BLOOD AND ITS IMPLICATION ON THE DIAGNOSIS OF NEGATIVE ENERGY BALANCE

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The monitoring of energy metabolism in the early part of lactation of high producing cows is essential for the management of the herd as a high fat mobilization can cause health problems and decreased reproduction performances. Blood and milk samples along with a gynecological examination were obtained on 61 cows from seven farms on a monthly basis starting from calving finishing at the pregnancy diagnostic. The fatty acids (FA) in the blood and the milk were analyzed by gas chromatography (GC). In order to characterize the level of fat mobilization and its link with the FA in the milk, the FA data sets were divided in categories based on plasma non esterified fatty acids (NEFA) ($< 0.4 \text{ mmol}\cdot\text{l}^{-1}$, $0.4\text{-}0.6 \text{ mmol}\cdot\text{l}^{-1}$ and $> 0.6 \text{ mmol}\cdot\text{l}^{-1}$) corresponding respectively to a low, mild and severe fat mobilization. A mixed procedure was applied to the data set (SAS 9.1). Fat in milk increased when NEFA increases ($40.7 \text{ g}\cdot\text{l}^{-1}$ vs $46.5 \text{ g}\cdot\text{l}^{-1}$ $P < 0.01$ respectively for cows with NEFA < 0.4 vs $> 0.6 \text{ mmol}\cdot\text{l}^{-1}$) but in the profile, the proportions of the milk C6-C14 FA produced *de novo* in the mammary gland, were significantly lower in fat mobilizing cows even in the cows with mild mobilization (15.1 vs 19.5% $P < 0.001$ in the < 0.4 vs $0.4\text{-}0.6 \text{ mmol}\cdot\text{l}^{-1}$ NEFA categories). However, with the increase of the fat content in the milk due to high NEFA concentration, the quantity of short chain FA was not significantly different ($7.9 \text{ g}\cdot\text{l}^{-1}$ vs $7.0 \text{ g}\cdot\text{l}^{-1}$ in the same categories). On the other hand, all the long chain FA in the milk was significantly increased with high NEFA content in blood. For example, the milk C18:1 was largely higher ($P < 0.001$) in proportion and content even in the mild mobilizing cows (30.4 vs 24.7% of FA and 15.1 vs $13.2 \text{ g}\cdot\text{l}^{-1}$ respectively for the $< 0.4 \text{ mmol}\cdot\text{l}^{-1}$ and the $0.4\text{-}0.6 \text{ mmol}\cdot\text{l}^{-1}$ NEFA categories). The C18:1 were the most promising FA in the milk to follow the energy balance in early calved cow. In fact, the excretion of the FA in the NEFA fraction was not linear. The C18:1 was the first FA excreted massively in the blood in case of high fat mobilization at the expense of the C18:0

and C18:2 FA ($R^2 = 0.57$). Moreover, the link between the C18:1 in milk and in the NEFA fraction was very high ($R^2 = 0.87$). Because the mammary gland takes directly long chain FA from the blood, especially from NEFA, milk sampling could be a useful method to diagnose cows with a negative energy balance in early lactation to prevent metabolic diseases.

Keywords. Energy metabolism, early lactation, NEFA.

MONITORING THE PREGNANCY STATUS OF DAIRY COWS USING MID-INFRARED SPECTRA OF MILK COLLECTED FROM MILK RECORDING

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Optimal fertility in dairy cattle is a major concern for farmers to ensure a sustainable milk production. Confirmation and monitoring of pregnancy are crucial to avoid extended number of days open and therefore increased production costs. Optimal monitoring of pregnancy has to be of sufficient accuracy but also easy to organize and as cheap as possible. Hence, the use of information directly collected in the context of milk recording programs, and especially the mid-infrared (MIR) spectra of milk, can be useful to develop a (pre-)screening tool. The MIR spectroscopy is a rapid, non-invasive and inexpensive method, which is widely and routinely used by milk labs to provide a prediction of milk composition. This study aims to investigate the opportunities to use directly the MIR spectra as an indicator of the pregnancy status of a given cow. MIR spectra and population records (*e.g.*, animal identification, calving dates, insemination dates) from milk recording programs of the Walloon Region in Belgium over the last three years have been used. The method is based on the comparison of the observed spectrum for a cow at a certain day with the expected spectrum of the same animal at the same day in milk if this cow was open. The resulting residual spectra (*i.e.*, difference between observed and expected spectra) are therefore the result of all effects, which were not taking account in the modeling of the expected spectra such

as the pregnancy status. Residual spectra are then used to build a predictive logistic equation to discriminate pregnancy. The obtained equation is finally validated on a testing dataset randomly selected from the original database. A total of 258,131 spectra from open cows have been used to perform modelling of all expected spectra of the whole database. Then, a total amount of 419,912 residual spectra have been obtained. Afterwards, 317,816 residual spectra have been selected as training dataset to build the predictive logistic equation. The testing dataset included 102,096 residual spectra (46,305 from open cows and 55,791 from pregnant cows). On the testing dataset the average error rate was 18.1%. Eighty four percents four of data belonging to open cows were correctly identified as open and 79.8% of the pregnant cows were correctly identified as pregnant. These results showed a high potential for providing a surveillance tool of the pregnancy status of a given cow at a given day using directly the MIR spectra of milk. Finally, these results and methodologies will be tested and implemented on real conditions, using a pilot farm network, in order to improve the usefulness of the tool to (pre-)screening status of pregnancy.

Keywords. Mid-infrared spectra, surveillance, pregnancy.

CORRELATION BETWEEN LEVELS OF B-HYDROXYBUTYRATE AND FATTY ACIDS IN BLOOD AND MILK AND ITS IMPACT ON KETOSIS DIAGNOSIS IN DAIRY COWS

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In high producing dairy cows, the onset of lactation is often linked with discrepancy between production needs and energy intake inducing a negative energy balance. In response to this energy

shortage fat mobilization occurs and production of ketones by the liver is stimulated. If excessively, accumulation of blood ketones occurs, leading to clinical and subclinical ketosis (SKC). SKC at herd level is difficult to diagnose. Poor production and reproduction performances are usually observed as an increased incidence of periparturient diseases in the herd (Suthar et al., 2013). Diagnosis methods include determination of beta-hydroxybutyrate (BHB) and increased non-esterified fatty acids (NEFA) resulting from body fat mobilization. BHB and NEFA could be dosed in blood of animals in late gestation and in early lactation. Post calving, cows presenting BHB over 1.2-1.4 mmol·l⁻¹ are considered SKC-cows while those presenting NEFA over 0.6 mg·l⁻¹ are labeled fat mobilizing cows. Development of non-invasive diagnosis techniques could be interesting to sample animals at a larger scale with lesser stress. The aim of this study was to verify whether blood and milk BHB values were correlated and whether diagnostic methods by milk analysis could be developed. Seventy-five cows out of eight selected Walloon dairy herds were followed up monthly from calving to pregnancy diagnosis regarding production and reproduction. At each visit (V), BHB and NEFA levels were determined in milk and blood. A maximum of 5 V was made. BHB was determined in blood using a cow-side test (Optium Xceed®, Abbott, Witney, UK) and in milk by a colorimetric test (Boehringer Mannheim, Ridgefield, CT). Blood NEFA and milk fatty acids were determined by gas chromatography (GC). Statistical analysis was performed by SAS 9.1. BHB levels in blood and milk were highly correlated ($r = 0.86$), indicating the possibility of diagnosis of SKC by milk sampling. The earliest the samples have been taken, the better the correlation is ($r = 0.95$ V1; $r = 0.91$ V2). Comparison of BHB with NEFA demonstrated a better correlation with milk BHB than with blood BHB (respectively 0.51 and 0.53 in milk vs 0.41 and 0.48 in blood for the V1 and V2 respectively). After the 2nd V, the correlation dropped to 0.38 (V3) and -0.14 (V4). Regarding C16:0 and C18:0 in milk, the best correlation with milk BHB was recorded in V2 (C16: 0.51; C18: 0.54) then dropped. These findings are likely due to the different mechanisms involved in the production of these metabolites. To conclude, dosage of milk BHB could be a good indicator for ketosis diagnosis taking into account that correlation with blood BHB and with NEFA is time-related.

Keywords. Subclinical ketosis, NEFA, dairy cows.

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Session 4 – Management indicator traits: contribution to animal breeding and benchmarking

Chair: SOPHIE MATTALIA (Institut de l'Élevage, France)

EXPLORING GENETIC INFORMATION CAPTURED BY MILK INFRARED PROFILES

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For many years infrared (IR) spectroscopy has been successfully used to routinely predict fat and protein content of milk. More recently there is a growing interest in predicting additional phenotypes based on milk IR profiles, *e.g.* milk fat composition, milk protein composition, milk coagulation, methane emission or energy balance. Once a prediction equation has been established these additional phenotypes can be obtained at virtually no additional costs. Although there is great value in exploring possibilities of predicting new phenotypes based on milk infrared profiles, there is also a need to gain more insight in the characteristics of the information captured by IR spectroscopy. An IR prediction equation can be based on direct relationships between specific frequencies and chemical bonds of milk components. However, prediction equations also might be based on indirect relations between infrared wavelengths and the component of interest. The distinction between direct and indirect information might have consequences for the range of conditions under which prediction equations are applicable. We used a genetic approach to explore the information on milk fatty acids that is captured by the milk IR profile. Bouwman (2014) compared results of a genome wide association study for a number of fatty acids based on gas chromatography (GC) with results based on IR predicted fatty acids. Considerable differences were observed between both association studies: regions detected based on IR predicted fatty acids were not detected based on GC fatty acids and regions detected based on GC fatty acids were not detected based on IR predicted fatty acids. A typical example was the region

on BTA26 containing the SCD1 polymorphism which showed a highly significant signal for C14:0 based on GC but which was not detected based on IR predicted C14:0. More recently we studied the effect of the SCD1 polymorphism on all 1,060 individual IR wavelengths (Wang, personal communication). No wavelengths were significantly affected ($p < 0.01$) by the SCD1 polymorphism. Duchemin et al. (2013) showed that the SCD1 polymorphism has significant effects on content of C10:0, C14:0 and C18:0 and highly significant effects of C10:1, C12:1, C14:1 and C16:1. Therefore, our results suggest that the IR profile contains little information on the content of these fatty acids. This confirms results by Eskildsen et al. (2014) who concluded that prediction of individual fatty acids relies on correlations with fat content rather than on direct relations with specific IR frequencies. IR prediction equations, which are mainly based on indirect associations, need close monitoring and might only work under strictly defined conditions (*e.g.* breed, season, lactation stage or feeding regime). Genetic analysis of IR spectra can provide insight in the information captured by milk infrared profiles.

Keywords. Milk infrared, genetics.

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USE OF INFRARED SPECTROSCOPY TO ENHANCE TECHNOLOGICAL AND NUTRITIONAL QUALITY OF MILK: GENETIC COMPONENTS OF MILK SPECTRA AND BREEDING VALUES ESTIMATES

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The aim of this study was to develop a general procedure for routine estimation of breeding values in the Italian Simmental cattle population for a number of innovative traits predicted by Fourier-transformed infrared (FTIR) spectroscopy, using a direct prediction method, where genetic analysis is directly conducted on milk FTIR spectral variables instead of FTIR predicted traits. Breeding values for all new traits, related to technological and nutritional quality of milk, were then derived from the genetic component of the spectra. The investigated traits were in total 62 and included: detailed milk fatty acid composition measured by 2D-GC, milk coagulation properties assessed by renneting meter analysis, pH, contents of major casein and whey protein fractions measured by RP-HPLC, cheese yield, curd composition and recovery rates of protein and fat in the curd, measured in individual micro-cheese making procedures, contents of major minerals by ICP-OES and of lactoferrin quantified by ELISA. FTIR calibrations were developed using more than 1,000 milk samples for all traits, with the exception of minerals (820 samples) and lactoferrin (635 samples). Calibration set included approximately 90% Simmental and 10% Holstein Friesian cows. A total of 100,272 milk spectra from 11,216 Italian Simmental cows were recorded in the routine milk recording of the Friuli-Venezia Giulia Milk Recording Agency (Italy). Principal component analysis of the unprocessed spectral data resulted in eight latent traits that explained 99% of the total spectral variability. Restricted maximum likelihood was used to estimate (co)variance components of the latent variables. Heritability for these eight latent traits ranged from 0.09 to 0.40. Variance ratios of the permanent environmental effect were between 0.10 and 0.25 and residual variance ratios ranged from 0.39 to 0.81. Variance components and the breeding values of original spectral transmittances were obtained by back transformation. Breeding values for the new milk traits were then predicted through FTIR calibrations as values correlated to the genetic component of the FTIR spectra. The procedure yielded estimates of breeding values for all the investigated traits for more than 10,000 Simmental animals. For each of the investigated traits, the correlation between the breeding values obtained with the direct approach (DIR_{EBV}) and those obtained from the genetic analysis of the FTIR-predicted traits (IND_{EBV}) was calculated. Values of the correlation between DIR_{EBV} and IND_{EBV} ranged from 0.36 to 0.99. This indicates that the use

of the spectra back transformation for genetic analysis may be feasible or not depending on the calibration equation.

Keywords. Breeding values, direct prediction, principal component analysis.

OCCURRENCE OF *STAPHYLOCOCCUS AUREUS* GENOTYPE B IN DAIRY FARMS AND CLINICAL SAMPLES IN BADEN-WÜRTTEMBERG

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Mastitis causes the greatest disease-related economic loss in dairy farms. *Staphylococcus aureus* is the most common cause of chronic mastitis worldwide. The infection usually proceeds subclinically and is spread at milking time. Following the studies of Fournier et al. (2008) and Graber et al. (2009) one particular genotype of *S. aureus*, *S. aureus* genotype B, is of outstanding interest due to its high contagiousity and increased pathogenicity. Detection of *S. aureus* is quite difficult due to the fact that the bacteria are shed cyclically. To prevent new infections, one of the most important steps is the monitoring of udder health. On population level, bulk tank milk (BTM) can be used to monitor the status within a herd. The objective of this study was to determine the occurrence of *S. aureus* especially *S. aureus* genotype B on farms in Baden-Württemberg (BW). For this purpose a highly sensitive and specific assay for the detection of *S. aureus* genotype B (GTB) in raw milk which was developed by Boss et al. (2011) was used. GTB is characterized by the presence of the *S. aureus* enterotoxin genes A (sea) and D (sed) and by a polymorphism within the leucotoxin E gene (lukEB) caused by a point mutation. Based on these facts, Boss et al. (2011) developed a qPCR assay for the detection of GTB in BTM. This assay has a high diagnostic sensitivity (90%) and specificity (100%). A BTM sample is defined as GTB-positive if qPCR for lukEB and sea and/or sed generates a positive result. Since January 2012, this assay (slightly modified and optimized) is used within a special project Gesundheitsmonitoring Rind (GMON) within Baden-Württemberg (BW). BTM of registered farms are monthly monitored for the three cow-associated

pathogens *S. aureus*, *Streptococcus agalactiae* and *Mycoplasma bovis*. If a sample is positive for *S. aureus* ($ct \leq 37$) the assay for GTB is additionally performed. Over a period of 15 months nearly 9,000 BTM samples were monitored and about 40% of these samples were genotyped. Furthermore, the GTB assay was used for 100 clinical samples from the service for udder health of BW, which were positive for *S. aureus* in bacteriology and confirmed by real-time PCR. The study shows that GTB is also common in BW. GTB was detected in 20% of all tested farms and in 15% of *S. aureus* positive clinical samples. These results indicate that *S. aureus* genotype B is also common in BW. The monitoring of the BTM using the described method revealed to be a very useful tool to detect and to prevent the spread of GTB within a herd. It also offers the possibility of intervention at an early stage of new infection after a herd is sanitized with the result of regression of tissue damage. Thus fewer cows are infected and antibiotics can be reduced. That all leads to less economic loss for the dairy sector.

Keywords. Mastitis, *Staphylococcus aureus*, genotyping.

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GENETIC VARIABILITY OF MID- INFRARED PREDICTED CHEESE YIELD IN HOLSTEIN FIRST-PARITY COWS IN WALLOON REGION OF BELGIUM

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Cheese yield is an important technological and economical parameter in cheese manufacturing at the farm level. Up to date, empirical or theoretical formulae allow estimating the theoretical cheese yield from fat, and casein or protein contents of milk. Since few years, several studies have demonstrated the usefulness of the mid infrared (MIR) spectrometry for the prediction of milk technological properties. Recently, two equations for the prediction of fresh and dry Individual Laboratory Cheese Yield (ILCYf and ILCYd) were developed. The cross-validation coefficients of determination of those two calibration equations are 0.74 and 0.81, respectively for ILCYf and ILCYd. The objective of this study was to estimate the genetic parameters for the MIR predicted fresh and dry individual laboratory cheese yield in first parity Holstein cows in Walloon Region of Belgium. These two equations were applied on the spectral database (FUTUROSPECTRE) generated during the Walloon routine milk recording. Based on the MIR spectrum, the standardized Mahalanobis distance (GH) of each milk sample was computed, separately for each trait, using the spectra of the final dataset used to obtain the calibration equation for the same trait. For each trait, separately, records with GH value higher than 3 or with a predicted value out of the range of the reference values in the final dataset mentioned before were discarded. This allows to work on predicted values based on samples encompassing the variability (spectral or range of reference values) really taken into account in the calibration equation. Only predictions recorded between the 5th and the 365th day in milk (DIM) were included in this study in order to use first lactation representative data. After editing, the dataset includes 109,249 and 109,536 predicted records of ILCYf and ILCYd, respectively, collected on 15,338 Holstein first-parity cows between January 2009 and November 2014. The ILCYf mean was 27.7 g coagulum·100 g⁻¹ milk (SD = 4.9) and ILCYf ranged from 11.5 to 50.9. Similarly, the ILCYd mean was 63.3 g dry matter of coagulum·100 g⁻¹ dry matter of milk (SD = 4.3) and ILCYd ranged from 43.3 to 82.4. The variances components were estimated by REML using single-trait random regression animal test-day model. Random regressions were performed with modified normalized second order Legendre polynomials and the model took into account the heterogeneity of the residual variances over days in milk. Estimated daily heritabilities ranged from 0.20 at 5th DIM to 0.49 at 240th DIM for ILCYf and ILCYd, thereby confirming the potential of selection. Further researches will study phenotypic and genetic correlations between predicted ILCYf and ILCYd and milk production traits.

Keywords. Cheese yield, daily estimated heritability, mid-infrared prediction.

GENETIC PARAMETERS FOR TOTAL, DIRECT AND INDIRECT LEVELS OF TOLERANCE TO CLINICAL MASTITIS IN DAIRY COWS UNDER FIELD CONDITIONS IN WALLONIA

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A structural equation system with two sets of random regression models was used to estimate total, direct and indirect milk losses associated with clinical mastitis. Milk loss mediated by the increase in somatic cell counts (SCC), itself due to the migration of phagocytes into the infected mammary gland, is a consequence of weak indirect tolerance and it is called "indirect loss". Milk loss not mediated by the increase in SCC is due to the negative effects of the pathogens and of the defense mechanisms not associated with SCC. It is a consequence of weak direct tolerance and it is called the "direct loss". The "total loss" is the sum of both types of loss. Data consisted of 3,070 milk and SCC test-day records on 660 diseased cows from 31 commercial dairy farms of the network "Observatory for Udder Health (OSaM)" that federates researchers, dairy associations and breeders in the Walloon region of Belgium. Expected values for total, direct and indirect losses in test-day milk were 265.50 g/week, 251.64 g/week and 13.38 g/week, respectively. The higher indirect than direct level of tolerance suggests cows were suffering subclinically before clinical signs appeared. Heritability was estimated at 26.08% for the total, at 6.25% for the direct and at 17.21% for the indirect milk losses. Heritability estimates are sufficient to provide a potential for genetic selection to improve indirect level of tolerance. To do so, we suggest breeding animals for which transmigration of a reduced number of short-lived phagocytes with a high killing ability is sufficient to get rid of the infection. If results are confirmed in other dairy populations, selection of indirectly tolerant animals would be an alternative to current therapy that aims at ensuring the well-being of diseased animal.

Keywords. Tolerance, mastitis, heritability.

CANADIAN EFFORTS TO ADVANCE THE UTILIZATION OF MILK MIR SPECTRA IN THE DAIRY CATTLE INDUSTRY

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Several projects in Canada are aiming to incorporate milk mid-infrared (MIR) spectra to develop predictions of milk components and cow status. Since early 2013, a proportion of milk spectral data from FOSS MIR machines at Canadian DHI partners, CanWest DHI (Guelph, ON) and Valacta (Sainte-Anne-de-Bellevue, QC), have been saved and transferred to the Canadian Dairy Network. The database is nearing 2 million saved spectra with roughly 50,000-60,000 added every month from the approximately 700,000 cows enrolled in milk recording programs in Canada. These spectra will be combined with Canada's collection of recorded cow reproduction and health events (including hoof health) and will be available for the prediction of future developed MIR-type traits. The University of Guelph is currently analyzing approximately 2,000 milk samples for their fatty acid profile, milk fat globule and casein micelle size, lactoferrin, calcium, casein, and phospholipid content to use as a reference for developing MIR prediction equations. An additional project measuring fatty acids and cholesterol content in the milk of 2,700 genotyped cows will aid in the refinement of these equations. MIR predicted milk component traits are to be used for the genetic and genomic evaluation of Canadian dairy cows and bulls with the objective of improving the nutritional value of milk and milk products for human consumption. A pending Canadian project measuring feed efficiency and methane emissions in dairy cattle will also incorporate MIR spectra as a possible means for prediction. Multiple milk samples from 430 cows

representing four dairy breeds from 45 herds across three Canadian provinces have been collected during routine milk recording. Samples were divided and portions were sent to a Canadian DHI laboratory to generate MIR spectral data, as well as the University of Guelph for component analysis. Thus far, analysis has been completed on 1,960 milk samples for MFG size, 2,787 for casein micelle size, 212 for calcium content, 964 for fatty acid profile, 149 for casein composition, and 105 for phospholipid content. Initial analysis with the available records shows variation existent in both the spectra and milk components of the examined milk. The quantity of individual and groups of fatty acids can be predicted with varying accuracies using partial least squares regression methods. It may also be possible to identify milk samples with either small or large milk fat globules. The various projects initiated in Canada address the many areas the MIR spectrum can be exploited. The implementation of MIR prediction of a variety of new milk component and cow traits through existing Canadian milk recording programs will greatly increase the information available for both cow management and genetic evaluation to further improve the Canadian dairy industry.

Keywords. Genetic improvement, nutritional value, health status.

GENETIC PARAMETERS FOR MILK CALCIUM CONTENT PREDICTED BY MID-INFRARED SPECTROSCOPY IN THREE FRENCH DAIRY CATTLE BREEDS

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The aims of this study were to develop an equation to estimate calcium content (Ca) in bovine milk, using

mid-infrared (MIR) spectroscopy and to determine Ca genetic parameters. To develop the Ca equation, 300 milk samples were selected from PhénoFinlait milkbank to cover a large range of breeding practices. The samples were chosen considering breeds, areas, seasons, lactation numbers, day in milk, protein level, and diets. Those samples were both analyzed by MIR and by atomic absorption spectrometry, which is the reference method for Ca measurement. Randomly chosen among the 300, 210 samples were used as calibration dataset and the remaining 90 were used as independent validation set. The determination coefficient of validation of the equation (Rv^2) reached 0.79, its residual standard deviation (sy,x) was 4% and the residual prediction deviation (RPD) was 2.2. Genetic parameters of Ca were estimated for the three French major dairy breeds (Prim'holstein [HOL], Montbéliarde [MON], Normande [NOR]). Calcium content equation was applied to 35,326 spectral records collected from 6,723 first lactation HOL cows, 28,508 spectral records collected from 5,590 first lactation NOR cows and 50,505 spectral records collected from 6,330 first lactation MON cows. Three different models were used to estimate genetic parameters: (1) an individual test-day repeatability model, (2) a lactation model, where the trait is the average of test-day records and (3) a test-day random regression model. The heritabilities of Ca estimated with lactation model were 0.44 in HOL, 0.74 in NOR and 0.70 in MON. The coefficients of genetic variation were 3.6%, 4.3% and 4.2% in HOL, NOR and MON respectively. Genetic correlation with protein content was positive, especially between Ca and casein content. Calcium content was negatively correlated to milk production. No significant correlations were observed between Ca and conformation traits. The relationship between Ca and functional traits such as fertility or longevity needs further investigations. Those results suggest that genetic selection for Ca is feasible.

Keywords. Calcium content, cow milk, mid-infrared (MIR) spectroscopy.

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THE GENETIC PARAMETERS AND HERITABILITY OF FATTY ACIDS IN UK NATIONAL HERDS PREDICTED USING MIR

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Routine collection and calibration of spectral data from mid-infrared (MIR) analysis of milk is an effective approach to predicting the fatty acid (FA) profile of milk samples from dairy cows, with advantages of cost and labor over gas chromatography. Originally published by Soyeurt et al. (2011), equations to quantify the concentration of a suite of FA's in milk based on MIR spectra have since been updated as part of an EU-funded project, OptiMIR, alongside a method of standardization to account for drift over time and milk-analysis machines. The objective of this study was to predict milk FA's from monthly milk MIR for over 88,000 cows from UK herds, collected between January 2013 – December 2014 with an average of 9.4 test dates. These data are those retained following edits to ensure the 10 focal FA's and FA groups were sampled in 0 and 50 weeks in milk (WIM), within three standard deviations of the overall mean for each fat type and with 10 records minimum per herd-year-season (HYS) and lactation. A linear model was used to model the data and accounted for month and year of record, age at first calving, lactation identifier and HYS of calving. Results showed that the heritability (h^2) of milk FA's varied across the FA's from 0.08 to 0.3 and across WIM. Milk yield is an important index used in selective breeding and its $h^2 = 0.30$. Here we estimate the h^2 of saturated fat as 0.36, short chain FA's as 0.35 and C16 as 0.32 and hence the usefulness for these as indices in selection. This research demonstrates the use of MIR-based FA predictions on a national level at routine milk recording and its potential use in future selection indices to lower the proportion of FA's with negative health associations in an otherwise nutrient rich product without effecting consumer consumption.

Keywords. Fatty acids, MIR, heritability.

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FTIR SPECTROSCOPY AND GENETIC SELECTION FOR IMPROVING BOVINE MILK FATTY ACID COMPOSITION

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Fourier Transform Infrared Spectroscopy (FTIR) has been proven a powerful tool to characterize chemical composition of milk in the dairy industry. Previous studies have shown that FTIR data calibrated against gas chromatography (GC) data from milk samples have the potential to give detailed prediction of milk fat composition with high precision (Afseth et al., 2010). Calibrations for selected fatty acids are now becoming commercially available. Therefore, FTIR can potentially be used as a routine method to determine milk fat profiles. The main aim of this study was to establish prediction models for fatty acid composition of bovine milk from Norwegian herds to be used for genome-wide association analysis (GWAS). For this purpose we have performed parallel FTIR analysis and GC fatty acid profiling of approximately 1,000 samples during recent years. Samples with a large variation in saturated fatty acids have been chosen allowing the establishment of reliable and stable models. Multivariate calibration was performed with cross-validated Partial Least Squares Regression relating 1,060 FTIR channels (X) to about 30 GC-determined fatty acids (Y), via about 20 latent variables, based on measurements from animal feeding experiments and routine milk analysis. A detailed analysis of cross-correlations between fatty acids obtained by reference data and predictions allowed an estimated of the stability of the calibration models (R^2 [C14:0] = 0.85, R^2 [C16:0] = 0.75, R^2 [C18:1 c9] = 0.94, R^2 [SAT] = 0.96, R^2 [MUFA] = 0.96, R^2 [PUFA] = 0.69). Sparse variable selection methods allowed the biological validation of the calibration models. In an on-going project at Norwegian University of Life Sciences, we collected 9 mio spectra from routine FTIR measurements of milk samples from practically all Norwegian cows since 2007. The established FTIR prediction models for fatty acids were applied to all 9 mio milk sample spectra and fatty acid composition was predicted. An analysis of the predicted fatty acid profiles revealed interesting seasonable and regional variations. Thus, month-to-month variations in fatty acids were observed in addition to farm-to-farm variations. This suggests variations of fatty acid composition of milk due to feed. Further, a genome-wide association analysis (GWAS) was performed

to reveal the genetic background for the fatty acid variation and to identify quantitative trait loci (QTL) affecting fatty acid composition. The GWAS detected QTLs for milk fatty acids on most chromosomes in the Norwegian Red cattle.

Keywords. Fourier Transform Infrared Spectroscopy, Partial Least Squares Regression, genome-based improvement, milk fat composition.

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GENETIC CORRELATIONS BETWEEN METHANE PRODUCTION AND MILK FATTY ACID CONTENTS OF WALLOON HOLSTEIN CATTLE THROUGHOUT THE LACTATION

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Methane (CH₄) from ruminal fermentation is the major greenhouse gas produced by dairy cattle, which contributes largely to climate change. Production of CH₄ also represents losses of gross energy intake. Therefore, there is a growing interest in mitigating these emissions. Acetate and butyrate have common bio-chemical pathways with CH₄. Because some milk fatty acids (FA) arise from acetate and butyrate, milk FA are often considered as potential predictors of CH₄. However, relationships between these traits remain unclear. Moreover, the evolution of the phenotypic and genetic correlations of CH₄ and milk FA across days in milk (DIM) has not been evaluated. The main goal of

this study was to estimate genetic correlations between CH₄ and milk FA contents throughout the lactation. Calibration equations predicting daily CH₄ production (g·d⁻¹) and milk FA contents (g·100 dl⁻¹ of milk) from milk mid-infrared (MIR) spectra were applied on MIR spectra related to Walloon milk recording. Data included 243,260 test-day records (between 5 and 365 DIM) from 33,850 first-parity Holstein cows collected in 630 herds. Pedigree included 109,975 animals. Bivariate (*i.e.*, CH₄ production and one of the FA traits) random regression test-day models were used to estimate genetic parameters of CH₄ production and seven groups of FA contents in milk. Saturated (SFA), short-chain (SCFA), and medium-chain FA (MCFA) showed positive averaged daily genetic correlations with CH₄ production (from 0.25 to 0.29). Throughout the lactation, genetic correlations between SCFA and CH₄ were low in the beginning of the lactation (0.11 at 5 DIM) and higher at the end of the lactation (0.54 at 365 DIM). Regarding SFA and MCFA, genetic correlations between these groups of FA and CH₄ were more stable during the lactation with a slight increase (from 0.23 to 0.31 for SFA and from 0.23 to 0.29 for MCFA, at 5 and 365 DIM respectively). Furthermore, averaged daily genetic correlations between CH₄ production and monounsaturated (MUFA), polyunsaturated (PUFA), unsaturated (UFA), and long-chain FA (LCFA) were low (from 0.00 to 0.15). However, these genetic correlations varied across DIM. Genetic correlations between CH₄ and MUFA, PUFA, UFA, and LCFA were negative in early lactation (from -0.24 to -0.34 at 5 DIM) and increased afterward to become positive from 15 weeks till the end of the lactation (from 0.14 to 0.25 at 365 DIM). Finally, these results indicate that genetic and, therefore, phenotypic correlations between CH₄ production and milk FA vary following lactation stage of the cow, a fact still often ignored when trying to predict CH₄ production from FA composition.

Keywords. Methane, fatty acid, genetic correlation.

GENETIC AND ENVIRONMENTAL EFFECTS ON INDIVIDUAL WAVENUMBERS OF BOVINE MILK INFRARED SPECTRA

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Fourier transform infrared (FTIR) spectroscopy is a powerful method, which can be used in the prediction of milk composition. Quantification is based on the principle that the absorption of infrared (IR) light differs by changes of relative amounts. Therefore variation in individual wavenumbers reflects variation in milk components. To increase our understanding of the information that is captured by the milk IR spectra we investigated genetic and environmental effects on individual wavenumbers. In the current study we analyzed IR wavenumbers of 1,759 Holstein Friesian cows on 359 herds in the Netherlands, collected during the winter period. An animal model was used to analyze each of the 1,060 wavenumbers. The average value of the wavenumbers ranged from 0.4 to 1.5. Genetic, herd and residual variances were estimated and in addition the significance of lactation stage on individual wavenumbers was quantified. Based on the estimated variance components we estimated the intra-herd heritability. Heritabilities of 1,060 IR wavenumbers ranged from 0 to almost 1 (0.99) indicating that the genetic background of IR wavenumbers differs considerably. One hundred seventy-seven wavenumbers had heritabilities lower than 0.2 and for 35 wavenumbers we estimated heritabilities larger than 0.7. The majority of the wavenumbers have moderate to high heritabilities ranging between 0.4 and 0.8. The wavenumbers with low heritabilities coincide with the wavenumbers, which are known to be related to water absorption. Herd effects explained between 2.2% to 52.4% of the total variance. Four hundred twenty-eight wavenumbers had herd effects ranging between 10% and 20%, while for 429 wavenumbers differences between herds explained between 20% and 30%. A substantial contribution of the herd variance to the total variance suggests that these wavenumbers are indicative for differences in feeding and management between herds. The fixed effect lactation stage had significant effects on 712 wavenumbers, which is two third of the total number of wavenumbers. The highest $-\text{Log}_{10}(\text{P-value})$ found for the effect of lactation stage is 14.1. The significant effects of lactation stage can be explained by the milk composition (*e.g.* fat and protein content) changing during lactation. The study demonstrated the different extents in the regions influenced by genetic and environmental effects. With improved knowledge of milk IR spectra, the more efficient prediction equations for milk composition can be created. The cows with favorable milk composition can be selected. Furthermore, due to the considerable herd effects, it is possible for IR spectra serving as indicators of farm managements.

Keywords. Milk infrared wavenumbers, heritability, herd effects.

Session 5 – Prospects and future challenges in analytical procedures to qualify fine milk composition

Chair: VINCENT BAETEN / GEORGES SINNAEVE (Walloon Agricultural Research Center, Belgium)

ON-FARM MILK COMPOSITION MEASUREMENT FOR COW HEALTH AND FERTILITY MONITORING

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As a result of the intensive genetic selection, the metabolism of modern dairy cows is mainly focused on milk production. While this evolution has led to a strong increase in the average milk production per lactation, it has also decreased the fertility and robustness of our dairy cows.

The profitability of a dairy farm greatly depends on the reproductive efficiency. In the past, a calving interval (CI) of 365 days was assumed to be optimal. Nowadays, taking into account the milk production persistence of modern dairy cows and the needed time for postpartum recovery, the optimum is considered to be around 385 days. As the average CI in Flanders is 418 days and an increase in the CI above the 395 was found to cost 2-3 € per cow per day, the current calving interval resembles a loss of about 50 € per cow per year, or 5,000 € for a farm of 100 dairy cows. This corresponds to an estimated loss over all Flemish dairy farms of 15 million € or 2.1% of the annual production

value of milk in Flanders. This impact is expected to increase further once the European milk quatum has been lifted.

The high production rates have a double, negative impact on the CI as they reduce both the oocyt quality and the expression of heat, leading to a lower insemination efficiency. This can be explained by the detrimental effects of the negative energy balance a cow undergoes in early lactation. At this moment, the energy output (milk) is larger than the input (feed), which causes the cow to mobilize the energy reserves stored in the body fat. This results in elevation of fatty acids (NEFA's), ketone bodies and other metabolites in the circulation to concentrations which can be detrimental for the quality of the ovules, leading to poor oocyt quality, delayed ovulation and less clear oestrous behavior. Because most farmers have less time to inspect their cows and detect the oestrous signs, determining the optimal insemination moment is difficult and ovulations are frequently missed. As a result, tight monitoring of the metabolic and fertility status of highly productive dairy cows is essential to reduce the CI. Moreover, if the negative energy balance is detected timely, proper action could be taken.

In lactating cows, there is a very intensive interaction between the blood circulation and milk production. This makes that the concentrations of many metabolites and hormones in the blood will be reflected in the milk composition. As cows are milked two to three times per day, analysis of the concentration and total production of the different milk components could be used as a diagnostic tool for the individual monitoring of the metabolic and fertility status of a dairy cow. Therefore, the aims of this research are to develop an online milk composition sensor for nutritional monitoring and a progesterone sensor for fertility monitoring, and to translate these signals into valuable information for the farmer.

To monitor the nutritional status of highly productive dairy cows, a dedicated Vis/NIR spectroscopic sensor for fat, protein and lactose measurement in milk has been elaborated which can be integrated on an automatic milking system. This sensor has been calibrated on a set of 234 raw milk samples and validated on another 116 raw milk samples from different Flemish farms ($n=19$) for which reference analyses had been performed by the Flemish Milk Control Center (MCC-Vlaanderen). The validation results were very promising with R^2 values of 0.994, 0.964 and 0.921 and prediction errors of 0.08%, 0.09% and 0.09% (all % w/w) for fat, protein and lactose, respectively. This sensor is now integrated on the automatic milking system at a test farm for online monitoring of the milk composition during milking.

In order to improve the detection of heat in dairy cows, it would be better to measure the hormone

levels related to ovulation and heat than the secondary symptoms such as increased activity. Even for cows that do not show any signs of heat, the hormone level will give the correct information on the reproductive status. Progesterone is a fertility hormone, produced by the corpus luteum, which is able to indicate accurately the reproduction and fertility status of a dairy cow. Therefore, monitoring this progesterone level would allow to accurately predict the moment of ovulation and thus the optimal moment of insemination. Moreover, the progesterone profile is a good indicator to confirm pregnancy and to detect ovarian abnormalities (*e.g.* cysts). Thanks to the diffusion process in the cow's udder, milk progesterone measurement provides an interesting alternative for blood sampling. Surface plasmon resonance (SPR) biosensors hold a great potential as diagnostic tools for a wide variety of applications since they can provide both an accurate quantification and real-time detection of target molecules. Despite of the high potential of this technique, it never resulted in a field device that can monitor the progesterone level of raw milk at the farm. In this research, a Fiber-Optic Surface Plasmon Resonance (FO-SPR) sensor has been elaborated which is sufficiently robust to be used on farm. A competitive bioassay has been designed for detection of low molecular weight molecules (here: progesterone) since the sensitivity of SPR sensors is determined by changes in weight near the sensor surface. In the competitive assay, a detection antibody is bound either to the target molecule from a sample or its derivative that is immobilized on the sensor surface. To improve the limit of detection, the signal of the bound detection antibody is amplified by use of gold nanoparticles functionalized with a secondary antibody. The resulting competitive bioassay has been shown to measure progesterone in raw milk with a detection limit of $0.5 \text{ ng}\cdot\text{ml}^{-1}$.

Each cow is unique and both the major milk components as well as the progesterone level show natural variation over time. Therefore, advanced data analysis algorithms are needed to monitor the acquired signals and translate these into valuable information for the farmer. In this way, the sensors can support the right management decisions. To reach this goal, data are managed on cow level, rather than on herd level, for example by means of synergistic control (SGC). This method combines an engineering process control (EPC) step for modeling the natural evolution in the measured signals with a statistical process control (SPC) step to detect anomalies. In anticipation of the integration of the FO-SPR biosensor on a dairy farm, this approach has already been evaluated on time-series of milk progesterone determined by means of ELISA. Moreover, milk progesterone levels were determined for 22 Holstein-Friesian dairy cows over a period of 47 days. From the analysis of these data it became

clear that there is a large variation in the progesterone profiles of different cows and that a fixed threshold on the progesterone level would not provide good heat detection results. However, the investigated approach which uses model parameters rather than a threshold allows to accurately describe the individual profiles and provide personalized detection of heat, pregnancy and abnormalities.

In the future, this approach for data management at the cow level will be applied to the data acquired with the FO-SPR progesterone sensor and the Vis/NIR milk composition sensor to develop a valuable decision support system for dairy farmers.

Keywords. Progesterone, nutritional status, management support.

DEVELOPMENT OF A NEW IMMUNORADIOMETRIC ASSAY FOR PREGNANCY-ASSOCIATED GLYCOPROTEINS (IRMA-PAG) ALLOWING PREGNANCY FOLLOW-UP IN CATTLE BY USING MILK SAMPLES

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Pregnancy-Associated Glycoproteins (PAGs) were first described as placental antigens that were also present in the peripheral circulation of the mother soon after implantation. They are synthesized by the mono- and binucleate trophoblastic cells, some of them being secreted in maternal blood from the moment when the conceptus becomes more closely attached to the uterine wall and formation of placentomes begins. In cattle, concentrations of PAG are detectable in maternal blood from day 28 to day 30 after fertilization. Milk concentrations are 20-30 times lower than in blood samples and cannot be measured by classical radioimmunoassay systems. In the present study we report the use of a highly sensitive immunoradiometric assay for PAG (PAG-IRMA) allowing pregnancy follow-up in cattle by measuring concentrations in milk samples. In the IRMA system, an antibody was coated to StarNunc tubes and used for the immobilization of the antigen. The sample (volume until 4 ml) was incubated in the tube. After incubation, the whole sample was eliminated by several washes. Thereafter, another

antibody bound to biotin (detection antibody) was added in order to quantify the antigen present in the sample. After a second wash, the reaction was revealed by adding a Streptavidin tracer (^{125}I). Purified bovine PAG 67kDa was used as standard at concentrations ranging from 100 to 50,000 $\text{pg}\cdot\text{ml}^{-1}$. Highly purified immunoglobulins (hp-Ig) were obtained from two distinct rabbit polyclonal antisera by using a specific affinity chromatography (anti-PAG 4B-Sepharose gel). The hp-Ig708 (purified from polyclonal antiserum raised against caprine PAG 55kDa+59kDa) was used as capture antibody (0.01 $\mu\text{g}/\text{tube}$). The hp-Ig727 (purified from polyclonal antiserum raised against purified boPAG67kDa) was used as detection antibody (1:8,000). Radiolabeled streptavidin (^{125}I -Strep; 50,000 $\text{cpm}\cdot 100\ \mu\text{l}^{-1}$) was used to reveal the Ab-Ag-Ab-Biot complexes. Milk was collected from pregnant cows ($n = 20$) during the whole duration of lactation until dry-off. Samples were frozen until assay. Before analysis, milk samples were thawed at 37 °C, centrifuged (2,500 x g) and fat was removed. Samples giving high PAG concentrations were serially diluted in order to fit with standard curve range. In pregnant cows, milk PAG concentrations increased from week 10 ($56.9 \pm 13.1\ \text{pg}\cdot\text{ml}^{-1}$) to week 11 ($93.5 \pm 20.4\ \text{pg}\cdot\text{ml}^{-1}$) and week 12 ($135.2 \pm 27.7\ \text{pg}\cdot\text{ml}^{-1}$). Thereafter, PAG concentrations increased regularly until week 32 ($2,177.6 \pm 496.2\ \text{pg}\cdot\text{ml}^{-1}$) and slightly decreased until dry-off at week 35 ($1,615.9 \pm 663.9\ \text{pg}\cdot\text{ml}^{-1}$). Immediately after parturition, PAG concentrations reached $5,615.3 \pm 615.7\ \text{pg}\cdot\text{ml}^{-1}$ and decreased continuously until week 11 postpartum ($36.6 \pm 2.1\ \text{pg}\cdot\text{ml}^{-1}$). In non-pregnant cows, concentrations remain lower than 40-50 $\text{pg}\cdot\text{ml}^{-1}$ at all time points. In conclusion, a new IRMA-PAG is available for quantitative measurement of PAG concentrations in cattle. This new test can be used for pregnancy diagnosis and follow-up in cattle.

Keywords. Pregnancy diagnosis, pregnancy-associated glycoprotein, dairy cattle.

VALIDATION OF A NEW IMMUNORADIOMETRIC ASSAY (IRMA) ALLOWING QUANTIFICATION OF PREGNANCY-ASSOCIATED GLYCOPROTEINS CONCENTRATIONS IN BOVINE MILK

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Pregnancy-Associated Glycoproteins (PAGs), also known as pregnancy-specific protein B (PSPB) are used since early eighties as pregnancy markers in cattle and other ruminant species. Until now, they are mainly assayed in plasma or serum samples by using radioimmunoassay or ELISA systems. The aim of this work was to develop a very sensitive and robust immunoassay offering the possibility to quantify PAG in milk samples: the PAG-immunoradiometric assay. In the IRMA system, an antibody was coated to StarNunc tubes and used for the immobilization of the antigen. The sample (volume until 4 ml) was incubated in the tube. After incubation, the whole sample was eliminated by several washes. Thereafter, another antibody bound to biotin (detection antibody) was added in order to quantify the antigen present in the sample. After a second wash, the reaction was revealed by adding a Streptavidin tracer (^{125}I). The validation of a highly sensitive immunoradiometric assay for PAG (PAG-IRMA) allowing PAG quantification in bovine milk is reported in details. Purified bovine PAG 67kDa was used as standard at the concentrations ranging from 100 to 50,000 $\text{pg}\cdot\text{ml}^{-1}$. Highly purified immunoglobulin hp-Ig708 (purified from polyclonal antiserum raised against caprine PAG 55kDa+59kDa) was used as capture antibody (0.01 $\mu\text{g}/\text{tube}$). The hp-Ig727 (purified from polyclonal antiserum raised against purified boPAG67kDa) was used as detection antibody (1:8,000). Radiolabeled streptavidin (^{125}I -Strep; 50,000 $\text{cpm}\cdot 100\ \mu\text{l}^{-1}$) was used to reveal the Ab-Ag-Ab-Biot complexes. Minimum detection limit was calculated as the mean concentration minus twice SD of 10 duplicates of the zero (B0) standard. Reproducibility was determined by calculating the intra- and inter-assay coefficients of variation (CV). The accuracy was determined by adding increasing quantities of purified boPAG67kDa (10, 20, 40 and 80 pg) to bovine milk containing low PAG concentrations. Specificity was tested regarding 16 different compounds including carbohydrates, aspartic proteinases, hormones and major milk proteins. These molecules were tested at dilutions ranging from 0.1 $\text{ng}\cdot\text{ml}^{-1}$ to 100 $\mu\text{g}\cdot\text{ml}^{-1}$. Parallelism was assessed by serially diluting pregnant cow milk containing relatively high PAG concentrations. Minimum detection limit was 4 $\text{pg}\cdot\text{ml}^{-1}$. Intra- and inter-assay CV ranged from 13.7 to 15.8%. Accuracy (recovery of added PAG) ranged from 86.3 to 104.8%. No cross reaction was observed with almost all tested compounds except lactoferrin (2.4%), rennin (2.5%), porcine pepsinogen (2.3%) and porcine pepsin (5.2%) at 100 $\mu\text{g}\cdot\text{ml}^{-1}$ (most of them at supra-physiological levels). Dilutions of

bovine milk containing detectable PAG concentrations were parallel to standard curve. In conclusion, the new PAG-IRMA is highly sensitive, accurate, precise and reproducible for PAG measurements in bovine milk samples. Parallelism test reveals an excellent ability to quantify PAG concentrations in milk of dairy cattle. This new test can be an alternative method useful for pregnancy diagnosis and follow-up in bovine species.
Keywords. Pregnancy-associated glycoprotein, immunoradiometric assay, dairy cattle.

UPLC®-MS/MS, AN ANALYTICAL TOOL FOR THE ACCURATE AND RAPID QUANTIFICATION OF PHYTOESTROGEN METABOLITES IN MILK

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The term “phytoestrogen” is used to define a wide variety of nonsteroidal compounds that occur naturally in many plants. When they are absorbed by cows, these polyphenolic compounds undergo biotransformation by specific bacteria present in the gastrointestinal tract. The resulting metabolites are absorbed in the organism and some are excreted in milk. The impact of these compounds on human health divides opinion. Some scientists believe that they might have adverse health effects. Others believe the opposite, especially with regard to their microbial metabolites, such as equol. Whatever one’s view on the subject, scientists need accurate, sensitive and rapid analytical methods in order to continue the research on clarifying the phytoestrogen issue. Cow’s milk is an animal product that is common in the human diet, and it is therefore important to evaluate its content of phytoestrogen metabolites. In order to study the human intake of equol *via* commercial milk, an analytical method was developed and validated following EMA/CVMP/VICH/463202/2009 guidelines. Enzymatic hydrolysis was used to release the equol. It was then extracted using double liquid/liquid extraction and analyzed

using UPLC®-MS/MS, with an analysis runtime of only 5 min. This analytical method produced a linear calibration curve with a high correlation coefficient ($R^2 \geq 0.996$) between 5 and 1,000 ng·ml⁻¹. Good intra- and inter-day precision (RSDs $\leq 5.3\%$ and $\leq 5.2\%$, respectively) and accuracy (bias $\leq 8.6\%$) were achieved. The recovery rate differed slightly among the different types of milk, ranging between $60.6 \pm 1.09\%$ and $82.3 \pm 5.21\%$. Good method repeatability was observed (RSDs $< 15\%$). There was neither a matrix effect nor a carry-over effect, and the sample extracts were stable during storage for at least 7 days at -21°C and 5°C . In order to apply the proposed method and obtain an initial estimate of equol concentration in cow's milk in Belgium, 44 samples of various brands found in several supermarkets and 5 raw milk samples from Walloon farms were analyzed. Equol was found in all the samples analyzed, with a concentration ranging from 10 to 50 ng·ml⁻¹ for conventional milk and from 70 to 130 ng·ml⁻¹ for organic milk. These results were compared with other European studies and the same trend was observed. The results obtained show the specificity, sensitivity and precision of this method for analyzing oestrogenic metabolite-equol in cow's milk. This study should be extended to include a greater number of samples and be implemented over a longer period to see if there is a correlation between livestock practice and/or sample origins with the equol content in milk. The addition of other phytoestrogen compounds with this method, using UPLC®-MS/MS technology, could also lead to more reliable monitoring of these compounds in dairy production.

Keywords. Equol, UPLC®-MS/MS, milk.

UNTARGETED MULTIVARIATE CHARACTERIZATION OF CONTAMINANTS IN POWDERED MILK

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Milk contamination can occur in many different ways. Natural ways of contamination include cow feces coming into direct contact with the milk, infection of the cow's udder (mastitis), cow diseases, bacteria that live on the skin of cows, etc. However, artificial contamination or adulteration can be also possible. The

most important case was the adulteration of milk by melamine. The first scandal erupted in China in 2008 when tainted milk killed at least six babies, and caused kidney stones and urinary tract problems in hundreds of thousands of young children. More recently, in 2011, more than 26 tons of melamine-tainted milk powder intended for use in ice cream were seized in southwestern China. In all these cases, melamine was added to milk products so they appear to have a higher protein level. Different studies have shown that all cases were linked to powdered products; no cases were associated with liquid milk. In this work, a combination of vibrational spectroscopy and multivariate analysis was used for detecting contamination and mislabeling (Baeten et al., 2014). Powdered milk samples containing different levels of contamination were analyzed by infrared spectroscopy, the resulting spectra were projected into a new untargeted chemometric tool, the moving window principal component analysis (PCA). With this tool, a moving window along the wavelength range in data is applied in order to build reduced PCA models (Liu et al., 2009) and calculate residuals. For each selected window in a calibration set, a PCA analysis is performed by fixing the number of principal components and applied to the validation or test set. Spectral score residuals in the calibration set are extracted and used to build thresholds. When a residual, at a certain wavelength, falls out the defined thresholds, the sample could be suspected of being abnormal and then indicating the possible presence of contaminants or mislabeling. A key point in all studies is to define the class "normal" and "abnormal" by fingerprint properties. In this work, this has been solved by the use of a local technique allowing, for each sample to be predicted, the selection of the most spectroscopic similar samples in the calibration set previous the application of the moving window PCA. In this study, the moving window PCA method is applied for the characterization of different agro food products using vibrational spectroscopic analysis tools as Near Infrared (NIR) or NIR Hyperspectral Imaging. The aim is taking advantage of the huge information comprised in the data generated by such techniques, which can promote the concept of data-driven discovery or untargeted analysis.

Keywords. Powdered milk, contaminant, untargeted detection, vibrational spectroscopy, chemometrics.

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PREDICTION OF PROTEIN CONTENTS OF COW MILK BY MID-INFRARED SPECTROSCOPY: IMPROVEMENTS MADE THROUGH THE LC/MS REFERENCE METHOD

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Milk protein composition has a real interest for both cheese-making and human nutrition. According to their protein profile, different milks can be more or less adapted to cheese production. The current methods used to determine the protein composition are quite time-consuming and expensive. Moreover, currently there is no standardized method to determine the individual protein composition. Mid-infrared (MIR) spectroscopy which is widely used for assessing milk quality and concentration of milk components, could be an interesting alternative to easily determine, and at a large scale, the protein composition. This study was carried out within the PhénoFinlait program and aimed (1) to develop a reference method to precisely and simultaneously determine the content of each individual milk protein using liquid chromatography coupled with mass spectrometry (LC/MS) and (2) to establish prediction equations of the milk protein composition from MIR spectra. Both qualitative and quantitative determinations of the individual proteins are very accurate using LC/MS. A first equation dataset had been established to estimate the detailed protein contents from the MIR spectra (Ferrand et al., 2012). However, the coefficients of determination (R^2_v) were lower than 0.80 ($RPD < 2.2$) for the different caseins and lower than 0.50 ($RPD < 1.5$) for the main two whey proteins. The LC/MS quantification method was further improved by (1) re-assigning the quantity of the main proteolysis products (γ -caseins) back to β -casein to obtain the original milk composition and (2) applying specific correction coefficients to the absorbance at 214 nm, taking into account the interference of aromatic amino acids. A standardization procedure (ISO/IDF) for this method is currently on-going. Finally, 271 individual cow milk samples from different

breeds (Holstein, Montbeliarde, and Normande) were analyzed again by the reference method and MIR spectroscopy to perform new calibrations. The most accurate predictions were obtained for total casein ($R^2_v = 0.96$, relative error = 2.5%, $RPD = 5.1$) and for individual caseins (α_{s1} -casein, α_{s2} -casein, β -casein) with a R^2 of validation ranging between 0.80 and 0.90 ($RPD > 2.5$) and a relative error ranging between 4 and 7%. For κ -casein, the equation is slightly less accurate with R^2_v of 0.73 ($RPD = 1.91$) and a relative error of 9.4%. For whey protein contents, the R^2_v equals to 0.70 ($RPD = 1.86$) and the relative error 9.4%. The content of α -lactalbumin remains poorly predicted. The performance of this new set of equations is encouraging and point out that MIR is applicable for predicting individual milk protein composition with acceptable accuracy to be used in selective breeding to enhance milk protein composition.

Keywords. Proteins, cow milk, mid-infrared (MIR) spectroscopy.

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Session 6 – Future challenges: genomics contributing to advisory tools?

Chair: NICOLAS GENGLER (University of Liege, Belgium)

USING GENOTYPES TO CONSTRUCT PHENOTYPES FOR DAIRY CATTLE BREEDING PROGRAMS AND BEYOND

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Modern dairying uses sophisticated data collection systems to maximize farm profitability. This has traditionally included information on cows and their environments, and now commonly includes genotype information from high-density single nucleotide polymorphism (SNP) panels. The US national database alone contains genotypes for 924,543 bulls and cows as of March 23, 2015, and many other countries are also genotyping animals. As the data continue to grow, the prospect of using genotypes to construct phenotypes directly, instead of measuring phenotypes on animals, becomes more attractive. There are many applications for this genomic information other than the prediction of breeding values. A notable recent application is the use of haplotypes in combination with next-generation sequencing data to identify causal variants associated with recessives. The methodology for identifying recessive haplotypes by searching for a deficit of homozygotes was first used in combination with sequence data to identify the causal variant (APAF1) associated with the HH1 haplotype. The US currently tracks 24 recessive haplotypes in four cattle breeds, and thanks to the work of several teams around the world the causal variants for 17 of them are known. The haplotypes include lethal recessive conditions, such as brachyspina, as well as hair coat color and polledness. There is growing interest in the latter to improve animal welfare and

increase economic efficiency, but the polled haplotype has a very low frequency (0.41%, 0.93%, and 2.22% in Brown Swiss, Holstein, and Jersey, respectively). Increasing haplotype frequency by index selection requires known status for all animals. Gene content (GC) for non-genotyped animals was computed using records from genotyped relatives. Prediction accuracy was checked by comparing polled status from recessive codes and animal names to GC for 1,615 non-genotyped Jerseys with known status. Ninety-seven percents ($n = 675$) of horned animals were correctly assigned GC near 0, and 3% ($n = 19$) were assigned GC near 1. Heterozygous polled animals had GC near 0 (52%, $n = 474$) and near 1 (47%; $n = 433$), although 3 animals were assigned a GC near 2. All homozygous polled animals ($n = 11$) were assigned GC near 2. Genotype information can also be combined with other data, such as milk spectral data, to predict phenotypes for traits that are expensive or difficult to measure directly. These data can be used for precision farm management, including early culling decisions, monitoring of animals at risk for health problems, and identification of efficient and inefficient cows. The most substantial challenge faced by many dairy managers will be the effective use of the new phenotypes that now are available.

Keywords. Animal health, genetic improvement, herd management, genotypes, phenotypes.