Mid-Infrared Spectroscopic Analysis of Raw Milk to Predict the Blood Plasma Non-Esterified Fatty Acid Concentration in Dairy Cows

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ABSTRACT

In high yielding dairy cattle, severe postpartum negative energy status is often associated with metabolic and infectious disorders that negatively affect production, fertility and welfare. Mobilization of adipose tissue associated with a negative energy status is reflected through an

increased level of non-esterified fatty acids (**NEFA**) in the blood plasma. Earlier, identification of a negative energy status through the detection of increased blood plasma NEFA concentration required laborious and stressful blood sampling. More recently there have been attempts to predict blood NEFA concentration from milk samples. This study aimed to develop and validate a model to predict the blood plasma NEFA concentration using milk mid-infrared (**MIR**) spectra that are routinely measured in the context of milk recording. To this end, blood plasma and milk samples were collected in weeks 2, 3 and 20 post-partum for 192 lactations in 3 different herds. The blood plasma samples were taken in the morning, while representative milk samples were collected during the morning and evening milk session on the same day. To predict the blood plasma NEFA concentration from the milk MIR spectra, partial least squares regression models were trained on part of the observations from the first herd. The models were then thoroughly validated on all other observations of the first herd and on the observations of the two independent herds to explore their robustness and wide applicability. The final model can accurately predict blood plasma NEFA concentrations below 0.6 mmol/L with a root mean square error of prediction (RMSE) of less than 0.143 mmol/L. However, for blood plasma with more than 1.2 mmol/L NEFA, the model clearly underestimates the true level. Additionally, it was found that morning blood plasma NEFA levels were predicted with a significantly higher accuracy (p = 0.009) using MIR spectra of evening milk samples compared to morning samples, with RMSEP values of respectively 0.182 and 0.197 mmol/L and R^2 values of 0.613 and 0.502. These results suggest a time delay between variations in blood plasma NEFA and related milk biomarkers. Based on the MIR spectra of evening milk samples, cows at risk for a negative energy status, indicated with detrimental morning blood plasma NEFA levels (> 0.6 mmol/L), could be identified with a sensitivity and specificity of respectively 0.831 and 0.800. As this model can be applied to millions of historical and future milk MIR spectra, it opens opportunities for regular metabolic screening and improved resilience phenotyping.

Key words: milk mid-infrared spectroscopy, blood plasma non-esterified fatty acid concentration, negative energy status, milk biomarker

INTRODUCTION

The transition from pregnancy to lactation in high-yielding dairy cows is typically accompanied by a negative energy status (**ES**) in which the energy requirement exceeds the energy input from feed. As severe negative ES increases the susceptibility to various health and fertility problems (Leblanc, 2010; Ospina et al., 2010a), the duration and degree of negative ES should be limited through preventive actions in combination with individual monitoring and imperative treatment.

To compensate for the energy deficit and maintain high milk production, adipose tissue is mobilized and non-esterified fatty acids (**NEFA**) are released in the blood. Hence, a blood plasma NEFA concentration above 0.6 mmol/L is generally used as an indicator for negative ES in dairy cattle (Ospina et al., 2010b). These high concentrations of circulating NEFA have a detrimental effect on the oocyte quality and the immune response of dairy cows (Leroy et al., 2005; Scalia et al., 2006). In the liver, part of the NEFA are oxidized completely to deliver energy or incompletely to produce ketone bodies (Adewuyi et al., 2005). Another portion of the NEFA is esterified to triglycerides and either stored in the liver or transported as lipoproteins to e.g. the alveolar epithelial cells of the udder tissue to synthetize milk fat. In this way, fatty acids (**FA**) and ketone bodies derived from the NEFA end up in the produced milk. Previous studies have demonstrated the use of milk biomarkers for monitoring negative ES in individual cows, e.g. through the measurement of certain FA (Van Haelst et al., 2008; Jorjong et al., 2014; Dórea et al., 2017), ketone bodies (Enjalbert et al., 2010), citrate and many more (Bjerre-Harpøth et al., 2012). In contrast to taking blood samples, milk sampling requires less labor and can be done without distressing the animals. Nevertheless, the reference techniques to measure these milk biomarkers are typically labor-intensive and costly (Jorjong et al., 2014).

A relatively straightforward and cost-efficient technique for milk analysis is mid-infrared (**MIR**) spectroscopy. As the covalent bonds of molecules in milk absorb MIR radiation at very specific wavenumbers, the concentrations of these milk components can be derived from the MIR absorbance spectra. Typically, multivariate linear models are trained to predict the milk constituents from the acquired spectra (De Marchi et al., 2014). Already for decades, this technique is accepted as the reference for accurate and routinely characterization of the main milk components in the context of milk recording (ISO, 2013; ICAR, 2019). Since the commercial introduction of Fourier-transform MIR spectrometers for milk analysis, milk MIR spectra can be obtained with a higher accuracy and repeatability. This opens opportunities for measuring minor milk components and milk biomarkers such as FA profiles (Rutten et al., 2009; Afseth et al., 2010; Soyeurt et al., 2011), protein composition (Franzoi et al., 2019), minerals (Soyeurt et al., 2009), ketone bodies and citrate (Grelet et al., 2016).

Recently, Benedet et al. (2019), Grelet et al. (2019) and Luke et al. (2019) developed models to predict the blood plasma NEFA concentrations from milk MIR spectra of individual dairy cows. However, the prediction performance of Grelet's model was poor (R^2 = 0.39), likely because it was built using a limited number (n = 234) of calibration samples (Grelet et al., 2019). Benedet's model performed better (R^2 = 0.52), however, like Grelet's model, it was not validated for a completely independent herd (Benedet et al., 2019; Grelet et al., 2019). Accordingly, the reported results might be overoptimistic compared to applying the model on the data of a new herd where the cows are managed differently. This was clearly illustrated by Luke et al. (2019) as the determination coefficient (R^2) of their model dropped from 0.61 for a randomly selected validation set, covering the same herds as the ones included in the calibration set, to 0.45 for a completely independent herd.

We hypothesized that a better prediction performance can be obtained through increasing the number of calibration samples and applying a very strict timing in the sampling of blood and milk samples relative to the diurnal pattern and the feeding schedule of the cows (Quiroz-Rocha et al.,

2010). To test this hypothesis, a high number of samples was collected following a strict protocol for blood and milk sample collection to obtain high quality data for training the prediction models. Additionally, it is investigated whether MIR spectra of morning or evening milk samples result in a better prediction of the NEFA concentration of the respective blood samples taken in the morning of that day. Finally, the performance of the prediction models is evaluated extensively on a completely independent validation set.

MATERIALS AND METHODS

Experimental Setup

The experimental protocol was approved by the Finnish Animal Experiment Board (ESAVI/5688/04.10.07/2013) and applied on 3 experimental herds in Finland: Luke Jokioinen (herd A), University of Helsinki in Viikki (herd B) and Luke Kuopio (herd C). All cows in these herds that calved for the first time in the period between September 2013 and October 2016 were included in the study, resulting in a total of 143 Nordic Red dairy cows from which 103 were in herd A, 24 in herd B and 16 in herd C. For 49 of these 143 cows, also the second lactation was included in the study period, thus resulting in a total of 192 lactations. A detailed description on the housing conditions, ration, feeding frequency and milking conditions and frequency during the experiment is given by Mäntysaari et al. (2019).

Data Collection

For each lactation, blood samples were taken from the coccygeal vein within one hour after the morning milking session, on two non-consecutive days in week 2 and in week 3 after calving, and once in week 20. This resulted in a total of 5 blood samples per lactation. Handling of the lactating cows prior to blood-sampling was minimized to reduce its effect on the blood plasma NEFA concentrations (Leroy et al., 2011). Blood was collected in 10 mL EDTA tubes and stored in ice

125 until centrifuged at -4°C for 15 min at 2,000 × *g*. Plasma samples were frozen and stored at -20°C
126 for later analysis of NEFA at the university of Helsinki (Salin et al., 2012). An enzymatic
127 colorimetric acyl-CoA synthetase (ACS)-acyl-CoA oxidase (ACOD) method [NEFA-HR(2) kit,
128 Wako Chemicals GmbH, Neuss, Germany] was used according to the manufacturer's instructions to
129 determine the blood plasma NEFA concentrations, further referred to as 'blood NEFA'. Intra- and
130 interassay coefficient of variation for blood NEFA determination were 1.61 and 3.53% for low
131 NEFA concentration (0.23 mmol/L) and 0.77 and 2.91% for high NEFA concentration (1.24
132 mmol/L).

133 Representative milk samples (± 30 mL) were collected during the morning and evening milking
134 sessions on the same days as the blood collection, providing a total of 10 milk samples per lactation.
135 The milk samples were stored at 4°C using a preservative (± 0.3 mg bronopol per ml milk, Broad
136 Spectrum Microtabs II, D and F Control Systems Inc., Dublin, CA). The MIR analyses (MilkoScan
137 FT6000 spectrometer, Foss, Hillerød, Denmark) were carried out by the Valio Ltd. milk laboratory
138 (Seinäjoki, Finland) according to ISO 9622:2013 (ISO, 2013). The MIR spectrum of each milk
139 sample consisted of 1060 values, representing the infrared light transmittance through 50 µm of
140 sample between wavenumbers 5010.2 and 925.7 cm^{-1} with a resolution of 4 cm^{-1}. The MIR spectra
141 were standardized following the procedure developed by Grelet et al. (2015). Because of data
142 storage problems, the MIR spectra of 152 morning milk samples and 183 evening milk samples got
143 lost. The resulting final dataset therefore included 808 and 777 MIR spectra for respectively
144 morning and evening milk samples (Table 1).

145

146 ***Prediction of the Blood Plasma NEFA Concentrations from Milk MIR Spectra***

147 The MIR spectra and NEFA concentrations were imported into R version 3.4.3 (R Core Team,
148 2017). Only the spectral regions from 2977 to 2768 cm^{-1}, 1800 to 1684 cm^{-1} and 1607 to 926 cm^{-1}
149 were used in the analysis. Moreover, the signal-to-noise ratio in the spectral regions between 3660

and 2977 cm^{-1}, and between 1684 and 1607 cm^{-1}, was considered too low due to substantial MIR absorption by the water molecules. The spectral regions above 3660 cm^{-1} and between 2768 and 1800 cm^{-1} were deleted because they do not contain significant spectral information on relevant milk components (Aernouts et al., 2011; Grelet et al., 2019). A principal component analysis (**PCA**) with maximum 20 principal components was used to identify potential outlier spectra. When both the *Q* residuals and the Hotelling T^2 statistic were above their 99% confidence limits, the spectrum was removed from the analysis (Bro and Smilde, 2014).

As blood samples were only taken once per cow per sampling day, while 2 milk samples were collected for respectively the morning and evening milking session of that day and cow, the number of blood NEFA analyses was half of the amount of milk MIR spectra. Accordingly, the same blood NEFA concentration was assigned to both the morning and the evening milk MIR spectrum of the respective cow and day. The combination of a morning milk MIR spectrum together with the respective blood NEFA concentration is further referred to as a morning observation, while the combination of an evening milk MIR spectrum together with the respective blood NEFA concentration is further referred to as an evening observation. The morning and evening observation of the same cow and day thus have the same blood NEFA concentration, while they have different milk MIR spectra. Next, about 60% of the morning and evening observations of herd A were allocated to the calibration set, while the remaining 40% of the observations of herd A and all observations of herds B and C were assigned to the validation set (Figure 1, step 1). Moreover, the observations of herd A were split 60/40 by applying the duplex selection method after ordering them on their blood NEFA concentration (Snee, 1977). This procedure assured that both sets had similar descriptive statistics. Observations for the same cow were treated as a block with all of them either in the calibration or validation set to prevent overoptimistic validation results in case of modeling cow-specific effects (Kemps et al., 2010).

The spectral pre-processing of the MIR spectra was a combination of (1) a logarithmic spectral transformation (Beer, 1852) or not; (2) a baseline correction, detrending, standard normal variates weighting or multiplicative scatter correction (Geladi et al., 1985; Barnes et al., 1989; Ruckstuhl et al., 2001) or none of those; (3) a first or second order Savitzky-Golay derivative with a second order polynomial filter and 10 different spectral window lengths (Savitzky and Golay, 1964) or no derivative and (4) mean centering. This resulted in 210 different combinations, as presented in Figure 1 (step 2) and described in detail in Aernouts et al. (2011). For each of these 210 combinations, a partial least squares regression (**PLSR**) model with up to 20 latent variables was built to predict the blood plasma NEFA concentrations, further referred to as 'predicted blood NEFA', from the pre-processed MIR spectra (Martens and Næs, 1987). A group-wise cross-validation (**CV**) with 20 groups, each containing spectra of 3 to 4 cows, was performed on the observations of the calibration set to obtain the root mean square error of cross-validation (**RMSECV**). We selected the smallest number of latent variables for which the PLSR model was not significantly worse compared to the same model with the number of latent variables resulting in the lowest RMSECV. The statistical comparison in this procedure was based on a one-sided paired *T*-test ($\alpha = 0.05$) applied on the absolute residuals of the cross-validated observations (Cederkvist et al., 2005). A similar approach was followed to select the best spectral pre-processing combination. Moreover, the PLSR models resulting from the 210 combinations were ranked by increasing RMSECV, and the one with the smallest number of latent variables and not being significantly worse compared to the model with the lowest RMSECV was selected. Again, a one-sided paired *T*-test ($\alpha = 0.05$) on the absolute residuals of the cross-validated observations was used to statistically compare the models (Cederkvist et al., 2005; Aernouts et al., 2011).

The selected pre-processing combination was applied on the MIR spectra to be used as an input for 4 different variable selection methods (Figure 1, step 3): variable importance in projection, jack-knife, reversed interval PLSR and forward interval PLSR (Norgaard et al., 2000; Westad and

Martens, 2000; Chong and Jun, 2005). Each of these 4 methods resulted in a set of most relevant wavenumbers for which a PLSR model with an optimal number of latent variables was built as described earlier. The performances of these 4 PLSR models were compared mutually and with the model that uses all wavenumbers. Finally, the set of wavenumbers related to the most parsimonious model whose prediction performance was not significantly worse (one-sided paired *T*-test, $\alpha = 0.05$) than that of the model with the lowest RMSECV was selected.

The final prediction model (Figure 1, step 4), together with the selected combination of spectral pre-processing techniques and the selected set of wavenumbers, was used to predict the NEFA concentrations of the observations in the validation set. Accordingly, an error or residual could be calculated for each observation of the validation set. Based on these residuals, the root mean square error of prediction (**RMSEP**), further referred to as the 'prediction error', was calculated for the entire validation set. Because this validation set is very diverse, containing morning and evening observations from 3 different herds with blood NEFA concentrations ranging from very low to very high, the RMSEP was also calculated for different subsets of the validation set, allowing for a better understanding of the prediction performance of the model under different situations. These subsets were defined based on a combination of the following features:

- Milking time: only morning observations, only evening observations or both morning and evening observations;
- Herd: observations from herd A, herd B, herd C or for the 3 herds together;
- NEFA range: observations with blood NEFA concentrations in the low (0 – 0.6 mmol/L), middle (0.6 – 1.2 mmol/L), high (1.2 – 2.0 mmol/L) or complete range (0 – 2 mmol/L). These ranges were defined like this because 0.6 mmol/L is generally considered as critical threshold (Ospina et al., 2010b) and because the blood NEFA concentration was always underestimated for true concentrations above 1.2 mmol/L.

The procedure described above (Figure 1) was initially followed to develop and validate a PLSR model that predicts the blood NEFA independent of the moment of milk sampling by training it on all the observations – both morning and evening – of the calibration set. This model is further referred to as the 'full model'. To evaluate the effect of restricting the calibration set to only morning or evening observations, 2 new models were trained following the same procedure as elaborated above, but with respectively only the morning or the evening observations of the calibration set for training the respective PLSR models. These models are further referred to as respectively the 'morning model' and the 'evening model'. All 3 models (full, morning and evening) were validated on the same observations – both morning and evening – of the validation set to allow for an objective comparison of the prediction performance.

The prediction performances of the 3 models were compared by applying a repeated-measures ANOVA on the absolute residuals for all the observations of the validation set. Moreover, 'model' was treated as a fixed effect, while 'sample' was specified as a random effect in the two-way ANOVA (Cederkvist et al., 2005). When the ANOVA test pointed out a significant effect ($\alpha = 0.05$) of the model, then the performance of the 3 models was compared bilateral using a Tukey HSD multiple comparison ($\alpha = 0.05$). The 3 models were compared for all the observations in the validation set, as well as the observations in the different subsets of the validation set. The model (full, morning or evening) which was not significantly different from the best model for most of the subsets of the validation set was identified as the most robust. This model was further evaluated on its ability to identify detrimental blood plasma NEFA concentrations (next section). Finally, a 4-way ANOVA analysis, with the model, the milking time, the herd, the NEFA range and all possible interactions as fixed factors, was applied on the absolute residuals for the observations of the entire validation set and subsets of the validation set. This analysis was not paired, so the samples could not be taken as a random factor. If one of the interactions was significant ($\alpha = 0.05$) then all possible combinations of the factors involved in these interactions were compared bilateral using the Tukey

HSD multiple comparisons. In absence of significant interaction for a factor, the effect of the factors could be interpreted separately. Moreover, if this factor had a significant (α = 0.05) influence on the performance, then the different levels within this factor were compared bilateral with the Tukey HSD multiple comparisons.

Identify Detrimental Blood Plasma NEFA Concentrations from Milk MIR Spectra

To evaluate whether the predicted blood NEFA concentrations can be used to identify detrimental blood NEFA levels (> 0.6 mmol/L), receiver operating characteristic (**ROC**) analyses were performed (Ospina et al., 2010b; Jorjong et al., 2014; Dórea et al., 2017). The ROC curves plot the true positive rate or sensitivity versus the true negative rate (= 1 – specificity) for different thresholds applied on the predicted blood NEFA concentration. Only the most robust model, the one that performed best according to the procedure described in the previous section, was subjected to this ROC analysis. A separate analysis was done for the morning and evening observations of the validation set. The R package *pROC* version 1.13.0 (Robin et al., 2011) was used to calculate the ROC curves, to apply binormal smoothing to the ROC curves, to calculate the 95% confidence intervals (**CI**) of sensitivities, specificities and area under the curve (**AUC**) of the smoothed ROC curves and to statistically compare the smoothed ROC curves. The CI were calculated with 100 000 bootstrap replicates to obtain a fair estimate of the second significant digit (Fawcett, 2006). Statistical two-sided pairwise comparisons (α = 0.05) between ROC were done based on the area under the curve (AUC) and based on the sensitivities at given specificities from 0 to 1 in steps of 0.01, both using the bootstrap method with 100,000 replicates.

RESULTS

Data Exploration

The MIR transmittance spectra of the 1585 milk samples included in this study are presented in the top part of Figure 2 as the black solid and dotted lines. Lipid absorption peaks can clearly be observed as dips in the MIR transmittance spectra around 2928 & 2858 cm^{-1}, 1745 cm^{-1}, 1455 cm^{-1} and 1157 & 1078 cm^{-1}, corresponding to respectively the C-H (alkyl) stretch, C=O (carbonyl) stretch, C-H bend and C-O stretch vibrations (Fox and McSweeney, 2006; De Marchi et al., 2009).

The Hotelling's T^2 and Q-statistics of the PCA model with 7 selected principal components and the scores for the first 2 principal components of that model were far beyond the 99% confidence limits for the spectra of sample 1445 (herd B, evening milking) and sample 1546 (herd C, morning milking), as shown in Appendix A1. Also, the raw transmittance spectra of these 2 outliers, illustrated with black dotted lines in top part of Figure 2, are clearly different from the other 1583 spectra, while the corresponding blood NEFA concentrations are not outlying. This suggests that these 2 samples have erroneous spectral measurements and were therefore removed from the dataset.

The reliability, accuracy, and robustness of spectroscopic calibrations are restricted to the range of the constituent of interest and the variation in measurement conditions taken into account during the calibration (Williams and Norris, 2001). The descriptive statistics of the blood NEFA concentrations linked to different subsets of milk MIR transmittance spectra are presented in Table 2. The entire calibration and validation set contain respectively 790 and 793 observations and they have a very similar mean, standard deviation and range for the blood NEFA. This table also illustrates the larger variability and range of the blood NEFA levels in herd A compared to herd B and C. Likely, this is the result of the higher number of blood samples being collected and analyzed (n = 658) and cows being monitored (n = 103) in herd A. Additionally, this might also be caused by differences in the genetic background and the management between the herds. The descriptive statistics for morning and evening samples in a same herd(s) are similar, but not exactly the same.

296 This is because for some of the blood plasma samples only the respective morning or evening milk
297 MIR spectra were collected and not both (Table 1).
298
299 ***Calibration on MIR Spectra of Morning and Evening Milk Samples (Full Model)***
300 The 790 morning and evening observations of the calibration set were used to build a PLSR
301 model (= full model) that relates the blood NEFA concentrations to the MIR transmittance spectra.
302 The best performance was obtained when the MIR transmittance spectra were pre-processed using a
303 2^{nd} order Savitzky-Golay derivative with a window length of 7 wavenumber variables, followed by
304 mean-centering. After this pre-processing step, the reversed interval PLSR method selected 117
305 wavenumbers that were most informative and resulted in the best model. The most informative
306 regions of the MIR spectra are indicated as the grey regions in Figure 2.
307 Figure 3a shows the RMSECV and the RMSEP as a function of the number of latent variables
308 included in the full PLSR model after applying the best pre-processing and selecting the best
309 wavenumbers. A separate RMSEP is provided for the morning and evening observations of the
310 validation set, respectively indicated with $RMSEP_M$ and $RMSEP_E$. The PLSR model with 6 latent
311 variables, indicated with the green triangle, was finally selected. This model complexity resulted in
312 nearly the minimum $RMSEP_M$ and $RMSEP_E$, confirming the right choice of number of latent
313 variables based on the cross-validation and illustrating the robustness of the full model. Figure 3a
314 clearly shows that the $RMSEP_E$ is smaller than the $RMSEP_M$ and that the latter is smaller than the
315 RMSECV.
316 The regression coefficients for the full model with 6 latent variables are presented with a green
317 solid line in the bottom part of Figure 2. The regression coefficients follow a relatively smooth
318 curve in function of the wavenumbers, which indicates that the PLSR model is not overfitting the
319 calibration data. High absolute values for the regression coefficients were obtained around 2950 cm^{-1},
320 1750 cm^{-1} and 1150 – 990 cm^{-1}, corresponding to important fat absorption bands: respectively the

fat B, fat A and C-O stretch vibrations (Afseth et al., 2010). As the PLSR model uses the 2nd derivative of the MIR spectra, some of the peaks in the regression coefficients are located at the flanks rather than the center of typical absorption peaks.

Figure 3b presents the predicted versus measured scatter plot for the full model with 6 latent variables. This figure illustrates that the prediction error of the full model varies a lot with the predicted blood NEFA concentration (y-axis), both for the cross-validated observations of the calibration set as well as for the morning and evening observations of the independent validation set. Additionally, the blood NEFA concentration is generally overestimated for true values (x-axis) between 0.2 and 0.55 mmol/L, while it is always underestimated for true concentrations above 1.2 mmol/L. The latter could explain why the $RMSEP_M$ and $RMSEP_E$ are lower than the RMSECV, as the validation set contains less observations with a very high blood NEFA concentration (Table 2). In Figure 3b, the predictions based on the evening observations of the validation set (blue crosses) are closer to the identity line compared to the ones based on the morning observations of the same set (red circles). This is the reason why the $RMSEP_E$ values are smaller than the $RMSEP_M$ values in Figure 3a and it suggests that the blood NEFA concentration in the morning can be predicted more accurately using MIR spectra of milk samples taken in the evening of that day rather than morning milk samples.

The prediction errors of the full model for different subsets of the validation set are summarized in Table 3. The heteroscedastic prediction error of the full model is clearly shown by the increasing RMSEP with increasing blood NEFA range (different horizontal sections of Table 3). Moreover, for the observations in the low blood NEFA range (0 – 0.6 mmol/L), the RMSEP values of the full model are all between 0.062 and 0.143 mmol/L, while for the middle blood NEFA range (0.6 – 1.2 mmol/L), the RMSEP values vary between 0.198 and 0.290 mmol/L. For the high blood NEFA range (1.2 – 2 mmol/L), the RMSEP values of the full model are between 0.620 and 0.793 mmol/L. Within the low, middle and high blood NEFA range, the RMSEP values do not differ

much between herds, illustrating that the model can be used for new herds as well (cfr. herd B and C). Compared to the morning observations of the validation set, the RMSEP values for evening observations are in most cases slightly higher for the low blood NEFA range, while they are clearly lower for the complete, middle and high blood NEFA ranges. The observations based on the RMSEP values described in this paragraph are similar to the ones based on the RMSECV values obtained from the cross-validation of the calibration samples (results not shown), confirming the robustness of the full model.

Calibration on MIR Spectra of Morning or Evening Milk Samples

The PLSR model trained on the morning observations of the calibration set (= morning model), as well as the one trained on the evening observations (= evening model) provided the best results after applying a 2nd order Savitzky-Golay derivative on the MIR transmittance spectra, followed by mean-centering. The optimal window length for the derivative was respectively 7 and 13 wavenumber variables. For both models, reversed interval PLSR proved to be the best variable selection method, resulting in respectively 92 and 126 retained wavenumbers (grey regions in Figure 2). Finally, 3 and 5 latent variables were selected for respectively the morning and evening PLSR model. The regression coefficients for the final morning and evening model are presented as respectively the red dashed and blue dotted lines in the bottom part of Figure 2. Both models have high absolute values for the regression coefficients in the regions near important MIR fat absorption bands, similar to the regression coefficients of the full model.

Analogues to the RMSEP values of the full model, the prediction errors of the morning and evening model for different subsets of the validation set are also provided in Table 3. The prediction performances of the three models (full, morning and evening) are compared for each subset of the validation set. Within each column (herd x milking time) and a specified blood NEFA range (complete, low, middle or high), RMSEP values with different subscripts indicate significant (α =

371 0.05) differences between the 3 models. Most subsets of herd C and some subsets involving herd B
372 indicated no significant difference between the models. For those subsets, it was found that the
373 statistical tests lacked power ($\beta > 0.4$) because of a too low number of samples. Therefore, the
374 further discussion of the model comparison was only based on the tests with sufficient power ($\beta <$
375 0.2), which all happened to indicate a statistical effect of the model. The first column of Table 3
376 presents the RMSEP values for all the observations of the validation set in each of the 4 specified
377 blood NEFA ranges.

378 In the complete range, the full model performs significantly better than the morning and the
379 evening model, while there is no significant difference between the morning and the evening model.
380 The full and evening models are not significantly different for the observations of the validation set
381 in the low blood NEFA range, while they are both significantly better compared to the morning
382 model. On the other hand, for the observations in the middle and high range, the morning model is
383 significantly better than the full model, while the latter is better than the evening model.

384 For the low, middle and high blood NEFA range, the same trends are reflected in the different
385 subsets of the validation set where the observations are split up per herd and/or milking time (Table
386 3, columns 2 to 9). For the complete blood NEFA range, the full model is significantly better
387 compared to the evening model for all validation subsets with only morning observations, while it is
388 significantly better compared to the morning model for all the subsets with evening observations.
389 Additionally, it was found that the blood NEFA concentrations predicted with the morning model
390 were on average 0.042 mmol/L higher compared to the predictions by the full model applied on the
391 same milk MIR spectra, while the evening model resulted in blood NEFA predictions which were
392 on average 0.048 mmol/L lower compared to the predictions by the full model. Given the fact that
393 low blood NEFA concentrations are generally overestimated by the models, while the high blood
394 NEFA concentrations are underestimated (Figure 3b), the morning model results in lower
395 predictions in the high NEFA range, while the evening model results in lower prediction errors for

the low NEFA range. This is also clearly reflected by the models' RMSEP values for the different blood NEFA ranges. As the models mainly rely on the absorption by fat-related covalent bonds (Figure 2), the offset between the models probably results from the difference in average fat content between the morning milk samples (4.3%) and the evening milk samples (5.1%) involved in the training. Taken all this into account, it was concluded that the full model is the most robust of the 3 models and is therefore further explored in the ROC analysis in the next section.

Apart from the comparisons between the full, the morning and the evening model for each of the subsets of the validation set, a single 4-way ANOVA analysis was performed on the residuals of the observations in these different subsets. As all but one of the two-way interactions between the ANOVA factors were significant, the effect of the individual factors could not interpret independently from the other factors involved in the interaction(s). Accordingly, all combinations of the factors involved in these interactions were compared bilateral using a Tukey HSD multiple comparison. This analysis mainly points out that the prediction errors for the middle and high blood NEFA range are significantly higher compared to the complete and low range, but that the absolute levels of these errors depend on the model, the farm and the milking time.

Table 3 can also be used to study the difference in prediction error when 1 of the 3 models is applied on the different herds, or on either morning or evening observations. The RMSEP values for the 3 herds, except for the evening observations of herd C, are very close to each other for the same blood NEFA range (low, middle or high) for either morning or evening observations. This illustrates that the models can be easily transferred to new herds. The RMSEP values for the subsets of evening observations in herd C should be interpreted with caution as each of them is based on a low number of observations ($n \leq 13$, Table 1). Comparing the prediction errors between morning and evening observations for respective subsets shows that the prediction errors for the morning observations are generally higher, especially for blood NEFA concentrations above 0.6 mmol/L (middle and high ranges). For the full model, a one-sided paired *T*-test applied on all the

observations of the validation set pointed out that the blood NEFA predictions are more accurate (p = 0.009) if the model is applied on evening milk MIR spectra. This confirms that the blood NEFA concentration in the morning is predicted more accurately from milk MIR spectra taken during the evening milk session of the same day.

The observations based on the RMSEP values described in this section are similar to the ones based on the RMSECV values obtained from the cross-validation of the calibration samples (results not shown), confirming the robustness of the models and the validity of this analysis.

Receiver Operating Characteristic Analysis of the Full Model

The smoothed ROC curves for the identification of detrimental blood NEFA concentrations based on the predictions of the full model are shown in Figure 4. Separate smoothed ROC curves are provided for the morning (red) and the evening observations (blue) of the validation set. The AUC of the smoothed ROC curves for the morning and evening observations are respectively 0.860 (95% CI: 0.815 – 0.901) and 0.898 (95% CI: 0.860 – 0.930). Accordingly, the AUC for the morning observations is significantly lower ($p < 0.001$) compared to the evening observations. Moreover, compared to the evening observations, the sensitivities for the morning observations are significantly lower in the range of specificities from 0.48 to 0.97. The average sensitivities are 0.752 and 0.573 for the morning observations and 0.831 and 0.690 for the evening observations (Figure 4) at specificities of respectively 0.8 and 0.9. Thus, cows with a detrimental blood NEFA concentration, as determined from their morning blood samples, can be detected more accurately using the MIR spectra of their milk collected during the evening milking session of that day. Moreover, it can identify 83 out of 100 cows with detrimental blood NEFA concentrations, while 20 out of 100 healthy cows will be wrongly classified as being at risk. Appendix A2 provides the mean values of the sensitivities and the 95% CI of the sensitivities and specificities at given

specificities from 0.7 to 0.95 (in steps of 0.05) for the morning and the evening observations of the validation set.

DISCUSSION

Morning and Evening Milk Samples

The validation of the PLSR models clearly indicates that, compared to morning milk, the MIR spectra of evening milk support more accurate predictions of the NEFA levels in blood taken in the morning of that day (Table 3, Figure 3 and Figure 4). Several studies have shown that blood NEFA follows a diurnal pattern with elevated levels from about 06:00 to 10:00 h in the morning, associated with a reduced energy intake during the night (Blum et al., 2000; Meier et al., 2010; Quiroz-Rocha et al., 2010). For this reason, extra attention was paid to the consistent timing of the blood and milk sampling. As the morning milking session was around 06:30 h, the majority of the period of expected elevated blood NEFA concentrations was after the morning milking session, thus mainly overlapping with the period in which the evening milk was produced. This likely introduced a time delay between the moment of elevated blood NEFA levels and the moment at which a change in the concentration of related milk biomarkers could be noticed, which is even further delayed by the metabolic processes in the liver that transfer NEFA into milk precursors and constituents (e.g. lipoproteins and ketone bodies). this delay would explain why the blood NEFA concentrations were predicted more accurately from MIR spectra of evening milk compared to morning milk as the blood samples were taken within 1 hour after the morning milking session, right in the middle of the time window of expected elevated blood NEFA levels. This hypothesis is also supported by the slightly higher NEFA levels predicted based on MIR spectra of evening milk samples compared to those based on the paired morning milk samples, especially for cows with detrimental blood NEFA concentrations (Figure 3b). It might be interesting for future research to study these dynamics more in detail by measuring the blood NEFA level at a frequent interval and

investigating the link with the MIR spectra of morning and evening milk samples on that day and the days after.

The fact that the morning blood NEFA concentration can be predicted more accurately when the full model is applied on MIR spectra of evening milk rather than morning milk suggests that the evening milk samples contain more information on the morning blood NEFA concentration and/or that the morning milk samples are more subject to interfering effects. Nevertheless, training the model on solely evening milk MIR spectra (evening model) did not improve the prediction performance compared to the full model, even not if only MIR spectra of evening milk samples are considered in the validation. Moreover, the performance of the evening model was worse if applied on MIR spectra of morning milk samples. This suggests that including morning milk MIR spectra in the calibration set makes the prediction model more robust for potential interfering parameters that vary independent of the cow's blood NEFA level. One of these interfering effects might be the total fat content in the milk, which is generally higher in evening milk compared to morning milk (Forsbäck et al., 2010). Apart from the results obtained in our study, the full model is probably more robust under practical conditions where farms have varying milking and feeding frequencies.

Prediction of Blood Plasma NEFA Concentration

The regression coefficients in Figure 2 show that the PLSR models primarily use information from the fat-related MIR absorption bands. During negative energy status, excessive amounts of NEFA are mobilized from the adipose tissue and part of them is transferred to the milk. These NEFA are particularly rich in long-chain fatty acids (**FA**), such as C18:1 FA (Jorjong et al., 2014). Dórea et al. (2017) found a nonlinear relation ($R^2 = 0.42$ and $p < 0.001$) between the concentrations of NEFA in the blood plasma and C18:1 FA in the milk fat. Moreover, the milk C18:1 FA increased nearly linearly with increasing blood NEFA for blood NEFA levels below 400 µEq/L, while the milk C18:1 FA concentration was practically constant for blood NEFA concentrations above 800

µEq/L. This suggests that the C18:1 FA concentration in milk fat saturates when the blood NEFA increases above a certain concentration. On the other hand, Jorjong et al. (2014) suggested a linear relation (R^2 = 0.383) between the concentrations of NEFA in the blood plasma and C18:1 *cis*-9 FA in the milk fat. However, their linear function slightly underestimated the milk C18:1 *cis*-9 FA for blood NEFA concentrations between 0.2 and 0.4 mmol/L, while it overestimated the milk C18:1 cis-9 FA for blood NEFA levels below 0.1 and above 0.9 mmol/L. Accordingly, the data of Jorjong et al. (2014) confirms the non-linear trend found by Dórea et al. (2017). In our study, the predicted blood NEFA concentrations versus the actual blood NEFA levels (Figure 3b) follows a very similar nonlinear trend as the milk C18:1 FA in the studies of Dórea et al. (2017) and Jorjong et al. (2014). Therefore, it is likely that our PLSR models largely rely on the MIR absorption by C18:1 and related FA in milk. Several researchers already explored MIR spectroscopy to predict the concentration of certain FA in milk, obtaining R^2 values for the prediction of C18:1 FA between 0.11 and 0.96 (Rutten et al., 2009; Afseth et al., 2010; Soyeurt et al., 2011). Mäntysaari et al. (2019) used the PLSR models developed by Soyeurt et al. (2011) to predict the milk FA concentrations from milk MIR spectra and accordingly studied the relation between the predicted milk FA and the blood NEFA concentration. It was found that C18:1 *cis*-9 and the sum of C18:1 FA in milk had the highest correlation (r = 0.73) with blood NEFA, confirming our hypothesis.

The predicted versus measured scatterplot in Figure 3b, as well as the RMSEP values in Table 3, clearly show that the accuracy of the prediction of the blood NEFA from milk MIR spectra is limited, especially if the blood NEFA concentration is high. The full model results in RMSEP values of 0.197, 0.182 and 0.190 mmol/L when evaluated on respectively morning observations, evening observations or a mixed set of morning and evening observations of the validation set. Taking into account the standard deviations of the blood NEFA concentration for the different sets (Table 2), the R^2 values are respectively 0.502, 0.613 and 0.558. Nevertheless, the RMSEP and R^2 values strongly depend, because of heteroscedasticity, on the proportion of observations with a high

blood NEFA concentration in the respective datasets. To account for this non-linear effect, we also explored non-linear models, such as convolutional neural networks, and a logarithmic transformation of the blood NEFA levels before applying PLSR without success. Moreover, using a more balanced calibration set with a similar number of observations with high and low blood NEFA levels through bootstrapping did not improve the performance of the prediction model either (results not shown). Because of the heteroscedasticity of the prediction error, benchmarking our results against earlier studies is challenging and should be done with caution.

Dórea et al. (2017) obtained RMSE values of 169 – 220 µEq/L (equivalent to µmol/L) and R^2 values of 0.080 – 0.457 for the prediction of blood NEFA levels for individual cows from different linear combinations or ratios of milk FA concentrations obtained from GLS analysis. As the descriptive statistics for the blood NEFA are very similar in their dataset and ours (Table 2), it is fair to compare the results of these 2 studies. The prediction errors reported by Dórea et al. are very close to the ones obtained in our study. Nevertheless, the performances of their models are only reported for the calibration set and thus might be overoptimistic. Additionally, the approach followed by Dórea et al. requires labor and cost intensive FA isolation and GLS analysis.

Mäntysaari et al. (2019) used a linear combination of C18:1 *cis*-9 and medium chain FA concentrations in milk, derived from evening milk MIR spectra, and lactation stage to predict the morning blood NEFA concentration, obtaining an R^2 of 0.61 and an RMSECV of 0.182 mmol/L. A similar approach using morning milk MIR spectra resulted in an R^2 of 0.52 and an RMSECV of 0.198 mmol/L. Although these results only represent the cross-validation of the model, and thus might be overoptimistic, they are in close agreement with the results obtained for the independent validation in our study.

Recently, Benedet et al. (2019), Grelet et al. (2019) and Luke et al. (2019) published PLSR models that predict the blood NEFA levels directly from the MIR spectra of raw milk samples. The prediction performance of Grelet's model (R^2 = 0.39 and RMSECV = 344 µeq/L) is only based on

545 cross-validation of the calibration set and should thus be confirmed on an external validation set
546 (Grelet et al., 2019). Still, their results are inferior to the ones obtained in our study due to the
547 higher prediction error by Grelet's model in the low blood NEFA range. Moreover, while our full
548 model is relatively accurate (RMSEP $\leq$ 0.143 mmol/L) in this range, Grelet's model generally
549 overestimates the low blood NEFA concentrations. In the high blood NEFA range, Grelet's model
550 performs similar to our models, both underestimating the blood NEFA concentration. As a result,
551 the prediction error of Grelet's model is nearly homoscedastic, but worse compared to our model,
552 especially in the low blood NEFA range. Benedet et al. (2019) obtained a PLSR model that
553 performed better, compared to Grelet's model, with an R^2 of 0.52 and a standard error of prediction
554 $\left[SEP = n\sqrt{RMSEP^2 - bias^2}/(n-1)\right]$ of 0.24 mmol/L for a randomly selected validation set.
555 Still, these results are slightly worse compared to the ones obtained in our study. In contrast to our
556 models, Benedet's model only uses wavenumbers between 1450 and 1000 cm^{-1} and thus ignores the
557 fat absorption bands at around 2928, 2858 and 1745 cm^{-1}. Additionally, it should be taken into
558 account that the model performance typically deteriorates when it is applied on a completely
559 independent herd, as illustrated by Luke et al. (2019). Moreover, the R^2 of Luke's model dropped
560 from 0.61 for a randomly selected validation set, covering the same herds as the calibration set, to
561 0.45 for a totally independent herd. The better performance of our full model is likely the result of a
562 higher number of calibration samples (n = 790) in combination with a well-controlled timing and
563 protocol for blood and milk sample collection (Quiroz-Rocha et al., 2010).
564
565 ***Receiver Operating Characteristic Analysis***
566 Unlike the regression analysis, in which the RMSE is subject to the effect of heteroscedasticity,
567 the result of the ROC analysis is less dependent on the relative number of observations with a high
568 blood NEFA concentration. Accordingly, these ROC analyses allow for a more objective
569 comparison among different studies. Dórea et al. (2017) obtained their best results to identify cows

570 with detrimental blood NEFA concentrations (≥ 600 µEq/L) based on the milk C13:0 FA using a
571 threshold of 0.036 g FA per 100 g milk fat. This resulted in an AUC, sensitivity and specificity of
572 respectively 0.90, 0.859 and 0.823. The ROC curve to detect detrimental blood NEFA levels based
573 on milk C18:1 *cis*-9 FA and reported by Jorjong et al. (2014) had a sensitivity of 0.75 and 0.5 at a
574 specificity of respectively 0.79 and 0.935. The full model obtained in our approach and applied on
575 the evening observations results in an AUC of 0.898 and a sensitivity of 0.831 at a specificity of
576 0.8. The same model applied on morning observations has an AUC of 0.860 and a sensitivity of
577 0.752 at a specificity of 0.8 (Appendix A2). Therefore, it can be concluded that our model, which
578 only requires MIR spectral analysis of raw milk, is not inferior compared to more complex
579 techniques that require characterization of certain FA in the milk fat. A similar approach followed
580 by Luke et al. (2019) to identify elevated blood NEFA levels resulted in a AUC values of 0.87 and
581 0.82, sensitivities of 0.73 and 0.25 and specificities of 0.81 and 0.90 for respectively a randomly
582 selected and a completely independent validation set. Thus, our model tends to be slight more
583 robust compared to the one of Luke et al. (2019).

584 Although the prediction accuracy is not excellent, the developed model can provide valuable
585 information to further improve genetics, nutrition and management of dairy cows. As it can be
586 applied on millions of historical and future milk MIR spectra, this approach can reveal detailed
587 information on the energy status of individual cows, herds and pedigrees. This could potentially
588 result in improved estimations of breeding values and the identification of specific genetic markers
589 for metabolic resilience.

590

591 **CONCLUSIONS**

592 In this study, we successfully predicted the blood NEFA level in individual dairy cows from
593 their milk MIR spectra. The best model was obtained after training on MIR spectra of both morning
594 and evening milk samples. The NEFA concentration of blood plasma samples taken in the morning

595 were predicted with a higher accuracy if the model was applied on MIR spectra of evening milk
596 samples. The obtained prediction accuracy is acceptable for low blood NEFA levels, but is
597 unsatisfactory if the blood NEFA concentration is high. Nevertheless, low and intermediate/high
598 blood NEFA levels could be discriminated well to identify 83 out of 100 cows with detrimental
599 blood NEFA levels, while only 20 out of 100 healthy cows are wrongly classified. This opens
600 opportunities for identifying cows at risk of a negative energy status and studying the metabolic
601 resilience of individual cows and pedigrees.
602

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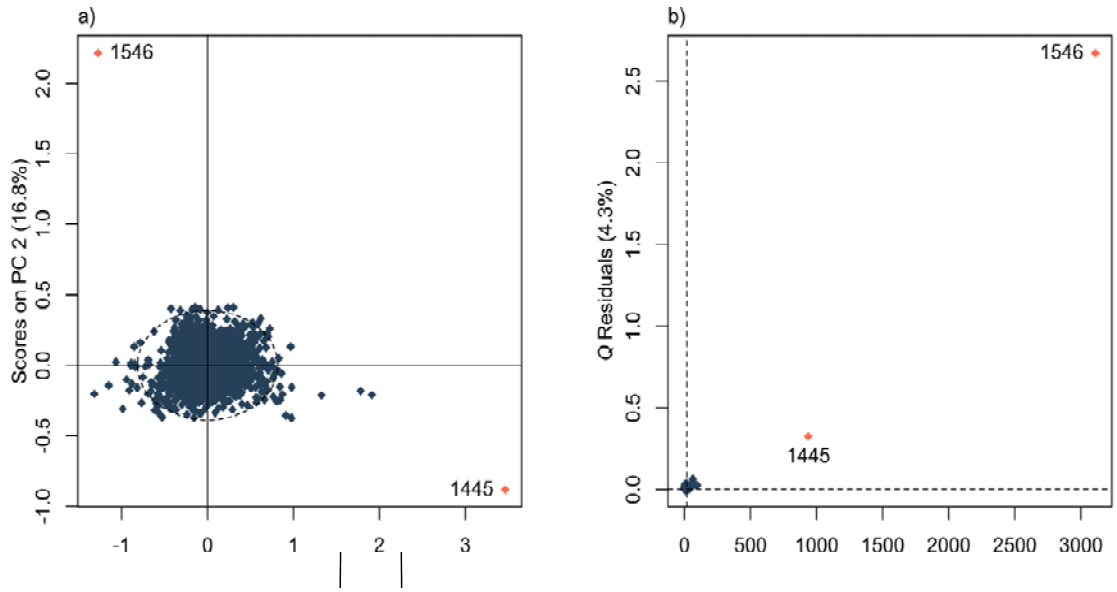
APPENDICES

Appendix A1 Figures of a) The scores plot of principal component (PC) 1 versus PC2 of the principal component analysis (PCA) on all 1585 mean-centered mid-infrared transmittance spectra. The dashed ellipse represents the 99% confidence limits of the scores on PC1 and PC2. b) The influence plot (*Q* residual versus Hotelling T^2 statistics) for the PCA model with 7 PC presenting the of the PCA. The dashed lines represent the 99% confidence limits on respectively the 2 statistics. In both figures, each dot represents a different sample spectrum and the red dots (with sample number) indicate potential outlier spectra.



Appendix A2 Table with the mean values of the sensitivities and the 95% confidence intervals (CI) of the sensitivities and specificities at given specificities for the morning and the evening observations of the validation set.

Sp	Morning observations			Evening observations		
	95% CI Sp	Se	95% CI Se	95% CI Sp	Se	95% CI Se
0.700	0.600 - 0.794	0.850	0.775 - 0.924	0.584 - 0.800	0.902	0.841 - 0.953
0.750	0.661 - 0.830	0.807	0.725 - 0.890	0.648 - 0.835	0.872	0.803 - 0.932
0.800	0.724 - 0.866	0.752	0.661 - 0.844	0.714 - 0.870	0.831	0.754 - 0.902
0.850	0.788 - 0.902	0.678	0.577 - 0.778	0.781 - 0.905	0.775	0.687 - 0.856
0.900	0.852 - 0.939	0.573	0.463 - 0.683	0.848 - 0.941	0.690	0.589 - 0.785
0.950	0.918 - 0.974	0.410	0.293 - 0.533	0.916 - 0.974	0.545	0.425 - 0.660

Sp = specificity; Se = sensitivity

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772

773 **TABLES**

774 **Table 1** Number of mid-infrared (MIR) transmittance spectra of morning and evening milk samples

775 available for the 3 different herds included in this study.

Milk samples	All herds	Herd		
		A	B	C
Morning	808	640	121	47
Evening	777	646	118	13

776 **Table 2** The descriptive statistics of the blood plasma non-esterified fatty acid (NEFA) concentrations linked to different subsets of milk mid-

777 infrared transmittance spectra.

Blood NEFA (mmol/L)	Calibration set (Herd A)			Validation set (Herd A, B and C)								
	Overall	Morning	Evening	Overall	Morning samples				Evening samples			
					All herds	Herd A	Herd B	Herd C	All herds	Herd A	Herd B	Herd C
Number	790	395	395	793	412	245	121	46	381	251	117	13
Mean	0.436	0.436	0.435	0.445	0.442	0.477	0.372	0.444	0.448	0.479	0.372	0.530
SD	0.296	0.296	0.296	0.286	0.280	0.316	0.187	0.246	0.292	0.324	0.188	0.298
Minimum	0.036	0.036	0.036	0.055	0.055	0.055	0.069	0.087	0.055	0.055	0.069	0.092
Maximum	1.951	1.951	1.951	1.748	1.631	1.631	1.080	1.256	1.748	1.748	1.080	1.033

Table 3 Root mean squared error of prediction (RMSEP) for the non-esterified fatty acid (NEFA) concentration in the blood plasma by the partial least squares regression models trained on morning and evening observations (= full model), only morning observations (= morning model) and only evening observations (= evening model) of the calibration set. The RMSEP values are provided for the different subsets (blood NEFA range, milking time and herd) of the validation set. Within each column and a specified blood NEFA concentration range, the RMSEP values with different subscripts indicate significant ($\alpha = 0.05$) differences between the models according to Tukey HSD multiple comparison, with a letter lower in the alphabetical order indicating a better model.

Blood NEFA range (mmol/L)	Model	Validation set - RMSEP (mmol/L)								
		Overall	Morning observations				Evening observations			
			All herds	Herd A	Herd B	Herd C	All herds	Herd A	Herd B	Herd C
Complete: 0 – 2	Full	0.190^{a}	0.197^{a}	0.220^{a}	0.135	0.204	0.182^{a}	0.199^{a}	0.140^{a}	0.167
	Morning	0.194^{b}	0.194^{a}	0.211^{a}	0.143	0.209	0.194^{b}	0.207^{b}	0.169^{b}	0.139
	Evening	0.206^{b}	0.225^{b}	0.252^{b}	0.161	0.220	0.183^{a}	0.195^{a}	0.156^{a,b}	0.162
Low: 0 – 0.6	Full	0.129^{a}	0.124^{a}	0.132	0.109	0.128	0.134^{a}	0.143^{b}	0.122^{a}	0.062
	Morning	0.158^{b}	0.141^{b}	0.145	0.129	0.152	0.174^{b}	0.184^{c}	0.161^{b}	0.071
	Evening	0.132^{a}	0.136^{a,b}	0.135	0.135	0.145	0.128^{a}	0.120^{a}	0.143^{a}	0.098
Middle: 0.6 – 1.2	Full	0.241^{b}	0.269^{b}	0.270^{b}	0.253^{a,b}	0.290^{a,b}	0.208^{a,b}	0.198^{a,b}	0.230	0.258
	Morning	0.211^{a}	0.234^{a}	0.232^{a}	0.218^{a}	0.277^{a}	0.185^{a}	0.174^{a}	0.219	0.206
	Evening	0.281^{c}	0.328^{c}	0.339^{c}	0.286^{b}	0.322^{b}	0.223^{b}	0.221^{b}	0.226	0.231
High: 1.2 – 2	Full	0.668^{b}	0.713^{b}	0.704^{b}		0.793	0.620^{b}	0.620^{b}		
	Morning	0.607^{a}	0.675^{a}	0.668^{a}		0.738	0.530^{a}	0.530^{a}		
	Evening	0.711^{c}	0.781^{c}	0.780^{c}		0.784	0.633^{b}	0.633^{b}		

784 # FIGURES

785 **Figure 1** Schematic overview of the methodology to build a partial least squares (PLSR) model to
786 predict the blood plasma non-esterified fatty acid concentration from milk mid-infrared spectra. CV
787 = cross-validation, RMSECV = root mean square error of cross-validation, RMSEP = root mean
788 square error of prediction.

789

790 **Figure 2** Top: Relative mid-infrared (MIR) transmittance spectra of the milk samples. The dotted
791 black lines (with sample number) indicate 2 potential outlier spectra. The grey regions indicate the
792 wavenumbers included in at least 1 of the 3 final partial least squares regression (PLSR) models
793 (full, morning or evening) to predict the blood plasma non-esterified fatty acid concentration after
794 applying a variable selection technique. Bottom: Regression coefficients for the 3 different PLSR
795 models constructed using a calibration set with MIR spectra of respectively i) morning and evening
796 milk samples (= full model, green solid), ii) only morning milk samples (= morning model, red
797 dashed) and iii) only evening milk samples (= evening model, blue dotted) of herd A.

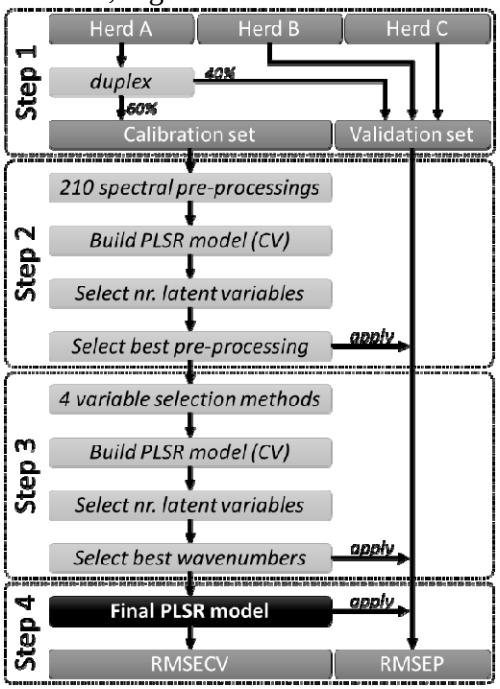
798

799 **Figure 3** The results of the partial least squares regression (PLSR) model trained on a calibration
800 set of mid-infrared transmittance spectra of milk samples collected during morning and evening
801 milking sessions (= full model) on herd A to predict the non-esterified fatty acid (NEFA)
802 concentration in the blood plasma of the respective cows for which blood was sampled in the
803 morning. a) Root mean squared error (RMSE) for the calibration set in cross-validation (CV) and
804 the morning (P_M) and evening (P_E) observations of the validation set (all 3 herds), in relation to the
805 number of latent variables of the PLSR model. The green triangle indicates the number of selected
806 latent variables (n = 6) for the final PLSR model. b) The predicted versus measured scatterplot for
807 the calibration set (herd A) in cross-validation (CV) and the morning (P_M) and evening (P_E)
808 observations of the validation set (3 herds).

809

810 **Figure 4** Smoothed receiver operating characteristic (ROC) curves for the identification of
811 detrimental blood plasma non-esterified fatty acid (NEFA) concentrations (≥ 0.6 mmol/L). The
812 NEFA concentrations were predicted with partial least squares regression models trained on
813 morning and evening observations (= full model) of the calibration set. The mean values (lines) and
814 95% confidence intervals (areas) for the smoothed ROC curves are provided for morning (red,
815 dashed) and evening observations (blue, dotted) of the validation set.

816 Aernouts, Figure 1



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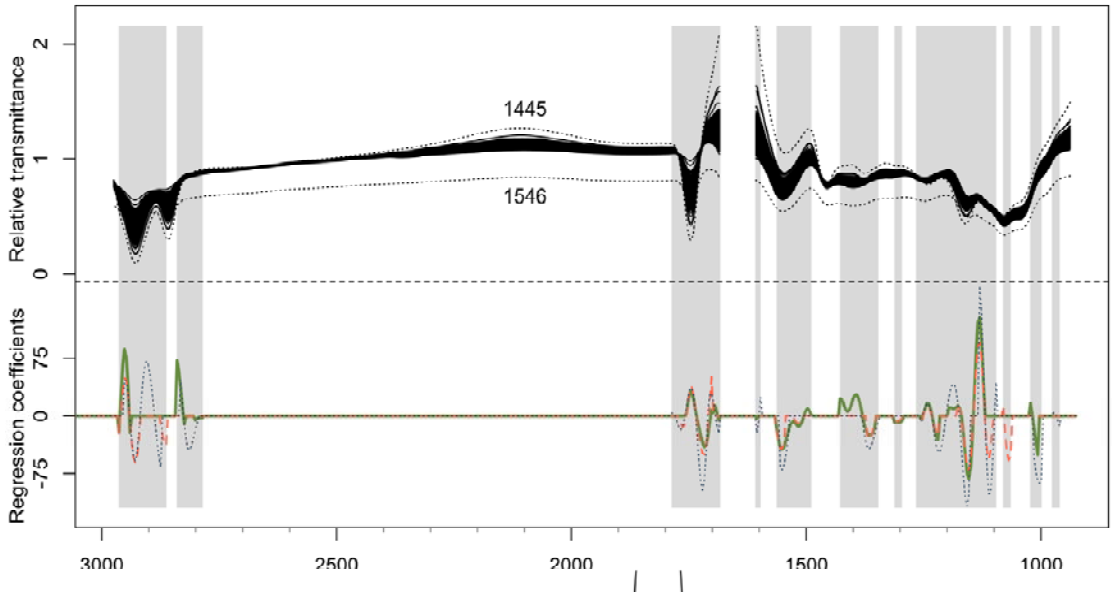
818 Aernouts, Figure 2



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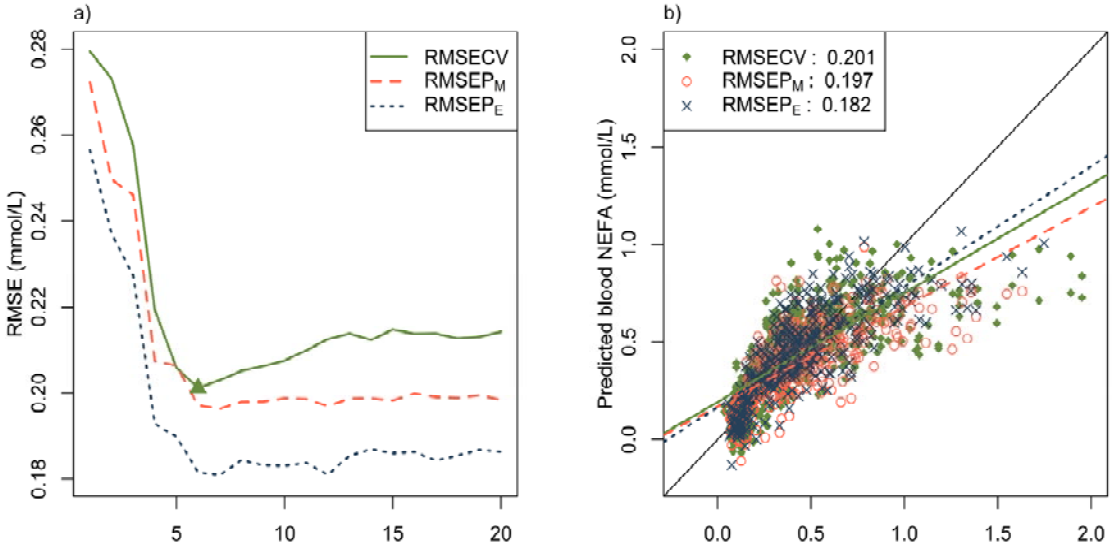
820 Aernouts, Figure 3



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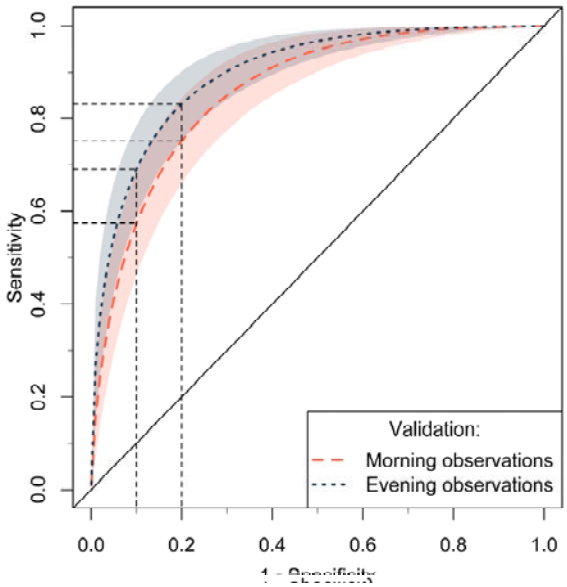
822 Aernouts, Figure 4



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