Near-Infrared Reflectance Spectroscopy (NIRS) detection to differentiate morning and afternoon milk based on nutrient contents and fatty acid profiles

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Abstract. Milking time is one of the factors that affect milk quality. The objective of this study was to differentiate morning milk from afternoon milk based on milk fatty acid profile and create a prediction model using Near-Infrared Reflectance Spectroscopy (NIRS). This study used explorative research and post-observation analysis. Milk sampling was collected from three different dairy farm locations in West Java Provinces (Pangalengan district of Bandung Regency, Cibungbulang District of Bogor Regency, and Tanah Sareal District of Bogor Municipality). Milk quality observed in this study included milk fat, protein, lactose, solid non-fat (SNF), and fatty acid compositions. Milk fat, protein, lactose, and SNF were analyzed using Lactoscan. Fatty acid compositions were identified using gas chromatography (GC). Sample spectrums were collected using NIRSflex 500. The difference between morning and afternoon milking was tested using a t-test carried out by SPSS ver. 25. Qualitative calibration of milk quality was conducted using NIRScal v5.6 by applying the cluster (CLU) method. The results from lactoscan and GC showed that milk fat, caprylic acid, and myristoleic acid, and total SFA were significantly different (Sig. (2-tailed) < 0.05) in morning and afternoon milk. However, NIRS failed to generate a sophisticated model for the milk quality differentiation, which shows a low Q-value (0.0011231). The quantitative analysis accurately produced milk fat and total SFA predictions but failed to accurately predict caprylic acid and myristoleic acid. This study concluded that morning milk could be differentiated from afternoon milk based on milk fat, caprylic acid, myristoleic acid, and total SFA content. The NIRS technology can differentiate between morning and afternoon milk based on quantitative calibration of total fat and SFA.

1. Introduction
Dairy milk has complete nutrition for human health and is frequently used as a nutrition standard. Dairy cattle milk contains 4.7% fat, 4.17% lactose, and 2.79% protein [1]. This composition varies greatly, primarily milk fat content. Riestanti et al. [2] reported a milk fat content of 3.16% from tropical lowland large farms, while Anzhany et al. [3] reported a milk fat content of 4.08% from small tropical scale highlands 4.49% from small tropical smallscale lowland dairy cattle farms. Milk composition can be
affected by feed, genetic, lactation stage, and milking time [4]. Generally, dairy farmers milk cows twice a day, in the morning and afternoon, producing different milk compositions [5]. Milk from morning milking has lower fat content than afternoon milking but has higher production [6]. Detection of the differences between morning and afternoon milking time is needed to provide better information for the consumer.

An accurate and rapid method to identify milk quality is essential because milk has perishable characteristics at room temperature. Milk spoils quickly, and the quality decrease sharply. One method that can be used to identify milk quality is the NIRS (Near Infrared Reflectance Spectroscopy) technique [7]. The NIRS has been used widely in the agriculture and food industry ranging from chemical feed compositions [8], ration digestibility [9], milk compositions, and milk fatty acid profile [2].

The NIRS had many advantages: maintain sample quality, easy sample preparation, and no chemical use [10]. The NIRS is used to identify the quantity of nutrients and is also used for qualitative analysis. For example, NIRS is used in differentiating organic milk from conventional milk [11]. The NIRS was also used to differentiate goat’, buffalo,’ and cow’s milk, milk from dairy with intensive pasture management [12], and milk from different locations of milking [13]. This technology might also be used to differentiate milk from morning and afternoon milking. This study aims to differentiate milk from morning and afternoon milking based on its nutrient content, especially fatty acid profiles, and to use NIRS qualitative and quantitative calibration to differentiate the milk based on the profiles.

2. Materials and methods

2.1. Milk sampling

Milk sample was collected from three dairy cattle areas in West Java, namely Pangalengan district of Bandung regency (24 farms), Tanah Sareal district of Bogor municipality (13 farms), and also Cibungbulang district of Bogor Regency (13 farms). From each farm, one lactating milk cow was chosen randomly. Milk sample was collected from the morning at 5.00–8.00 a.m. and afternoon at 1.00–5.00 p.m. milking. About 500 mL of milk was collected using a plastic bottle in the middle of manual milking from cow’s teats directly and stored at four °C before being sent to the laboratory for analysis.

2.2. Analyzed milk qualities

Milk’s components were analyzed using lactoscan to measure milk fat, solid non-fat (SNF), lactose, and protein. Milk fatty acid was analyzed using Chromatography Gas (GC-7820A, Agilent Technologies) following a similar procedure as Martha et al. [14]. The procedure consisted of three steps, namely extraction of milk fat, methylation, and GC quantification. Milk samples were let at room temperature for 5 minutes and homogenized manually. The 100 µL milk sample was pipetted into a screw-cap tube and added with 2 mL of H2SO4 2.5 % in methanol. The tube was agitated for 2 minutes with vortex. To complete the lipid extraction, the tube was let at a -20°C freezer overnight. The fat extract was methylated by warming the tube in a 75°C water bath for two hours and adding 2 mL of a saturated NaCl solution and 1 mL isooctane. The tube was agitated for 30 s and centrifuged at 2000 rpm for 5 minutes. The upper layer isooctane was collected and used later in GC injection. HP-5 capillary column (30 m length, 0.320 mm diameter, 0.25 µm film thickness) from Agilent Technologies, Palo Alto, CA, USA, was used to separate fatty acid. The injector and detector temperature was set at 250°C. Helium was used as carrier gas with a 1.3 mL/minute flow rate. The temperature program used as follows: initial temperature 35°C for 2 minutes, increased to 100°C (30°C /minute), increased to 195°C (10°C /minute, 5 minutes hold), increased to 205°C (7°C /minutes, 9 minutes hold), and increased to 240°C (3°C /minute, 7 minutes hold). The amount of 1 µL prepared sample was injected into GC with a split ratio
of 10:1. Data were recorded in a computer output device installed with GC Chem Station Software integrator version B.03.02 (Agilent Technologies).

2.3. Analyzed milk composition and fatty acid profile using NIRS
Before it is ready for use, the NIRS instrument (Buchi NIRFlex N-500 Solids Cell, made in Switzerland) was warmed up for 15 minutes and tested for its system suitability by running automatic SST using NIRSware operator. After that, external and internal references were scanned. Spectrum collection was done thrice for each sample. To the collected spectra, chemo-metric results were inputted with the help of the NIRSware Management Console. Qualitative calibration and validation were done using qualitative cluster calibration regression and validation set. While quantitative calibration and validation were done using the partial least squares (PLS). Calibration and validation were conduction using NIRCal V5.6 software. The software automatically divided the spectra into 2/3 for calibration and 1/3 for validation using block-wise methods. This calibration and validation process compares the chemometric data with NIRS prediction values. The database resulting from the calibration and validation can be used as a standard reference.

2.4. Statistical analysis
This study used explorative and post observative methods. The data were analyzed using descriptive statistics. After the normality test based on the Kolmogorov Smirnoff method, paired t-test was conducted using SPSS ver. 25. Calibration and validation data on NIRS using Qualitative Cluster Calibration (CLU) and quantitatively using the partial least squares (PLS) regressions.

3. Results and discussion
3.1. Composition of milk nutrient
The milk components with different milking times are shown in Table 1. The milk component consists of fat, solid non-fat (SNF), lactose, and protein. The result shows that milk fat from morning milking was significantly different from afternoon milking. However, there were no significant differences found in milk SNF, lactose, and protein. The average milk SNF found in this research was lower than the national milk quality standard [15]. Milk protein from morning milking was also slightly lower. The lower milk component in morning milking is due to the dilution effect. Longer milking time produces a higher milk production but lower milk component, especially milk fat, which is significantly lower in morning milk [16]. Besides milking time, milk fat is also affected by feeds used. High use of forage proportion in ration increases milk fat content due to the high molar acetic acid produced in the rumen used in fat milk synthesis [17].

| Parameter | Morning milking      | Afternoon milking | Sig. (2-tailed) | SNI 3141.1: 2011 |
|-----------|----------------------|-------------------|-----------------|-----------------|
| Fat (%)   | 3.803 ± 1.678b       | 4.409 ± 1.192a    | 0.006           | Min 3.0         |
| SNF (%)   | 7.603 ± 0.519        | 7.677 ± 0.577     | 0.432           | Min 8.0         |
| Lactose (%) | 4.175 ± 0.289        | 4.214 ± 0.317     | 0.444           | -               |
| Protein (%) | 2.787 ± 0.190        | 2.815 ± 0.212     | 0.413           | Min 2.8         |

Different superscripts between morning and afternoon milking of the same parameters indicated significantly different (Sig. (2-tailed) < 0.05), SNF = solid non-fat, SNI = Indonesian national standard
3.2. Fatty acid profile of morning and afternoon milking time

Table 2. The average level of milk fatty acid from GC analysis.

| Fatty acid, % total fat milk | Morning milking | Afternoon milking | Sig. (2-tailed) |
|-----------------------------|-----------------|------------------|----------------|
| C6:0                        | 1,184 ± 0.818   | 1,065 ± 0.653    | 0.279          |
| C8:0                        | 0.728 ± 0.790   | 0.533 ± 0.363    | 0.033*         |
| C10:0                       | 1,114 ± 0.788   | 0.946 ± 0.629    | 0.111          |
| C11:0                       | 0.005 ± 0.051   | 0.017 ± 0.167    | 0.508          |
| C12:0                       | 1,896 ± 1.477   | 1,680 ± 1,339    | 0.301          |
| C13:0                       | 0.005 ± 0.044   | 0.020 ± 0.196    | 0.450          |
| C14:0                       | 3,634 ± 3,427   | 4,000 ± 3,284    | 0.460          |
| C14:1                       | 1,915 ± 3,294   | 1,042 ± 1,823    | 0.027*         |
| C15:0                       | 0.533 ± 0.505   | 0.557 ± 0.720    | 0.794          |
| C15:1, cis10                | 0.383 ± 0.472   | 0.397 ± 0.451    | 0.836          |
| C16:0                       | 16,324 ± 10,138 | 15,248 ± 8,936   | 0.446          |
| C16:1, cis9                 | 0.514 ± 0.469   | 0.534 ± 0.445    | 0.768          |
| C17:0                       | 0.406 ± 0.474   | 0.733 ± 2.897    | 0.287          |
| C17:1, cis10                | 0.477 ± 0.497   | 1.350 ± 8.383    | 0.320          |
| C18:0                       | 2,163 ± 1,782   | 2,128 ± 1,915    | 0.900          |
| C18:1, cis9                 | 32,123 ± 20,126 | 34,395 ± 20,768  | 0.452          |
| C18:1, trans9               | 0.372 ± 1.001   | 0.447 ± 1.043    | 0.621          |
| C18:2, trans                | 3,816 ± 3,711   | 3,109 ± 4,350    | 0.237          |
| C18:2, cis                  | 5,651 ± 3,912   | 5,651 ± 3,912    | 0.856          |
| C18:3                       | 0.054 ± 0.167   | 0.021 ± 0.114    | 0.118          |
| γ-C18:3                     | 0.039 ± 0.185   | 0.019 ± 0.179    | 0.460          |
| C20:0                       | 0.036 ± 0.145   | 0.238 ± 2,108    | 0.362          |
| C20:1, cis 11               | nd              | 0.008 ± 0.080    | 0.319          |
| C20:2, cis 11,14            | 0.088 ± 0.266   | 0.033 ± 0.087    | 0.061          |
| C20:3, cis8,11,14           | 0.007 ± 0.063   | nd              | 0.319          |
| C20:4, cis 5,8,11,14        | 0.020 ± 0.188   | nd              | 0.323          |
| C21:0                       | 0.002 ± 0.019   | 0.001 ± 0.009    | 0.685          |
| C22:0                       | 0.082 ± 0.303   | 0.051 ± 0.174    | 0.392          |
| C23:0                       | 0.013 ± 0.115   | 0.002 ± 0.016    | 0.338          |
| C24:0                       | nd              | 0.00013 ± 0.001  | 0.319          |
| CLA                         | 1,113 ± 0.935   | 1,009 ± 0.831    | 0.837          |
| Σ SFA                       | 34.978 ± 8.304  | 37.728 ± 7.595   | 0.044          |
| Σ UFA                       | 64,129 ± 8.514  | 61,233 ± 6.679   | 0.027          |
| Σ MUFA                      | 52,230 ± 11,059 | 51,191 ± 7,411   | 0.516          |
| Σ PUFA                      | 14,382 ± 4,439  | 12,888 ± 4,997   | 0.065          |

Different superscripts between morning and afternoon milking of the same parameters indicated significantly different (Sig. (2-tailed) < 0.05); CLA = conjugated linoleic acid; SFA = saturated fatty acid; UFA = unsaturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.
Milk fatty acid profiles of morning and afternoon milking time are shown in Table 2. The results show significant differences in caprylic acid (C8:0) and myristoleic acid (C14:1) between morning and afternoon milking. Caprylic acid is one medium-chain fatty acid which easy to absorb than long-chain fatty acid [18]. The level of caprylic acid on milk is around 1.39 g 100 g\(^{-1}\) of total milk fat; caprylic acid is also classified as easy to evaporate [19]—caprylic acid milk fat produced by the endogenous and exogenous process. The endogenous process occurs by elongating short-chain fatty acid [20], while the exogenous process is affected by the feeding system. Dairy cattle ration with a high proportion of good quality forage stimulates rumen microbial to produce short-chain fatty acid (acetate, propionic, and butyric acid) that can be used as a precursor for caprylic acid synthesis through the elongation process. Besides that, the total of neutral detergent fiber (NDF) on feed also affects acetate and butyric acid availability [21]. The lactation stage influenced the production of caprylic acid through the endogenous process [22].

Milk from morning milking has a higher level of myristoleic acid. Myristoleic acid is classified as an unsaturated fatty acid that reduces cholesterol levels in the blood and stabilizes protein for the immune. Myristoleic in milk is produced by the endogenous synthesis of myristic acid (C14:0). It has a function on the desaturation process in \(\Delta^9\) desaturase [23]. Some factors that affect the myristoleic acid level in milk are the estrous cycle. Myristoleic acid is a fatty acid produced from bio-hydrogenation of myristic acid with the stearoyl-CoA desaturase enzyme. This enzyme also affects fatty acid compositions [24].

The average CLA found in this research was lower than Riestanti et al. [2] and Anzhany et al. [3], which studied milk CLA content from a specific location. The SFA found in this study was also lower than Riestanti et al. [2] and Anzhany et al [3], which indicated a high proportion of UFA. High UFA proportion in milk fat mainly in the form of MUFA. Not all fatty acids were found in the morning and afternoon milking. The C20:1, cis 11, and C24:0 were only found in the afternoon milk, while C20:3, cis8,11,14, and C20:4, cis 5,8,11,14 were only detected in morning milk. The difficulties in detecting the milk fat in the morning and afternoon milk are due to the very low concentration of the milk fatty acids (< 0.02%). The low proportion of long-chain fatty acids that contained C > 21 in this research was not detected in Riestanti et al. [2] and Anzhany et al. [3] due to different milk sample origins. In this research, the milk samples were collected from both highland and lowland, while in Riestanti et al [2], the milk samples were collected from lowland in contrast to Anzhany et al. [3] collected sample from highland. Total SFA was significantly higher in afternoon milk than morning milk which indicated morning milk was healthier than afternoon milk. Saturated fatty acids (SFAs) have been linked to cardiovascular risk, obesity, and some cancers, especially C12:0, C14:0, and C16:0 [8].

### 3.3. Qualitative and quantitative calibration of milk using NIRS

The NIRS Spectra collected from milk fatty acids are shown in Figure 1. The graph shows an overlapping spectrum between morning and afternoon milking with a narrow band. Although chemical analysis detected different milk fat content, caprylic acid (C8:0) and myristoleic acid (C14:1) between morning and afternoon milk, the NIRS qualitative calibration failed to produce a satisfaction prediction. This condition might be caused by a low concentration of caprylic acid (C8:0) and myristoleic acid (C14:1) as a percentage of total milk fat (<2%). It might need other distinctive fatty acids with a high proportion in milk as a marker to detect the difference, such as detection of milk from organic and conventional systems that were distinctly different in \(\Delta^9\)-FA content [11].

The summary statistic of the qualitative calibration and validation set for milking time is shown in Table 3. Each milk spectra calibration and validation set produced a low score of mean, standard deviation, and Q-value (0.0011231). These values indicated the inability of the NIRS to differentiate in the morning and afternoon milking based on milk fat, milk fat, caprylic acid, myristoleic acid, and total SFA. The significance of qualitative calibration on NIRS produces a Q value that closed to 1.
inability of NIRS to differentiate morning and afternoon milking qualitatively due to narrow-spectrum produced, which come from a bit of difference between the marker used (milk fat, caprylic acid, and myristoleic acid and total SFA). Considerable variation between the nutrient content due to different feeds type used and milk origin. It might also cause an insignificant difference. Uneven samples distribution and air bubbles in milk samples can also result in a considerable variation of results. This condition also makes lower Q-value, and the NIRS failed to detect the difference of milk from morning and afternoon milking time. A similar condition is also found in the qualitative analysis based on Laser-Induced Breakdown Spectroscopy (LIBS) [25]. Milk samples in this study have a similar composition which causes qualitative analysis to fail to differentiate milk from morning and afternoon milking time.

Figure 1. Spectra collection of milk-based on NIRS analysis.

Table 3. Spectra calibration and validation of milk.

| Milking time | Calibration | Validation | Q-value |
|--------------|-------------|------------|---------|
|              | n  | Mean     | Standard deviation | n | Mean | Standard deviation |
| Morning      | 200 | 0.000539 | 0.00011189 | 100 | 0.00128 | 0.00295 | 0.0011231 |
| Afternoon    | 200 | 0.000583 | 0.00014142 | 100 | 0.00106 | 0.00211 |

Quantitative calibration, validation, and external validation to differentiate milk from morning and afternoon milking are shown in table 4. The data shows that calibration of milk fat, C8:0, C14:0, and total SFA produced high $R^2_C$ except for C8:0. However, $RPD > 1.5$ was only produced in the calibration of milk fat and total SFA. Validation fails to improve the prediction accuracy.

External validation of the model using an independent sample set produced SEP/SEL below 1 for all parameters. The accuracy model found in this study was lower than Despal et al. [8]. Accuracy of NIRS quantitative analysis were accepted based on $R^2_C > 0.5$, $RPD > 1.5$ and $RPL (SEP/SEL) < 2.5$ criteria. In this case, total fat and SFA can be detected accurately using the NIRS and can differentiate milk from morning and afternoon milking.
Table 4. Quantitative NIRS calibration and validation to differentiate morning and afternoon milk.

| Parameters | Milk fat | C8:0  | C14:0 | Total SFA  |
|------------|---------|-------|-------|-----------|
| Calibration |        |       |       |           |
| n          | 201     | 201   | 201   | 201       |
| Mean       | 4.303   | 0.034 | 0.284 | 1.970     |
| Range      | 0.085–7.305 | 0.0–0.054 | 0.0–0.459 | 0.037–4.848 |
| SD         | 1.199   | 0.010 | 0.087 | 0.997     |
| SEC        | 0.583   | 0.011 | 0.087 | 0.610     |
| R2C        | 0.809   | 0.462 | 0.504 | 0.627     |
| RPD        | 2.058   | 0.927 | 1.009 | 1.637     |
| Validation |        |       |       |           |
| n          | 99      | 99    | 99    | 99        |
| Mean       | 4.130   | 0.034 | 0.284 | 1.964     |
| Range      | 0.085–6.98 | 0.0–0.049 | 0.0–0.456 | 0.037–4.848 |
| SD         | 1.169   | 0.008 | 0.076 | 0.933     |
| SEP        | 0.552   | 0.011 | 0.090 | 0.616     |
| R2V        | 0.817   | 0.369 | 0.420 | 0.594     |
| RPD        | 2.118   | 0.746 | 0.845 | 1.515     |
| External Validation |        |       |       |           |
| NIRS       | 3.728±0.971 | 0.032±0.007 | 0.291±0.056 | 1.329±0.477 |
| CWC        | 3.838±1.215 | 0.039±0.014 | 0.128±0.163 | 1.511±0.546 |
| R          | 0.823   | 0.462 | 0.164 | 0.277     |
| SEP        | 0.690   | 0.013 | 0.164 | 0.617     |
| SEL        | 1.580   | 0.024 | 0.240 | 0.770     |
| SEP/SEL    | 0.437   | 0.530 | 0.683 | 0.802     |

C8:0 = caprilic acid, C14:0 = myristoleic acid, SFA = saturated fatty acid, SD = standard deviation, SEC (P) = standard error calibration (validation), R2C (V) = coefficient determination for calibration (validation), RPD = residual predictive deviation, AVG = average, NIRS = Prediction by NIRS, CWC = Conventional Wet Chemical; R = coefficient correlation, SEP (L) = standard error procedure (laboratory)

4. Conclusion
This study concluded that morning milk could be differentiated from afternoon milk based on milk fat, caprylic acid, myristoleic acid, and total SFA content. Morning milk is healthier than afternoon milk. The NIRS technology can differentiate between morning and afternoon milk based on quantitative calibration of total fat and SFA.

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