https://doi.org/10.17221/114/2025-CJAS

Lactoferrin content determined in bovine milk by HPLC and mid-infrared spectrometry – Relation to udder health and potential for detection of milk adulteration

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Citation: Nejeschlebová H., Hanuš O., Bartáková K., Parsons C., Samková E., Vorlová L., Thompson G., Hasoňová L., Konečný R., Navrátilová P., Klimešová M. (2025): Lactoferrin content determined in bovine milk by HPLC and mid-infrared spectrometry – Relation to udder health and potential for detection of milk adulteration. Czech J. Anim. Sci., 70: 467–479.

Abstract: Lactoferrin (LF) is a multifunctional glycoprotein linked to udder health in dairy cows. This study aimed to develop a calibration model for LF quantification using mid-infrared spectrometry (MIR-FT), with ion-pair reversed-phase high-performance liquid chromatography (HPLC) as the reference method. Two sets of individual milk samples (A: n = 120; B: n = 91) were collected from five dairy farms in the Czech Republic. Set A included a higher proportion of samples with somatic cell count (SCC) above 300 000 cells/ml to ensure broad LF variability. After merging both sets and removing six outliers, a final calibration set C (n = 205) was created. The developed model achieved a cross-validated coefficient of determination of 0.588 7 and a standard error of cross-validation of 67.33 mg/l. Correlation analysis showed that several milk parameters correlated with LF determined by MIR-FT in patterns typical of mastitis (SCC: r = 0.450; lactose: r = –0.364; free fatty acids: r = 0.621; electrical conductivity: r = 0.442), indicating potential for MIR-FT in evaluating mammary gland health. The feasibility of using LF as an indicator of milk adulteration by artificial SCC reduction through centrifugation was also assessed. Two sample sets (n = 20 and n = 68) were analysed, each containing normal bulk tank milk and bulk tank milk supplemented with abnormal milk. Centrifugation caused minimal changes in LF determined by both HPLC and MIR-FT (maximum 3.27%) while SCC decreased by nearly 50%, suggesting that LF may serve as a marker for detecting artificial SCC reduction. However, practical application of MIR-FT for accurate LF determination is limited by the achieved validation parameters and the high expanded uncertainty (114.7 mg/l). The method is therefore more suitable for monitoring relative LF changes in milk than for determining exact LF content.

Keywords: calibration model; dairy cow; mastitis; milk centrifugation; milk composition; somatic cell count

Supported by the National Agency for Agricultural Research of the Czech Republic (Project Nos. QK21010326 and QK21010212).

https://doi.org/10.17221/114/2025-CJAS

Lactoferrin (LF) is an iron-binding glycoprotein that belongs to the transferrin family. It was first identified in bovine milk by Sorensen and Sorensen (1939) and its presence has since been confirmed in a number of mammals. LF is present in mucosal secretions (milk, saliva, tears, nasal secretions, bile etc.) and it is also found in secondary granules of neutrophil leukocytes and blood plasma (Giansanti et al. 2016).

LF is particularly known for its antibacterial effect against various pathogens (Bullen et al. 1972; Arnold et al. 1982). To the contrary, LF has been found to promote the growth of some probiotic bacteria of the genera *Bifidobacterium* and *Lactobacillus* (Chen et al. 2013, 2017). Further, biological properties of LF are antiviral, antifungal, immunomodulatory, anti-inflammatory or anticarcinogenic activity (Superti 2020).

The richest source of LF is found in the milk of mammals. LF content varies among different species (Giansanti et al. 2016; Abd El-Hack et al. 2023). Within the species, it is considerably influenced by the stage of lactation. For mature milk of healthy cows, LF content ranging from 31.8 mg/l to 485.6 mg/l with a mean of 115.4 ± 67.4 mg/l was found (Cheng et al. 2008). For cow's colostrum, 30 mg/l to 10 200 mg/l of LF was found. In the course of 72 h from the start of colostrum production, the amount of LF drops sharply. An increase follows in the later stages of lactation. Before drying off, the LF content is comparable to LF content in colostrum (Valk-Weeber et al. 2020).

LF is involved in the immune response of the mammary gland to microbial infection, and its level in milk usually increases if a cow is affected by mastitis. It was suggested that the level of LF increase is specific to the mastitis pathogen, because different mastitis pathogens induce different response of the mammary gland (Chaneton et al. 2008). Hagiwara et al. (2003) found LF content in dairy cows with subclinical mastitis from 7 mg/l to 3 600 mg/l, and an increasing trend in LF content with an increase in the somatic cell count (SCC) score. SCC and major milk components are often studied parameters related to LF regarding dairy cow's udder health (Hagiwara et al. 2003; Cheng et al. 2008; Soyeurt et al. 2012). It could be beneficial to investigate also the relationship of other milk parameters to LF, e.g. of electrical conductivity (EC), freezing point (FP), urea or free fatty acids (FFA). Further, the relationship between LF and the health status of the mammary gland might be used to detect an artificial SCC reduction by raw milk centrifugation. This is a practice used on some dairy farms in order to meet legislative requirements for SCC limits, or to achieve stricter limits for SCC resulting from supplier-customer contracts. By using this practice, the milk supplier violates Regulation (EU) No. 1308/2013 of the European Parliament and the Council that lays down: "Milk means exclusively the normal mammary secretion obtained from one or more milkings without either addition thereto or extraction therefrom." Moreover, such a practice is immoral, as milk from ill animals is knowingly supplied for human consumption, which poses a risk to consumers. The use of the information on LF content for the detection of milk centrifugation is based on the assumption that after centrifugation the SCC will decrease, but the LF content will remain the same or close to the original milk. A strikingly high LF content in milk with low SCC could therefore point to this manipulation.

Therefore, rapid LF determination in milk presents the possibility of collecting data during routine milk recording and milk payment analysis, and their further exploitation for determining the health status of animals, milk quality and authenticity or the nutritional value of milk. For LF determination in milk, mostly methods of simple radial immunodiffusion, enzyme-linked immunosorbent assay (ELISA), and high-performance liquid chromatography (HPLC) have been described (Zhang et al. 2021). However, these methods are time-consuming and labour-intensive. Today's preferred method for routine determination of milk composition (including minor components) is Fourier transform mid-infrared spectrometry (MIR-FT), which is widely used in laboratories for milk recording and payment testing. The possibility of LF determination by the MIR-FT method was mentioned previously by Soyeurt et al. (2007, 2012). ELISA was used as a reference method for LF determination in both studies.

Based on the previously mentioned, the aim of this study was *i*) to develop a calibration model for LF determination in milk using HPLC as a reference method; *ii*) to examine the relationship between LF content and selected milk parameters in relation to udder health, and *iii*) to investigate the possibility of using LF content to detect artificial SCC reduction in raw milk by centrifugation.

https://doi.org/10.17221/114/2025-CJAS

MATERIAL AND METHODS

Calibration (reference) sample set and development of a calibration model for LF determination in milk by MIR-FT

Sampling. Sampling was carried out according to the milk recording guidelines. The samples were cold-preserved after collection (≤6 °C). Three sets of samples A, B and C were created. In set A, the characteristics of milk indicators (especially SCC) were deliberately influenced to increase the likelihood of subclinical mastitis occurrence through targeted animal selection. The sampling of dairy cows in set B was random. Set C was obtained by merging the sets A and B.

Samples of set A were collected from four dairy cattle farms in the Czech Republic, specifically in the region of northern Moravia. The characteristics of dairy herds are in Table 1. Samples were collected within regular milk recording controls during a period of approximately one year (from December 2022 to September 2023). A total of 120 milk samples were collected over four sampling sessions from Czech Fleckvieh and Holstein cows (Czech Fleckvieh and Holstein in the winter feeding period and Czech Fleckvieh and Holstein in the summer feeding period), 30 samples were taken at each sampling. Thus, when creating a reference set of milk samples, the sampling represented the average conditions of dairy cows in the Czech Republic in terms of the influence of season, breed and milk yield. In each sampling, one-third of samples from cows in the first lactation and two-thirds of samples from the cows in the second or higher lactation (not more than in the sixth lactation) were included. Samples were collected from day 10 to day 200 after calving. Cows were selected according to results of somatic cell count (SCC) on the previous milk recording control day (i.e. two weeks before sampling). The selection was performed so that, approximately, one-third of the samples were taken from cows that showed SCC lower than 300 000 cells/ml, while two-thirds of the cows showed SCC above 300 000 cells/ml, with a maximum of 5 000 000 cells/ml, on the previous control day. This approach was followed to ensure the necessary greater SCC variability (for calibration reasons) in the reference sample set.

Samples in set B were collected on a dairy cattle farm located in the Vysočina region of the Czech Republic, at an altitude of 485 m with an average annual temperature of 7.4 °C. The Holstein dairy cows are kept in free housing with straw bedded cubicles and fed TMR all the year round. The average annual milk yield is 11 553 kg. Individual milk samples were collected from August to November (2021–2023) and in March 2024. Samples were collected from dairy cows up to day 20 after calving (variable lactation order 1–6). A total of 91 samples were taken during eight samplings, 4 to 15 samples were obtained within each sampling. Average number of samples at one sampling was 11.4.

Milk analysis. For all milk samples, LF (mg/l), fat (%), crude protein (protein; %), casein (%), lactose monohydrate (lactose; %), solids-not-fat (SNF; %), urea (mg/100 ml), FFA (mmol/100 g of fat), citric acid (CA; %), SCC (cells/ml), EC (mS/cm) and FP (°C) were determined.

Fat, protein, casein, lactose, SNF, urea, FFA and CA were simultaneously determined by the MIR-FT method using DairySpec FT milk analyser (Bentley Instruments, Chaska, MN, USA). The instrument is regularly calibrated according to the results of reference methods. Samples were run after warming to 40 °C, and mixed by inverting

Table 1. The main characteristics of dairy cow herds for selecting milk samples of the reference set for lactoferrin determination using a reference (HPLC) and routine (MIR-FT) method

Herd number	Breed	Altitude (m)	Number of dairy cows	Milk yield per standard (305 days) lactation (kg)	Feeding season	Average annual temperature (°C)
1	CF	375	120	7 583	S	7.3
2	CF	530	297	5 941	W	7.4
3	H	258	439	12 105	S	9.2
4	H	262	277	9 557	W	8.8

CF = Czech Fleckvieh; H = Holstein; HPLC = ion-pair reversed-phase high-performance liquid chromatography; MIR-FT = Fourier transform mid-range infrared spectrometry; S = summer; W = winter

https://doi.org/10.17221/114/2025-CJAS

the vial five times prior to introduction into the cell via the DairySpec FT high-pressure pump and homogenizer valve. To collect the mid-IR transmission spectra, the CaF_2 cell with an approximately 20 µm pathlength using 20 co-added scan averages at 8 cm^{-1} resolution was used. Absorption spectra were collected for analysis relative to a DI water background spectrum and they were the results of the average of two samplings per milk vial.

SCC was determined by flow cytometry on Somacount 300 (Bentley Instruments, Chaska, MN, USA) in set A and on Fossomatic 7 (Foss Analytical A/S, Hillerød, Denmark) in set B. EC was determined on a HI5321-02 conductometer (Hanna Instruments, Woonsocket, RI, USA). FP was determined with a CryoStar automatic cryoscope (Funke-Gerber, Berlin, Germany).

Ion-pair reversed-phase HPLC was used as the reference method for LF determination. The samples were centrifuged (3 000 rpm/15 min/5 °C) to remove the fat from the surface. For whey separation, precipitation with 10% acetic acid to a pH of 4.6 was used. After centrifugation, the whey was filtered through a nylon membrane filter (0.22 µm) into vials for HPLC determination. Lactoferrin from bovine milk (Sigma Aldrich, St. Louis, MO, USA) was used as a reference standard; 10 mg was weighed into a 10 ml volumetric flask and made up to 10 ml with mobile phase (water/acetonitrile/trifluoroacetic acid). The LF determination was performed with an Alliance 2695 liquid chromatograph with a PDA 2996 detector (Waters, Milford, MA, USA) and a Poroshell 300SB-C8 column, 2.1 × 75 mm, 5 µm (Agilent Technologies, Santa Clara, CA, USA). A gradient elution and mobile phase flow rate of 1.0 ml/min (water/acetonitrile/trifluoroacetic acid) were used, the column temperature was 50 °C, the injection volume was 5 µl. Analytes were detected at 205 nm. To collect and evaluate the data, Empower 2 software (Waters, Milford, CA, USA) was used.

Calibration dataset and calibration model. MIR-FT transmission absorption spectra and the LF reference values determined by HPLC (LF HPLC) were used to construct a Partial Least Squares (PLS) chemometric model with Unscrambler X (Camo Analytics/AspenTech, Bedford, MA, USA) software. Spectral pretreatment consisted in subtracting a constant background value from each spectrum relative to a temperature-insensitive region near 1 940 cm^{-1}. PLS regression employed a Kernel algorithm. The initial spectral regions used were approximately 980–1 580 cm^{-1}, 1 723–1 800 cm^{-1}, and 2 800–2 940 cm^{-1}. Cross-validation using randomly selected samples was used to generate a standard error of cross-validation (SECV) which was used as the merit function for further model refinement. Six sample outliers (five outliers from set A, one outlier from set B) were excluded from the original 211 sample dataset based on their excessive residual Y-variances. Spectral points were removed based on the weighting of the model B-coefficients and also to reduce the influence of the identified noisy spectral regions. Successful x-axis point deletions were confirmed by improved SECV values. The number of principal components in the final model was chosen by noting the SECV, software recommendation, and monitoring for evidence of overfitting in the B-coefficients. A cross-validated coefficient of determination (R^2_{CV}) was calculated to assess the agreement between LF HPLC and LF determined by MIR-FT (LF MIR-FT).

Descriptive statistics including arithmetic mean (hereinafter referred to as mean), standard deviation, coefficient of variation, minimum and maximum were calculated for parameters of sets A, B and a combined set C (LF HPLC, fat, protein, casein, lactose, SNF, urea, FFA, CA, SCC, log SCC, EC and FP). SCC values were used in their original and logarithmically transformed form for the calculations. For SCC, the geometric mean was also calculated. Calculated SCC statistics – mean, standard deviation, and geometric mean – were rounded to the nearest thousand. MS Excel 2013 (Microsoft Corporation, Redmond, WA, USA) was used for these calculations.

Relation of LF content to selected milk parameters in association with udder health

For set C, the relationship between LF, determined by both LF HPLC and LF MIR-FT, and between selected milk parameters (fat, protein, casein, lactose, SNF, urea, FFA, CA, SCC, log SCC, EC and FP) was evaluated, based on the correlation analysis performed. Significance of the Pearson correlation coefficient and *P*-value was assessed at conventional confidence interval probability levels (ns) $P > 0.05$; (s) $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$). MS Excel 2013 (Microsoft Corporation, Redmond, WA, USA) was used for the calculations.

https://doi.org/10.17221/114/2025-CJAS

LF determination as a tool for detection of artificial SCC reduction in milk by centrifugation

Two sets of milk samples (n = 20 and n = 68) were obtained, both of which consisted of normal bulk tank milk and bulk tank milk with the addition of abnormal milk (individual milk of dairy cows with subclinical mastitis, with SCC above 900 000 cells/ml). Both sets were collected in the region of northern Moravia in the Czech Republic. For the set of n = 20, the milk was collected from three herds of Holstein and Czech Fleckvieh dairy cows (at a ratio of 1 : 1). The sampling was carried out in July, August, September and October 2024. For the set of n = 68, the milk was collected from four herds of Holstein and Czech Fleckvieh dairy cows (at a ratio of 1 : 1). The sampling was carried out from June 2023 to October 2024, which eliminated the seasonal effect.

For all samples, a volume of 30 litres was centrifuged in a Motor Sich-100 dairy centrifuge (Motor Sich, Zaporizhzhia, Ukraine). The centrifuge was set to an approximately 50% reduction of SCC. Fat, protein, casein, lactose, SNF, urea, SCC, LF HPLC and LF MIR-FT were determined in the original and centrifuged milk in the set of n = 20. In the set of n = 68, only SCC and LF MIR-FT were determined. The used analytical methods were identical to those for the analysis of set C above. SCC was determined in all samples by flow cytometry on Somacount 300 (Bentley Instruments, Chaska, MN, USA).

Descriptive statistics (mean, standard deviation, coefficient of variation, minimum, maximum) were calculated for the sets of n = 20 and n = 68. Between the milk parameters in the original and centrifuged milk, a paired t-test was performed, and the coefficient of determination, mean difference, its relative value, and the standard deviation of the differences were calculated. Calculated SCC statistics – mean, standard deviation, mean difference and the standard deviation of the differences – were rounded to the nearest thousand. All calculations were performed in MS Excel 2013 (Microsoft Corporation, Redmond, WA, USA).

Estimation of measurement uncertainty

The expanded combined uncertainty of LF measurement by the MIR-FT method was estimated. The individual components of uncertainty were identified and quantified as measurement repeatability and measurement accuracy. To calculate repeatability, a sample set of raw bulk tank milk samples (n = 10) collected from 10 Holstein farms in the Vysočina region in 2024 was used. Repeatability, based on pairs of duplicate measurements, was calculated using the following equation:

$$u_A = \sqrt{\frac{\sum_{i=1}^{n} a_i^2}{2n}} \quad (1)$$

where:

u_A – standard deviation expressing the repeatability of LF MIR-FT measurements;

a_i – difference between the results of duplicate LF MIR-FT measurements of the i^{th} sample;

n – number of measurement pairs.

For the calculation of accuracy, values of LF HPLC and LF MIR-FT from dataset C (n = 205) were used, after excluding samples with LF HPLC values lying outside the interval of one standard deviation (106.8 mg/l) from the mean (127.6 mg/l). Accuracy was calculated according to the following equation:

$$u_B = \sqrt{\frac{\sum_{i=1}^{n} \left(x_i - r_i\right)^2}{n-1}} \quad (2)$$

where:

u_B – standard deviation of the differences between the results of LF MIR-FT and LF HPLC;

r_i – LF HPLC measurement of the i^{th} sample;

x_i – LF MIR-FT measurement of the i^{th} sample;

n – number of samples.

The combined standard uncertainty (u_c) was calculated according to the equation:

$$u_c = \sqrt{u_A^2 + u_B^2} \quad (3)$$

The expanded combined uncertainty (U) was determined as the product of the combined standard uncertainty u_c and the coverage factor k, according to the equation:

$$U = 1.96 u_c \quad (4)$$

The value of k =1.96 was chosen to correspond to a 95% confidence level of the result. The expanded combined uncertainty is expressed in the same units as the measured quantity (mg/l).

https://doi.org/10.17221/114/2025-CJAS

RESULTS AND DISCUSSION

Calibration (reference) sample set

The characteristics of the milk parameters of sets A, B and C are shown in Table 2. The highest mean LF HPLC was observed in set A (A 157.6 ± 122.9 mg/l vs B 89.3 ± 64.3 mg/l vs C 127.6 ± 106.8 mg/l), which corresponds to the chosen sampling methodology. For set A, two-thirds of the samples with SCC above 300 000 cells/ml were selected (based on the SCC from the previous milk recording), whereas for set B, the selection was random. Cheng et al. (2008) reported that the increase of LF in milk occurs only when SCC score (SCS) 4 is exceeded (141 000 cells/ml ≤ SCS 4 < 283 000 cells/ml), i.e. at the point when the probability of mammary gland infection is increased. It is therefore logical that sets A and C, which contained a higher proportion (32.5% and 26.1%, respectively) of samples with SCC above 283 000 cells/ml than set B (18.7%), also showed a higher content of LF and a more even distribution of LF (Table 3). To ensure a sufficient number of samples with higher LF content in the reference set, it is advisable to choose a procedure for the sample collection where approximately two-thirds of the milk samples come from cows with SCC above 300 000 cells/ml. It can be assumed that the smaller the reference set is, the more important it is to follow this procedure.

However, there are many sources of variation in LF content. The calibration set of Soyeurt et al. (2007) showed the mean SCS of 3.03 ± 1.74 and mean LF of 253.72 ± 206.37 mg/l determined by ELISA. SCS 3 includes samples with SCC equal to or greater than 71 000 cells/ml and less than 141 000 cells/ml. In our study, the mean SCC in sets A, B and C was 568 000 ± 1 087 000 cells/ml, 512 000 ± 2 039 000 cells/ml and 543 000 ± 1 573 000 cells/ml, respectively. The mean LF in all three sets was lower than in both studies of Soyeurt et al. (2007, 2012). The distinctive variation in mean LF can therefore be caused e.g. by the difference in reference methods used or proportions of breeds present. In our study, Holstein and Czech Fleckvieh cows accounted for 72% and 28% of the calibration set, respectively. In the study of Soyeurt et al. (2007), Brown-Swiss (2.8%), dual-purpose Belgian Blue (11.6%), Holstein (46.3%), Jersey (3.8%), Montbeliarde (11.0%), Non-Holstein Red and White (4.5%), Normande (13.2%)

Table 2. Descriptive statistics of milk parameters in sets A (n = 115), B (n = 90), and C (n = 205)

Milk parameter	Set A					Set B					Set C				
	mean	SD	CV	min	max	mean	SD	CV	min	max	mean	SD	CV	min	max
Fat	4.02	0.73	18.2	2.41	6.13	5.15	0.95	18.4	3.40	7.95	4.52	1.00	22.2	2.41	7.95
Protein	3.50	0.38	10.8	2.74	4.52	3.39	0.40	11.8	2.63	4.44	3.45	0.39	11.3	2.63	4.52
Casein	2.74	0.44	16.0	1.71	3.94	2.56	0.42	16.5	1.82	3.71	2.66	0.44	16.5	1.71	3.94
Lactose	4.87	0.23	4.66	4.09	5.26	4.88	0.28	5.77	3.81	5.44	4.87	0.25	5.17	3.81	5.44
SNF	9.11	0.37	4.01	8.32	10.0	8.98	0.45	5.06	7.60	10.0	9.05	0.41	4.54	7.60	10.0
Urea	20.8	8.72	41.9	6.02	42.3	25.3	10.6	42.0	6.13	50.6	22.8	9.83	43.2	6.02	50.6
FFA	0.63	0.09	14.6	0.42	0.96	0.53	0.08	14.7	0.23	0.76	0.58	0.10	16.8	0.23	0.96
CA	0.22	0.04	19.3	0.11	0.38	0.22	0.05	21.8	0.12	0.35	0.22	0.04	20.4	0.11	0.38
SCC	568k	1 087k	192	3k	5 889k	512k	2 039k	398	12k	18 271k	543k	1 573k	290	3k	18 271k
Log SCC	5.111	0.833	–	3.477	6.770	4.993	0.629	–	4.079	7.262	5.059	0.751	–	3.477	7.262
EC	4.56	0.36	7.80	3.83	5.76	4.19	0.35	8.42	2.73	5.38	4.40	0.40	9.03	2.73	5.76
FP	–0.536	0.006	1.19	–0.550	–0.518	–0.528	0.019	3.66	–0.552	–0.422	–0.533	0.014	2.65	–0.552	–0.422
LF HPLC	158	123	78.0	21.5	793	89.3	64.3	71.9	21.6	432	128	107	83.7	21.5	793

SCC geometric means: A = 129 000; B = 98 000; C = 115 000 cells/ml

CA = citric acid content (%); casein = casein content (%); CV = coefficient of variation (%); EC = electrical conductivity (mS/cm); fat = fat content (%); FP = freezing point (°C); HPLC = ion-pair reversed-phase high-performance liquid chromatography; k = thousand; lactose = lactose monohydrate content (%); LF HPLC = lactoferrin content determined by HPLC (mg/l); max = maximum; mean = arithmetic mean; min = minimum; protein = crude protein content (%); SCC = somatic cell count (cells/ml); SD = standard deviation; SNF = solids-not-fat content (%); urea = urea content (mg/100 ml), FFA = free fatty acid content (mmol/100 g of fat)

https://doi.org/10.17221/114/2025-CJAS

Table 3. Absolute and relative distribution of lactoferrin (mg/l) measured by HPLC at different intervals for sample sets A (n = 115), B (n = 90), and C (n = 205)

Interval	Set A		Set B		Set C	
	absolute	relative (%)	absolute	relative (%)	absolute	relative (%)
[21; 100]	44	38.3	58	64.4	102	49.8
(100; 200]	42	36.5	29	32.2	71	34.6
(200; 300]	15	13.0	1	1.1	16	7.8
(300; 400]	8	7.0	1	1.1	9	4.4
(400; 500]	4	3.5	1	1.1	5	2.4
(500; 600]	1	0.9	0	0.0	1	0.5
(600; 700]	0	0.0	0	0.0	0	0.0
(700; 800]	1	0.9	0	0.0	1	0.5
Total	115	–	90	–	205	–

HPLC = ion-pair reversed-phase high-performance liquid chromatography

and cows of unknown breed (6.8%) were present. In Soyert et al. (2012), the proportion of breed types was not listed. Previously, it was shown that LF can be influenced by breed. Krol et al. (2010) revealed that LF in milk increases in the order of Holstein, Jersey and Simmental. Similar results were also noted by Soyeurt et al. (2007). In our study, the majority of cows were of the Holstein breed and no Jersey cows were present. It is therefore possible that breed was the source of variation for different LF content in our study and the studies of Soyeurt et al. (2007, 2012).

Development of a calibration model for LF determination in milk by MIR-FT

The calibration dataset (set C) contained 205 samples with the mean LF HPLC and standard deviation of 127.6 ± 106.8 mg/l. Nine principal components were used for the developed calibration model. SECV was 67.33 mg/l and R^2_{CV} was 0.588 7 (Figure 1). Previously, Soyeurt et al. (2007, 2012) investigated the possibility of LF analysis by the MIR-FT method. In both studies, ELISA was used as a reference method for LF determination and MilkoScan FT6000 instruments (Foss Analytical A/S, Hillerød, Denmark) were used to record infrared spectra. In their pilot study, Soyeurt et al. (2007) used a calibration set of 69 cow's milk samples from the Walloon region of Belgium. They reached R^2_{CV} of 0.75 and SECV of 103.93 mg/l. The number of PLS factors used was seven. In a subsequent larger study (Soyeurt et al. 2012), the calibration set was made up of 2 499 cow's milk samples from herds in Belgium, Ireland and Scotland. R^2_{CV} was 0.71 and SECV was 50.55 mg/l. It is obvious that in our study, we reached slightly worse R^2_{CV} in comparison with both mentioned studies of Soyeurt et al. (2007, 2012). SECV was better in our study than in the pilot study of Soyeurt et al. (2007), but worse than in their second study (Soyeurt et al. 2012).

A possible reason for this variation can be the lower mean LF determined by HPLC in our study than the mean LF determined by ELISA in both studies of Soyeurt et al. (2007, 2012). The quantity of the analysed constituent can affect R^2_{CV}. The higher the concentration, the higher the R^2_{CV} (Soyeurt et al. 2006, 2012). This distinctive varia-

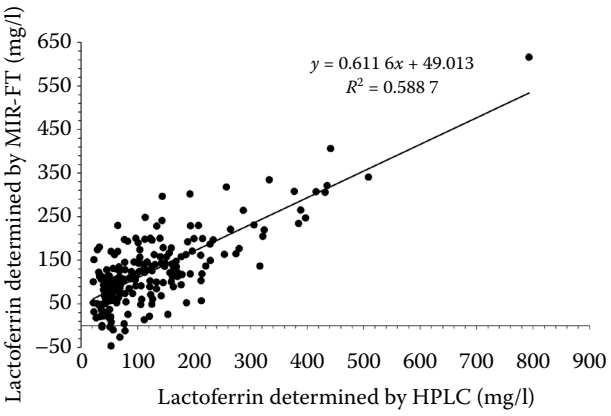

Figure 1. Accuracy plot. Lactoferrin determined by HPLC (reference lactoferrin) and lactoferrin determined by MIR-FT (predicted lactoferrin), n = 205

HPLC = ion-pair reversed-phase high-performance liquid chromatography; MIR-FT = Fourier transform mid-range infrared spectrometry

https://doi.org/10.17221/114/2025-CJAS

tion in mean LF can be caused by the difference in reference methods used or proportions of breeds present as previously discussed in the present study.

Relation of LF content to selected milk parameters in association with udder health

Mastitis is linked to alterations in milk composition, which is important for its diagnosis, particularly its subclinical form (Hamann and Zecconni 1998; Ogola et al. 2007). Previously, an increase in LF content during mastitis was reported (Hagiwara et al. 2003; Cheng et al. 2008). We investigated the relationship between milk indicators and between LF determined by both the reference method and the indirect method (Table 4).

Considering major milk components, fat was negatively correlated with LF MIR-FT (–0.155; $P < 0.05$). For LF HPLC, there was only a tendency of LF to increase with decreasing fat content. Fat may decrease due to reduced fat synthesis caused by mastitis (Bansal et al. 2005; Bochniarz et al. 2023). However, different studies offer contradictory results of fat relationship to LF. While Soyeurt et al. (2020) noted a positive correlation of fat and LF (0.11), Cheng et al. (2008) reported no correlation.

Table 4. Correlation coefficients between milk parameters and lactoferrin determined by HPLC (reference lactoferrin) and MIR-FT (predicted lactoferrin), $n = 205$

Milk parameter	LF HPLC (r)	LF MIR-FT (r)
Fat	–0.113^{ns}	–0.155*
Protein	0.361***	0.462***
Casein	0.440***	0.525***
Lactose	–0.312**	–0.364***
SNF	0.117^{ns}	0.180**
Urea	–0.410***	–0.436***
FFA	0.501***	0.621***
CA	–0.227**	–0.246**
SCC	0.450***	0.505***
Log SCC	0.555***	0.534***
EC	0.444***	0.442***
FP	–0.178**	–0.132^{ns}

$P > 0.05$ (ns); *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

CA = citric acid content (%); casein = casein content (%); EC = electrical conductivity (mS/cm); fat = fat content (%); FP = freezing point (°C); HPLC = ion-pair reversed-phase high-performance liquid chromatography; lactose = lactose monohydrate content (%); protein = crude protein content (%); r = Pearson correlation coefficient; SCC = somatic cell count (cells/ml); SNF = solids-not-fat content (%); urea = urea content (mg/100 ml), FFA = free fatty acid content (mmol/100 g of fat)

Further, protein and casein correlated positively with both LF HPLC ($r = 0.361$ and $r = 0.440$, respectively; $P < 0.001$) and LF MIR-FT ($r = 0.462$ and $r = 0.525$, respectively; $P < 0.001$). PA similar positive association was reported by Cheng et al. (2008), who found $r = 0.482$ ($P < 0.001$). Likewise, Soyeurt et al. (2020) reported a positive correlation (0.28). This can probably be explained due to the dynamics of milk composition during lactation. After a decrease in the first stage of lactation, protein increases until the end of lactation (Schutz et al. 1990). Likewise, LF increases in the late stage of lactation (Cheng et al. 2008). Casein may decrease because of mastitis (Bochniarz et al. 2023). However, no tendency of LF to increase with decreasing casein was observed in our study.

Lactose was negatively correlated with LF HPLC ($r = –0.312$; $P < 0.01$) and LF MIR-FT ($r = –0.364$; $P < 0.001$). Similar results were demonstrated by Cheng et al. (2008) and Niero et al. (2023), who also reported a negative correlation of lactose and LF ($r = –0.183$; $P = 0.049$ and $r = –0.33$; $P < 0.001$, respectively). This is probably related to the fact that in addition to metabolism and energy balance, the health of the mammary gland is an important factor that affects the ability of the mammary gland to synthesize lactose. These findings are consistent with research conducted by Bobbo et al. (2017) and Antanaitis et al. (2021), who demonstrated a decrease in lactose with an increase in SCC. They also found lower lactose in milk that contained mastitis pathogens than in milk that was free of mastitis pathogens.

Urea was negatively correlated with LF HPLC ($r = –0.410$; $P < 0.001$) and LF MIR-FT (–0.436; $P < 0.001$). By contrast, Niero et al. (2023) reported an insignificant correlation coefficient ($r = –0.02$; $P > 0.05$) between urea and LF. However, the health status as a source of variation in milk urea content was observed previously. Freitas et al. (2017) reported a significant negative correlation coefficient ($r = –0.128\ 43$; $P < 0.05$) for the relationship between urea nitrogen and SCC. Similarly, Meyer et al. (2006) noted that a one-unit increase in logarithmically transformed SCC [ln(SCC + 1)]

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is accompanied by a 0.29 mg/dl decrease in milk urea nitrogen.

FFA correlated positively with LF HPLC ($r = 0.501$; $P < 0.001$) and LF MIR-FT ($r = 0.621$; $P < 0.001$). The impact of mastitis on FFA was discussed by Hanus et al. (2016). They found a 121.5% increase in FFA levels in milk linked to mastitis. Additionally, they noted that a rise in SCC of 1 000 000 cells/ml leads to a rise in FFA of 0.433 mmol/100 g of fat.

CA was negatively correlated with LF HPLC ($r = -0.227$; $P < 0.01$) and LF MIR-FT ($r = -0.246$; $P < 0.01$). The association between the mammary gland health and CA content was previously discussed by Oshima and Fuse (1981). They observed a reduction in CA levels in the milk of dairy cows affected by subclinical mastitis. The degree of reduction was proportional to the severity of mastitis. This was supported by the results of the study of Johnzon et al. (2018), who observed a significant decrease in CA content in blood plasma in dairy cows with experimentally induced mastitis.

SCC was correlated positively with LF HPLC ($r = 0.450$; $P < 0.001$) and LF MIR-FT ($r = 0.505$; $P < 0.001$). Cheng et al. (2008) reported a positive correlation of SCS and LF ($r = 0.375$), but not of SCC and LF. Niero et al. (2023) also reported a correlation of SCS score and LF ($r = 0.40$; $P < 0.001$). A positive correlation of SCC and LF (0.21) was reported by Soyeurt et al. (2020). SCC is considered an essential diagnostic tool for the early detection of subclinical and clinical mastitis, as it is mainly influenced by the current infection and other factors (lactation, age of the dairy cow, stress etc.) usually have only a small influence (Harmon 1994).

EC and LF HPLC showed a positive correlation ($r = 0.444$; $P < 0.001$), likewise EC and LF MIR-FT ($r = 0.442$; $P < 0.001$). EC is known as a frequently used mastitis predictor (Norberg et al. 2004). Hamann and Zecconi (1998) explained that the increase in EC of milk due to mastitis is caused by alterations in the balance of cations, anions, and lactose. In the affected quarter, there is a decrease in lactose and K^+ levels, while Cl^- and Na^+ concentrations increase.

FP correlated negatively with LF HPLC ($r = -0.178$; $P < 0.01$). For LF MIR-FT there was only a tendency of LF to increase with decreasing FP ($r = -0.132$; $P > 0.05$). The negative correlation of LF and FP corresponds to the previously described phenomenon, when FP decreases with increasing SCC and EC (Hanus et al. 2010). This can be explained by the fact that during mastitis, the lactose content in milk typically decreases and is replaced by ions in order to maintain the osmotic pressure. As a result, the FP decreases and EC increases.

LF determination as a tool for detection of artificial SCC reduction in milk by centrifugation

Table 5 describes the effect of milk centrifugation on the results of selected milk parameters for the set of $n = 20$. As expected, SCC decreased after centrifugation. This decrease averaged 43.8% and was statistically significant ($P < 0.001$). Likewise, statistically significant differences were found in fat ($P = 0.001$), casein ($P = 0.001$), lactose ($P = 0.001$), urea ($P < 0.001$), LF HPLC ($P < 0.05$) and LF MIR-FT ($P < 0.001$) between original and centrifuged milk. However, unlike SCC these were minimum differences that were within the limits of the repeatability of the measurements. For fat, casein, lactose and LF MIR-FT, the change was always less than 1% of the value of the given milk parameter in the original milk, for urea and LF HPLC there was a change of 2.98% and 3.27%, respectively. LF HPLC as well as LF MIR-FT showed a close relationship in milk before and after centrifugation. For LF HPLC, 99.3% of the variability of LF content in milk after centrifugation was due to variations of LF in the original milk. For LF MIR-FT, it was 99.9%.

The experiment was then verified on a larger set of samples ($n = 68$), where only SCC and LF MIR-FT were determined. Table 6 shows that, similarly to the set of $n = 20$, there was also a reduction in SCC, in this case by 48.5% ($P < 0.001$). A statistically significant difference ($P < 0.001$) was also found in LF MIR-FT before and after centrifugation of milk. However, the change was minimum again, i.e. 0.61%. LF MIR-FT in original milk was closely correlated with LF MIR-FT in centrifuged milk, 99.8% of the variability in LF MIR-FT in the centrifuged milk was due to variations in LF MIR-FT in the original milk.

LF HPLC results, considered as more significant for the biological essence of the matter, were used to carry out the following qualified estimate of the LF distribution in milk. The reduction of somatic cells by 43.8% (537 000 cells/ml; $P < 0.001$; 100% = original milk) through centrifugation caused the removal of 3.27% of LF (8.88 mg/l; $P < 0.05$) present on cell

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Table 5. The effect of milk centrifugation on the results of some milk parameters, n = 20 (statistically significant differences are printed in italics)

Milk parameter	SCC		Fat		Protein		Casein		Lactose		SNF		Urea		LF HPLC		LF MIR-FT	
Milk	O	C	O	C	O	C	O	C	O	C	O	C	O	C	O	C	O	C
Mean	1 226k	689k	3.79	3.81	3.55	3.55	2.97	2.96	4.77	4.78	8.92	8.92	20.3	19.7	271	263	241	242
SD	1 576k	1 029k	0.51	0.52	0.24	0.24	0.26	0.26	0.17	0.17	0.17	0.16	5.35	5.34	142	132	36.8	37.0
CV	129	149	13.5	13.7	6.83	6.70	8.83	8.65	3.59	3.64	1.85	1.82	26.4	27.1	52.4	50.4	15.3	15.3
Min	104k	12k	2.50	2.51	3.03	3.02	2.46	2.43	4.42	4.43	8.46	8.45	10.5	9.93	86.1	82.7	169	169
Max	5 022k	3 500k	4.74	4.75	3.89	3.87	3.44	3.40	5.02	5.04	9.18	9.17	28.7	28.3	613	551	301	302
R^2	96.8		99.8		99.7		99.7		99.8		98.9		99.8		99.3		99.9	
d	–537k		0.02		0.00		–0.01		0.01		0.00		–0.61		–8.88		1.63	
D	–43.8		0.61		–0.03		–0.43		0.14		0.04		–2.98		–3.27		0.68	
sd	593k		0.03		0.01		0.01		0.01		0.02		0.41		15.2		1.23	
P-value	*<0.001*		*0.001*		0.739		*0.001*		*0.001*		0.414		*<0.001*		*0.017*		*<0.001*	

C = centrifuged milk; casein = casein content (%); CV = coefficient of variation (%); D = relative mean difference, 100% = parameter in original milk (%); d = mean difference (the mean of the parameter in centrifuged milk minus the mean of the parameter in original milk); fat = fat content (%); HPLC = ion-pair reversed-phase high-performance liquid chromatography; k = thousand; lactose = lactose monohydrate content (%); LF HPLC = lactoferrin content determined by HPLC (mg/l); LF MIR-FT = lactoferrin content determined by MIR-FT (mg/l); max = maximum; mean = arithmetic mean; min = minimum; O = original (uncentrifuged) milk; protein = crude protein content (%); R^2 = coefficient of determination (%); SCC = somatic cell count (cells/ml); SD = standard deviation; sd = standard deviation of differences; SNF = solids-not-fat content (%); urea = urea content (mg/100 ml)

membranes and endoplasmic reticula of neutrophil leukocytes. This corresponds to a reduction of 0.000 000 017 mg of LF (8.88/537 000 000) associated with one larger-than-average somatic cell in cow's milk. The remaining LF (a total of 96.7%) stays freely dissolved in the solution or bound to the remaining somatic cells and is not affected by the centrifugation procedure used in our experiment. Therefore, it can be estimated that 94.3% of LF is free in the solution, while the remaining approx. 5.7% is bound, under the given conditions, to the organelles of somatic cells (on the right side of the distribution 3.27/43.8 × 50 = 3.77%; due to the justified assumption of a lower content of LF in smaller cells on the left side of the data size distribution 3.77/2 = 1.89%; 1.89 + 3.77 = 5.66%).

It is to note that the centrifugation of milk causes only a minimum change in LF HPLC and LF MIR-FT; the previously proven relationship of SCC to LF in milk could thus be used for the detection of raw milk treatment by centrifugation. For further work, a limit value for LF indicating the use of centrifugation should be derived based on the analysis of LF and SCC in a large set of original and centrifuged milk samples.

Estimation of measurement uncertainty

Two partial components were considered in the estimation of the expanded combined uncertainty of LF measurement by the MIR-FT method – a repeatability component and an accuracy component.

The dataset for repeatability calculation (n = 10) exhibited the following descriptive statistics for LF MIR-FT: the mean of 151.4 mg/l, standard deviation of 25.1 mg/l, coefficient of variation of 16.6%, minimum of 106.3 mg/l, and maximum of 190.3 mg/l.

The reduced dataset (n = 183) used to calculate the accuracy exhibited the following descriptive statistics for LF HPLC: the mean of 98.1 mg/l, standard deviation of 55.1 mg/l, coefficient of variation of 56.1%, minimum of 21.5 mg/l, and maximum of 234.1 mg/l.

The repeatability component and the accuracy component were calculated as 2.14 mg/l and 58.48 mg/l, respectively. The expanded combined uncertainty of LF measurement by the MIR-FT method was estimated to be 114.7 mg/l. It follows that the repeatability component contributes only minimally to the overall uncertainty, while the main contribution arises from the difference between the

https://doi.org/10.17221/114/2025-CJAS

Table 6. The effect of milk centrifugation on SCC results and lactoferrin content determined by MIR-FT, n = 68 (statistically significant differences are printed in italics)

Milk parameter	SCC		LF MIR-FT	
Milk	O	C	O	C
Mean	591k	304k	228	230
SD	968k	614k	31.0	31.1
CV	164	202	13.6	13.5
Min	38k	4k	169	169
Max	5 022k	3 500k	308	310
R^2	96.5		99.8	
d	–287k		1.39	
D	–48.5		0.61	
sd	383k		1.30	
P-value	*<0.001*		*<0.001*	

C = centrifuged milk; CV = coefficient of variation (%); D = absolute value of relative mean difference, 100% = parameter in original milk (%); d = mean difference (the mean of the parameter in centrifuged milk minus the mean of the parameter in original milk); k = thousand; LF MIR-FT = lactoferrin content determined by MIR-FT (mg/l); max = maximum; mean = arithmetic mean; min = minimum; MIR-FT = Fourier transform mid-range infrared spectrometry; O = original (uncentrifuged) milk; R^2 = coefficient of determination (%); SCC = somatic cell count (cells/ml); SD = standard deviation; sd = standard deviation of differences

results obtained by the LF HPLC and LF MIR-FT methods, i.e. from the accuracy component.

The achieved expanded combined uncertainty of 114.7 mg/l limits the applicability of the MIR-FT method for the precise quantification of LF, particularly when used to assess compliance with established limits (e.g. decisions concerning centrifuged vs uncentrifuged milk). The method, however, may be more suitable for monitoring relative changes in LF levels (e.g. those associated with the cow's health status, the course of lactation, or milk processing).

CONCLUSION

A calibration model for rapid LF determination in bovine milk by the MIR-FT method was developed, using HPLC as the reference method. The variability of LF values in the calibration set was effectively increased by deliberately including a higher proportion of samples from cows with an assumed occurrence of subclinical mastitis, based on SCC values above 300 000 cells/ml.

Correlation analysis between LF (determined by both HPLC and MIR-FT) and several milk parameters (SCC, fat, lactose, urea, FFA, CA, EC and FP) revealed relationships that are associated with mastitis. These findings indicate that MIR-FT may serve as a potential tool for assessing the udder health in dairy cows.

Centrifugation of milk reduced SCC by nearly 50%, while LF content determined by both methods changed only minimally, suggesting the potential of LF as an indicator of artificial SCC reduction in milk adulteration.

The expanded combined uncertainty of LF measurement by the MIR-FT method was estimated to be 114.7 mg/l, which limits the use of the method for absolute quantification. Nevertheless, MIR-FT could be suitable for monitoring relative LF changes related to the animal's health status, lactation stage, or milk processing.

In summary, owing to its speed, simplicity, and good repeatability, the MIR-FT method represents a valuable complementary tool to the reference HPLC method for LF determination in bovine milk.

Conflict of interest

The authors declare no conflict of interest.

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Received: July 26, 2025
Accepted: October 15, 2025
Published online: November 28, 2025