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Determination of nutritional health indexes of fresh bovine milk using near infrared spectroscopy

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SUMMARY: Bovine milk is one of the most complete foods that exist. During the last decades, milk FA have shown to improve human health due to the reduction in risk of cardiovascular disease and related pathologies. The aim of this study was to evaluate the feasibility of near infrared spectroscopy (NIRS) reflectance analysis to predict the nutritional value, fatty acid (FA) composition, and health index of fresh milk from dairy cows of pastoral systems. The prediction of Atherogenicity and Thrombogenicity indexes, along with other FA ratios in fresh milk samples by NIRS were precise and accurate. In addition, the calibration model obtained by NIRS provides an opportunity for the routine quantification of milk's healthy FA such as omega-3 and conjugated linoleic acid (CLA), with applications in the dairy industry for food labeling, and at the farm level for management of the dairy cow's diet.

KEYWORDS: *Chemometrics; Conjugated linoleic acid; Dairy fat; Health indexes; Human nutrition.*

RESUMEN: ***Determinación de los índices de salud nutricional de la leche fresca de bovino mediante espectroscopía de infrarrojo cercano.*** La leche bovina es uno de los alimentos más completos que existe. Durante la última década, se ha demostrado que los ácidos grasos de la leche pueden mejorar la salud humana, a través de la reducción del riesgo de enfermedades cardiovasculares y patologías asociadas. El objetivo de este estudio fue evaluar la factibilidad del análisis de reflectancia NIRS para predecir valor nutricional, composición de ácidos grasos e índices de salud de leche fresca de vacas de sistemas lecheros pastoriles. La predicción por NIRS del índice aterogénico y trombogénico, de ácidos grasos en muestras de leche fresca, fueron precisos. Por tanto, el modelo de calibración obtenido por NIRS representa una oportunidad para la cuantificación rutinaria de los ácidos grasos saludables de la leche como omega-3 y CLA, con aplicaciones en la industria lechera para el etiquetado nutricional y a nivel de lechería para el manejo de la alimentación de las vacas.

PALABRAS CLAVE: *Ácido linoleico conjugado; Grasa láctea; Índices de salud; Nutrición humana; Quimiometría.*

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1. INTRODUCTION

Bovine milk is one of the most complete foods that exists. It provides energy as lactose lipids, protein, and vitamins and minerals. Particularly, milk fat is made up of a complex mixture of lipids that mainly include triglycerides, phospholipids, and cholesterol; and it is considered an essential milk constituent in terms of its nutritional value, technological aptitude for manufacturing dairy products, and the palatability that it delivers to dairy products (Rodríguez-Alcalá *et al*., 2009).

During the last decades, it has been shown that milk FA improves human health (Shingfield *et al*., 2013) due to a reduction in the risk of atherosclerosis, hypercholesterolemia and other factors related to cardiovascular disease (Salter, 2013). Nonetheless, from a nutritional perspective, the effects of individual conjugated linoleic acids (CLA) are not well elucidated due to the difficulty in isolating individual CLA isomers. Therefore, most studies have used predominantly 18:2*cis*-9, *trans*-11 (9,11 CLA or rumenic acid) or 18:2*trans*-10, *cis*-12 (10,12 CLA) within a mixture of CLA isomers and other FA. Both 9,11 CLA and 10,12 CLA are the most abundant CLA isomers in milk, accounting for approximately 85 and 10% of all CLA isomers naturally present in milk, respectively (Den Hartigh, 2019). Recent research suggests that the beneficial effects of CLA are mainly related to rumenic acid (c9t11, RA) and its precursor (t11 18:1, vaccenic acid), and that RA and t10c12 would exert different physiological effects (Gómez-Cortés *et al*., 2018). Moreover, conjugated linoleic acids have been shown to reduce the risk of cardiovascular disease, type 2 diabetes, rheumatoid arthritis, asthma, degenerative diseases associated with age, and some types of cancer (Preble *et al*., 2019). At the same time, 10,12 CLA has shown an anti-lipogenic effect in lipogenic tissues such as liver, mammary and adipose tissue (Park and Pariza, 2007). Omega-3 FA are essential FA that are found in bovine milk, and possess well-known anti-inflammatory properties (Den Besten *et al*., 2013). The contents in healthy or beneficial FA in bovine milk depend mostly on the composition of the dairy cow's diet, with greater milk CLA and n-3 FA when the diet is based on pasture grazing as compared to mix diets with preserved forage and grains (Morales *et al*., 2015).

In addition, milk is also an important source of saturated FA, especially whole milk and high-fat dairy products (e.g., cream, butter). Saturated FA have been claimed to be harmful due to the association between saturated fat intake and cardiovascular disease. However, this harmfulness has been recently challenged by new research (Siri-Tarino *et al*., 2010). In this sense, there is evidence that dietary exposure to whole dairy products can substantially affect several health conditions, even chronic disease by reducing risk in later life (Markey *et al.,* 2014; Givens, 2020). Yet, it is now unclear whether saturated FA are harmful or not to human health, and therefore the use of low-cost indexes that may better characterize the diet in human population studies is timely.

Ulbricht and Southgate (1991) proposed two indexes that characterize the atherogenic and thrombogenic potential of the diet based on the content in saturated (SFA) and unsaturated FA, in addition to the polyunsaturated (PUFA) to SFA ratio. The atherogenic (AI) and thrombogenic index (TI) consider the effects of FA on human health, as well as the probability of an increase in incidence of injuries such as atheroma and/or thrombus formation (Pilarczyk *et al*., 2015). Another index regarding the profile of FA is the n-6 to n-3 ratio, which is a numerical balance between these FA, as n-6 and n-3 have distinct metabolic pathways, both necessary for physiological functions (Simopoulos, 2002).

For the analysis of this type of compounds, gas chromatography has traditionally been used as a technique of proven specificity and robustness even when it requires considerable time, highly trained personnel, use of many solvents and reagents, and therefore, is an expensive analysis.

In the last decades, new instrumental methods that are as robust and reliable as conventional methods have been developed. One of them is Near-Infrared Spectroscopy (NIRS), a method that captures the reflectance spectrum of a sample in a range of 780-2500 mm, corresponding to NIR. Briefly, the radiant energy of a sample is absorbed, according to the vibration frequency of the molecules present, which generates an overtone in the spectrum (Conzen, 2006). Vibrations in C-H, O-H, N-H chemical bonds produce reflectance signals which serve to identify the relative proportion of each element in the analyzed sample (Cécillon *et al*., 2009). NIRS technology has been reported to be a rapid, consist-

ent, and inexpensive tool for predicting authenticity control, sensory evaluation, rheological and technological properties, and physical attributes in solid, dry, paste, and liquid samples in diverse matrices (Porep *et al*., 2015). NIRS has been used in the dairy industry for over 30 years, in liquid and oven-dried milk samples for the analysis of major components (fat, protein, lactose, moisture, etc.) without sample pre-treatment, and recently for FA composition in liquid and dry milk (Coppa *et al*., 2010; Coppa *et al*., 2014). In addition, the prediction of AI or TI by the use of NIR has only been reported by Nuñez-Sanchez *et al*. (2016), Nuñez-Sanchez *et al*. (2020) and Llano-Suarez *et al*. (2018).

The objective of this study was to evaluate the feasibility of NIRS reflectance analysis to predict the nutritional value, FA composition, and health indexes of fresh milk from cows of pastoral systems.

2. MATERIALS AND METHODS

2.1. Milk sampling collection

A total of 175 fresh milk samples were used in this study, obtained from 2 dairy farm studies conducted in Los Lagos Region, Chile, between 2018 and 2020 as follows.

Set 1: Between October 2018 and March 2019, 133 bulk tank milk samples were collected from a dairy farm in Fresia, Los Lagos Region, Chile (41 ° 28′18 ″ S 72 ° 56′12 ″ W). During this period, the tested dairy farm had 43 lactating Holstein Friesian cows with an average production of 21 L/cow/day. Their diet was composed of grazed pasture [*Lolium Perenne* (54%), *Lolium Trifolium* (34%), *Holcus Lanatus* (7%), Trifolium repens (2%) and 3% of other species] and 2 kg of a pelleted concentrate (2 kg/cow/day).

Set 2: Between August 2019 and February 2020, 42 milk samples were collected from milk collection trucks at a dairy processing facility in La Araucanía region. The sample collection period represented the pasture-grazing period of the year, and all milk sampled from the trucks was produced in pasture-grazing based dairies.

All milk samples collected were kept at 4°C during transport to the NIR spectroscopy laboratory of INIA-Remehue. Each fresh milk sample was registered by NIRS and subsequently stored at -80 ° C until FA analysis by gas chromatography was carried out.

2.2. Fatty acids analysis

The derivatization of milk fat was performed with 3 mL of fresh milk at room temperature using the double fat extraction method of chloroform and methanol 1:1 (Kramer *et al*., 2008). For FA methylation, a base-catalyzed methylation procedure with sodium methoxide was used (i.e. 0.5N methanolic base #33080, Supelco Inc., Bellefonte. PA) as described by Cruz-Hernandez *et al*. (2006). Prior to methylation, 1 mL of internal standard was added to the sample for FA quantification (1 mg/mL of 23:0 methyl ester, n-23-M, Nu-Chek Prep Inc., Elysian. MN. USA). The contents in FA methyl esters (FAME) were expressed as g per 100 g of FAME quantified and as mg of FAME per 100 mL of fresh milk.

The FAME were analyzed using a GC equipped with a flame ionization detector (GC-2010 Plus; Shimadzu®. Kyoto. Japan), a capillary column (SP-2560; 100 m × 0.25 mm (i.d.) with 0.2-µm film thickness; Supelco Inc., Bellefonte. PA. USA) and an ionic liquid capillary column (SLB-IL111; 100 m × 0.25 mm (i.d.) with 0.2-µm film thickness; Supelco Inc., Bellefonte. PA. USA) to confirm the identification of several biohydrogenation intermediates such as CLA isomers and other *trans* FA (Delmonte *et al*., 2011). The samples were analyzed with two GC temperature programs that plateaued at 175 °C and 150 °C (Kramer *et al*., 2008). Hydrogen was used as carrier gas in both columns, with a constant flow rate of 1 mL/min. and the injector and detector temperatures were set at 250 °C.

For peak identification, one reference standard (GLC463), individual FAMEs (21:0. 23:0. 26:0), and a CLA mixture (9c,11t-/8t,10c-/11c,13t-/10t,12c-/8c,10c-/9c,11c-/10c,12c-/11c,13c-/11t,13t-/10t,12t-/9t,11t-/8t,10t-18:2; UC-59M) were used, all of which were obtained from Nu-Chek Prep Inc. (Elysian, MN, USA). Isomerized mixtures of linoleic (18:2*n*−6) and linolenic (18:3*n*−3) acids were purchased from Sigma-Aldrich, and branched-chain FA (BCFA) were identified using a bacterial FAME mixture from Matreya (Pleasant Gap, PA, USA).

2.3. Health index calculations

Atherogenic (AI) and Thrombogenic (TI) indexes were calculated as in Ulbricht and Southgate (1991), as follows:

$$AI = [12:0 + (4 \times 14:0) + 16:0] / [\Sigma \text{ Monounsaturated (MUFA)} + \text{PUFA-}n6 + \text{PUFA-}n3]$$

$$TI = (14:0 + 16:0 + 18:0) / [(0.5 \times \Sigma\text{MUFA} + 0.5 \times \text{PUFA-}n6 + 3 \times \text{PUFA-}n3) + (\text{PUFA-}n3 / \text{PUFA-}n6)]$$

2.4. NIRS and chemometric analysis

For spectral analysis, 175 fresh milk samples in reflectance mode were scanned using NIR spectroscopy (MPA-FT NIR, Bruker Optik GmbH, Ettlingen, Germany). Spectral data were transformed to absorbance (A) according to the equation: A = log10 (1/R), where R is the reflectance obtained at each wavenumber from 12.000-4.000 cm^{-1} (NIR region) with 16 cm^{-1} resolution and 64 scans (Figure 1). Partial least-squares regression (PLSR) with leave-one-out (LOO) cross validation was performed to fit predictive models using chemometrical software OPUS version 6.5 (Bruker Optik GmbH, Melvyn Becerra Cia. Ltda). For the cross-validation method, the selection of validation samples was based on spectral information as described by Conzen, (2006).

In addition, external validation was performed with a random subset of 10 samples which were not included in the calibration and cross validation method.

The software OPUS was used to apply different preprocessing to spectra:vector normalization (VN), multiplicative scatter correction (MSC), straight line subtraction (SLS), first derivative (FD), and second derivative (SED). Outliers were identified and removed during the calibration process to improve precision and model performance.

The criteria used in choosing the best prediction model considered: i) low root mean square error of cross validation (RMSECV), ii) high coefficients of determination in cross-validation (R^2cv), iii) root mean square error of estimation (RMSEE); iv) residual predictive deviation (RPD: ratio between the

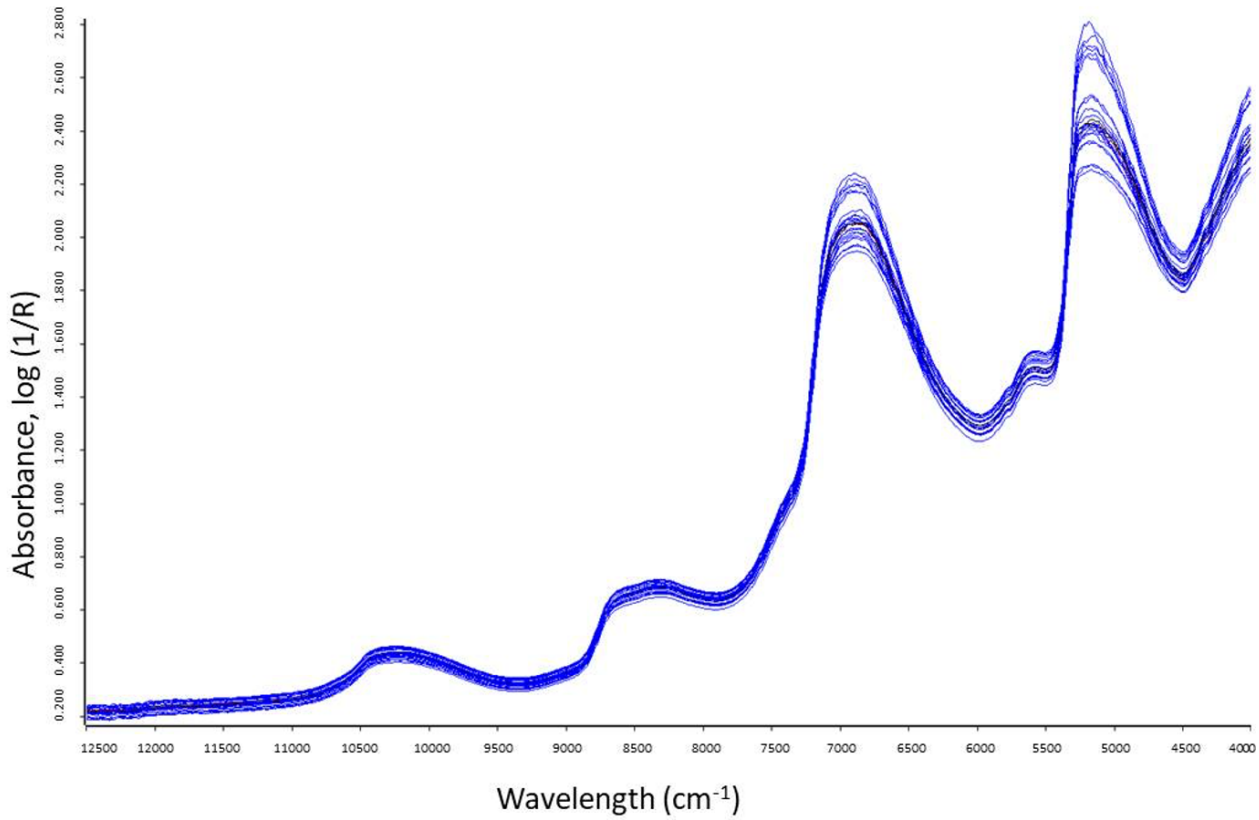

Figure 1. Average absorbance (log (1/R)) of near-infrared spectra for liquid milk NIR calibration results. PLS: Number of PLS factors; Treatment: [(MSC:Multiplicative Scatter Correction); (VN:Vector Normalization); (FD+VN: First Derivate + Vector Normalization); (COE: Constant Offset Elimination); (NSDP: No Spectral Data Preprocessing)]; R_c^2: Coefficient of determination of the calibration set; RMSEE: root mean square error of estimation; RPD: residual prediction deviation; R_{cv}^2: Coefficient of determination of cross validation; RMSECV: root mean square error of cross validation.

standard deviation -SD- of the reference values and the error of prediction), and v) number of factors (Conzen, 2006).

In this study the criteria for selection were the lowest RMSECV, the lowest number of PLS factors and the highest RPD. Small error of cross validation was desired, as this would result in greater RPD values, and a better prediction model.

3. RESULTS

3.1. Milk fatty acid composition

The fatty acid methyl ester contents in the milk of the 2 sets of samples used in this study are expressed as % of total FAME (g/100g) and mg FAME per 100 mL of milk and shown in Table 1. The ratio of n-6 to n-3 was 1.69 ± 0.5 with a range between 1.14 and

TABLE 1. Fatty acid methyl esters (FAME) and health indexes of milk samples quantified by gas chromatography.

FAME[a]	**g/100g FAME**			**mg FAME /100mL milk**		
	Mean ± SD	Min	Max	Mean ± SD	Min	Max
C 8:0 Caprylic acid	0.6 ± 0.3	0.0	1.3	26.7 ± 16.6	0.0	82.6
C 10:0 Capric acid	2.7 ± 0.5	1.0	4.1	107.6 ± 35.2	34.3	282.1
C 12:0 Lauric acid	3.8 ± 0.5	2.8	5.4	151.7 ± 44.0	85.7	396.9
C 14:0 Myristic acid	12.6 ± 0.9	10.1	15.5	503.4 ± 144	319.8	1382.2
C 16:0 Palmitic acid	31.4 ± 0.3	25.4	36.8	1260.4 ± 373.3	734.6	3506
C 18:0 Stearic acid	10.3 ± 1.3	7.3	14.7	411.8 ± 112.6	252	1044
C 9-18:1 Oleic acid	18.8 ± 1.5	14.4	23.7	753.2 ± 204.6	435.3	1968.5
10 *trans*-18:1	0.32 ± 0.16	0.16	1.61	12.6 ± 7.20	5.3	62.3
11 *trans*-18:1	2.15 ± 0.67	1.03	4.83	86.1 ± 29.94	32.6	200
Linoleic acid (LA)	1.33 ± 0.31	0.88	2.49	53.2 ± 20.61	23.2	162
Linolenic acid (LNA)	0.71 ± 0.10	0.41	1.09	28.2 ± 8.24	15.4	87.3
Eicosapentaenoic acid	0.07 ± 0.02	0.00	0.11	3.02 ± 1.08	0.06	10.3
Docosahexaenoic acid	0.10 ± 0.03	0.04	0.31	4.23 ± 1.52	1.36	12.3
Total n-6	1.50 ± 0.35	0.99	2.84	60.3 ± 24.4	26.0	1867
Total n-3	0.91 ± 0.13	0.54	1.35	36.2 ± 11	20	115
∑ CLA	1.12 ± 0.27	0.57	2.33	44.7 ± 15.5	15.5	129
Saturated FA (SFA)	66.7 ± 2.29	59.3	72.6	2692 ± 777	1658	7366
Monounsaturated FA (MUFA)	28.3 ± 1.84	23.1	32.9	1138 ± 311	648	3009
Polyunsaturated FA (PUFA)	2.42 ± 0.38	1.75	3.64	96.5 ± 32.6	46	3012
Unsaturated FA (UFA)	30.7 ± 1.96	25.7	35.9	1234 ± 340	694	3311
Hypercholesterolaemic FA (HFA)	47.7 ± 2.94	38.9	54.8	1932 ± 570	1160	5284
∑ Odd and branched FA	4.10 ± 0.34	3.36	4.93	163 ± 43.9	102.6	448
Health Indexes						
n-6/n-3	1.69 ± 0.5	1.14	5.01	1.69 ± 0.5	1.19	5.01
UFA/SFA	0.46 ± 0.05	0.35	0.60	0.46 ± 0.05	0.35	0.60
HFA/UFA	1.57 ± 0.2	1.09	2.13	1.57 ± 0.2	1.09	2.13
h/H	0.49 ± 0.1	0.34	0.72	0.49 ± 0.1	0.34	0.72
LA/LNA	1.91 ± 0.6	1.23	5.41	1.91 ± 0.6	1.23	5.41
Thrombogenic index (TI)	3.09 ± 0.3	2.16	3.88	3.09 ± 0.3	2.25	3.98
Atherogenic index (AI)	2.86 ± 0.4	1.96	4.07	2.84 ± 0.4	1.96	4.07
PUFA/SFA	0.04 ± 0.01	0.03	0.06	0.04 ± 0.01	0.03	0.06
MUFA/SFA	0.43 ± 0.04	0.32	0.55	0.43 ± 0.04	0.32	0.55

[a]SFA: total saturated fatty acids; MUFA: total monounsaturated fatty acids; total PUFA: polyunsaturated fatty acids; UFA: total unsaturated fatty acids (MUFA+PUFA); HFA: hypercholesterolaemic FA (Σ C12:0+C14:0+C16:0); h/H: hypocholesterolaemic/hypercholesterolaemic ratio [C18:1n-9+C18:2n-6+C20:4n-6+C18:3n-3+C20:5n-3+C22:5n-3+C22:6n-3)/(C14:0+C16:0) (Santos-Silva *et al*., 2002); Σ Odd and branched FA (Σ C11:0; isoC14:0; isoC15:0; anteisoC15:0; C15:1; iso C16:0; C17:0; isoC18:0; C17:1); EPA: Eicosapentaenoic acid (20:5n-3); DPA: Docosapentaenoic acid (22:5n-3); LA: Linoleic acid (18:2n−6) (LA); LNA: Linolenic acid (18:3n−3); n-6/n-3: ratio omega 6 FA/omega 3FA; Atherogenic Index (AI)= [12:0 + (4 × 14:0) + 16:0] / (ΣMUFA + PUFA-*n6* + PUFA-*n3*) Ulbricht and Southgate (1991); Thrombogenic Index (TI)= (14:0 + 16:0 + 18:0) / [(0.5 × ΣMUFA + 0.5 × PUFA-*n6* + 3 × PUFA-*n3*) + (PUFA-*n3* / PUFA-*n6*)] Ulbricht and Southgate (1991). For the calculation of total conjugated linoleic acids (Σ CLA), all the CLA isomers identified in fresh milk samples by GC were summed.

5 g/100g of FAME. The TI and AI were 3.09 ± 0.3 and 2.86 ± 0.4 g/100g of FAME, respectively, with a range of 2.16 to 3.88 and 1.96 to 4.07 for TI and AI, respectively.

3.2. NIR models

NIR spectral features. Average absorbance of NIR spectra for liquid milk presented two bands with maxima at 7500-6400 cm^{-1} and 5400-4900 cm-1 related to O-H first overtone and O-H combination band (Figure 1).

NIR calibration. The calibration statistics for individual FA and groups of FA and indexes used to evaluate the nutritional health properties of food (considering the potential negative or positive effects) in fresh bovine milk are detailed in Table 2. The coefficient of determination in the calibration sets fluctuated between 0.76 and 0.95. The RPD values varied between 2.1 and 4.3. The number of PLS factors values varied between 2 and 10. All pre-preprocessing methods used were different and in accordance to the main functional groups of FA and health related indexes. The relation between NIRS prediction and composition obtained by the reference methods for all main functional groups of FA and health indicators are shown in Figures 2a and 2b.

External validation. Table 3 shows the means of the residuals and the Root Mean Standard Error (RMSE) obtained from the external validation. The levels of significance obtained were between 0.09 for 10 *trans*-18:1 and 0.88 for LA. Therefore, no differences between the spectroscopic and chromatographic method were detected.

4. DISCUSSION

4.1. Milk fatty acid composition

Regarding the FA composition of milk, the FAME contents in this study were in the same range reported previously for Holstein Friesian's milk from Chilean dairies (Morales *et al*., 2015; Vargas-Bello *et al*., 2015), in milk from dairy processing facilities in Southern Chile (Pinto *et al*., 2002), and in pasture-grazing based dairies from other regions (Nantapo *et al*., 2014). For instance, total SFA (66.6 g/100 g of FAME) was in the same range reported for grazing animals by Morales *et al*. (2015); 67.19 g/100 g, and for TMR-fed Holstein Friesian cows of the control group of Vargas-Bello *et al*. (2015); 68.2 g/100 g.

Our reported milk FAME content is not comparable to other studies where diet relies on a total mixed ration based on preserved forages and grains because diet and fresh forage inclusion in the diet have a major impact on the FA profile of milk (Sun and Gibs, 2012). Although our study only included milk samples of Holstein Friesian cows, greater variances and range of milk FA observed in other studies could be explained, in lesser magnitude, by the inclusion of other dairy breeds. In this sense, Coppa *et al*. (2014) reported milk FA composition for a heterogeneous productive system that included five different dairy cow breeds present in northwest Italy, with total milk SFA and total CLA (64.28 and 0.93 g/100 g of FA, respectively) below our reported values (66.7 and 1.12 g/100 g FA, respectively). On the contrary, this last study reported greater values for MUFA, PUFA, and n-6/n-3 (29.63; 5.17; 3.42 g/100 g of FA, respectively) than our study, which could be explained by the use of TMR and a more diverse set of cow genetics that included Jersey cows.

The nutritional and health indexes PUFA/SFA, n-6/n-3, AI, and TI, are commonly used to evaluate the nutritional value and effects of edible products on consumer health. In general, a ratio of dietary PUFA to SFA above 0.45 and a ratio of *n-6/n-3* below 4.0 are expected to reduce the risk of diseases such as coronary heart disease and cancer (Simopoulos, 2002). Furthermore, the low PUFA/SFA ratio (0.04 g/100g FAME) reported in this study was due to the high SFA content in the two sets of milk samples analyzed. The *n-6/n-3* ratio obtained in the current study (1.69 g/100 g of FAME) is lower than the ratio reported by Morales *et al*. (2015), most likely because of differences in pasture botanical composition. Indeed, some authors have indicated that the PUFA/SFA ratio may not be adequate to evaluate the nutritional value of dietary fat, as it ignores the effects of MUFA and also, some SFA have no effect on plasma cholesterol (Orellana *et al*., 2009).

Milk atherogenic and thrombogenic indices in cattle from different breeds and feed and management systems have been previously reported. Kuczyńska *et al*. (2012) and Pilarczyk *et al.* (2015) reported AI of 2.1 and 2.5 in dairy cows from a pasture-based and a total mixed-ration-based dairy system, respectively, values which are slightly lower than those reported in our study. Nantapo *et al*.

Table 2. Statistical descriptors for the partial least-squares regression (PLSR) predictions for fatty acid methyl esters (FAME) in milk samples and corresponding health indexes.

FAME[a]	#PLS	Treatment	Spectral region (nm)	R_C^2	RMSEE	RPD_C	R_{CV}^2	RMSECV	RPD_{CV}	Range	N
10 *trans*-18:1	6	VN	12489.5-7498.3; 4605.4-4242.9	0.84	0.9	2.5	0.76	1.1	2.0	5.3 - 21.1	96
11 *trans*-18:1	4	FD+VN	6102-5446.3	0.78	9.7	2.1	0.74	10.2	2.0	36.81 - 138.6	104
Linoleic acid (LA)	9	COE	12489.5-7498.3;6102-5446.4;4605.4-4242.9	0.94	4.2	3.9	0.84	6.3	2.5	28.52 - 131.4	118
Linolenic acid (LNA)	9	COE	9295.7-6094.3; 5454-4844.6	0.82	2.6	2.4	0.73	3.2	1.9	19.58 - 61.69	99
Eicosapentaenoic acid	5	MSC	9997.7- 7498.3; 6102- 5770.3	0.77	0.4	2.1	0.71	0.4	1.9	1.40 - 6.35	127
Docosahexaenoic acid	2	MSC	12489.5- 7498.3; 6102- 5446.3	0.82	0.4	2.3	0.80	0.5	2.2	1.66 - 8.11	110
***Total n*-6**	7	VN	12489.5-7498.3; 6102-5446.3; 4428-4242.9	0.91	5.4	3.4	0.82	7.5	2.4	36.78 - 155.9	120
***Total n*-3**	4	MSC	12489.5-7498.3; 5454-4242.9	0.79	3.8	2.2	0.75	4.1	2.0	24.52 - 74.47	105
∑ CLA	6	VN	12489.5-7498.3; 6102-5446.3	0.89	4.4	3.0	0.82	5.3	2.4	22.68 - 104.7	116
Saturated FA (SFA)	5	MSC	12489.5-7498.3	0.89	190	3.0	0.80	249	2.3	1679 - 5442	132
Monounsaturated FA (MUFA)	4	MSC	12489.5-7498.3	0.90	86.9	3.1	0.85	102	2.6	727.8 - 2531	129
Polyunsaturated FA (PUFA)	7	VN	12489.5-7498.3; 6102-5446.3; 4428-4242.9	0.90	9.5	3.1	0.81	12.5	2.3	61.3 - 241.9	127
Unsaturated FA (UFA)	7	NSDP	12489.5-7498.3; 6102-5446.3	0.81	127	2.3	0.71	155	1.9	773.2 - 2773	150
Hypercholesterolaemic FA (HFA)	5	MSC	12489.5-798.3	0.87	155	2.8	0.77	201	2.1	1160 - 3859	140
∑ Odd and branched FA	5	MSC	12489.5-5446.3	0.81	13.4	2.3	0.77	14.5	2.1	113.1 - 307.4	102
Health Indexes											
***n*-6/*n*-3**	8	VN	12489.5-7498.3;6102-5770.3;4605.4-4242.9	0.91	0.1	3.3	0.77	0.1	2.1	1.288 - 2.662	110
UFA/SFA	8	COE	12489.5-9990; 6102-5446.3	0.94	0.0	3.9	0.79	0.0	2.2	0.379 - 0.576	109
HFA/UFA	7	COE	12489.5-7498.3; 6102-5770.3	0,86	0.1	2.7	0,76	0.1	2.1	1.16 - 1.96	114
h/H	6	COE	12489.5-7498.3; 6102-5446.3	0,83	0.0	2.4	0,71	0.0	1.9	0.34 - 0.59	107
LA/LNA	5	VN	12489.5-6094.3; 4605.4-4242.9	0,85	0.1	2.6	0,70	0.1	1.8	1.45 - 2.79	102
Thrombogenic index (TI)	9	NSDP	12489.5-7498.3; 6102-5446.3; 4428-4242.9	0,93	0.8	3.6	0,78	0.1	2.2	2.45 - 3.59	100
Atherogenic index (AI)	9	NSDP	12489.5-7498.3; 6102-5446.3 ;4428-242.10	0,95	0.1	4.3	0,81	0.1	2.3	1.96 - 3.69	109
PUFA/SFA	10	NSDP	9997.7- 7498.3; 6102-5446.3; 4428-4242.9	0,92	0.0	3.5	0,84	0.0	2.5	0.03 - 0.05	101
MUFA/SFA	6	NSDP	12489.5-7498.3; 6102-5446.3	0,76	0.0	2.1	0,62	0.0	1.6	0.36 - 0.49	100

[a]SFA: total saturated fatty acid; MUFA: total monounsaturated fatty acids; total PUFA: polyunsaturated fatty acids; UFA: total unsaturated fatty acid (MUFA+PUFA); HFA: hypercholesterolaemic FA (sum of C12:0. C14:0. and C16:0); h/H: hypocholesterolaemic/hypercholesterolaemic ratio [C18:1n-9+C18:2n-6+C20:4n-6+C18:3n-3+C20:5n-3+C22:5n-3+C22:6n-3)/(C14:0+C16:0) (Santos-Silva *et al*., 2002); Σ Odd and branched FA (Σ C11:0; isoC14:0; isocC15:0; anteisoC15:0; C15:1; iso C16:0; C17:0; isoC18:0; C17:1); EPA: Eicosapentaenoic acid (20:5n-3); DPA: Docosapentaenoic acid (22:5n-3); LA: Linoleic acid (18:2n−6) (LA); LNA: Linolenic acid (18:3n−3); n-6/n-3: ratio omega 6 FA/omega 3FA; Atherogenic Index (AI)= [12:0 + (4 × 14:0) + 16:0] / (ΣMUFA + PUFA-*n6* + PUFA-*n3*) Ulbricht and Southgate (1991); Thrombogenic Index (TI)= (14:0 + 16:0 + 18:0) / [(0.5 × ΣMUFA + 0.5 × PUFA-*n6* + 3 × PUFA-*n3*) + (PUFA-*n3* / PUFA-*n6*)] Ulbricht and Southgate (1991). For the calculation of total conjugated linoleic acids (Σ CLA), all the CLA isomers identified in fresh milk samples by GC were summed. #PLS: Number of PLS factors; Treatment: ((MSC:Multiplicative Scatter Correction); (VN:Vector Normalization); (FD+VN: First Derivate + Vector Normalization); (COE: Constant Offset Elimination); (NSDP: No Spectral Data Preprocessing))R_c^2: Coefficient of determination of the calibration set; RMSEE: root mean square error of estimation; RPD: residual prediction deviation; R_{cv}^2: Coefficient of determination of cross validation; RMSECV: root mean square error of cross validation.

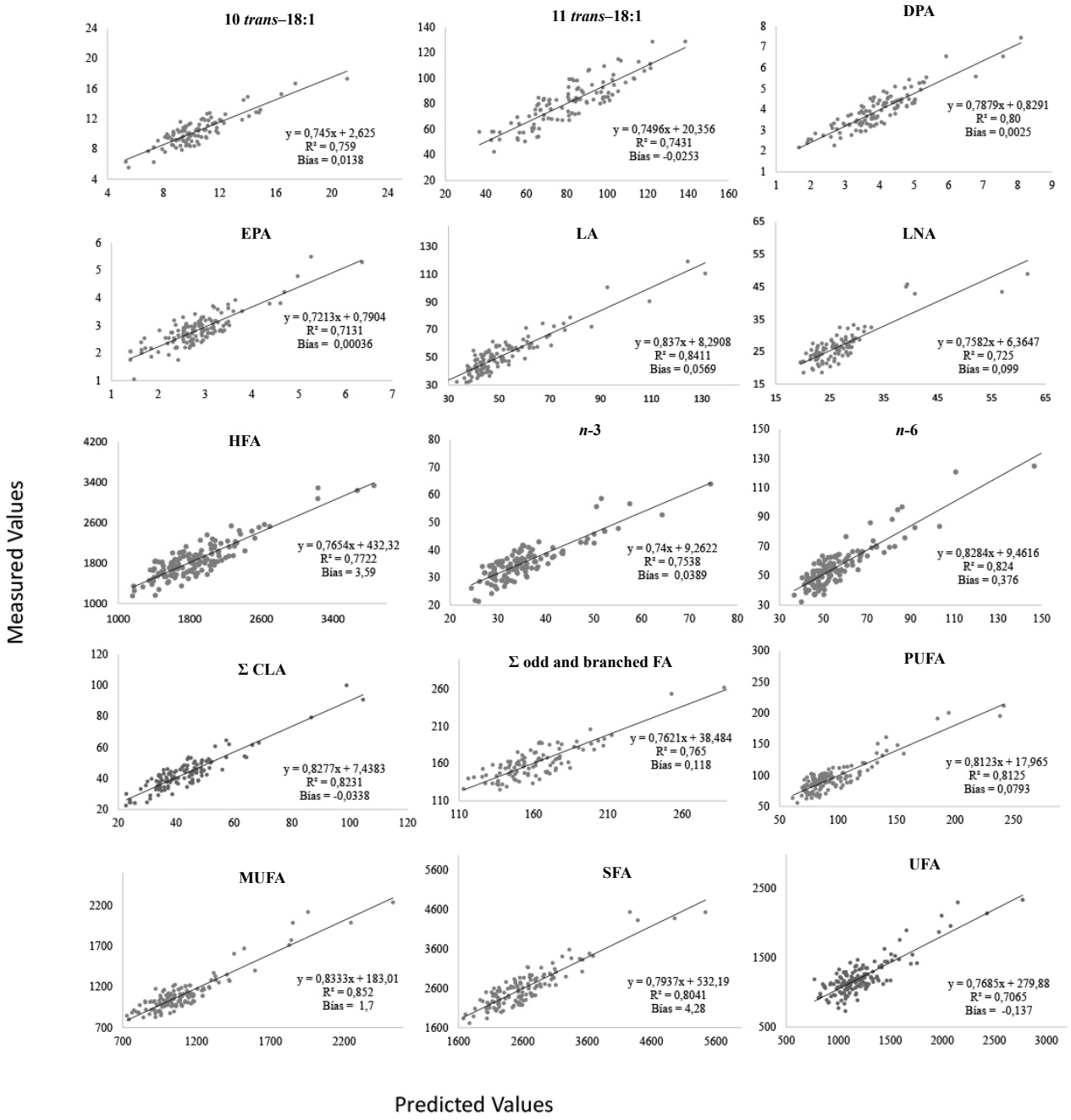

FIGURE 2A. Validation model performance for the correlation of values obtained in the laboratory with respect to those predicted by NIRS for a) 10 *trans*-18:1; b) 11 *trans*-18:1; c) Docosahexaenoic acid (DPA); d) Eicosapentaenoic acid (EPA); e) Linoleic acid (LA); f) Linolenic acid (LNA); g) Hypercholesterolaemic FA (HFA); h) Total n-3; i) Total n-6; j) ∑CLA; k) ∑ odd and branched FA; l) Polyunsaturated fatty acids (PUFA); m) Monounsaturated FA (MUFA); n) Saturated FA (SFA) and, o) Unsaturated FA (UFA) in fresh bovine milk.

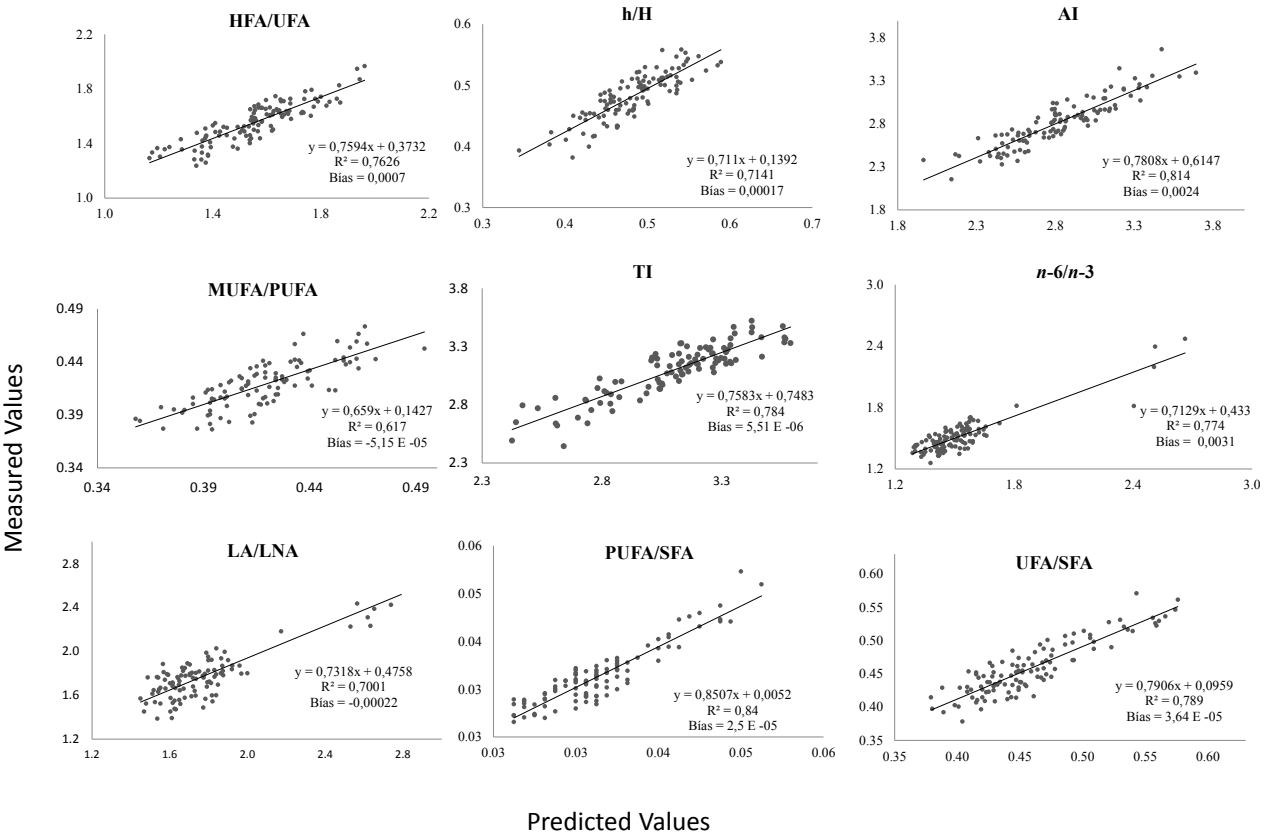

FIGURE 2B. Validation model performance for the correlation of nutritional and health indexes obtained in the laboratory with respect to those predicted by NIRS for a) HFA/UFA; b) h/H; c) AI; d) MUFA/PUFA; e) TI; f) n-6/n-3; g) LA/LNA; h) PUFA/SFA and i) UFA/SFA in fresh bovine milk.

(2014) reported milk AI values of 4.08 and 5.13 for Jersey and Friesian x Jersey cows, respectively. In Tarentaise and Montbeliarde cows, Ferlay *et al*. (2006) reported an AI of 3.14 and 3.43. On the other hand, the TI reported in the present study is within the range of that reported by Vargas-Bello *et al*. (2015), and below that of Thanh and Suksombat (2015), who reported 4.11.

4.2. NIR models

NIR spectral features. Average absorbance of NIR spectra for liquid milk presented small bands corresponding to FA and fat contents and appeared at 8900-7450 cm^{-1} and 6000-5300 cm^{-1}, associated with the first and second overtones from C-H stretching vibration of methyl (-CH_3), methylene (-CH_2-), and ethenyl (-CH=CH-). In addition, the absorption bands of bovine liquid milk used in this study were similar to those reported by Coppa *et al*. (2010) and Llano-Suaréz *et al*. (2018).

NIR calibration results. For model evaluation, Williams (2014) proposed R^2 y RPD as parameters that serve to classify NIR models into excellent or good, when R^2 fall above 0.91 or between 0.9 and 0.82, respectively. However, when R^2 falls between 0.81 and 0.66, the model will predict approximate values, and only simple discrimination of low, medium and high when R^2 is between 0.65 and 0.5. The RPD value is a measure of comparison between the standard error of the predicted values with the deviation of the references data, and therefore will evaluate the NIR calibration model (Williams, 2014; Nuñez *et al*., 2016). Therefore, RPD above 4 and 3 are considered excellent and good, respectively, while values between 2.9 and 2 are acceptable for detection, between 2 and 1.5 acceptable for discrimination between low and high concentration, and below 1.5 are not useful.

Regarding R_{CV}^2 statistics, DPA, ∑CLA, SFA, MUFA, PUFA, n-6, AI, PUFA/SFA and LA, showed

Table 3. Level of significance, residual mean and root mean square error (RMSE) of milk fatty acid ethly esters (FAME) and corresponding health indexes. (n=10)

FAME[a]	p (Level of significance)	Residual mean	RMSE	R^2	Bias	Slope
10 *trans*-18:1	0.09	4.62	6.5	0.49	3.02	0.13
11 *trans*-18:1	0.44	22.4	27.8	0.61	2.20	0.43
Linoleic acid (LA)	0.88	9.24	10.8	0.81	0.56	0.95
Linolenic acid (LNA)	0.41	5.99	7.81	0.67	0.99	0.47
Eicosapentaenoic acid	0.17	0.77	0.96	0.72	0.43	0.55
Docosahexaenoic acid	0.22	0.44	0.56	0.94	022	0.89
Total n-6	0.38	9.22	12.8	0.84	3.18	0.98
Total n-3	0.64	5.74	7.69	0.83	1.21	0.67
∑ CLA	0.44	7.09	9.11	0.83	-1.20	0.87
Saturated FA (SFA)	0.36	3389	351.3	0.94	150.00	0.83
Monounsaturated FA (MUFA)	0.33	90	109.4	0.98	70.70	0.89
Polyunsaturated FA (PUFA)	0.45	11.3	14.7	0.92	2.10	1.08
Unsaturated FA (UFA)	0.36	124	143.5	0.94	61.40	0.84
Hypercholesterolaemic FA (HFA)	0.15	255	273.4	0.92	126.00	0.86
∑ Odd and branched FA	0.45	19.8	21.7	0.86	2.43	0.81
Health Indexes						
n-6/*n*-3	0.17	0.32	9.11	0.01	0.14	0.00
UFA/SFA	0.23	0.03	0.04	0.58	-0.01	0.69
HFA/UFA	0.23	0.03	0.04	0.74	0.02	1.00
h/H	0.84	0.01	0.02	0.94	0.00	1.01
LA/LNA	0.72	0.36	0.45	0.37	0.06	0.18
Thrombogenic index (TI)	0.81	0.11	0.13	0.85	0.01	0.97
Atherogenic index (AI)	0.48	0.14	0.15	0.90	0.04	0.85
PUFA/SFA	0.14	0.00	0.00	0.51	0.00	0.47
MUFA/SFA	0.27	0.02	0.03	0.73	0.01	0.52

[a]SFA: total saturated fatty acid; MUFA: total monounsaturated fatty acids; total PUFA: polyunsaturated fatty acids; UFA: total unsaturated fatty acid (MUFA+PUFA); HFA: hypercholesterolaemic FA (sum of C12:0. C14:0. and C16:0); h/H: hypocholesterolaemic/hypercholesterolaemic ratio [C18:1n-9+C18:2n-6+C20:4n-6+C18:3n-3+C20:5n-3+C22:5n-3+C22:6n-3)/(C14:0+C16:0) (Santos-Silva *et al*., 2002); Σ Odd and branched FA (Σ C11:0; isoC14:0; isocC15:0; anteisoC15:0; C15:1; iso C16:0; C17:0; isoC18:0; C17:1); EPA: Eicosapentaenoic acid (20:5n-3); DPA: Docosapentaenoic acid (22:5n-3); LA: Linoleic acid (18:2n−6) (LA); LNA: Linolenic acid (18:3n−3); n-6/n-3: ratio omega 6 FA/omega 3FA; Atherogenic Index (AI)= [12:0 + (4 × 14:0) + 16:0] / (ΣMUFA + PUFA-*n6* + PUFA-*n3*) Ulbricht and Southgate (1991); Thrombogenic Index (TI)= (14:0 + 16:0 + 18:0) / [(0.5 × ΣMUFA + 0.5 × PUFA-*n6* + 3 × PUFA-*n3*) + (PUFA-*n3* / PUFA-*n6*)] Ulbricht and Southgate (1991). For the calculation of total conjugated linoleic acids (Σ CLA), all the CLA isomers identified in fresh milk samples by GC were summed.

good predictive capacity, with R_{CV}^2 values between 0.80 and 0.85. The R_{CV}^2 for EPA, n-3, n-6/n-3, TI, UFA, UFA/SFA, HFA, HFA/UFA, h/H, LNA, LA/LNA, 10 *trans*, 11 *trans*, and ∑ odd and branched FA were very acceptable with values from 0.7 to 0.8. The ratio MUFA/SFA had a R_{CV}^2 of 0.6. Therefore, this model is considered not suitable to be used; however, discriminations between high, medium and low concentrations could still be made. Regarding our data, RPD values ranged from 1.6 to 2.5, and were in accordance with those detailed above when comparing R_{CV}^2 values.

Possible explanations for our low NIR prediction for individual FA and some ratios may include: i) Low FA variability in the sample sets used in this study (Table 1) which may have limited their prediction from NIR spectra. The generation of a successful statistical model requires wider data sets, or data sets covering a wide range of concentrations. When random samples are used for the purpose of calibration, as was the case in this study, performance may be constrained by narrow data sets. Also, ii) the complexity of the aqueous matrix of liquid milk (minerals in solution, proteins in a colloidal dispersion, and lipids in emulsion), hinder the NIR analysis (Marinori *et al.*, 2013). The highwater content of milk in the fresh state could limit the detection capacity of other constituents, since the absorption bands of water in NIRS are strong. Calibration models could be improved by the

use of a bigger set of samples and with a greater range in concentration of analyzed FA.

Our results suggest that water content can often mask NIR signals and generate a limited predictive model (Reeves, 2000). It is necessary to work on reducing the difference between RMSEE and RMSECV values, which will serve to strengthen the calibration models (González-Sáiz *et al*., 2007).

The R^2_{CV} and RPD obtained in this study for SFA, MUFA, PUFA, n-6, n-3, n6/n3, AI, LNA and 10 *trans* were higher than those reported by Núñez-Sanchez *et al*. (2016), in calibration models using dry and fresh samples of goat milk measured in reflectance and transflectance mode, respectively. The R^2_{CV} obtained by Andueza *et al*. (2013) in samples of dry goat milk were higher than our reported R^2_{CV}, except for n6 (0.26), LA (0.42), LNA (0.49) and ∑odd and branched fatty acids (0.38). In addition, our study obtained greater R^2_{CV} and RPD values than those reported by Núñez-Sanchez *et al*. (2020) for EPA, DPA, MUFA, n-6, n-3 and h/H in thawed ewe's milk samples.

As compared to our study, the models generated by Coppa *et al.* (2010) with samples of fresh cow's milk measured in transflectance mode obtained lower R^2 coefficients in set validations for: ∑CLA (0.60); PUFA (0.65); n-6 (0.00); n-3 (0.20) and ∑odd and branched FA (0.57). Similarly, Coppa *et al*. (2014), using samples of fresh cow's milk in reflectance mode, reported validation R^2 values for ∑CLA (0.71); MUFA (0.79); PUFA (0.77), n-6 (0.43); n-3 (0.72) and n-6/n-3 (0.66). In addition, our study obtained greater RCV2 and RPD values than those reported by Llano-Suárez *et al*. (2018) for SFA, MUFA, and PUFA in liquid milk samples.

In addition, only some authors have determined AI and TI by NIRS: i) Nuñez-Sanchez *et al*. (2016) in oven-dried goat milk samples in reflectance mode (AI=0.77; TI=0.74) and liquid goat milk samples in transflectance mode (AI=0.68; TI=0.75); ii) Llanos-Suárez *et al*. (2018) in liquid milk samples using a near infrared handheld spectrometer (AI=0.85; TI=0.89); and iii) Nuñez-Sanchez *et al*. (2020) in thawed ewe's milk samples previously oven-dried on glass fiber filters (AI=0.75; TI=0.87). In comparison to these reports, our calibration models presented greater R^2_{CV} (AI=0.95; TI=0.93) and RPD (AI=4.3; TI=3.6) values.

External validation. Finally, the robustness of our model was checked with an independent subset of 10 milk samples which were not included initially in the calibration set of samples. Briefly, these samples were analyzed both by GC, and by NIR, and resulting NIR prediction values were compared to values obtained by GC with a Student t-test for paired values. There were no differences between the predicted values by NIRS and the values reported by gas cromatography obtained for this set of 10 samples which were not included in the calibration model (P=0.05). Therefore, it can be concluded that the NIRS method provides significantly identical data to the reference data for individual FA, groups of FA and health indexes.

Therefore, the development of a fast, reliable method to routinely monitor milk FA individual or as FA groups (PUFA, MUFA, etc) could be applied on a larger scale in the dairy industry in order to promote farmers, and improve dairy systems regarding factors which affect milk's FA composition. The use of NIRS as a rapid method provides an opportunity for the routine quantification of healthy milk FA such as omega-3 and CLA, with applications in the dairy industry for food labeling, and at the farm level for management of the dairy cow's diet.

5. CONCLUSIONS

The coefficient of determination of the calibration sets fluctuated between 0.76 and 0.95, while RPD values varied between 2.1 and 4.3. The R^2_{CV} and RPD statistics were proven to have an excellent predictive capacity of the models for DPA, ∑CLA, TSFA, TMUFA, TPUFA, n-6, AI, PUFA/SFA and LA. The results obtained for EPA, n-3, n-6/n-3, TI, UFA, UFA/SFA, HFA, HFA/UFA, h/H, LNA, LA/LNA, 10-*trans*, ∑odd and branched FA, and 11-*trans* displayed very acceptable R^2_{CV} and RPD statistics but it was not possible to generate a robust calibration model for MUFA/SFA. Also, based on the external validation, it can be stated that NIRS can predict individual and grouped FA, as well as health indexes based on FA content in fresh milk samples. Therefore, models of NIRS calibration can be used for predicting the nutritional and health values of fresh milk from cows from pastoral systems.

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