Comparison of Diffuse Reflectance Fourier Transform Mid-Infrared and Near-Infrared Spectroscopy with Grating-Based Near-Infrared for the Determination of Fatty Acids in Forages

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Diffuse reflectance Fourier transform mid infrared (FTMIR) and near-infrared spectroscopy (FTNIR) were compared to scanning monochromator-grating-based near-infrared spectroscopy (SMNIR), for their ability to quantify fatty acids (FA) in forages. A total of 182 samples from thirteen different forage cultivars and three different harvest times were analyzed. Three calibration analyses were conducted for lauric (C12:0), myristic (C14:0), palmitic (C16:0), stearic (C18:0), palmitoleic (C16:1), oleic (C18:1), linoleic (C18:2), and α-linolenic (C18:3) acids. When all samples were used in a one-out partial least squares (PLS) calibration, the average R^2 were FTNIR (0.95) > SMNIR (0.94) > FTMIR (0.91). Constituents C18:2 and C16:0 had among the highest R^2 regardless of the spectroscopic method used. The FTNIR did better for C12:0, C14:0, and C18:3. The SMNIR did better for C16:0, C16:1, C18:0, C18:1, and C18:2. A second set of calibrations developed with half of the samples as the calibration set and the rest as the validation set showed that all the methods produce acceptable calibrations, with calibration R^2 above 0.9 for most constituents. However, the SMNIR had a better average calibration relative error than the FTNIR, which was slightly better than the FTMIR. A third set of calibration equations developed using 100 random PLS runs with the 182 samples split randomly also shows that the three spectral methods are satisfactory for predicting FA. It is not clear whether any of the spectral methods is distinctly better than another. Calibration R^2 and validation R^2 were higher for most FA with the SMNIR than the FTMIR and FTNIR.

KEYWORDS: Diffuse reflectance; fatty acids; forage; Fourier transformed; infrared; mid-infrared; near-infrared; NIR; spectroscopy

INTRODUCTION

Over the last several decades, near-infrared diffuse reflectance spectroscopy (NIR) has become a premier method for the rapid analysis of agricultural products ranging from grains and feedstuffs to manures (*1–3*). Recently, Foster et al., (*4*) demonstrated that scanning monochromator-grating-based NIR (SMNIR) is valuable for the quantitative analysis of fatty acids (FA) in a heterogeneous set of forage samples, showing that a single set of calibrations developed with several forage species was able to reliably predict FA composition of individual plant species.

Diffuse reflectance Fourier transform mid-infrared spectroscopy (FTMIR) has not been extensively examined for the analysis of such materials because of the belief that specular reflection causes spectral distortions, which requires that materials be diluted with KBr to concentrations of 5% or less (*5–7*). However, work with forages (*8–10*), grains (*11*), and soils (*12–14*) has demonstrated that FTMIR on ground, nondiluted materials can perform as well as, or even outperform, NIR for the rapid analysis of such samples (*15*).

The same chemometric procedures, such as principal component analysis (PCA) and partial least squares analysis (PLS) are required to extract the relevant information from FTMIR as from NIR spectra (*16–19*). Although FTMIR has been shown

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to outperform NIR for soils (*12–14*) and some measures such as digestibility in forages (*9*), very few direct comparisons have been carried out for most determinations of interest. Also, most NIR research on agricultural products has been performed using scanning monochromators such as the grating-based NIRSystems 6000 series, and not Fourier transform (FT) spectrometers. While FT instruments are capable of superior resolution compared to instruments such as the NIRSystems model 6500 (Foss-NIRSystems, Eden Prairie, MN), they also have lower signal to noise ratios and often scan a much smaller sample area.

The objective of this research was to compare the ability of FTMIR, with two modes of NIR, FTNIR, and SMNIR, to determine FA in forages.

MATERIALS AND METHODS

Experiment Design and Generation of the Plant Material. Thirteen different forage cultivars belonging to 11 species of grasses, legumes, and forbs were grown in the greenhouse under controlled conditions as detailed in Foster et al. (*4*). The plant species and cultivars included borage (*Borago officinalis L.*), Lancelot plantain (*Plantago lanceolata* L.), Trical 102 triticale (*Triticale hexaploide Lart.*), Kentucky 31 tall fescue (*Festuca arundinacea Schreb.*), Seville perennial ryegrass (*Lolium perenne L.*), Benchmark orchard grass (*Dactylis glomerata L.*), Huia white clover (*Trifolium repens L.*), fodder galega (*Galega orientalis Lam.*), forage turnip (*Brassica rapa L.*), forage rape (*Brassica napus L.*), and three chicory cultivars (*Cichorium intybus L.*), Grasslands Puna, Forage Feast, and INIA le Lacerta. The plants were grown in five randomized complete blocks with one replicate per block. Treatments included the 13 forages, and three harvest times. A total of 182 samples from a possible total of 195 were used in the analyses due to missing samples. At each harvest, the stems plus leaves were frozen in liquid nitrogen and then lyophilized. Freeze-dried tissue was ground with a Wiley mill (2 mm), then ground with a cyclone mill (0.5 mm) before chromatographic and spectral analyses (*4*).

The forage FA were extracted and methylated using the procedure of Sukhija and Palmquist (*20*), and analyzed by GC and identified as described in Foster et al. (*4*). The FA detected on the sample set were lauric acid (C12:0), myristic acid (C14:0), myristoleic acid (C14:1), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and α-linolenic acid (C18:3). Each sample and reference material was prepared and analyzed in duplicate, weighing 250 mg into sample prep tubes. FA losses during preparation were corrected using an internal standard, C17:0 (heptadecanoic acid). Potential oxidative changes were minimized with the addition of butylated hydroxytoluene (~0.01% final) and storage of prepared solutions at −85 °C. Coefficients of variation (CV) were calculated for each pair of duplicates. If CV exceeded 10% for C16:0, C18:0, C18:1, C18:2, or C18:3, the sample was reanalyzed. If CV exceeded 20% for C12:0, C14:0, C14:1, or C16:1, the sample was reanalyzed.

Spectroscopy. All samples were scanned as dried ground samples in the FTNIR and FTMIR on a Digilab (Varian, Inc., Palo Alto, CA) FTS 7000 Fourier transform spectrometer equipped with a lead selenide (PbSe) detector and a quartz beam splitter for the NIR range, and a deuterated triglycine sulfate detector and KBr beam splitter for the FTMIR. The test sample sizes were 0.16 cm^3 (approximately 70 mg). Spectra were obtained in diffuse reflectance mode using a Pike (Pike Technologies, Madison, WI) AutoDIFF auto-sampler with sulfur and KBr as background samples for the FTNIR and FTMIR, respectively. All spectra were collected as pseudo-absorbance (log [1/reflectance]) as compared to absorbance which is log [1/transmittance]). Spectral data were collected at 4 cm^{-1} resolution (64 co-added scans per spectrum) from 10000 to 4000 cm^{-1} and 4000 to 400 cm^{-1} for the FTNIR and FTMIR, respectively.

Spectra were also collected using a NIRSystems model 6500 near-infrared scanning monochromator equipped with a sample transport module. The SMNIR chamber dimensions allowed for 25.7 cm^3 of sample to be scanned. The actual weight of each sample varied
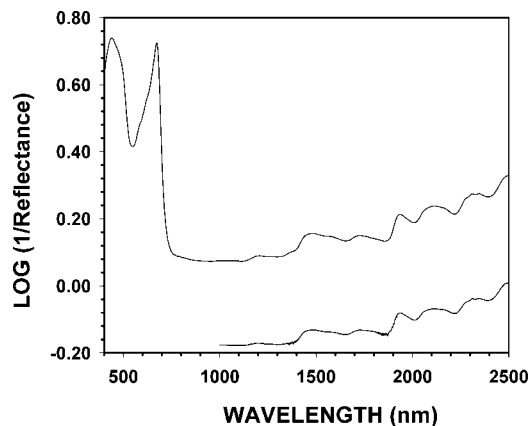

Figure 1. Near-infrared spectrum of all 182 forage samples obtained by averaging spectra obtained on Fourier transform near-infrared spectrometer (bottom spectrum, data converted from wavenumbers to nm) and from the scanning monochromator (top spectrum).

predominantly with the species, and was not determined. Samples were scanned from 400 to 2498 nm (25000 to 4003 cm^{-1}) with data collected every 2 nm (1050 data points per spectra) at a nominal bandwidth of 10 nm using a ceramic background and computed as log(1/reflectance).

Chemometrics/Calibration Development/Statistical Analysis. Spectra were examined qualitatively using GRAMS/AI Ver. 7.02 (Thermo Galactic, Salem, NH). Calibration development was performed using partial least squares (PLS) regression with a SAS® (SAS, Institute, Inc., Cary, NC) program that was published previously (*18, 19, 21*) and is available on the WEB at (www.nirpublications.com/software/index.html). A variety of math pretreatments (1st and 2nd derivatives with gaps of 1, 2, 4, 8, 16, 32, and 64 data points, multiplicative scatter) were used to determine the best calibration method for each analyte based on one-out cross validations. In addition, all spectra were mean-centered and variance scaled. Results are reported for the final calibrations resulting in the highest R^2 and lowest root mean squared deviation (RMSD). Further details on the pre-treatments may be found in Reeves and Delwiche (*18*).

The first set of calibration equations were developed with all 182 samples in a one-out PLS calibration. A second set of calibration equations was developed by splitting the samples into two calibration: validation sets as in Foster et al. (2006). The available samples were divided into two sets by assigning every other pot to the calibration or validation set. The pot number sequence was arranged by plant species and harvest time, so this method provided a nearly even allocation of each plant species and harvest between the two sets. This procedure resulted in one subset of 92 samples (subset A: calibration set 1, validation set 2), and another subset of 90 samples (subset B: calibration set 2, validation set 1). A third calibration approach involved 100 random PLS runs of each spectral dataset with the samples split randomly (122 for the calibration set and 60 for the validation set), using all spectral data points for each of the three sets of spectra.

Summary statistics and correlation analysis were performed using SAS Proc Means and Proc Freq (*21*).

RESULTS AND DISCUSSION

Spectra. Figure 1 shows the average NIR spectra of the 182 samples studied. As shown in the data from the scanning monochromator, the SMNIR spectrum has spectral information in the visible region due to color from pigments, etc., but overall does not have sharp spectral features. The FTNIR spectrum appears overall similar to the SMNIR spectrum except for missing the data in the shorter wavelength range and showing noise between 1400 and 1500 nm and again between 1900 and 2000 nm (Fig. **1**). The FTNIR sample cups are open, and it is possible that the noise might have been due to water vapor. The lower absorption seen in the FTNIR spectrum is due to the use of sulfur as opposed to ceramic for the background standard.
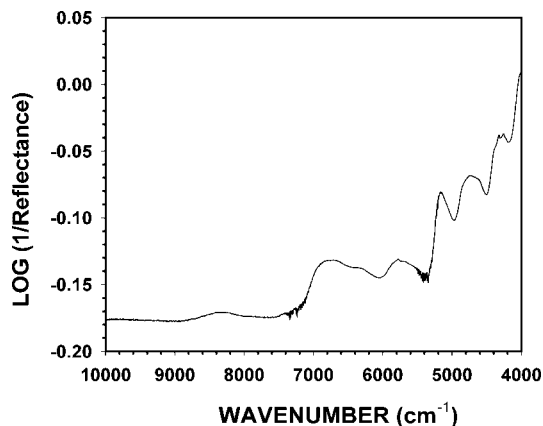
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Figure 2. Near-infrared spectrum in wavenumbers of all 182 forage samples obtained by averaging spectra obtained on Fourier transform near-infrared spectrometer.
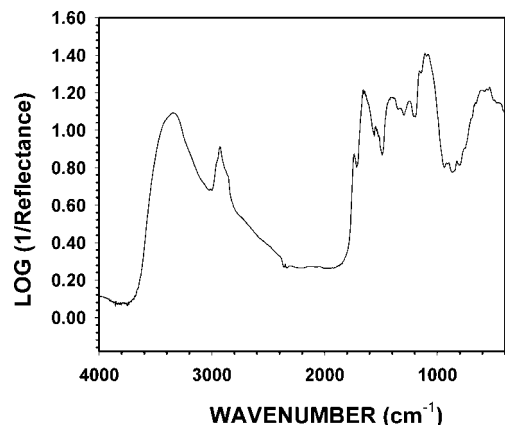

Figure 3. Mid-infrared spectra of all 182 forage samples obtained by averaging spectra obtained on Fourier transform mid-infrared spectrometer.

Table 1. Fatty Acid (FA) Composition of 13 Forage Varieties Studied Determined by Gas Chromatography[a]

FA	N[b]	mean	std dev[b]	minimum	maximum
subset A: calibration set 1, validation set 2					
C12:0	92	28.2	19.54	0	79.3
C14:0	92	415.24	111.56	207.37	660.21
C14:1	92	340.26	366.04	8.11	2291.7
C16:0	92	5413.2	1345.1	2476.2	7647.1
C16:1	92	755.86	321.07	172.17	1412
C18:0	92	400.92	220.44	139.62	1054
C18:1	92	763.63	497.75	162.79	2530.9
C18:2	92	5567.1	2275.6	1798.3	11247
C18:3	92	21940	7948.1	6741.5	38304

FA	N	mean	std dev	minimum	maximum
subset B: calibration set 2, validation set 1					
C12:0	90	30.3	25.9	0	182.17
C14:0	90	409.83	112.46	201.73	667.22
C14:1	90	354.02	385.14	9.17	2285.6
C16:0	90	5293.7	1343.1	2426	7851.7
C16:1	90	721.7	305.69	159.37	1390.9
C18:0	90	398.84	214.51	129.27	987.69
C18:1	90	702.33	439.73	171.32	2379.5
C18:2	90	5495.5	2307.4	1845.7	11788
C18:3	90	21838	8627	6797	54991

FA	N	mean	std dev	minimum	maximum
combined sample set, subset A plus subset B					
C12:0	182	29.24	22.87	0	182.17
C14:0	182	412.56	111.73	201.73	667.22
C14:1	182	347.06	374.63	8.11	2291.7
C16:0	182	5354.1	1341.8	2426	7851.7
C16:1	182	738.97	313.16	159.37	1412
C18:0	182	399.89	216.93	129.27	1054
C18:1	182	733.31	469.67	162.79	2530.9
C18:2	182	5531.7	2285.3	1798.3	11788
C18:3	182	21889	8267.9	6741.5	54991

[a] Data in mg Kg^{-1} dry matter basis. [b] *N* is the number of samples included in the calibration. Std Dev is standard deviation.

The NIR spectrum obtained from the FT instrument is also shown in **Figure 2**, but in wavenumbers as it was originally obtained. The spectrum is similar to the FTNIR spectrum of **Figure 1**, although close comparisons of the two show some differences due to the nonlinear transformation of the two scales (wavenumbers and nm, e.g., 1000–1500 nm is a gap of 3333 cm^{-1}, whereas 2000 to 2500 nm is only a gap of 1000 cm^{-1}). The average FTMIR spectrum in **Figure 3** shows more distinct spectral features than the corresponding FTNIR spectrum.

Sample Composition. Table 1 shows the FA composition of the forage varieties from GC data for all 182 samples and for the two sample subsets used as calibration and validation sets. The range of concentrations among constituent fatty acids was large, thus the sample set was appropriate to test how FTMIR, SMNIR, and FTNIR calibrations perform in determining the FA composition of forages. Lauric acid (C12:0) was usually present in small amounts and had one of the largest coefficients of variation, possibly because concentrations were close to the detection limits of the GC, and the potential of this FA to volatilize during the extraction/derivatization procedure. Splitting of the sample set into two subsets for use in calibration and validation sets resulted in two data sets with similar mean values and standard deviations. However, note that the maximum concentrations for C12:0, and C18:3 varied considerably (**Table 1**). In each case, the calibration based on 92 samples (calibration set 1) determined samples beyond its range of calibration samples, something that is not recommended.

Data in **Table 2** show the correlations between the various FA for the entire data set of 182 samples. The highest correlations were 0.87 ($R^2 = 0.76$) between C16:0 and C16:1, 0.85 between C16:1 and C18:3, and 0.81 between C16:0 and C18:2. Most correlations (29 of 36) were below 0.7, indicating that determination of one FA strictly from its correlation with another would not often result in good prediction values.

Calibrations Using All 182 Samples. Results obtained using spectra from all three spectrometers and all 182 samples in a one-out PLS calibration show that the three spectroscopic methods performed similarly in predicting FA concentrations (**Table 3**). The root mean squared deviation between measured and predicted values (RMSD) gives an index of the calibration prediction error. In general, the smaller the RMSD, the better the calibration's ability to predict unknowns. Lauric acid (C12:0) had the largest RMSD/mean values in all calibrations regardless of spectral range or instrument (FT or scanning monochromator), due to it's high coefficient of variation in the wet chemistry determinations (**Table 1**). The R^2 is the proportion of variance in the FA data accounted for by the calibration. The ideal calibration will have a low relative error of validation, and high validation R^2. Lauric acid (C12:0) had the lowest R^2 in all spectral types, whereas C18:2 and C16:0 were among the top R^2 in all spectral types (**Table 3**).The average R^2 for the

Table 2. Correlation of Fatty Acids (FA) Concentrations in 13 Forage Varieties[a]

FA	C14:0	C14:1	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
C12:0	N.S.	0.40	−0.32	−.24	−0.28	N.S.	−0.34	N.S.
C14:0		−0.51	0.69	0.67	N.S.	0.38	0.48	0.77
C14:1			−0.52	−0.45	−0.40	−0.26	−0.31	−0.40
C16:0				0.87	0.15	0.60	0.81	0.79
C16:1					N.S.	0.49	0.75	0.85
C18:0						N.S.	−0.22	−0.21
C18:1							0.56	0.36
C18:2								0.70

[a] $N = 182$. All values significant at $P < .05$ or greater, except where noted by N.S.

Table 3. Calibrations statistics for individual fatty acids (FA) using all 182 samples in a one-out partial least squares calibration[a]

			calibration results based on fourier transform mid-infrared spectra				
FA	deriv	scatter	gap	factors	R^2	RMSD	RMSD/mean
C12:0	2nd	STR	32	5	0.70	12.4	0.42
C14:0	2nd	STR	8	6	0.91	34.2	0.08
C14:1	2nd	MSC	16	10	0.94	29.7	0.09
C16:0	2nd	MSC	16	8	0.95	313	0.06
C16:1	1st	STR	8	9	0.93	80.1	0.11
C18:0	2nd	STR	16	12	0.95	50.6	0.13
C18:1	2nd	MSC	16	9	0.93	120.4	0.16
C18:2	2nd	MSC	32	12	0.96	459.8	0.08
C18:3	1st	MSC	4	8	0.92	2323.9	0.11
			average all fatty acids:		0.91	380.5	0.14
			average, C12:0 excluded:		0.94	426.5	0.10

			results based on 182 fourier transform near-infrared spectra				
FA	deriv	scatter	gap	factors	R^2	RMSD	RMSD/mean
C12:0	1st	MSC	8	6	0.89	7.6	0.26
C14:0	1st	STR	8	9	0.99	9.4	0.02
C14:1	2nd	STR	16	6	0.94	94.4	0.27
C16:0	1st	STR	4	6	0.98	210.8	0.04
C16:1	none	MSC	0	12	0.93	84.3	0.11
C18:0	1st	STR	8	8	0.95	49.7	0.12
C18:1	1st	STR	32	9	0.94	114.4	0.16
C18:2	1st	MSC	4	7	0.97	413.2	0.07
C18:3	1st	MSC	8	6	0.95	1903.2	0.09
			average all fatty acids:		0.95	320.8	0.13
			average, C12:0 excluded:		0.95	359.9	0.11

			results based on grating scanning monochromator near-infrared spectra				
FA	deriv	scatter	gap	factors	R^2	RMSD	RMSD/mean
C12:0	2nd	MSC	64	8	0.79	10.4	0.36
C14:0	1st	MSC	4	10	0.94	27	0.07
C14:1	2nd	MSC	8	11	0.94	95	0.27
C16:0	2nd	STR	8	12	0.99	160.6	0.03
C16:1	2nd	STR	4	11	0.98	48.8	0.07
C18:0	2nd	STR	4	10	0.96	41.7	0.10
C18:1	2nd	STR	4	12	0.97	79.7	0.11
C18:2	2nd	STR	4	11	0.98	306	0.06
C18:3	2nd	STR	4	7	0.92	2393.9	0.11
			average all fatty acids:		0.94	351.5	0.13
			average, C12:0 excluded:		0.96	394.1	0.10

[a] deriv is the derivative (1st, 2nd, or none), scatter is the scatter correction (no scatter correction STR or multiplicatve scatter correction MSC), gap is the number of gap for derivative, factors = number of factors, RMSD = root mean squared difference.

complete FA set (C12:0 included) was FTNIR (0.95) > SMNIR (0.94) > FTMIR (0.91) (**Table 3**). The average RMSD for all FA was FTNIR (320.8) < SMNIR (351.5) < FTMIR (380.5). Excluding C12:0 increased the average R^2 for the NIR and FTMIR, and the average RMSD of all spectral types, making results from the three methods virtually identical with average R^2 ranging from 0.94–0.96. The R^2 values for individual FA indicate that the three methods provide similar results for C14:1, the FTNIR did better for C12:0, C14:0, and 18:3, and

the SMNIR did better for C16:0, C16:1, C18:0, C18:1, and C18:2. However, the differences between spectral types were often small (**Table 3**).

Calibration Results Based on Independent Validation Sets. A second set of calibration equations was computed for each spectroscopic method using one half of the samples, while the remaining samples were used as an independent validation set. We computed the calibration and validation relative errors by dividing the RMSD for the calibration and validation results

Table 4. Results Using Fourier Transform Mid-Infrared Spectra with ~50% of Samples as the Calibration Set and ~50% as an Independent Validation or Test Set[a]

fatty acid	deriv	scatter	IGAPS	fact	CALR2	RMSD	calibration RMSD/mean	VALR2	VRMSD	VBIAS	validation RMSD/mean
					results from calibration set 1 (*n* = 92) with validation set 1 (*n* = 90)						
C12:0	none	MSC	0	11	0.93	5.3	0.19	0.54	17.60	1.06	0.58
C14:0	1st	STR	8	7	0.99	9.6	0.02	0.85	44.40	−0.58	0.11
C14:1	none	STR	0	14	0.94	90.7	0.27	0.82	163.40	27.98	0.46
C16:0	none	MSC	0	9	0.90	417.9	0.08	0.89	454.50	−50.74	0.09
C16:1	1st	MSC	32	5	0.91	93.8	0.12	0.81	134.80	−19.84	0.19
C18:0	1st	MSC	16	6	0.91	64.8	0.16	0.66	125.20	−0.63	0.31
C18:1	none	MSC	0	13	0.97	86.1	0.11	0.80	196.70	−14.99	0.28
C18:2	none	MSC	0	12	0.96	482.7	0.09	0.83	959.20	152.09	0.18
C18:3	none	STR	0	14	0.94	1871.3	0.09	0.74	4380.00	134.66	0.20
					results from calibration set 2 (*n* = 90) with validation set 2 (*n* = 92)						
C12:0	1st	STR	1	5	0.91	7.6	0.25	0.67	11.40	−2.21	0.40
C14:0	2nd	STR	32	6	0.97	17.9	0.04	0.82	47.80	4.85	0.12
C14:1	none	STR	0	12	0.92	111.5	0.32	0.72	206.20	−31.04	0.61
C16:0	none	STR	0	13	0.97	230.2	0.04	0.86	534.10	101.52	0.10
C16:1	none	MSC	0	12	0.96	57.6	0.08	0.78	156.80	37.53	0.21
C18:0	2nd	MSC	32	4	0.85	83.1	0.21	0.75	113.40	−6.67	0.28
C18:1	none	MSC	0	11	0.92	123.7	0.18	0.75	249.30	0.53	0.33
C18:2	1st	MSC	32	7	0.96	457.7	0.08	0.83	942.00	−19.07	0.17
C18:3	1st	MSC	8	4	0.93	2253.2	0.10	0.73	4181.80	385.65	0.19
	sum (average) of RMSD/mean C12:0 to C18:3 for both calibrations:						2.43 (0.13)				
	sum (average) of RMSD/mean C14:0 to C18:3 for both calibrations:						1.99 (0.12)				
				sum (average) of validation RMSD/mean C12:0 to C18:3 for both validations:							4.79 (0.27)
				sum (average) of validation RMSD/mean C14:0 to C18:3 for both validations:							3.81 (0.24)

[a] deriv is the derivative (1st, 2nd, or none), scatter is the scatter correction (STR is no correction or straight spectra, MSC is the multiplicative scatter correction), IGAPS = number of gap for derivative, fact is the number of factors, CALR2 is the calibration R^2, RMSD is the root mean squared difference, VALR2 is the validation R^2, VRMSD is the root mean squared difference for the vaidation set, VBIAS is the validation bias.

Table 5. Results Using Fourier Transform Near-Infrared Spectra with ~50% of Samples as the Calibration Set and ~50% as an Independent Validation or Test Set[a]

fatty acid	deriv	scatter	IGAPS	fact	CALR2	RMSD	calibration RMSD/mean	VALR2	VRMSD	VBIAS	validation RMSD/mean
					results from calibration set 1 (*n* = 92) with validation set 1 (*n* = 90)						
C12:0	1st	STR	16	6	0.90	6.2	0.22	0.54	17.60	1.77	0.58
C14:0	1st	STR	32	6	0.88	39.3	0.10	0.88	39.00	−3.83	0.10
C14:1	1st	STR	4	11	0.96	69.4	0.20	0.88	137.20	22.52	0.39
C16:0	2nd	STR	8	7	0.97	245.0	0.05	0.93	357.40	2.60	0.07
C16:1	none	MSC	0	11	0.92	92.7	0.12	0.91	90.50	−7.94	0.13
C18:0	1st	MSC	8	9	0.92	62.6	0.16	0.87	77.90	2.77	0.20
C18:1	1st	STR	4	6	0.92	140.4	0.18	0.90	143.80	−2.35	0.21
C18:2	1st	STR	2	7	0.95	511.2	0.09	0.92	677.30	146.74	0.12
C18:3	1st	MSC	32	11	0.94	2002.9	0.09	0.86	3306.40	497.74	0.15
					results from calibration set 2 (*n* = 90) with validation set 2 (*n* = 92)						
C12:0	1st	MSC	1	2	0.44	19.3	0.64	0.73	10.30	−1.91	0.36
C14:0	1st	MSC	16	6	0.90	34.9	0.09	0.87	41.00	1.75	0.10
C14:1	2nd	STR	16	10	0.96	72.8	0.21	0.88	129.40	−11.14	0.38
C16:0	2nd	MSC	64	8	0.97	249.4	0.05	0.89	466.50	−9.58	0.09
C16:1	1st	STR	2	7	0.97	56.3	0.08	0.86	122.30	10.74	0.16
C18:0	2nd	MSC	32	12	0.96	41.0	0.10	0.85	85.80	−7.09	0.21
C18:1	2nd	MSC	16	10	0.97	80.4	0.12	0.88	171.80	0.39	0.23
C18:2	2nd	STR	32	13	0.98	359.7	0.07	0.91	749.40	−30.00	0.14
C18:3	1st	STR	1	6	0.92	2381.6	0.11	0.87	2957.30	−144.47	0.14
	sum (average) of RMSD/mean C12:0 to C18:3 for both calibrations:						2.66 (0.15)				
	sum (average) of RMSD/mean C14:0 to C18:3 for both calibrations:						1.80 (0.11)				
				sum (average) of validation RMSD/mean C12:0 to C18:3 for both validations:							3.73 (0.21)
				sum (average) of validation RMSD/mean C14:0 to C18:3 for both validations:							2.79 (0.17)

[a] deriv = derivative (1st, 2nd or none), Scatter = scatter correction (STR = no correction or straight spectra, MSC = multiplicative scatter correction), IGAPS = number of gap for derivative, fact = number of factors, CALR2 = calibration R², RMSD = root mean squared difference, VALR2 = validation R², VRMSD is the root mean squared difference for the vaidation set, VBIAS is the validation bias.

by the mean value of the analytes for each set. Averaging RMSD values can be deceiving due to differences in the amounts of each constituent, e.g. a material at high concentration would have a higher RMSD even if the R^2 for the calibration was identical to another constituent with a much lower mean concentration, therefore dividing the RMSD by the mean value removes this problem. Calibration and validation statistics are presented in **Tables 4**, **5**, and **6** for FTMIR, FTNIR, and

SMNIR, respectively. The spectroscopic methods varied in their ability to accurately predict concentrations of individual FA. For example, the FTMIR had a lower RMSD/mean than the other methods for predicting C14:0 in both calibration sets. On the other hand, FTNIR had a lower RMSD/mean than SMNIR for predicting C14:1 in both calibration sets, and SMNIR had a lower RMSD/mean than FTNIR for the prediction of C18:2 in both calibration sets. The calibration quality of C18:1 varied

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Table 6. Results Using Scanning Monochromator Near-Infrared Spectra with ~50% of Samples as the Calibration Set and ~50% as an Independent Validation or Test Set[a]

fatty acid	deriv	scatter	IGAPS	fact	CALR2	RMSD	calibration RMSD/mean	VALR2	VRMSD	VBIAS	validation RMSD/mean
						results from calibration set 1 ($n = 92$) with validation set 1 ($n = 90$)					
C12:0	1st	STR	32	11	0.90	6.1	0.22	0.68	14.80	1.36	0.49
C14:0	2nd	MSC	8	7	0.95	25.6	0.06	0.91	34.20	−1.47	0.08
C14:1	none	MSC	0	12	0.88	126.0	0.37	0.87	141.70	12.30	0.40
C16:0	1st	STR	1	8	0.98	183.1	0.03	0.97	227.90	−21.70	0.04
C16:1	2nd	MSC	16	11	0.96	64.7	0.09	0.94	77.70	−15.72	0.11
C18:0	2nd	STR	8	11	0.97	37.4	0.09	0.93	58.10	−1.85	0.15
C18:1	2nd	STR	8	13	0.98	73.8	0.10	0.89	162.50	5.52	0.23
C18:2	2nd	STR	8	13	0.99	256.1	0.05	0.95	550.30	170.86	0.10
C18:3	2nd	STR	4	7	0.95	1709.1	0.08	0.84	3483.70	224.74	0.16
						results from calibration set 2 ($n = 90$) with validation set 2 ($n = 92$)					
C12:0	1st	MSC	8	2	0.54	17.6	0.58	0.74	10.00	−1.17	0.36
C14:0	1st	STR	8	8	0.94	28.2	0.07	0.93	30.00	−0.70	0.07
C14:1	2nd	MSC	8	8	0.93	104.2	0.29	0.88	126.00	−14.19	0.37
C16:0	1st	STR	1	6	0.98	188.8	0.04	0.97	256.50	25.97	0.05
C16:1	2nd	STR	8	13	0.98	38.5	0.05	0.93	86.20	3.42	0.11
C18:0	2nd	STR	8	9	0.95	48.8	0.12	0.90	71.10	−1.52	0.18
C18:1	2nd	STR	16	11	0.92	122.1	0.17	0.87	178.90	15.87	0.23
C18:2	2nd	STR	8	13	0.99	270.2	0.05	0.94	592.40	−143.84	0.11
C18:3	2nd	STR	8	9	0.91	2643.9	0.12	0.89	2816.80	−416.14	0.13
	sum (average) of RMSD/mean C12:0 to C18:3 for both calibrations:						2.58 (0.14)				
	sum (average) of RMSD/mean C14:0 to C18:3 for both calibrations:						1.78 (0.11)				
						sum (average) of validation RMSD/mean C12:0 to C18:3 for both validations:					3.36 (0.19)
						sum (average) of validation RMSD/mean C14:0 to C18:3 for both validations:					2.52 (0.16)

[a] deriv is the derivative (1st, 2nd, or none), scatter is the scatter correction (STR is no correction or straight spectra, MSC is the multiplicative scatter correction), IGAPS is the number of gap for derivative, fact is the number of factors, CALR2 is the calibration R^2, RMSD = root mean squared difference, VALR2 is the validation R^2, VRMSD is the root mean squared difference for the vaidation set, VBIAS is the validation bias.

between subsets, with FTNIR having a lower calibration error and RMSD than SMNIR in subset A, but the opposite being the case with subset B. The sum of the RMSD/mean for all FA other than C12:0, including both calibration sets, was FTMIR > FTNIR > SMNIR, indicating that the calibrations using data from the SMNIR were overall better than the FTNIR which, in turn, was better than the FTMIR, but none of the spectroscopic methods was unacceptable. For instance, differences in validation R^2 between SMNIR and FTNIR were usually small, although SMNIR had slightly better R^2 for C16:0, C16:1, and C18:0 (**Tables 4**, **5**, and **6**). The FTNIR had a higher validation R^2 than FTMIR for C14:1, C18:0, C18:1, and C18:3 in both calibration sets. The SMNIR had a higher validation R^2 than FTMIR for C14:1, C16:1, C18:0, C18:1, and C18:3 in both calibration sets (**Tables 4**, **5**, and **6**). The validation RMSD/mean for C16:0, C16:1, C18:0, and C18:2 was higher for the FTMIR than the SMNIR (**Tables 4**, **5**, and **6**), underscoring the differences in R^2 and error terms above. The SMNIR had a better relative error of the validation for several FA relative to FTNIR, especially for C16:0.

There were some relatively large biases (the mean differences between measured and predicted values), especially with C18:2 and C18:3 (**Tables 4**, **5**, and **6**), which might be due to the unbalanced data subsets as previously illustrated in **Table 1**. In all three sets of spectral data, there are indications of overfitting to the calibration set as indicated by the poorer performance of the validation set versus the calibration set. The FTMIR provides more spectral information than the FTNIR, whereas the FTNIR may have more than the SMNIR, thus the different spectroscopic methods overfit in the order FTMIR > FTNIR > SMNIR. If we combine the overfitting pattern with the results based on all 182 samples, it is not clear whether one method (spectrometer) is better than another.

Calibrations with the Samples Split Randomly. Table 7 summarizes the calibration and validation statistics for calibration equations developed with 100 random PLS runs with all

182 samples split randomly. This resource-intensive computation is valuable in showing the robustness of the calibrations from each spectral set. The RMSD/mean were not computed due to the random nature of the data sets. The data from the SMNIR, FTNIR, and FTMIR indicate that the three methods provide comparable and acceptable results, although the SMNIR overall did better in terms of errors and R^2 for the validation set. Most FA (except C14:1) had a lower mean calibration RMSD and validation RMSD using the SMNIR compared to the FTNIR and FTMIR. The FTMIR had smaller validation RMSD and calibration RMSD values than FTNIR for most FA, but the opposite was true for C14:0 and C18:1, which had lower calibration RMSD in the FTNIR. The mean calibration R^2 and validation R^2 were higher for most FA when using the SMNIR than the FTMIR and FTNIR, with the exception of C14:0 and C14:1. The FTMIR calibrated better than the FTNIR for C16:1, while the FTNIR calibrated better than the FTMIR for C14:0. The mean validation R^2 was higher for all FA without exception for the SMNIR than either the FTMIR and FTNIR. The FTMIR had higher validation R^2 than the FTNIR for all FA.

Robust calibrations should have similarly high R^2 values for the calibration and validation sets. In this respect, the SMNIR did better than the FTMIR for most FA except C18:1 and C18:3. The FTNIR results are less robust than either of the other two spectroscopic methods (**Table 7**).

After analyzing the results of the three calibration sets, we conclude that the FTMIR and FTNIR data are comparable to the SMNIR in terms of their potential to build calibrations for fatty acids in forage materials. The slightly better performance of the SMNIR can be explained by the difference in sample area scanned because the SMNIR instrument allows considerably more sample to be analyzed using the sample transport module. The spectroscopic method of choice for estimating FA concentrations in forages may not necessarily be the one that provides the best calibration accuracy. Other analytical aspects

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Table 7. Summary of 100 Random Runs with All 182 Samples Split Randomly (~2/3 for the Calibration Set and ~1/3 for the Validation Set)[a]

F. acid	deriv	scatter	IGAPS	MNNUM	MNRMSD	MNVRMSD	MNCALR2	MNVALR2	MNCALR2-MNVALR2
				summary of results using SMNIR spectra					
C12:0	2nd	STR	32	4.8	12.0	13.1	0.71	0.69	0.028
C14:0	1st	STR	2	6.4	28.3	36.1	0.93	0.90	0.036
C14:1	2nd	STR	8	9.7	92.2	145.8	0.94	0.86	0.078
C16:0	1st	STR	1	7.2	192.6	250.2	0.98	0.97	0.013
C16:1	2nd	STR	8	11.5	49.6	83.3	0.97	0.93	0.043
C18:0	2nd	STR	8	11.8	38.5	65.7	0.97	0.91	0.059
C18:1	2nd	STR	8	12.3	91.9	164.2	0.96	0.87	0.088
C18:2	2nd	STR	8	12.9	313.2	558.5	0.98	0.94	0.040
C18:3	2nd	STR	8	9.7	2276.1	3230.4	0.92	0.85	0.067
	minimum excluding C12:0:			6.4	28.3	36.1	0.92	0.85	0.013
	maximum excluding C12:0:			12.9	2276.1	3230.4	0.98	0.97	0.088
				summary of results using mid-infrared spectra					
C12:0	1st	STR	16	4.7	12.0	13.7	0.71	0.65	0.061
C14:0	2nd	MSC	64	6.2	37.6	44.8	0.88	0.84	0.043
C14:1	1st	MSC	1	7.1	79.3	142.3	0.95	0.86	0.094
C16:0	none	STR	0	11.5	327.7	415.7	0.94	0.91	0.032
C16:1	none	MSC	0	10.7	85.2	110.7	0.92	0.88	0.047
C18:0	1st	MSC	8	9.9	55.5	84.3	0.93	0.85	0.082
C18:1	2nd	MSC	32	5.9	142.9	176.8	0.91	0.85	0.052
C18:2	2nd	STR	32	11.4	445.7	717.2	0.96	0.90	0.058
C18:3	1st	MSC	8	7.6	2404.3	3256.8	0.91	0.85	0.067
	minimum excluding C12:0:			5.9	37.6	44.8	0.88	0.84	0.032
	maximum excluding C12:0:			11.5	2404.3	3256.8	0.96	0.91	0.094
				summary of results using FTNIR spectra					
C12:0	none	MSC	0	9.7	9.9	15.9	0.80	0.56	0.239
C14:0	1st	STR	8	6.7	15.6	47.3	0.98	0.82	0.152
C14:1	none	STR	0	13.3	108.5	171.9	0.91	0.80	0.112
C16:0	1st	MSC	16	5.3	316.7	511.9	0.94	0.86	0.081
C16:1	none	STR	0	9.5	107.0	156.2	0.87	0.76	0.112
C18:0	1st	MSC	16	6.2	65.4	119.6	0.91	0.70	0.207
C18:1	none	MSC	0	12.4	111.0	221.7	0.94	0.78	0.164
C18:2	none	MSC	0	12.6	543.5	1010.7	0.94	0.81	0.132
C18:3	1st	MSC	32	5.2	2758.0	4623.2	0.89	0.69	0.194
	minimum excluding C12:0:			5.2	15.6	47.3	0.87	0.69	0.081
	maximum excluding C12:0:			13.3	2758.0	4623.2	0.98	0.86	0.207

[a] deriv is the derivative 1st, 2nd, or none, scatter is the scatter correction (STR is the none or straight spectra, MSC is the multiplicative scatter correction), IGAPS is the number of gap for derivative. MNNUM is the mean number of factors, MNRMSD is the mean root mean squared difference for the calibration set, MNVRMSD is the mean RMSD for the validation set, MNCALR2 is the mean R^2 for the calibration set, MNVALR2 is the mean R^2 for the validation set.

are also important factors to consider when results are otherwise comparable. For example, for the FTMIR spectrometer, it is easier to load the samples, scans are completed more quickly, the spectra are easier to interpret, and the sample holders are easier to clean. The FTMIR can be more robust with large sample sets, possibly because of the greater information content, which in turn helps to avoid overfitting.

SAFETY

Acetyl chloride and methanolic-HCL were used during the fatty acid analysis. Keep acetyl chloride away from water, alcohols, amines, strong oxidizing agents, and strong bases and all heat sources and flames. When preparing methanolic-HCL, work in a fume hood and wear appropriate NIOSH/MSHA approved respirator, chemical-resistant (rubber) gloves, goggles, face shield, and long-sleeved lab coat.

ABBREVIATIONS USED

C12:0, lauric acid; C14:0, myristic acid; C16:0, palmitic acid; C18:0, stearic acid; C16:1, palmitoleic acid; C18:1, oleic acid; C18:2, linoleic acid; C18:3, α-linolenic acid; CALR2, calibration R^2; CV, coefficient of variation; DERIV, derivative; FA, fatty acids; FACT, number of factors; FT, Fourier Transform; FTMIR, Fourier transform mid-infrared; FTNIR, near infrared spectroscopy; GC, gas chromatography; IGAPS, number of gap

for derivative; MNCALR2, mean R^2 for the calibration set; MNNUM, mean number of factors; MNRMSD, mean root mean squared difference for the calibration set; MNVRMSD, mean RMSD for the validation set; MNVALR2, mean R^2 for the validation set; NIR, near-infrared diffuse reflectance spectroscopy; PCA, principal component analysis; PLS, partial least squares analysis, RMSD, root mean squared difference; SMNIR, scanning monochromator-grating-based near infrared spectroscopy; Std Dev, standard deviation; VALR2, validation R^2; VBIAS, validation bias; VRMSD, root mean squared difference for the validation set.

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