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Quantification of Fatty Acids in Forages by Near-Infrared Reflectance Spectroscopy

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Near-infrared reflectance spectroscopy (NIRS) was evaluated as a possible alternative to gas chromatography (GC) for the quantitative analysis of fatty acids in forages. Herbage samples from 11 greenhouse-grown forage species (grasses, legumes, and forbs) were collected at three stages of growth. Samples were freeze-dried, ground, and analyzed by GC and NIRS techniques. Half of the 195 samples were used to develop an NIRS calibration file for each of eight fatty acids, with the remaining half used as a validation data set. Spectral data, collected over a wavelength range of 1100−2498 nm, were regressed against GC data to develop calibration equations 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. Calibration equations had high coefficients of determination for calibration (0.93−0.99) and cross-validation (0.89−0.98), and standard errors of calibration and cross-validation were <20% of the respective means. Simple linear regressions of NIRS results against GC data for the validation data set had r^2 values ranging from 0.86 to 0.97. Regression slopes for C12:0, C14:0, C16:0, C18:0, C16:1, C18:2, and C18:3 were not significantly different ($P = 0.05$) from 1.0. The regression slope for C18:1 was 1.1. The ratio of standard error of prediction to standard deviation was >3.0 for all fatty acids except C12:0 (2.6) and C14:0 (2.9). Validation statistics indicate that NIRS has high prediction ability for fatty acids in forages. Calibration equations developed using data for all plant materials accurately predicted concentrations of C16:0, C18:2, and C18:3 in individual plant species. Accuracy of prediction was less, but acceptable, for fatty acids (C12:0, C14:0, C18:0, C16:1, and C18:1) that were less prevalent.

KEYWORDS: Near-infrared reflectance spectroscopy; forage; lauric acid; linoleic acid; α-linolenic acid; myristic acid; palmitic acid; palmitoleic acid; oleic acid; stearic acid

INTRODUCTION

Fatty acids in ruminant diets influence the fatty acid composition of meat and milk derived from the animals (*1*−*7*). In turn, fatty acids in animal products affect the flavor of the products (*1*, *8*) and the health of consumers (*9*, *10*). Linoleic acid and α-linolenic acid, which are precursors of beneficial conjugated linoleic acids and omega-3 fatty acids, respectively, in meat and milk from ruminants (*3*, *11*), are prevalent in forages (*12*). However, plant genus, species, stage of development, environment, and management influence the fatty acid composition of herbage (*13*−*15*). Selecting and managing pasture species to increase concentrations of desirable fatty acids in animal products has been a goal of several research investigations (*5*, *13*−*16*) and is of particular interest to producers of pasture-finished beef and operators of pasture-based dairies.

Determination of the fatty acid composition of forages is typically accomplished by gas chromatography (GC) (*17*). This procedure involves time-consuming extraction, derivatization, and chromatography steps and requires hazardous chemicals, expensive equipment, and a skilled analytical technician. Near-infrared reflectance spectroscopy (NIRS) is a rapid, nondestructive procedure that could provide an alternative to GC for the analysis of fatty acids in forages. This empirical procedure involves using various mathematical treatments to generate a regression equation relating the infrared spectral data for a set of samples with data obtained by GC. This calibration equation is then used to predict values for the reference technique (GC) using infrared spectra. Standardization procedures allow calibration equations developed on one instrument to be used successfully on another instrument (*18*, *19*).

Forage crude protein, neutral detergent fiber, acid detergent fiber, and in vitro organic matter digestibility are now frequently measured with NIRS (*20*−*24*), and NIRS calibration equations have been developed for lactic acid and volatile fatty acids in fresh, undried silages (*17*, *25*). The ability to predict concentrations of long-chain saturated and unsaturated fatty acids in forages by NIRS has not been assessed. NIRS has been evaluated as a means to estimate fatty acid composition of oil seeds, including rapeseed (*Brassica napus* L.) (*26*), mustard

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Table 1. Age and Developmental Stage of Forage Plant Materials Harvested for Fatty Acid Determinations

species	scientific name	cultivar	plant type	days after seeding/developmental stage[a] harvest 1	harvest 2	harvest 3
borage	*Borago officinalis* L.	common	forb	31/leaf	51/stem	70/flower
plantain	*Plantago lanceolata* L.	Lanceolot	forb	43/leaf	64/leaf	84/leaf
chicory	*Cichorium intybus* L.	Grasslands Puna	forb	38/leaf	57/leaf	77/leaf
chicory	*Cichorium intybus* L.	Forage Feast	forb	38/leaf	57/leaf	77/leaf
chicory	*Cichorium intybus* L.	INIA le Lacerta	forb	38/leaf	57/leaf	77/leaf
triticale	*Triticale hexaploide* Lart.	Trical 102	grass	38/leaf	57/leaf	77/leaf
tall fescue	*Festuca arundinacea* Schreb.	Kentucky 31	grass	38/leaf	57/leaf	77/leaf
perennial ryegrass	*Lolium perenne* L.	Seville	grass	38/leaf	57/leaf	77/leaf
orchardgrass	*Dactylis glomerata* L.	Benchmark	grass	38/leaf	57/leaf	77/leaf
white clover	*Trifolium repens* L.	Huia	legume	43/stolon	64/stolon	84/flower
fodder galega	*Galega orientalis* Lam.	common	legume	43/stem	64/stem	84/stem
forge turnip	*Brassica rapa* L.	Barkant	forb	31/leaf	51/leaf	70/leaf
forage rape	*Brassica napus* L.	Barnapoli	forb	31/leaf	51/stem	70/stem

[a] Developmental stages: leaf, plants without any stem present; stem, vertical axis present with clearly visible internodes; stolon, horizontal axis present with clearly visible internodes; flower, at least one open flower present.

(*Brassica carinata* Braun) (*27*), ironweed [*Vernonia galamensis* (Cass.) Less.] (*28*), soybean [*Glycine max* (L.) Merr.] (*29*), sunflower (*Helianthus annuus* L.) (*30*, *31*), sesame (*Sesamum indicum* L.) (*32*), and oats (*Avena sativa* L.) (*33*). In most cases, calibration equations developed for seeds of *Brassica* spp. (*27*, *34*) and sunflower (*30*, *31*) accurately predicted concentrations of oleic acid (C18:1), linoleic acid (C18:2), and a-linoelenic acid (C18:3). Estimates of C18:2 in Brassicacea, based on calibration equations developed with seeds from several different genera (*35*), and C18:3 in oat groats (*33*) were not reliable. Success in predicting concentrations of palmitic acid (C16:0) and stearic acid (C18:0) was generally moderate to poor (*26*−*35*), but good results were obtained for these fatty acids in sunflower meal, oil, and husked seeds (*30*) and for C16:0 in oat groats (*33*). Pérez-Vich and colleagues (*30*) also achieved good results for pamitoleic acid (C16:1) in sunflower oil, meal, and husked seeds. Quantification of fatty acids in soybean seeds (*29*) and ironweed seeds (*28*) by NIRS was not successful.

Velasco and co-workers (*34*) reported that NIRS calibration equations developed for seeds of one *Brassica* species were not suitable for analysis of seeds of other species of the genus, but accuracy increased when a number of different species within the genus were included in the calibration sample set. Reliability also increased when samples used to develop the NIRS calibration equations exhibited a range of values for a particular constituent (*27*, *28*, *35*); however, reliability decreased when the calibration set included large taxonomic diversity (*35*).

Forages that perform in one location or season are often not productive under other conditions. Moreover, forages may be grasses, legumes, or forbs, and mixtures of these plant types are generally present in pastures and hay fields. Developing separate NIRS calibration equations for fatty acids for every forage species is not practical. Calibration equations for indicators of nutritive value of hay have been developed using grass and legume mixtures and found to provide good results when the parameters being quantified are ones that are based on specific chemical entities (*20*, *36*). Thus, the objective of the current study was to determine the feasibility of using NIRS to estimate fatty acid composition of forages and, more specifically, to determine whether one set of calibration equations, developed using multiple forage species, can predict fatty acid composition of individual forage species.

MATERIALS AND METHODS

Plant Growth and Sample Preparation. Plant materials representing a range of traditional and novel forages (grasses, legumes, and forbs, **Table 1**) were grown from seed under greenhouse conditions from mid-January through mid-March, 2002. White plastic pots (30-cm diameter) were filled with commercial potting medium. Slow-release fertilizer [Osmocote 15−9−12 plus minor nutrients (Scotts, Marysville, OH), 25 g per pot] was then added to each pot and incorporated by hand to a depth of 8 cm. Pots were thoroughly moistened with tap water prior to seeding with 30−50 seeds per pot to achieve pure stands. Pots were maintained with temperature limits of 13 °C minimum and 25 °C maximum. Supplemental light (metal halide) was used as necessary to provide 12 h of light per day. Plants were thinned to 25 plants per pot within 3 weeks after sowing. Additional nutrient (same type and amount used at seeding) was surface-applied 60 days after seeding. Tap water was applied via an automated irrigation system to maintain adequate soil moisture. Pots were arranged as a randomized complete block with tables representing blocks. A total of 195 pots (experimental units) represented the 11 plant species, 3 harvests, and 5 blocks. The initial harvest of a plant material took place when visual examination indicated that plants had reached an average of 95% cover across all replicates (**Table 1**). Second and third harvests took place at 3-week intervals thereafter. Shoots (including any stem and all leaves) were frozen immediately in liquid nitrogen and then maintained at −85 °C or below until lyophilized. Herbage from the second and third harvests was cut to lengths of 5−8 cm prior to submersion in liquid nitrogen to facilitate drying. Freeze-dried tissue was chopped to a particle size of 2 mm with a Wiley mill. A subsample was ground with a cyclone mill to pass a 0.5-mm screen and was mixed thoroughly before being used for chromatographic and spectral analyses. All ground samples were stored under a nitrogen atmosphere at −85 °C until analyzed.

Fatty Acid Extraction and Quantification by GC. Fatty acids were extracted and methylated using the one-step procedure of Sukhija and Palmquist (*17*). Briefly, internal standard [heptadecanoic acid (0.4 mg mL^{-1} in hexane), Matreya, Pleasant Gap, PA] and 5% methanolic-HCl were added to forage samples in screw-cap tubes, and the mixture was heated at 70 °C for 2 h. Following the addition of potassium carbonate to neutralize the solution and hexane to dissolve chlorophyll, tubes were centrifuged, and the hexane layer was collected and passed through a Supelclean ENVI-carb (Supelco, Bellefonte, PA) solid-phase extraction tube containing 0.25 g of anhydrous sodium sulfate. Samples collected in amber vials were amended with butylated hydroxytoluene and stored at −80 °C until analyzed. Each set of samples extracted included an orchardgrass check sample that was used for correction of set-to-set variations. The check sample was taken from a bulk, oven-dried and milled (0.5-mm particle size) herbage collected from an ongoing field experiment.

Separation and quantification of fatty acid methyl esters were accomplished with a Hewlett-Packard (Wilmington, DE) model 6890 GC equipped with electronic pneumatics control, model 7683 automatic liquid sampler, flame ionization detector, and ChemStation data system. Fatty acid methyl esters in hexane (2 μL) were introduced by split injection (50:1 ratio) onto a WCOT fused silica, chemically bonded

capillary column (Chrompack CP-select CB for FAME, 100 m long, 0.25 mm inside diameter, 0.39 mm outside diameter, 0.25-μm film thickness; Varian, Walnut Creek, CA). Helium was used as the carrier gas at a constant flow rate of 3 mL min^{-1}. The temperature gradient (70−250 °C) consisted of the following steps: 70 °C for 1 min; increase to 135 °C at 90 °C min^{-1}, hold for 1 min; increase to 160 °C at 1.5 °C min^{-1}, hold for 0.5 min; increase to 185 °C at 1 °C min^{-1}, hold for 0.5 min; increase to 195 °C at 60 °C min^{-1}, hold for 5.5 min; increase to 250 °C at 90 °C min^{-1}, hold for 3 min. Total run time was 54.7 min. Injector temperature was 280 °C; detector temperature was 300 °C. Fatty acids were identified according to their retention times using reference standards (GLC-63B, Nu-Chek-Prep, Elysian, MN). Calibration curves generated with these standards were used to quantify fatty acid methyl esters in forage extracts. Chromatography of α-linolenic acid methyl ester and γ-linolenic acid methyl ester standards (Nu-Chek-Prep) confirmed that the GC column separated these two compounds and that the GC data for α-linolenic acid in borage were not confounded by γ-linolenic acid that was also present.

NIRS Calibration Equation Development and Validation. Ground samples were scanned on a FOSS (Eden Prairie, MN) model 6500 NIRS system (firmware version 156) using WinISI Winscan software (version 1.50) (Infrasoft International, State College, PA) to collect and analyze the data and perform the calibrations and cross validations. Spectra were collected over a wavelength range of 400−2498 nm in 2-nm increments, and data were recorded as the logarithm of the reciprocal of reflectance and standardized to FOSS Master Instrument number 1272. Reflectance values input to the calibration procedure ranged from 1100 to 2498 nm in 8-nm increments, a range that should allow deployment of calibrations over a range of standardized NIRS systems. Scan files were merged with GC data and then split into two sets (A and B) by assigning every other pot to the A or B set. This procedure provided as even a distribution as possible of each plant material between the two sets. Calibrations were developed for each fatty acid within set A and validated against set B and vice versa. This procedure resulted in larger validation sets (ratio of validation to calibration samples of 1:1) than is often recommended (1:3) (*37*). The large validation sets were needed for adequate power in statistical evaluation of residuals for the 11 plant species as described below.

Modified partial least squares was the regression method used for calibration development (*18*). Six calibration equations were developed for each fatty acid in each set (A and B). These six calibrations resulted from the evaluation of three derivative pretreatments applied with and without scatter correction. The three derivative pretreatments evaluated for each calibration were coded as follows: 1, 4, 4, 1; 2, 4, 4, 1; and 2, 8, 6, 1, where the first digit is the order of the derivative, the second is the gap over which the derivative is calculated, the third is a smoothing factor indicating the number of data points in a running average, and the fourth is secondary smoothing (*38*). When performed, scatter correction utilized standard normal variance (SNV) and detrending (DT) mathematical procedures (*39*). The optimal number of terms in each calibration was determined by cross-validation. This procedure obtained validation errors by partitioning the calibration set into six cross-validation groups. Several derivatives were evaluated, and the best-fit equation was selected, taking into account different statistics such as the coefficient of determination for calibration (R^2), the standard error of the cross-validation (SECV, the standard deviation of the differences between measured and predicted chemical components for the cross-validation), and the ratio of the standard deviation (SD) of the reference data to the SECV (SD/SECV) (*37*, *40*). To evaluate the prediction ability of the calibration models, several statistics were used: the coefficient of determination between measured and predicted chemical components for the calibration (R^2), cross-validation [one minus the variance ratio (1-VR)], and external validation (r^2) samples; the standard error of calibration (SEC), cross-validation (SECV), and prediction (SEP) (the standard deviation of the differences between measured and predicted chemical components for the calibration, cross-validation, and external validation samples, respectively); the ratio of SEP to the SD of the reference data [RPD, calculated by dividing the SD of reference values used in the validation by the SEP]; the ratio of the SEP to the range in validation reference data (RER, calculated by dividing the range by the SEP); bias, the average difference between measured and predicted chemical components for the validation samples; and the unexplained error, the SEP corrected for bias [SEP(C)] (*37*, *40*). The calibration monitoring procedure implemented in the WinISI software was used for validation of the equations. The procedure defines two control limits to determine if a meaningful bias is occurring and if a meaningful increase in unexplained error is occurring (*41*). Output includes bias limits and values for global (GH) and neighborhood (NH) spectral distances.

The calibration equation with the lowest SECV among the six equations generated for each fatty acid in each subset of samples was selected for validation. The two sets of samples (A and B) provided two equations for each fatty acid. Calibrations derived and selected from subset A data were used to predict fatty acid content of subset B samples and vice versa. Simple linear regressions of NIRS predicted values against GC reference values were used to validate and compare calibration performance of the two equations evaluated for each fatty acid. Equations with the higher validation r^2 for each fatty acid were selected.

Statistical Analysis. Residual differences between NIRS and GC fatty acid determinations of the validations were analyzed to determine significant species bias. The GLM procedure of SAS (*42*) was used to assess mean residuals of each species for each fatty acid. Dunnett's Procedure (*42*) was used to test ($P = 0.05$) the residuals of each species against a dummy "control" variable having a mean of zero and a standard deviation equal to the standard deviation of the residuals of the fatty acid under evaluation. A significant mean residual indicated the calibration tended to over- or underestimate fatty acid concentration for that species.

RESULTS AND DISCUSSION

NIRS calibration equations were developed for lauric acid (C12:0), myristic acid (C14:0), C16:0, C18:0, C16:1, C18:1, C18:2, and C18:3 using from 83 to 93 data points representing 11 different plant species. Calibration statistics are given in **Table 2**. The best equations for all fatty acids employed second-derivative spectra. Equations for C14:0, C16:0, and C18:3 were developed without scatter correction. Standard normal variance and detrend scatter corrections were used for C12:0, C18:0, C16:1, C18:1, and C18:2. Mean concentrations of C12:0, C14:0, C18:0, C16:1, and C18:1 [30−778 mg kg^{-1} dry matter (DM)] were lower than mean concentrations of C16:0, C18:2, and C18:3 (5347−21670 mg kg^{-1} DM). Standard deviations ranging from 25 (C16:0) to 65% (C18:1) of the respective means are indicative of the variability in fatty acid composition of the plant materials used. Only C18:0 ($R^2 = 0.93$) had a coefficient of determination for calibration of <0.95. Calibration equations with coefficients of determination values of >0.9 are considered to have excellent accuracy (*19*). Coefficients of determination calculated during equation cross-validation ranged from 0.89 to 0.93 for low-concentration fatty acids (C12:0, C14:0, C18:0, C16:1, and C18:1). The fraction of the constituent variance explained by the calibration during cross-validation (1-VR) for C16:0, C18:2, and C18:3 was 0.98, 0.96, and 0.93, respectively. The SEC and SECV represented similar fractions of the mean for each fatty acid and were <10% of the mean for C14:0, C16:0, C16:1, C18:2, and C18:3 and <20% of the mean for C12:0, C18:0, and C18:1. Low SECV values and SD/SECV ratios that were ≥3.0 for all of the fatty acids show that the prediction accuracy of the calibration equations is good. Although forage and oilseeds differ dramatically in chemical composition, the SD/SECV ratios obtained for forage samples were comparable to or exceeded those for C16:0, C18:0, C18:1, and C18:2 in husked achenes of sunflower [SD/SECV = 1.4 (C16:0), 2.2 (C18:0), 2.9 (C18:1), and 3.3 (C18:2)] (*31*) and mustard seeds [SD/SECV = 2.4 (C16:0), 2.3 (C18:0), 4.2 (C18:1), and 4.2 (C18:2)] (*27*), for which predicted concentrations of C18:1 and C18:2 were considered to be accurate.

Table 2. Near-Infrared Reflectance Spectroscopy Calibration and Cross-Validation Statistics for Quantification of Eight Fatty Acids in a Variety of Forage Materials[a]

fatty acid	math treatment[b]	scatter correction[c]	N	mean (mg kg^{-1})	SD[d] (mg kg^{-1})	R^{2e}	1-VR[f]	SEC[g] (mg kg^{-1})	SECV[h] (mg kg^{-1})	SD/SECV
lauric (C12:0)	2, 8, 6, 1	SNV and DT	83	30	18	0.95	0.89	4	6	3.0
myristic (C14:0)	2, 4, 4, 1	none	89	411	108	0.95	0.91	24	32	3.4
palmitic (C16:0)	2, 4, 4, 1	none	92	5347	1342	0.99	0.98	149	194	6.9
palmitoleic (C16:1)	2, 4, 4, 1	SNV and DT	93	707	305	0.98	0.96	40	59	5.2
stearic (C18:0)	2, 4, 4, 1	SNV and DT	90	391	222	0.93	0.90	56	71	3.1
oleic (C18:1)	2, 8, 6, 1	SNV and DT	93	778	502	0.96	0.93	94	134	3.7
linoleic (C18:2)	2, 4, 4, 1	SNV and DT	90	5521	2146	0.98	0.96	281	434	4.9
α-linolenic (C18:3)	2, 4, 4, 1	none	91	21670	7555	0.96	0.93	1551	1957	3.9

[a] Plant materials are listed in **Table 1**. Data are expressed on a dry matter basis. [b] Math treatment designations: derivative order, gap, first smoothing, and second smoothing, respectively. [c] Scatter corrections were none or standard normal variance (SNV) and detrend (DT) transformations. [d] SD, standard deviation of the reference data. [e] R^2, coefficient of determination for calibration. [f] 1-VR, one minus the variance ratio (the ratio of unexplained variance to total variance), is the coefficient of determination for cross validation. [g] SEC, standard error of the calibration. [h] SECV, standard error of the cross validation.

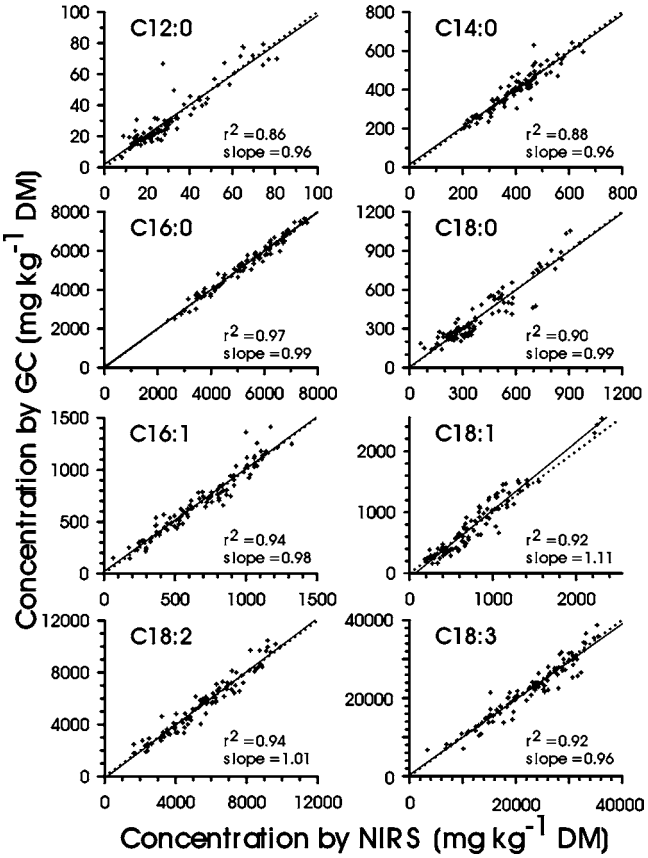

Figure 1. Prediction plots for forage fatty acid concentrations in external validation of near-infrared reflectance spectroscopy calibration equations. Dotted reference lines represent 1:1 relationships between NIRS and GC determinations of fatty acid concentration. C12:0, lauric acid; C14:0, myristic 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; DM, dry matter. Validation statistics are reported in **Table 3**.

The selected calibration equations described in **Table 2** were applied to independent validation data sets containing 85−96 data points. Plots of GC values against NIRS estimates for fatty acid concentrations of the validation data sets are shown along with coefficients of correlation in **Figure 1**. Other validation statistics are given in **Table 3**. Coefficients of correlation ranged from 0.86 for C12:0 to 0.97 for C16:0. Simple linear regressions for C12:0, C14:0, C16:0, C18:0, C16:1, C18:2, and C18:3 had slopes = 1.0 ($P = 0.05$) and intercepts = 0.0 ($P = 0.05$). The regression equation for C18:1 had a slope (1.11) significantly greater than 1.0 ($P = 0.05$) and an intercept (−75 mg kg^{-1} DM) significantly less than 0.0 ($P = 0.05$); therefore, NIRS predictions for this fatty acid would tend to underestimate the concentration at higher levels and to overestimate the concentration at lower levels. Estimates in the middle of the concentration range would be little affected by this systematic error, especially for a relationship with a high r^2 (0.92) and a relatively low SEP (146 mg kg^{-1}). The bias was small, ranging from 0.05% of the mean concentration for C14:0 and C18:0 to 2.3% of the mean concentration for C12:0. A negative bias for C14:0, C16:0, C18:0, C18:2, and C18:3 indicates that NIRS determinations will overestimate the concentrations of these fatty acids, whereas the positive bias for C12:0, C16:1, and C18:1 will result in underestimates for these constituents. The absolute value of the bias for each fatty acid is less than the bias limit, indicating that the calibration equations are acceptable.

For all of the fatty acids, SEP and SEP(C) were virtually identical; therefore, there is no unexplained error in the performance of the calibration equations. The SEP for each fatty acid was much lower than the corresponding SD for the reference method, and the RPD was >3.0 for all fatty acids except C12:0 (RPD = 2.6) and C14:0 (RPD = 2.9). The RER was >13 for all fatty acids except C12:0 (RER = 10.4) and C14:0 (RER = 11.6). These validation statistics confirm that the calibration equations give reliable predictions of concentrations of C16:0, C18:0, C16:1, C18:1, C18:2, and C18:3. Both the RPD and the RER statistics indicate that NIRS has less value as an analytical tool for C12:0 and C14:0, which occur at low concentrations in forages, but is suitable for rough screening of such samples (*37*, *40*). The RPD for C16:0, C18:0, C18:1, and C18:2 in forages equaled or exceeded the RPD for corresponding fatty acids in canola seeds [2.7 (C16:0), 2.3 (C18:0), 2.5 (C18:1), 4.2 (C18:2)] (*43*) and seeds from several *Brassica* species [1.9 (C16:0), 1.7 (C18:0), 3.7 (C18:1), 1.8 (C18:2)] (*35*). The RPD for forage C18:3 (3.6) was less than that obtained in oilseed investigations conducted by Daun and co-workers (*43*) (RPD = 6.2) and Velasco and colleagues (*35*) (RPD = 4.7), but interpretation of the statistic (*37*, *40*) is the same for all of the studies. NIRS data for C12:0, C14:0, and C16:1 are not available in the oilseed literature. The high RPD (6.2) observed for C16:0 in forages may reflect the prevalence of this fatty acid in herbage (16%) (*44*) compared to oilseeds (4−5%) (*26*, *34*, *35*, *43*, *45*). Mean GH values of 1.0 or 1.1 for the individual fatty acids indicate that spectra for the forage samples are quite similar. Mean NH values of 0.2 or 0.3 confirm the redundancy of the samples.

Table 3. Validation Statistics of Near-Infrared Reflectance Spectroscopy Calibration Equations for Quantification of Fatty Acids in Forage[a]

fatty acid	*N*	range (mg kg^{-1})	mean (mg kg^{-1})	SD (mg kg^{-1})	SEP (mg kg^{-1})	SEP(C) (mg kg^{-1})	RPD	RER	bias (mg kg^{-1})	bias limit (mg kg^{-1})	mean GH	mean NH
C12:0	85	6–79	31	18	7	7	2.6	10.4	0.7	2.9	1.1	0.3
C14:0	94	202–642	413	109	38	38	2.9	11.6	–0.2	19.1	1.0	0.2
C16:0	94	2426–7653	5371	1339	216	217	6.2	24.2	–9	123.1	1.0	0.2
C18:0	96	140–1054	400	218	70	71	3.1	13.1	–0.2	40.7	1.1	0.2
C16:1	93	146–1412	708	305	79	78	3.9	16.0	14	35.5	1.0	0.2
C18:1	96	163–2531	770	496	146	146	3.4	16.2	10	94.2	1.1	0.2
C18:2	96	1782–10467	5564	2178	537	540	4.1	16.2	–5	295.8	1.1	0.2
C18:3	96	6742–38712	21843	7647	2122	2097	3.6	15.1	–384	1204.0	1.1	0.2

[a] Plant materials are listed in **Table 1**. Fatty acid concentrations are expressed on a dry matter basis. Range, mean, and standard deviation (SD) values are for the reference (GC) method. GH, global spectral distance; NH, neighborhood spectral distance; RER, ratio of the standard error of prediction to the range in validation reference data, calculated as range/SEP (*3*, *40*); RPD, ratio of standard error of prediction to standard deviation of the reference data, calculated as SD/SEP (*3*, *40*); SEP, standard error of prediction; SEP(C), standard error of prediction corrected for bias; bias, mean of validation differences.

Table 4. Mean Bias in Prediction of Concentrations of Fatty Acids in Individual Plant Materials Using Near-Infrared Reflectance Spectroscopy Calibration Equations Developed with Multiple Plant Species[a]

	mean bias							
plant material	C12:0	C14:0	C16:0	C18:0	C16:1	C18:1	C18:2	C18:3
borage	–2	1	109	5	12	12	–208	–1908
plantain	7	13	–15	21	–20	20	–269	–467
chicory								
Grasslands Puna	1	14	3	–30	–3	39	465	426
Forage Feast	2	16	–83	31	15	0	–154	–675
INIA le Lacerta	1	–22	141	–46	58	–82	395	228
triticale	1	11	–194	2	11	–15	–230	357
tall fescue	–1	–1	24	–14	44	75	47	–946
perennial ryegrass	0	–9	61	–17	–48	–18	28	–532
orchardgrass	–1	–32	40	2	55	–12	289	1304
white clover	10*	12	–117	–44	67	188*	–22	–976
fodder galega	–5	–3	–127	13	–36	–57	–154	–3248*
forage turnip	0	–4	185	16	30	–83	–412	–366
forage rape	–1	4	–219	60	–19	25	119	944

[a] Samples of each plant material represent three different harvest dates (**Table 1**). An asterisk (*) indicates significant difference from 0 ($P < 0.05$).

Fatty acid concentration in forages is influenced by a variety of factors, including species and stage of growth (*15*). We therefore examined the ability of NIRS equations, developed using multiple plant materials, to accurately predict concentrations of the fatty acids in herbage from individual plant species (**Table 4**). Analysis of residual differences between NIRS and GC fatty acid determinations of the validation data set indicated a significant ($P = 0.05$) species bias only for C12:0 and C18:1 in white clover and C18:3 in fodder galega. The small quantity of fodder galega seeds available for this study resulted in limited quantities of herbage at designated harvest times, and some samples were insufficient for NIRS analysis, resulting in a smaller number of calibration and validation samples for this species compared to other species in this study. This situation, combined with the validation bias for C18:3 (**Table 3**), may account for the species bias in fodder galega for C18:3. Forage samples represented three different periods of growth, but only borage and white clover developed from the vegetative stage at the initial harvest to the flowering stage by the final harvest (**Table 1**). The white clover bias, which results in underestimation of C12:0 and C18:1 concentrations by NIRS, could reflect differences in fatty acid composition of tissue comprising white clover samples from the three harvests; however, a growth-stage effect was not apparent with borage.

The equations reported here demonstrate the potential for using NIRS as a means to predict concentrations of individual fatty acids in forages. The NIRS calibration equations developed using greenhouse-grown herbage samples representing a variety of traditional and nontraditional forage species and different plant types and harvest intervals (**Table 1**) give good estimates for C16:0, C18:2, and C18:3, which account for an average of 95% of total plant fatty acids. Estimates for the other, less prevalent, fatty acids (C12:0, C14:0, C18:0, C16:1, and C18:1) are acceptable, but tend to be less accurate. Calibration equations should cover the complete range of variability; therefore, the usefulness of the equations developed in this study depends on incorporation of data for field-grown samples representing a wide range of plant species, developmental stages, environmental conditions, and herbage processing procedures. Because pastures and hay fields typically contain a number of different plant species, the value of the NIRS procedure also rests upon its ability to accurately predict fatty acid concentrations in herbage mixtures. The most accurate way to analyze species mixtures is to include plants of all forage types in the calibration (*36*). We are currently acquiring the additional data needed to refine our calibration equations to make them more robust. Our goal is to develop broadly applicable equations and use them to identify plant materials and forage management strategies that will maximize availability of polyunsaturated fatty acids for grazing ruminants.

Quantification of the fatty acid composition in our study was performed using samples that had been frozen immediately upon harvest and lyophilized. These sample-handling procedures can be accommodated in research investigations, but are not practical for producers who submit samples to commercial forage testing laboratories. The results of Boufaïed et al. (*15*), who quantified fatty acids in timothy with GC using both lyophilized and oven-dried (55 °C, 2 days) samples, indicated that oven-drying

reduced fatty acid concentrations by 20−30%, relative to freeze-drying, but differences related to species, cultivar, plant type, growth stage, nitrogen fertilization, and growth period were still evident. Application of NIRS procedures to oven-dried fresh herbage may, therefore, be useful in identifying relative differences in fatty acid concentrations in forages collected from pastures, providing data needed to make decisions regarding pasture management. We anticipate that NIRS will also be valuable in determining fatty acid concentrations in conserved forages. NIRS procedures are used routinely to assess the nutritive value of hays (*20*−*23*). Results enable producers to match forage quality to livestock requirements. Determination of fatty acid composition of conserved herbage using NIRS could be easily incorporated into forage testing protocols. With appropriate calibration equations, a single scan can be used to determine traditional forage quality indicators such as crude protein, fiber content, and digestibility, as well as fatty acid composition (*20*, *36*). A simplified standardization procedure using a single sealed sample of representative material has been developed to correct for spectral differences between master and satellite instruments (*18*). Garcia-Olmo and co-workers (*19*) demonstrated that satisfactory standardization of a satellite instrument could be achieved with unsealed samples such that equations to predict fatty acid composition can be used on another instrument of the same manufacturer with better accuracy and reproducibility than can be achieved with GC analysis.

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; DM, dry matter; DT, detrend; GC, gas chromatography; GH, global spectral distance; NH, neighborhood spectral distance; NIRS, near-infrared reflectance spectroscopy; RER, ratio of the standard error of prediction to the range in validation reference data (range/SEP); RPD, ratio of standard error of prediction (validation) to standard deviation of the reference data (SD/SEP); SD, standard deviation; SEC, standard error of calibration; SECV, standard error of cross-validation; SEP, standard error of prediction; SEP(C), standard error of prediction corrected for bias; SNV, standard normal variance; VR, variance ratio; 1-VR, coefficient of determination for cross-validation.

SAFETY

Acetyl chloride reacts violently with water. Keep reagent away from water, alcohols, amines, strong oxidizing agents, strong bases, and all heat sources and flames. When preparing methanolic-HCL, work in a fume hood and wear appropriate NIOSH/MSHA approved respirator (organic vapor, acid gas cartridge), chemical-resistant (rubber) gloves, ANSI-approved chemical worker's goggles, full-length face shield, and long-sleeved lab coat.

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