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Near-Infrared Reflectance Spectroscopy (NIRS) Assessment of $\delta^{18}O$ and Nitrogen and Ash Contents for Improved Yield Potential and Drought Adaptation in Maize

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The oxygen isotope composition ($\delta^{18}O$), accumulation of minerals (ash content), and nitrogen (N) content in plant tissues have been recently proposed as useful integrative physiological criteria associated with yield potential and drought resistance in maize. This study tested the ability of near-infrared reflectance spectroscopy (NIRS) to predict $\delta^{18}O$ and ash and N contents in leaves and mature kernels of maize. The $\delta^{18}O$ and ash and N contents were determined in leaf and kernel samples from a set of 15 inbreds and 18 hybrids grown in Mexico under full irrigation and two levels of drought stress. Calibration models between NIRS spectra and the measured variables were developed using modified partial least-squares regressions. Global models (which included inbred lines and hybrids) accurately predicted ash and N contents, whereas prediction of $\delta^{18}O$ showed lower results. Moreover, in hybrids, NIRS clearly reflected genotypic differences in leaf and kernel ash and N contents within each water treatment. It was concluded that NIRS can be used as a rapid, cost-effective, and accurate method for predicting ash and N contents and as a method for screening $\delta^{18}O$ in maize with promising applications in crop management and maize breeding programs for improved water and nitrogen use efficiency and grain quality.

KEYWORDS: Ash content; $\delta^{18}O$; oxygen isotope composition; maize; mineral content; near-infrared reflectance spectroscopy; plant nitrogen

INTRODUCTION

The use of integrative physiological traits is a valuable tool in breeding programs assisted by analytical selection for improving yield potential and stress adaptation of cereals (*1*−*3*). Among these integrative traits, oxygen stable isotope signature (expressed for example as a composition, $\delta^{18}O$) and mineral accumulation (measured as ash content) in plant organic matter have been proposed as indirect methods for assessing the photosynthetic and transpirative performance of crops. The $\delta^{18}O$ of plant matter reflects the isotopic composition of source water, the evaporative enrichment due to transpiration, and the biochemical fractionation during synthesis of organic matter (*4*). The accumulation of minerals in leaves provides information on transpirative gas-exchange activity, whereas mineral content in mature kernels can be related to photosynthetic and retranslocation processes occurring during grain filling of cereals such as wheat (*5*, *6*) and maize (*7*). Recent studies have demonstrated the utility of the oxygen isotope signature and mineral accumulation in leaves and kernels (measured as ash content) to assess the yield of maize (*Zea mays* L.) genotypes better suited to different water conditions (*7*−*9*).

High nitrogen content is a desirable trait for improving grain quality; for example, it is targeted in the development of quality protein maize (QPM) (*10*, *11*). Additionally, N content in vegetative tissues is of interest to water and nitrogen use efficiency breeding programs because of its effect on leaf photosynthesis, which determines final grain yield (*12*).

However, despite the potential value of these analyses, the refined technical skills required, together with the high cost of oxygen isotope analysis (over U.S. $15 per sample), the slowness of mineral and N content determination, and the destructive nature of reference methods often limit their use, especially in early generations of breeding programs when many genotypes must be screened and seed may be scarce.

Near-infrared reflectance spectroscopy (NIRS) is a chemometric technique that combines spectroscopy and mathematics to rapidly produce indirect, quantitative estimates of the concentrations of OH-, NH-, CH-, or SH-containing compounds. Compared to wet chemistry procedures, NIRS requires simple sample preparation methods, is fast, accurate, and highly repeatable, and can be a nondestructive and, most importantly, inexpensive technique (< U.S. $1 per sample) facilitating simultaneous analysis of multiple traits (*13*, *14*). NIRS is currently used to assess feed and food quality traits in various crop species including maize, wheat, sorghum, and soybean. Such NIRS assessments include not only organic plant compounds, such as total nitrogen, moisture, fiber, carbohydrates, and amino acids (see ref *15* and references cited herein), but also inorganic compounds such as

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Table 1. Detailed Pedigree of the Drought-Tolerant Inbred Lines Derived from the La Posta Sequía (LPS) Population and the Hybrids Generated by Crossing These Lines with the Tropical Testers CML-449 and CML-495 Used in the Experiments Conducted during the 2007 and 2008 Dry Seasons

hybrids	inbred lines
CML-495 × CML-449[a,b]	La Posta Seq C7-F103-3-1-1-1-B-B-B[a]
La Posta Seq C7-F103-3-1-1-1-B-B × CML-495[a],	La Posta Seq C7-F103-2-6-1-1-B-B-B[a]
La Posta Seq C7-F103-2-6-1-1-B-B × CML-495[a,b]	La Posta Seq C7-F103-3-2-1-1-B-B-B[a]
La Posta Seq C7-F103-3-2-1-1-B-B × CML-449[a,b]	La Posta Seq C7-F180-3-1-1-1-B-B-B[a]
La Posta Seq C7-F180-3-1-1-1-B-B × CML-449[a,b]	La Posta Seq C7-F236-1-2-1-B-B[a]
La Posta Seq C7-F236-1-2-1-B-B x CML-449) × DTPWC9-F32-1-5-1-B-B[a,b]	La Posta Seq C7-F31-2-4-1v-1-B-B-B[a]
La Posta Seq C7-F31-2-4-1-1-B-B × CML-449[a,b]	La Posta Seq C7-F64-1-1-1-1-B-B-B[a]
La Posta Seq C7-F64-1-1-1-1-B-B × CML-449[a,b]	La Posta Seq C7-F64-2-3-1v-2-B-B-B[a]
La Posta Seq C7-F64-2-3-1-2-B-B × CML-449[a,b]	La Posta Seq C7-F64-2-6-1-2-B-B-B[a]
La Posta Seq C7-F64-2-6-1-2-B-B × CML-495[a,b]	La Posta Seq C7-F64-2-6-2-1-B-B-B[a]
La Posta Seq C7-F64-2-6-2-1-B-B × CML-449[a],	La Posta Seq C7-F64-2-6-2-2-B-B-B[a]
La Posta Seq C7-F64-2-6-2-2-B-B × CML-449[a,b]	La Posta Seq C7-F71-1-2-1-1-B-B-B[a]
La Posta Seq C7-F64-2-6-2-2-B-B × CML-495[a,b]	La Posta Seq C7-F86-3-1-1-1-B-B-B[a]
La Posta Seq C7-F71-1-2-1-1-B-B × CML-449[a,b]	La Posta Sequia C7-F125-1-3-1-B-B[a]
La Posta Seq C7-F86-3-1-1-1-B-B × CML-495[a,b]	La Posta Sequia C7-F55-3-2-1-B-B[a]
La Posta Sequia C7-F125-1-3-1-B × CML-449[a,b]	
La Posta Sequia C7-F55-3-2-1-B × CML-449[a,b]	
La Posta Sequia C7-F32-3-1-1-B × CML-449[b]	
La Posta Seq C7-F96-1-2-1-3-B × CML-495[b]	
La Posta Seq C7-F96-1-6-2-1-B-B × CML-495[b]	
Puma (Asgrow, Monsanto)[a,b]	

[a] Experiment 2007. [b] Experiment 2008.

minerals because most of these elements are associated with organic or hydrated molecules (*15*, *16*). Stable isotopes such as the stable carbon isotope composition ($\delta^{13}C$) of plant materials (*17*−*19*) and soils (*19*−*21*) can also be estimated by the NIRS technique; recently, Kleinebecker et al. (*22*) accurately predicted $\delta^{13}C$ and nitrogen isotope composition ($\delta^{15}N$) and N content using NIRS by combining data from a number of species in a wide range of growing conditions. Although $\delta^{18}O$ can be incorporated into organic molecules with NIR absorbance, to our knowledge, $\delta^{18}O$ has not been assessed using NIRS. This is despite the potential of $\delta^{18}O$ to replace other integrative traits such as $\delta^{13}C$ in the breeding for drought resistance in C_4 crops, such as maize (*7*).

Drought is a major constraint to maize yields, particularly in the tropics (*23*). NIRS may be useful not only in assessing differences in quality traits but also in providing data on breeding for genotypic differences in grain yield and stress adaptation. This could involve not just determining $\delta^{18}O$ but also traits typically associated with quality such as N and mineral contents in vegetative tissues and grains. NIRS's utility may be limited in breeding for yield and stress adaptation, as the above traits show greater variability under environmental stresses than among genotypes (*5*, *7*, *8*, *24*). This is particularly evident for $\delta^{18}O$, given the relatively low range of differences associated with genotypic variability. Nevertheless, the use of NIRS for selection may have some advantages, because it makes selection before planting much more feasible, saving time and resources. For example, in the case of the QPM maize breeding program at the International Maize and Wheat Improvement Center (CIMMYT), screening S_2 (second generation of inbreeding) nurseries before planting could reduce total costs by up to 20% (*11*).

The first objective of the research reported here was to test whether NIRS can be used to predict $\delta^{18}O$ and ash and N contents in maize leaves and kernels. Second, we evaluated the potential use of NIRS as a genotypic selection tool with applications in maize breeding programs for improved water and nitrogen use efficiency and grain quality.

MATERIALS AND METHODS

Plant Material and Growth Conditions. Two experiments were conducted at CIMMYT's experiment station in Tlaltizapán, Mexico (18° 41′ N, 99° 07′ W, 940 m above sea level) during the 2007 and 2008 dry seasons (November−May). In 2007, a set of 15 contrasting tropical maize (*Z. mays* L.) inbred lines derived from the La Posta Sequía (LPS) population, 17 single hybrids (resulting from single crosses generated by crossing LPS lines with the tropical testers CML-449 and CML-495), and one commercial hybrid (cv. Puma), used as a check, were grown under field conditions and subjected to three different water regimes: full irrigation (WW), intermediate stress (IS), and severe stress (SS). Plants received ca. 1500, 675, and 325 mm of water for the WW, IS, and SS treatments, respectively. In 2008 the same hybrids and check used in 2007, together with an extra hybrid from the same population, were grown under the same field and received ca. 1510, 481, and 331 mm of water for the WW, IS, and SS treatments, respectively (**Table 1**). Trials for each condition during the 2007 and 2008 seasons were randomized in a complete block design with three replications per genotype. Growing conditions for experiments conducted during 2007 and 2008 are detailed in Araus et al. (*9*).

Oxygen Isotope Composition and Ash and Total Nitrogen Content Analyses. Reference methods were performed in only leaf and kernel samples from the 2007 season. Leaf samples were collected from one entire plant per plot 2 weeks after anthesis before the onset of senescence. Kernel samples from the entire plot were collected at maturity. Both leaf and kernel samples were oven-dried at 60 °C for 48 h, after which they were milled using a cyclotec mill (manufactured by Tecator Höganäs) with a 0.5 mm sieve.

The $^{18}O/^{16}O$ ratios of kernel and leaf samples were analyzed at the Colorado Plateau Stable Isotope Laboratory (CPSIL). Samples of about 0.3 mg and reference materials were weighed into silver capsules and analyzed via pyrolysis over glassy carbon at 1350 °C using a Thermo-Electron TC/EA (thermochemical elemental analyzer) interfaced via a CONFLO-II to a gas IRMS (isotope ratio mass spectrometer) Thermo Electron Delta Plus XL (Finnigan MAT, Bremen, Germany). Results were expressed as $\delta^{18}O$ values, using two secondary standards (IAEA 601 and IAEA 602) calibrated against Vienna Standard Mean Oceanic Water (VSMOW) with an analytical precision of about 0.3‰.

The same samples were used for ash content determination [AACC Method 08-01 (*25*)]. Briefly, 2 g of dry mass was placed in preweighed porcelain crucibles. Samples were burnt in a muffle furnace for 6−8 h at 600 °C. Then, the mineral residue was weighed. Results were expressed as percentage of dry mass (%).

The total N content of the same kernels and leaf samples was analyzed at CPSIL using an Elemental Analyzer (Carlo Erba 2100, Milan, Italy). Results were expressed as percentage of dry mass (%).

NIR Measurements and Spectrum Acquisition. The same kernel and leaf samples used for $\delta^{18}O$ and ash and N content determination

Table 2. Composition of the Calibration and Validation Global Sample Sets (Including Inbred Lines and Hybrids) and Specific Sample Sets for Hybrids for N, Ash, and $\delta^{18}O$ in Kernels and Leaves Obtained by Reference Methods[a]

	calibration					validation				
trait	*n*	mean	SD	range	CV	*n*	mean	SD	range	CV
Global Models										
$N_{kernels}$	121	1.81	0.22	1.26−2.54	12.2	128	1.83	0.25	1.15−2.62	13.7
N_{leaves}	139	1.56	0.22	0.99−2.05	14.1	149	1.57	0.23	0.92−2.17	14.6
$ash_{kernels}$	126	1.44	0.24	0.92−2.04	16.7	128	1.45	0.25	0.91−2.10	17.3
ash_{leaves}	146	14.2	2.89	8.78−21.5	20.4	148	13.92	2.88	8.75−21.4	20.7
$\delta^{18}O_{kernels}$	123	31.8	1.33	28.0−35.0	4.2	128	31.73	1.43	27.9−35.0	4.5
$\delta^{18}O_{leaves}$	141	32.9	1.20	29.2−38.3	3.6	147	33.04	1.31	28.5−36.6	4.0
Hybrid Models										
$N_{kernels}$	68	1.75	0.24	1.2−2.3	13.7	69	1.75	0.25	1.15−2.24	13.9
N_{leaves}	77	1.48	0.21	0.99−2.17	14.2	81	1.49	0.21	0.92−1.95	14.3
$ash_{kernels}$	68	1.38	0.26	0.92−2.10	18.8	69	1.37	0.26	0.91−1.88	19.2
ash_{leaves}	76	14.7	2.86	10.4−21.4	19.5	81	14.85	2.99	10.0−21.5	20.1
$\delta^{18}O_{kernels}$	68	31.0	1.04	28.0−33.5	3.4	69	30.91	1.09	27.9−33.5	3.5

[a] *n*, number of samples; SD, standard deviation; CV, coefficient of variation.

together with kernel samples from the 2008 season were analyzed using a scanning monochromator NIRSystems 6500 spectrometer (Foss NIRSystems, Inc., Silver Spring, MD). For each sample, about 4 g of ground material was placed in a small ring cup (37 mm diameter) with a quartz glass cover, and the NIR reflectance spectra were determined at 2 nm intervals from 400 to 2500 nm. The spectrophotometer was controlled through its bundled software (ISI Software, Intrasoft International, LLC), and spectral data were recorded as log $1/R$ (where R is reflectance). Because of the small amount of kernel samples from SS treatments obtained in 2007, some samples were not used for NIRS determination.

Development of NIRS Calibration and Validation Curves. To compile calibration and validation sets with similar distribution for each of the studied traits, ash, N, and $\delta^{18}O$ data from each of the sets (all samples and hybrids) were sorted by ascending reference values, and stratified samples consisting of half the samples for each trait were selected systematically from the whole range of values. The remaining samples were used for validation (see **Table 2**). Sample sets and calibration and validation statistics were chosen using WinISI software, version 3.0 (Infrasoft International, LLC). Calibration equations were developed using a modified partial least-squares (MPLS) method. A cross-validation method was used to determine the number of factors in the regression models and to avoid overfitting. Four cross-validation segments were used. The scatter correction of standard normal variant and detrend (SNV-D) was applied (*26*). The mathematical treatment was set to 1,4,4,1, where the first number is the degree of the derivative, the second is the gap between data points for subtraction, and the third and fourth are the number of data points used for smoothing (*27*). The results of the calibration calculation were monitored by checking the T, global H (GH), and X outliers. Outliers with $T > 2.5$ and GH and $X > 10$ were not considered for calibration development. Two outlier elimination passes were made.

Global calibration models combining inbred line and hybrid data were then constructed for leaf and kernel ash, N content, and $\delta^{18}O$. Additionally, specific calibration models for leaf and kernel ash and N content and kernel $\delta^{18}O$ were developed using samples from hybrids.

The standard error of calibration (SEC), the coefficient of determination in calibration (R^2_c), and the standard error of cross-validation (SECV) were calculated. The ratio of performance deviation (RPD), defined as the ratio between the standard deviation (SD) of the calibration population and the SECV, was used to test the accuracy of the calibration models. Moreover, to evaluate the predictive ability of the models, we calculated the standard error of prediction (SEP), slope, and coefficient of determination in validation (R^2_v). As the quality and robustness of a NIRS calibration can be judged by the SEP and SD/SEP (*28*), we calculated the ratio between the SD and SEP for each trait.

Statistical Analyses. Two-way analysis of variance (ANOVA) was performed using the general linear model procedure to calculate the effects of water regime and genotype on the studied parameters. Within each experiment, means were compared by the least significant difference (LSD) multiple-range test ($P < 0.05$). Data were analyzed by the SPSS 15.0 statistical package (SPSS Inc., Chicago, IL).

Table 3. Descriptive Statistics of the Calibration and Validation Global Sample Sets (Including Inbred Lines and Hybrids) and Specific Sample Sets for Hybrids for Leaf and Kernel N and Ash Contents and Kernel Oxygen Isotope Composition ($\delta^{18}O$)[a]

	calibration						validation			
trait	terms	*T* outliers	SEC	R^2_c	SECV	RPD	SEP	R^2_v	SD/SEP	slope
Global Models										
$N_{kernels}$	4	6	0.06	0.93	0.06	3.66	0.09	0.87	2.76	0.99
N_{leaves}	9	8	0.06	0.92	0.07	3.14	0.11	0.73	2.09	0.96
$ash_{kernels}$	4	1	0.11	0.76	0.12	1.87	0.13	0.74	1.96	0.94
ash_{leaves}	10	1	0.50	0.97	0.61	4.73	0.81	0.92	3.46	0.96
$\delta^{18}O_{kernels}$	8	4	0.61	0.79	0.78	1.71	0.98	0.54	1.45	0.86
$\delta^{18}O_{leaves}$	5	6	0.80	0.55	0.88	1.36	1.06	0.36	1.24	0.78
Hybrid Models										
$N_{kernels}$	3	1	0.07	0.91	0.08	2.98	0.08	0.88	3.81	1.11
N_{leaves}	5	4	0.06	0.90	0.07	2.60	0.09	0.79	2.76	1.00
$ash_{kernels}$	3	1	0.10	0.86	0.11	2.36	0.14	0.71	2.51	0.95
ash_{leaves}	7	5	0.38	0.98	0.52	5.5	0.67	0.95	5.61	0.96
$\delta^{18}O_{kernels}$	3	1	0.60	0.67	0.69	1.50	0.65	0.64	1.69	1.10

[a] Terms, number of PLS terms; R^2_c, determination coefficient of calibration; RPD, ratio of performance deviation; SEC, standard error of calibration; SECV, standard error of cross calibration; SEP, standard error of prediction.

RESULTS AND DISCUSSION

Reference Sample Distribution. Water regime and genotype significantly affected leaf and kernel $\delta^{18}O$ and ash and N contents in both hybrids and inbred lines, with the water regime exerting the greater effect, as revealed by ANOVA (see the Supporting Information, Tables S1 and S2). **Table 2** shows the number of samples, mean SD, and range, as well as the coefficient of variation (CV) of the calibration and validation data sets used to construct the global and hybrid-specific models. Calibration data sets for each of the studied traits shared a similar range of mean and SD values with validation models. The CV exceeded 10% for ash and N contents in both leaves and kernels, with the leaf ash content showing the largest CV (ca. 20%). In contrast, CV values of <4.5% were observed for the kernel and leaf $\delta^{18}O$.

NIRS Calibration and Validation Development. In global calibration models, higher determination coefficients of calibration (R^2_c) were observed for leaf and kernel ash and N contents compared with leaf and kernel $\delta^{18}O$ (**Table 3**). Nevertheless, all of the calibration regressions showed highly significant R^2_c ($P < 0.001$) and relatively low SEC values. On the basis of the RPD values, calibrations for leaf and kernel ash and leaf N contents showed good predictions, whereas the rest of the calibrations would be useful for screening purposes (*29*). Global models accurately predicted leaf and kernel N content with $R^2_v > 0.7$ and SD/SEP > 2 (**Table 3**; **Figure 1a**,**b**), leaf ash content with $R^2_v = 0.92$ and SD/SEP = 3.46, and ash content in kernels with $R^2_v = 0.74$ and SD/SEP = 1.96 (**Table 3**; **Figure 1c**,**d**). In addition, the predictive ability for $\delta^{18}O$ was lower compared with models predicting ash and N contents, with values in kernels of $R^2_v = 0.54$, SEP = 0.96‰, and SD/SEP = 1.45 and in leaves of $R^2_v = 0.36$, SEP = 1.06‰, and SD/SEP = 1.24 (**Table 3**; **Figure 1e**,**f**).

Kernel $\delta^{18}O$ and maize quality traits (including N and ash contents in leaves and kernels) are potential traits for assessing yield performance and drought resistance in maize hybrids grown under different water conditions (*7*, *8*, *12*). Thus, specific calibra-

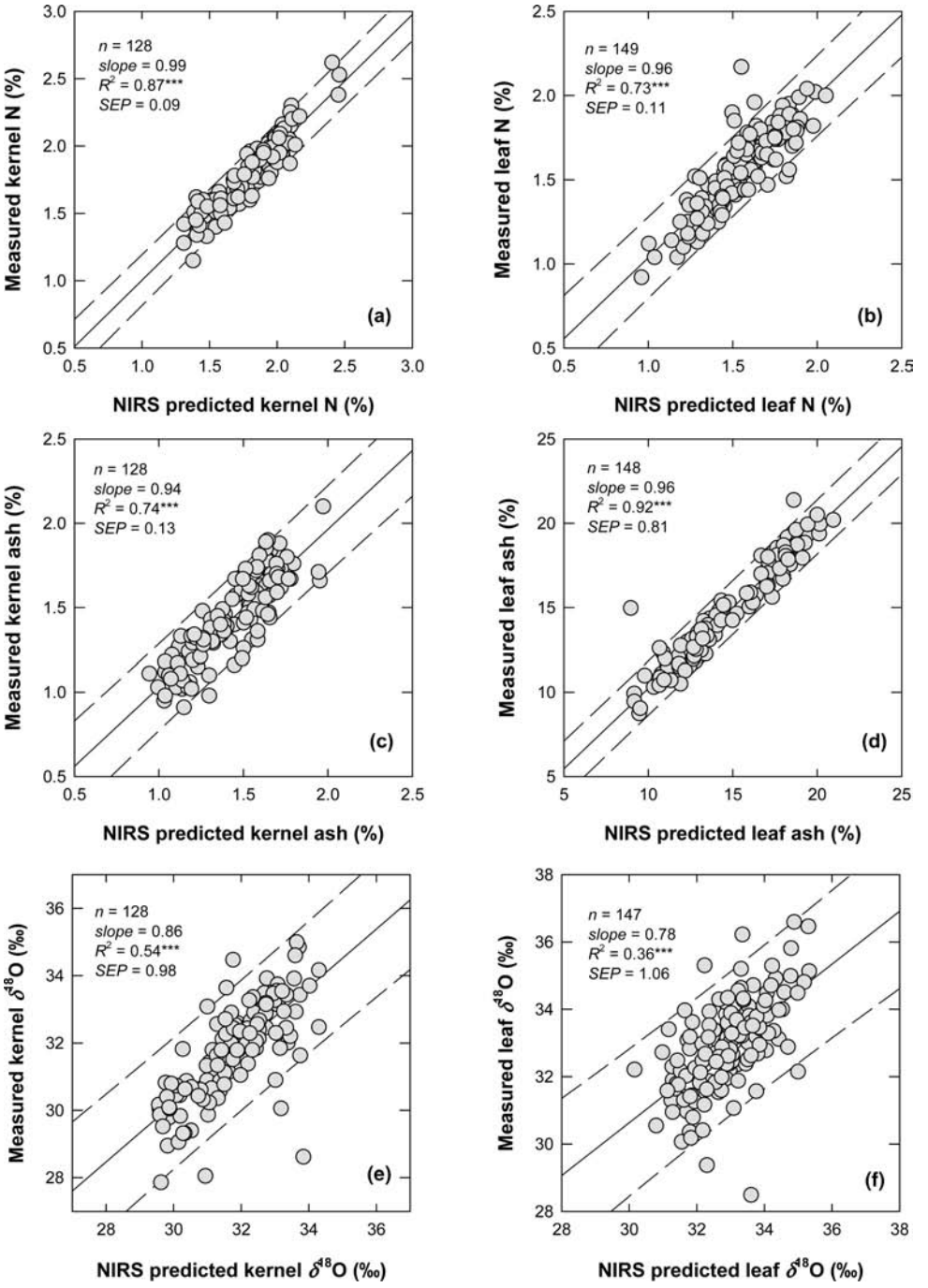

Figure 1. Relationships between the measured and the NIRS-predicted values in global models including lines and hybrids for (**a**) kernel nitrogen (N) content, (**b**) leaf N content, (**c**) kernel ash content, (**d**) leaf ash content, (**e**) kernel oxygen isotope composition (δ^{18}O), and (**f**) leaf δ^{18}O. Dashed lines represent the prediction intervals ($P < 0.05$). *n*, number of samples; R^2, determination coefficient; SEP, standard error of prediction.

tion and validation models for the kernel and leaf N and ash contents and the δ^{18}O of kernels were developed for hybrids (**Table 3**). The accuracy of calibrations for leaf and kernel N and ash for hybrids alone slightly improved on the global model combining inbred lines and hybrids (**Table 3**). RPD values showed good predictions for leaf and kernel N and ash contents. Consequently, the predictive ability of leaf and kernel N and ash contents for hybrids alone also slightly increased compared with global models, with an R^2_v of 0.88, SEP = 0.07%, and SD/SEP of 3.81 for kernel N content, an R^2_v of 0.79, SEP = 0.09%, and SD/SEP of 2.76 for leaf N content (**Table 3**; **Figure 2a**,**b**), an R^2_v of 0.71, SEP = 0.14%, and SD/SEP of 2.51 for kernel ash content, and an R^2_v of 0.95, SEP = 0.67%, and SD/SEP of 5.61 for leaf ash content (**Table 3**; **Figure 2c**,**d**). The predictive ability of δ^{18}O of kernels using specific models for hybrids improved compared to general models, according to the decrease in SEP (from 0.98 to 0.65‰) and the increase in the SD/SEP ratio (from 1.45 to 1.69), although the RPD values remained low (**Table 3**).

Farmers grow mainly hybrids; therefore, evaluation of their performance is more relevant than evaluation of the performance of parental lines because the relationship between line and hybrid performance is not very strong (*29*). However, evaluating lines is also of interest because the study of heterosis (the superior performance of heterozygous hybrid plants over their homozygous parental inbred lines) might open opportunities for increasing yield potential and stress adaptation (*9*).

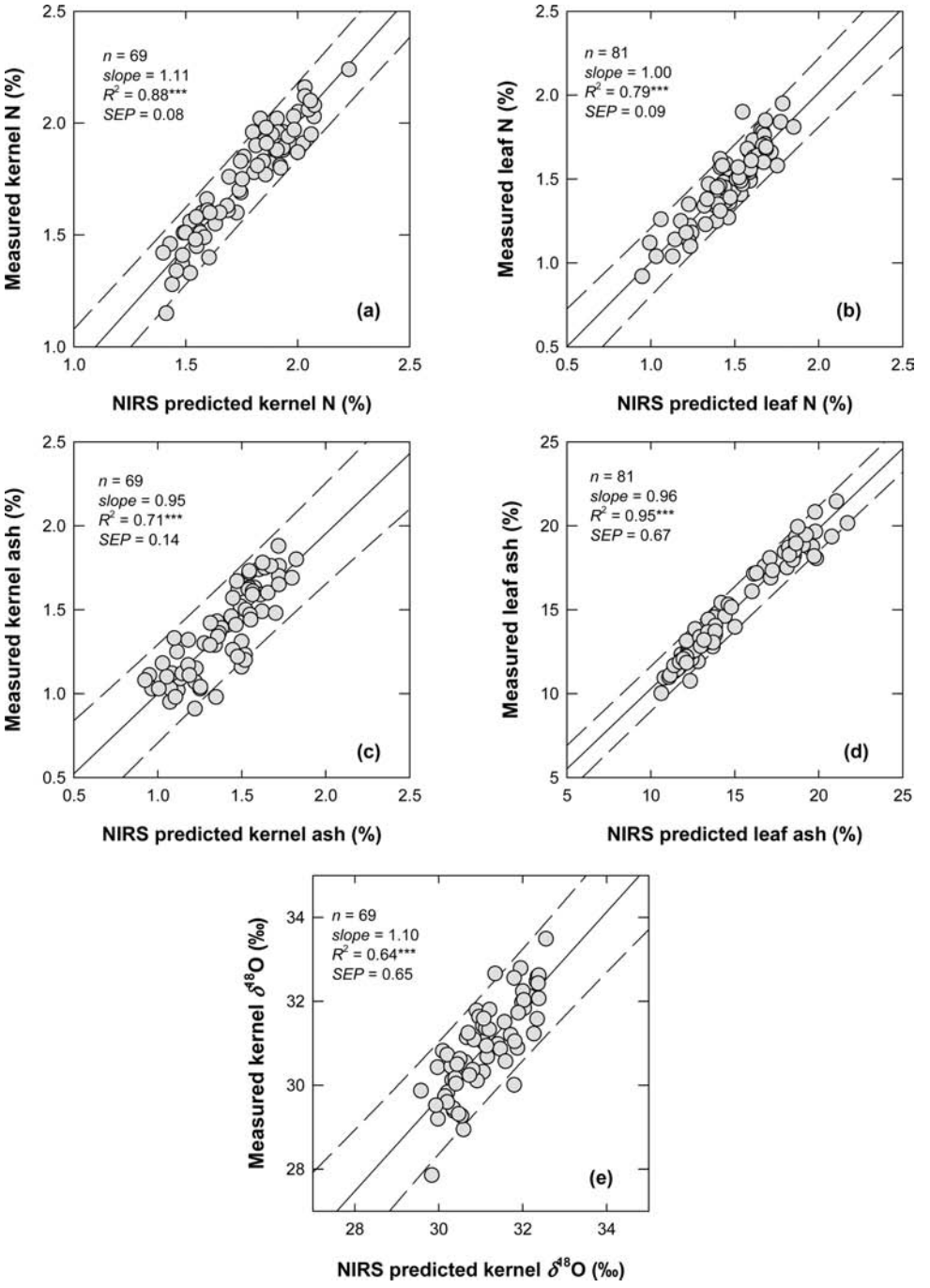

Figure 2. Relationships between the measured and the NIRS-predicted values in hybrids for (**a**) kernel N content, (**b**) leaf N content, (**c**) kernel ash content, (**d**) leaf ash content, and (**e**) kernel oxygen isotope composition ($\delta^{18}O$) in hybrids. Dashed lines represent the prediction intervals ($P < 0.05$). *n*, number of samples; R^2, determination coefficient; SEP, standard error of prediction.

Leaf ash content predictions in both hybrids and lines together and hybrids alone showed a high accuracy ($R^2_v > 0.90$) and SD/SEP > 3.5). This could be explained by numerous causes due to the large range of variation observed in this trait across samples and treatments, about 12% (i.e., CV of 20%), and because of the relevance of the chemical composition of this trait, constituting between 9 and 21% of the dry weight of the sample. This is supported by the lower R^2_v values observed for ash content in kernels (constituting only on average 1.5% of the kernel dry weight). Our results are in accordance with a number of studies that predicted leaf ash content in different grass species with R^2 of 0.97−0.98 and SEP of 0.73−0.98% (*30*, *31*), pasture samples (R^2 of 0.88 and SEP of 0.51%) (*32*), and bread wheat kernels (SEP of 0.14%) (*33*). Nevertheless, it should be noted that ash content has no NIR signature. The NIR calibration is based on correlations with organic molecules associated with minerals that have an NIR absorbance pattern that may vary among species (*15*, *16*, *33*).

The ability of NIRS to determine N content in both leaves and kernels is well documented in the literature (*14*). The strong N−H absorptions in the NIR region and the relatively high concentrations of N in plant tissues (here 1−2.5% of dry weight) are the primary causes for these accurate predictions (*33*). In contrast, the predictive ability of NIRS for determining leaf $\delta^{18}O$ (global models) and kernel $\delta^{18}O$ (global models and hybrids alone) was lower (as assessed by the lower R^2_v and SD/SEP) compared with the other traits studied. This can be explained in part because of

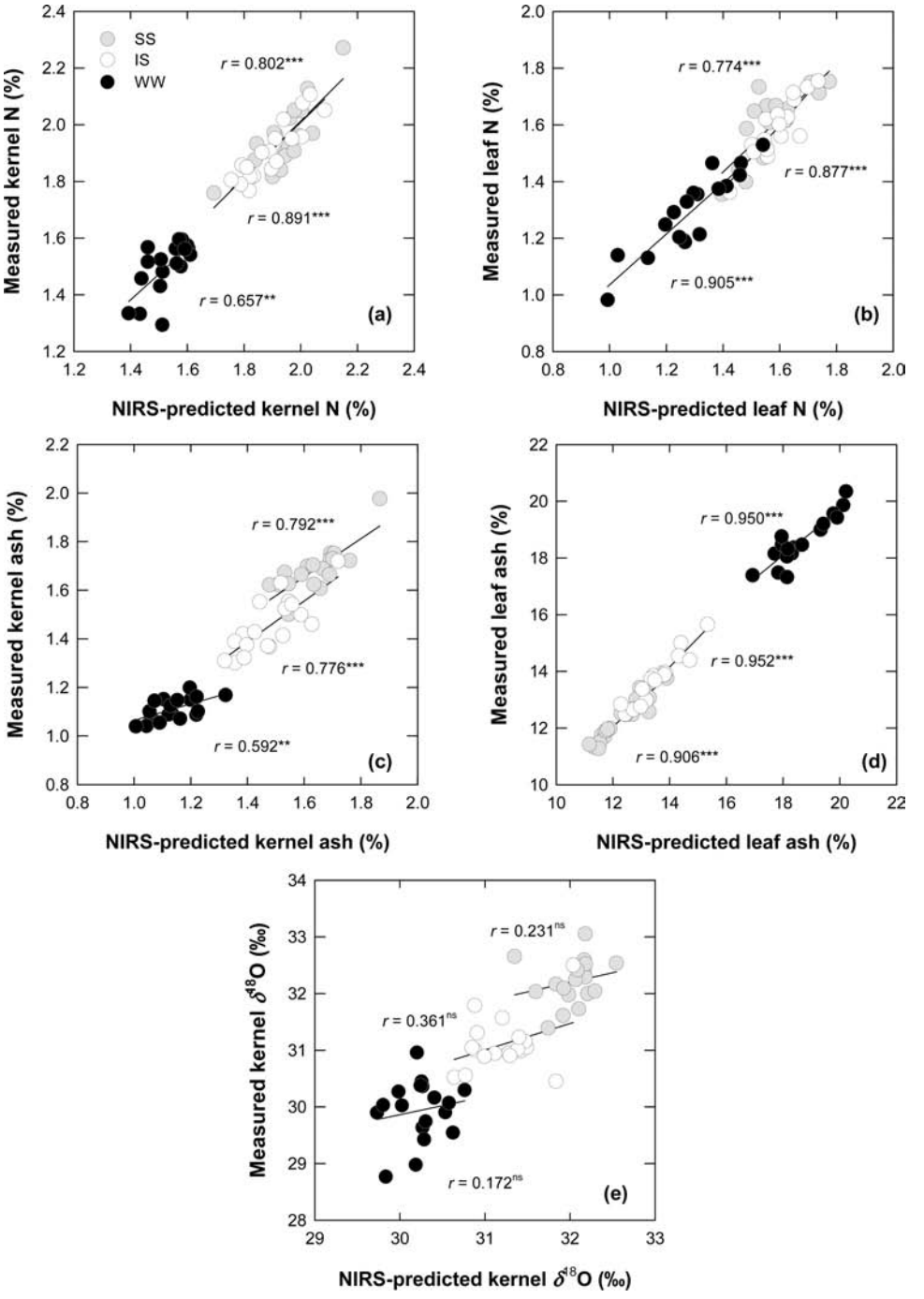

Figure 3. Relationships between the measured and the NIRS-predicted values in hybrids for (**a**) kernel N content, (**b**) leaf N content, (**c**) kernel ash content, (**d**) leaf ash content, and (**e**) kernel oxygen isotope composition ($\delta^{18}O$). Within each water treatment (WW, IS, and SS), each symbol represents the average of the three replicates of each of 18 genotypes assayed. ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$; ns, not significant.

the low range of variation observed for leaf and kernel $\delta^{18}O$. In fact, coefficients of variation for leaf and kernel $\delta^{18}O$ were lower than 4.5%, compared with the other traits studied for which CV ranged between 11 and 20%, showing a lower range of variation.

On the other hand, the improvement in the calibrations and validations for kernel $\delta^{18}O$ using specific models for hybrids, as compared with the model combining both inbred lines and hybrids, may be due to differences in the chemical compositions of hybrid kernels versus kernels of inbred lines (e.g., lower N and higher starch content in hybrids), which probably impairs the establishment of a global model for $\delta^{18}O$. Finally, and probably most importantly, the NIRS prediction of stable isotope composition relies on variation of parameters such as the amounts of starch, protein, and water indirectly associated with the stable isotope signature and which are quantified by NIRS (*18*). In this way it is well-known that water availability not only has a strong influence on the oxygen isotope composition (*4*, *7*, *8*, *34*) but is also likely to affect chemical characteristics of kernels related to grain quality (see ref *18* and references herein) that may be associated with variation in $\delta^{18}O$. Whereas $\delta^{18}O$ has not been assessed before in any crop using NIRS, other stable isotopes, such as $\delta^{13}C$ and $\delta^{15}N$, have been predicted by NIRS in different plant species. $\delta^{13}C$ has been predicted in wheat kernels with a R^2 of 0.82 and a RMSEP of 0.55‰ (*19*). Similarly, $\delta^{13}C$ was predicted in alfalfa leaves and several grass species with a R^2 of 0.69−0.93 and a RMSEP of 0.35−0.59‰ (*17*). More recently,

Table 4. Mean and SD Values for the Measured Grain Yields and Measured and NIRS-Predicted Kernel N and Ash Contents and $\delta^{18}O$ in Hybrids Grown under Three Different Water Regime in 2007 and 2008[a]

	experiment 2007	experiment 2008
kernel N (%)	*measured*	*NIRS-predicted*
SS	1.96 ± 0.13a	1.99 ± 0.09a
IS	1.89 ± 0.13b	1.96 ± 0.12a
WW	1.49 ± 0.12c	1.66 ± 0.12b
kernel ash (%)		
SS	1.69 ± 0.12a	1.91 ± 0.12a
IS	1.45 ± 0.16b	1.88 ± 0.17a
WW	1.11 ± 0.11c	1.45 ± 0.17b
kernel $\delta^{18}O$ (‰)		
SS	32.2 ± 0.6a	32.0 ± 0.4a
IS	31.2 ± 0.7b	31.9 ± 0.5a
WW	29.9 ± 0.7c	30.5 ± 0.5b
grain yield (Mg ha^{-1})		*measured*
SS	0.4 ± 0.2c	0.8 ± 0.5c
IS	1.5 ± 0.7b	1.8 ± 0.8b
WW	7.3 ± 1.5a	9.6 ± 1.2a

[a] Within each experiment, values with different letters are significantly different ($P < 0.05$) according to the LSD test.

$\delta^{13}C$ has been predicted in a number of Chilean species with a R^2 and SEP ranging between 0.79 and 0.89‰ and between 0.64 and 0.44‰, respectively, and $\delta^{15}N$ with a R^2 of 0.96 and SEP ranging between 1.42 and 1.99‰ (*22*). Therefore, our predictions of $\delta^{18}O$ in hybrid kernels, and also the predictions of kernel and leaf $\delta^{18}O$ using the whole data set (hybrids and inbred lines), have statistical performance similar to those reported in the literature for prediction of isotopic composition by NIRS. Nevertheless, even though the analytical precision of IRMS for $\delta^{13}C$ and $\delta^{15}N$ obtained in the former studies was about 0.1−0.15‰, it should be noted that the precision attained for $\delta^{18}O$ was about 0.3‰. Such results are in line with differences in the precision of ^{13}C, ^{15}N, and ^{18}O analyses for cereals published in other recent papers (see, e.g., refs *34*−*36*). Moreover, although previous NIRS studies on stable isotopes have reported SEPs that are about 3−10 times higher than the accuracy in the analytical measurements (*17*, *18*, *22*), the SEP was only 2−3 times higher in our work. Our study, however, focused on only one species (maize), and the aim was to assess genotypic differences for these traits within each growing condition, rather than to compare one or several species across different growing conditions. Therefore, if the objective is to use NIRS for plant breeding, it may be difficult to develop highly accurate calibration and prediction models. Despite this, NIRS assessment of $\delta^{18}O$ may be useful for plant ecology and crop management studies, when different growing conditions are compared; the range of genotypic values for the above traits was usually smaller than in studies aimed at implementing this analytical technique in plant ecology (see, e.g., ref *22*) or crop management, when different growing conditions and one or several species are compared. The interest in NIRS to detect differences in kernel $\delta^{18}O$ stems from the potential usefulness of $\delta^{18}O$ in maize breeding. It has been shown that $\delta^{18}O$ integrates the evaporative conditions throughout the crop cycle (*4*), and it has been proposed as a proxy method for assessing the environmental and genotypic differences related to drought resistance and yield potential in maize (*7*, *8*).

Besides the predictive ability of NIRS for determining N and ash contents and $\delta^{18}O$, its usefulness as a selection tool in maize breeding was also tested. **Figure 3** shows that NIRS not only was able to predict ash and N contents in leaf and kernel maize samples across water treatments but also clearly reflected genotypic differences within each water treatment. This can be observed with the highly significant relationships ($P < 0.001$) between the measured and the NIRS-predicted leaf and kernel traits (N and ash contents) within each water regime (WW, IS, and SS) across the 18 hybrid genotypes assayed (**Figure 3a**−**d**). Thus, according to our results, NIRS provides breeders with a powerful tool to select maize genotypes with desirable quality properties such as N and ash contents. Ash content in leaves and kernels and N content in leaves have been previously shown to be useful selection criteria for drought resistance and nitrogen use efficiency in maize hybrids (*7*), whereas increased kernel N content is a targeted trait in QPM maize breeding (*10*, *11*). On the other hand, although NIRS clearly reflected differences in kernel $\delta^{18}O$ across water regimes with adequate accuracy, genotypic differences within each growing condition were weakly predicted (**Figure 3e**). As explained before, the lower predictive ability of the models for $\delta^{18}O$ compared with the models for ash and N contents and the reduced range of variation in kernel $\delta^{18}O$ across (CV < 4.5%) and within each water regime (CV < 2.2%) may explain such results.

In addition, calibrations obtained for ash, N, and $\delta^{18}O$ of kernels for hybrids from the experiment conducted in 2007 were tested using grain from the experiment performed in 2008; ash and N contents and $\delta^{18}O$ were predicted in kernel samples from hybrids grown under three contrasting water regimens in 2008. Although no reference tests were performed in those kernel samples, the resulting NIRS-predicted traits (kernel ash and N contents and kernel $\delta^{18}O$) showed values that were in line with those obtained in 2007 (**Table 4**). NIRS-predicted kernel ash contents ranged between 1.2 and 2.6%, kernel N contents between 1.4 and 2.5%, and kernel $\delta^{18}O$ values between 29.5 and 34.0‰. Moreover, according to what is reported in the literature and according to the results obtained in 2007, $\delta^{18}O$ values decreased with increasing water availability (*4*, *7*−*9*), and the proportion of minerals (ash) and N in kernels increased with drought (*9*), showing clear differences between irrigated and water-limited treatments.

We conclude that NIRS can be used as a rapid, cost-effective, and sufficiently accurate method for predicting ash and N contents and for screening $\delta^{18}O$ in leaf and kernel maize samples. However, as clearly indicated by Foley et al. (*14*), NIRS is not supposed to replace IRMS for determining isotopic compositions of plant material; ultimately, data must be confirmed by IRMS. Nevertheless, owing to the high costs and time requirements of IRMS analyses, NIRS can be used efficiently in early generations of maize breeding programs when thousands of genotypes must be screened. This would help breeders to select maize genotypes with desirable leaf and kernel attributes, such as ash and N contents or $\delta^{18}O$ values, associated with superior grain quality and/or better yield performance and drought adaptation. Furthermore, one of the benefits of NIRS is the use of whole grains for measurements; this nondestructive technique is suitable for research when a limited amount of seed is available (*37*). Further calibrations using whole grain are needed to improve the applicability of NIRS to breeding. There is also growing interest in the field-level application of NIRS by use of hand-held spectroradiometers for in situ and in vivo determination of desirable plant traits (*2*).

ABBREVIATIONS USED

CV, coefficient of variation; $\delta^{18}O$, oxygen isotope composition; IRMS, isotope ratio mass spectrometry; NIRS, near-infrared reflectance spectroscopy; MPLS, modified partial least-squares; R^2_c, determination coefficient of calibration; R^2_v, determination coefficient of validation; RPD, ratio of performance deviation; SD, standard deviation; SEC, standard error of calibration; SECV, standard error of cross-validation; SEP, standard error of prediction.

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Supporting Information Available: Supplemental Tables 1 and 2 report the genotypic means for the leaf and kernel $\delta^{18}O$ and ash and N contents and the ANOVA in both hybrids and inbred lines, respectively. This material is available free of charge via the Internet at http://pubs.acs.org.

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