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Near-Infrared Reflectance Spectroscopy (NIRS) for Protein, Tryptophan, and Lysine Evaluation in Quality Protein Maize (QPM) Breeding Programs

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ABSTRACT: Quality protein maize (QPM) has approximately twice the tryptophan (Trp) and lysine (Lys) concentrations in protein compared to normal maize. Because several genetic systems control the protein quality of QPM, it is essential to regularly monitor Trp and/or Lys in breeding programs. Our objective was to examine the potential of near-infrared reflectance spectroscopy (NIRS) to enhance the efficiency of QPM research efforts by partially replacing more expensive and time-consuming wet chemistry analysis. More than 276 maize samples were used to develop NIRS models for protein content (PC), Trp, and Lys. The standard error of prediction (SEP) for the calibration and the coefficient of determination for validation (R^2_v) were 0.26 and 0.96 for PC, 0.005 and 0.85 for Trp, and 0.02 and 0.75 for Lys. When the NIRS models were used to evaluate 266 S2 lines from five QPM breeding populations, the coefficients of determination between NIRS and the chemical data were 0.94, 0.76, and 0.80 for PC, Trp, and Lys, respectively. Therefore, the NIRS models can be used to support the QPM breeding efforts.

KEYWORDS: NIRS, *Zea mays*, quality protein maize, tryptophan, lysine

INTRODUCTION

Traditionally, maize breeding programs have focused on increased stability and yield potential under abiotic and biotic stresses. In the past few years, biofortification programs designed to increase the nutritional quality of maize for human and animal consumption has received more attention.[1,2] Quality protein maize (QPM) has increased amounts of two essential amino acids, lysine (Lys) and tryptophan (Trp), and provides increased nutritional value for protein-deficient populations who depend upon maize as a staple food.[3−5] The development of biofortified maize with high provitamin A and zinc concentrations is also a priority in international programs, such as HarvestPlus.[2] Additionally, food and chemical industries are demanding more quality parameters in maize grain requirements. For example increasing protein or oil content will provide a more valuable product for livestock and poultry feeds. New industrial uses of maize grain, such as the conversion of starch to ethanol, have also introduced new quality requirements.[1,6] Thus, the physical and chemical characterization of grain is an important element of any modern maize breeding program.

Limitations of chemical analysis include costs, time, and method robustness. Most QPM breeding programs routinely monitor Trp and/or Lys concentrations in protein by colorimetric methods or high-performance liquid chromatography (HPLC).[7] The colorimetric method is less complex than HPLC but requires >20 h to complete analysis because of overnight digestion procedures. Labor needs and costs can be significant, especially when large numbers of samples are screened, as is necessary in the early stages of breeding programs.[7]

Near-infrared reflectance spectroscopy (NIRS) is a technique that combines spectroscopy and mathematics to rapidly produce indirect, quantitative estimates of concentrations of OH-, NH-, CH-, or SH-containing compounds. In comparison to wet chemistry procedures, NIRS requires none or simple sample preparation methods and is a rapid and relatively inexpensive technique that facilitates the analysis of several traits simultaneously.[8] Spectral data are correlated with biochemical components obtained by standard methods. However, NIRS is an indirect method that requires development and validation of calibrations by analysis of a large number of samples covering the range of variability for each trait and with more or less uniform distribution between extreme values.[9,10] Attempts to use NIRS for amino acid quantification in cereals date to 1978, when Rubenthaler and Bruinsma [11] successfully developed a NIRS calibration for the Lys content in wheat and barley. More recently, Fontaine et al.[12] developed NIRS calibrations for Lys and Trp, among other amino acids, using 258 and 156 temperate maize samples, respectively. The results showed that NIRS data were more accurate than amino acid estimation based on crude protein regression data, but the usefulness of these calibration curves was very limited, as indicated by a ratio of performance deviation (RPD) values lower than 3, and because the ratios between the standard deviation (SD) of the reference values and the standard error of prediction for the validation curves were not published.[1,12]

Multiple genetic systems control and modify the protein quality of QPM;[5,13] therefore, Trp or Lys monitoring is required to ensure and maximize genetic gain in breeding programs.[7,14] In addition, most QPM breeding programs focus on tropical or subtropical environments typical of many developing countries.

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Table 1. Reference Values, NIRS Calibration, and Cross-Validation Statistics for PC, Trp, and Lys in the Calibration Set of Maize

	N	range	mean	SD[a]	R^2_c[b]	SEC[c]	R^2_{cv}[d]	SECV[e]	RPD[f]
PC	276	7.8−15.1	11.4	1.2	0.93	0.31	0.78	0.32	3.79
Trp	756	0.02−0.12	0.07	0.01	0.87	0.005	0.73	0.006	2.61
Lys	424	0.20−0.59	0.36	0.07	0.93	0.01	0.77	0.02	3.34

[a] SD = standard deviation. [b] R^2_c = coefficient of determination in calibration. [c] SEC = standard error of calibration. [d] R^2_{cv} = coefficient of determination in cross-validation. [e] SECV = standard error of cross-validation. [f] RPD = ratio of performance deviation.

Our objective, therefore, was to develop and validate NIRS calibration curves for protein content (PC), Lys, and Trp for tropical and subtropical QPM germplasm.

MATERIALS AND METHODS

Plant Material and Sample Preparation. A total of 272 samples from the germplasm bank of the International Maize and Wheat Improvement Center (CIMMYT) were used for the development of NIRS calibrations. These samples included seed of landraces, inbred lines, open pollinated varieties (OPVs), and hybrids; samples were different in grain color (white, yellow, and blue), environmental adaptation (highland, tropical, and subtropical), and endosperm types (non-QPM and QPM). Additional samples from the active QPM breeding programs at CIMMYT were included to increase the range of values for developing the calibration curves for Lys (n = 221) and Trp (n = 480). In addition, 62 inbred lines and hybrids from CIMMYT QPM breeding programs for mid-altitude and tropical environments, in Africa and Mexico, were used for independent validations.

A total of 50−100 seeds were milled for each maize sample using a cyclotec mill (manufactured by Tecator Hoganes) with a 0.5 mm sieve. A sub-sample of the flour obtained from each sample was used for wet chemical analysis, and the remnant flour was used for NIRS analysis. The milled flour was stored in a dark area at room temperature in glass flasks.

Chemical Analysis To Obtain the Reference Values. The nitrogen content was determined using the Technicon Autonalyzer II Kjeldahl method (industrial method 334-74), and the PC was estimated using the conversion factor 6.25.[15] Lys determination in maize grain used the colorimetric method described by Tsai et al.[16] and Galicia et al.,[15] and Trp determination used the colorimetric method described by Nurit et al.[7] Each 10 samples was included as an internal standard to verify the accuracy of the methods. The standard error for laboratory values using those methodologies is 0.03, 0.04, and 0.01 for PC, Trp, and Lys, respectively.

NIRS Analysis. The milled material was scanned twice (2−3 g per sample) by NIRS monochromator model FOSS 6500 (FOSS NIRSystems, Inc., Silver Spring, MD) using small ring cups (internal diameter of 35 mm and depth of 8 mm). Spectra were collected between 400 and 2500 nm, registering the absorbance values log(1/R) at 4 nm intervals for each sample.

Mathematical Procedures for Calibrations and Validation. Calibration equations for PC, Trp, and Lys were developed using WinISI III software from Infrasoft International (FOSS NIRSystems, Inc., Silver Spring, MD). Calibration models were developed using modified partial least-squares (MPLS) regression and cross-validation techniques. Prior to the PLS regression, spectra were pretreated by applying a first-derivative transformation defined by 2,4,4,2 for PC and 1,4,4,1 for Trp and Lys, where the first number is the degree of the derivative, the second number is the gap between data points for subtraction, and the third and fourth numbers are the data points used for smoothing.[17] The results of the calibration calculation were monitored by checking the t outliers with t > 2.5, GH, and X outliers >10; samples with t > 2.5 were deleted from the sample file.[17] About 2% of the samples was left out for PC, and 5% was left out for Trp and Lys.

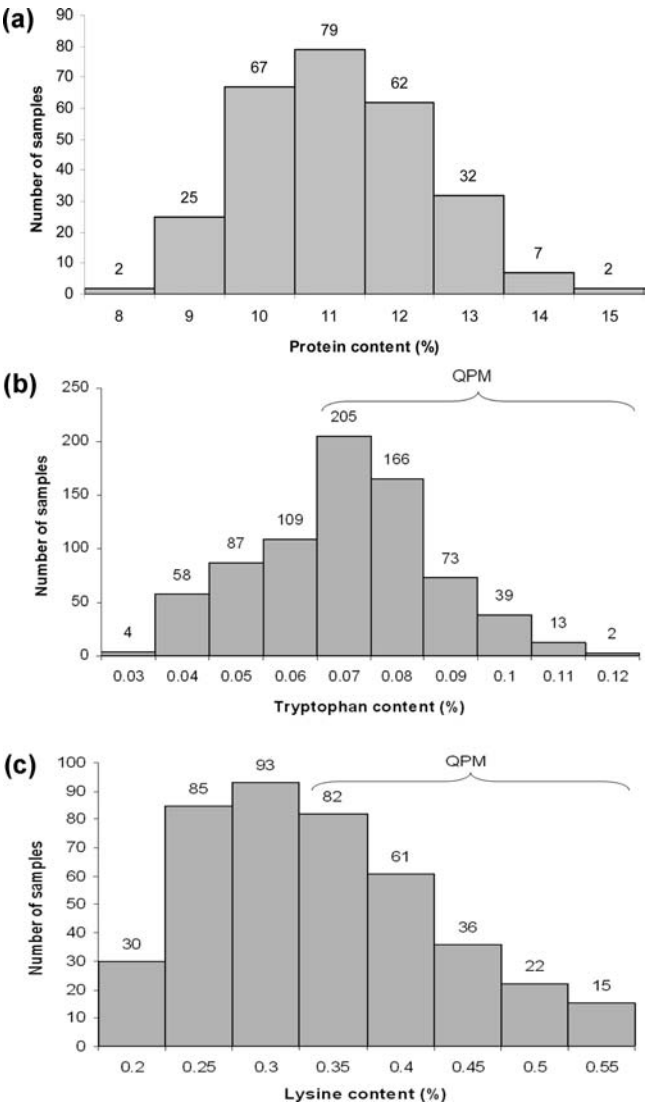

Figure 1. Distribution of the (a) PC (n = 276), (b) Trp content (n = 756), and (c) Lys content (n = 424) from laboratory analyses.

The SD between NIRS and reference determinations for the calibration [standard error of calibration (SEC)] and validation sets [standard error of prediction (SEP)] were calculated. We also calculated the coefficient of determination of calibration (R^2_c) and the coefficient of determination of validation (R^2_v) (the fraction of the variance of the reference values explained by the variance of NIRS determinations).

The RPD was calculated as the ratio between the SD of the reference value and standard error of cross-validation (SECV). RPD is indicative

Table 2. Reference Values and External Validation Statistics of the NIRS Calibrations for PC and Trp and Lys Contents in Maize

	N	range	mean	SD[a]	R^2_v [b]	SEP[c]	SD/SEP
PC	62	7.8−12.6	9.8	1.3	0.96	0.26	6.30
Trp	62	0.05−0.11	0.08	0.01	0.85	0.005	2.60
Lys	48	0.20−0.41	0.27	0.04	0.75	0.02	2.14

[a] SD = standard deviation. [b] R^2_v = coefficient of determination in validation. [c] SEP = standard error of prediction.

of the usefulness of the NIRS calibrations. If this ratio exceeds a value of 3, the calibration equation is very meaningful, whereas for values below 2, its applicability is limited.[12,17] In addition, we determined the ratio between the SD and the SEP of each trait because the quality and robustness of a NIRS calibration can also be judged by the SEP and SD/SEP; a SD/SEP less than 2 indicates an unsuitable calibration.[1]

QPM Germplasm Screening. PC and Trp and Lys contents were estimated by wet chemistry and using the developed NIRS calibrations for three sets (N = 72, 86, and 21) of S2 lines derived from QPM × QPM crosses and two sets (N = 42 and 45) derived from non-QPM × QPM crosses from the tropical lowland breeding program of CIMMYT. Each S2 line consisted of the bulked seed derived from self-pollinating a single F2 plant. All samples were white maize and were grown at the Agua Fria experiment station of CIMMYT in Puebla, Mexico (20° 27′ 18.3″ N/97° 38′ 28.8″ W). A total of 50−100 seeds of each sample (S2 line) were visually selected using a light box to ensure an appropriate endosperm modification level for QPM by excluding grains with completely opaque or normal endosperm phenotype.[14] The selected grains were subsequently ground, and the milled flour samples were divided into subsamples for NIRS scanning and chemical analyses.

One factor analysis of variance (ANOVA) was used for a statistical comparison of the values obtained by NIRS and wet chemistry.

RESULTS AND DISCUSSION

Reference Sample Distribution. The reference values in the calibration set for PC, Lys, and Trp were distributed over a wide range (Table 1 and Figure 1) because both normal and QPM maize samples were included; QPM has more than 0.07% Trp and more than 0.34% Lys. The wide range of PC values is also explained by the use of samples from different locations, including farmers' fields where different agronomic crop management practices were applied. The coefficients of variation were 11, 24, and 22% for PC, Lys, and Trp, respectively, which were small and thereby contributed to the robustness of the calibrations.

NIRS Calibration Development. NIRS calibration equations, developed on the basis of 276, 756, and 424 samples for PC, Trp, and Lys, respectively, had high coefficients of determination for calibrations (R^2_c = 0.87−0.93) and slightly lower coefficients of determination for cross-validations (R^2_{cv} = 0.73−0.78) (Table 1). However, the difference between R^2_c and R^2_{cv} was minor, indicating that the calibrations were homogeneous. The SEC and SECV were small (SECV/mean = 2.8, 8.2, and 6.3% for PC, Trp, and Lys, respectively). On the basis of the RPD values, all calibrations can provide meaningful estimates of PC, Trp, and Lys for breeding programs (Table 1).

Independent Validation. An independent validation of the calibrations was performed using 62 samples for PC and Trp and 48 samples for Lys from the tropical and subtropical breeding programs of CIMMYT in Mexico and Ethiopia. The coefficients of determination for independent validation (R^2_v) were larger than those measured for the cross-validations (Tables 1 and 2). The SD/SEP ratio for PC was 6.3, indicating excellent quality of the calibration. For Trp and Lys, SD/SEP ratios were between 2 and 3, indicating that the calibrations were satisfactory (Table 2). Considering both the SD/SEP ratios and the R^2_v values, our results show that reliable selection for PC, Trp, and Lys is possible by NIRS.

Comparison of NIRS Calibrations with Protein Regression. The concentrations of most amino acids in grain are strongly correlated with crude PC, which implies that most of them could be estimated from reference protein values. We therefore calculated the regressions for Trp and Lys with protein (Figure 1) and compared these to the NIRS calibrations. The coefficients of determination for the linear regressions of Trp and Lys to PC were quite small for the 276 samples used in the calibration set (Table 3); Lys versus PC had a R^2 of 0.13, and Trp versus PC had a R^2 of 0.04. When samples were divided into QPM and non-QPM according to their protein quality, the R^2 values of the linear regression for Trp and PC and Lys and PC were much larger (although still too small to be of practical use) for the QPM than for the non-QPM samples (Table 3).

Estimation of PC and Trp and Lys Contents in QPM Breeding Samples. Means and ranges of the values obtained for PC, Trp, and Lys by NIRS and chemical analyses for the five sets of S2 lines are presented in Table 4. The correlation between the PC of reference (RD) and NIRS data was very strong (average R^2 = 0.94), with no significant variation of PC observed among populations ($p < 0.0001$).

The overall R^2 for Trp measured by chemical analysis relative to NIRS methods was 0.76; however, correlations between RD and NIRS were significantly larger for the S2 of the non-QPM × QPM populations (R^2 = 0.88 and 0.91) compared to the S2 of the QPM × QPM populations (R^2 = 0.57, 0.70, and 0.77) (Table 4). Estimates of Trp by NIRS were significantly smaller ($p < .0001$) than estimates obtained by the colorimetric method; however, this does not affect the ranking of the breeding samples.

A R^2 of 0.80 was obtained for Lys values estimated by NIRS and RD obtained by the colorimetric method. Similar to Trp, the correlations between estimates by the two methods were smaller for S2 of QPM × QPM compared to S2 of non-QPM × QPM (Table 4). More importantly, no statistically significant difference was observed between means estimated by the two methods.

Breeding programs devoted to developing QPM are being implemented in several countries around the world,[5] and these programs require robust, fast, and inexpensive laboratory methods to screen for PC, Lys, and/or Trp. We recently developed a colorimetric method for Trp determination, which has proven to be very useful where laboratories with spectrophotometer analysis are in place.[7] The NIRS calibrations described herein for PC, Lys, and Trp offer a fast and simple screening option because extractions and chemical reactions are not required. However, their application will be limited to laboratories where NIRS equipment and software are available. Use of NIRS for Trp

Table 3. Coefficient of Determination for Linear Regression of Amino Acids to Crude Protein in the Validation Sample Set

	all samples (n = 276)			QPM samples (n = 97)			non-QPM samples (n = 179)		
	Trp	Lys	PC	Trp	Lys	PC	Trp	Lys	PC
Trp		0.82	0.04		0.73	0.33		0.51	0.11
Lys			0.13			0.57			0.21

Table 4. Means and Ranges of PC and Trp and Lys Contents in Five Maize Populations

			mean ± SD; range			mean ± SD; range			mean ± SD; range		
breeding material		n	% PC (C)[a]	% PC (N)[b]	R^2[c] (% PC)	% Trp (C)	% Trp (N)	R^2 (% Trp)	% Lys (C)	% Lys (N)	R^2 (% Lys)
S2 QPM × QPM	population 1	72	9.9 ± 1.2; 7.9−13.6	9.9 ± 1.4; 7.5−13.7	0.92	0.09 ± 0.01; 0.07−0.12	0.08 ± 0.01; 0.06−0.12	0.7	0.38 ± 0.04; 0.26−0.44	0.36 ± 0.05; 0.24−0.5	0.65
	population 2	86	9.7 ± 1.1; 6.9−12.5	9.8 ± 1.2; 6.8−12.8	0.92	0.11 ± 0.01; 0.08−0.13	0.08 ± 0.01; 0.06−0.09	0.57	0.33 ± 0.04; 0.26−0.44	0.36 ± 0.05; 0.25−0.46	0.73
	population 3	21	8.5 ± 0.9; 6.9−10.2	8.8 ± 0.8; 6.9−10.5	0.94	0.09 ± 0.02; 0.06−0.13	0.07 ± 0.01; 0.05−0.09	0.77	0.34 ± 0.05; 0.24−0.44	0.34 ± 0.05; 0.24−0.42	0.89
	population 4	42	10.4 ± 0.9; 7.3−11.9	10.3 ± 1; 7.1−11.9	0.92	0.10 ± 0.02; 0.06−0.14	0.08 ± 0.01; 0.05−0.11	0.88	0.37 ± 0.07; 0.24−0.49	0.37 ± 0.06; 0.24−0.45	0.88
S2 non-QPM × QPM	population 5	45	9.6 ± 1.7; 7.4−14.7	9.6 ± 1.8; 7.0−13.9	0.98	0.11 ± 0.02; 0.06−0.15	0.09 ± 0.01; 0.07−0.12	0.91	0.40 ± 0.07; 0.25−0.57	0.39 ± 0.07; 0.28−0.54	0.87

[a] C = data obtained by chemical analysis. [b] N = data obtained by NIRS. [c] R^2 = correlation between NIRS and chemical data.

Table 5. Costs and Time Comparison between the NIRS and Glyoxylic Acid Methods[7] for Trp Determination in Maize Grains[a]

		glyoxylic acid method	NIRS	costs (U.S. $)[b]
sample preparation	grinding	2 min	2 min	2
	deffating[c]	10 h	0 h	1.8
protein hydrolysis[c]		18 h	0 h	1
sample analysis	chemical reaction[c] and calculations	1 h	0 h	2
	sample scanning in NIRS	0 h	1 min	0.5

[a] Estimates are for analysis of one sample. [b] Costs from the maize nutritional quality laboratory at CIMMYT. [c] Time invested includes sample aliquoting and sample cooling time.

analysis is inexpensive and fast; Trp analysis by wet chemistry is 3 times more expensive and requires 19 h compared to 3 min using NIRS (Table 5). However, use of NIRS requires a substantial investment in equipment, which depending upon the manufacturer can range from U.S. $60 000 to U.S. $100 000. If NIRS is used in large breeding programs, where thousands of samples are analyzed per year or where multiple traits can be analyzed by NIRS, then the equipment will pay for itself in a short period of time.

Fontaine et al.[12] developed NIRS calibrations for amino acids in maize, including Trp and Lys, with R^2_v of 0.86 and 0.72, respectively; however, as they concluded, the applicability of those calibrations is limited because of the very low RPD values. Using a large and diverse set of samples for calibration (Table 1) and validation chemical analysis methods,[7,15] we have developed and present robust calibrations for Trp, Lys, and PC screening of QPM, with RPD values greater than 2.6 and SD/SEP for the validations greater than 2.1. Samples used to develop the calibrations included a wide range of material from the germplasm collection at CIMMYT, ensuring broad applicability of the NIRS models developed. Additionally, when we used those calibrations to evaluate QPM breeding samples, the data obtained were precise and indicated that NIRS can be confidently used for screening the thousands of samples that must be evaluated every cycle in typical breeding programs (Figure 2 and Table 4). Because NIRS allows for simultaneous estimation of PC, Trp, and Lys, breeders can select genotypes with protein quality of specific or overall greatest interest. We recommend verifying the accuracy of extreme values by chemical analysis, especially for very advanced breeding material.

In wheat, barley, triticale, maize,[12] and soybean,[18] most of the amino acid concentrations are strongly correlated with crude protein, which implies that the content of most of them could be derived from known reference protein values. However, this correlation depends upon not only the levels of the amino acid of interest but also the set of samples studied, and in general, the accuracy of the correlation method is low.[18] Because QPM involves genetic changes to the typical protein profile of maize, it is not surprising that PC should fail to consistently predict Trp or Lys concentrations. In maize, Fountaine et al.[12] found a RSQpc (fraction of explained variance for linear crude protein regression) of 0.93 for leucine (Leu) and isoleucine (Ile), while

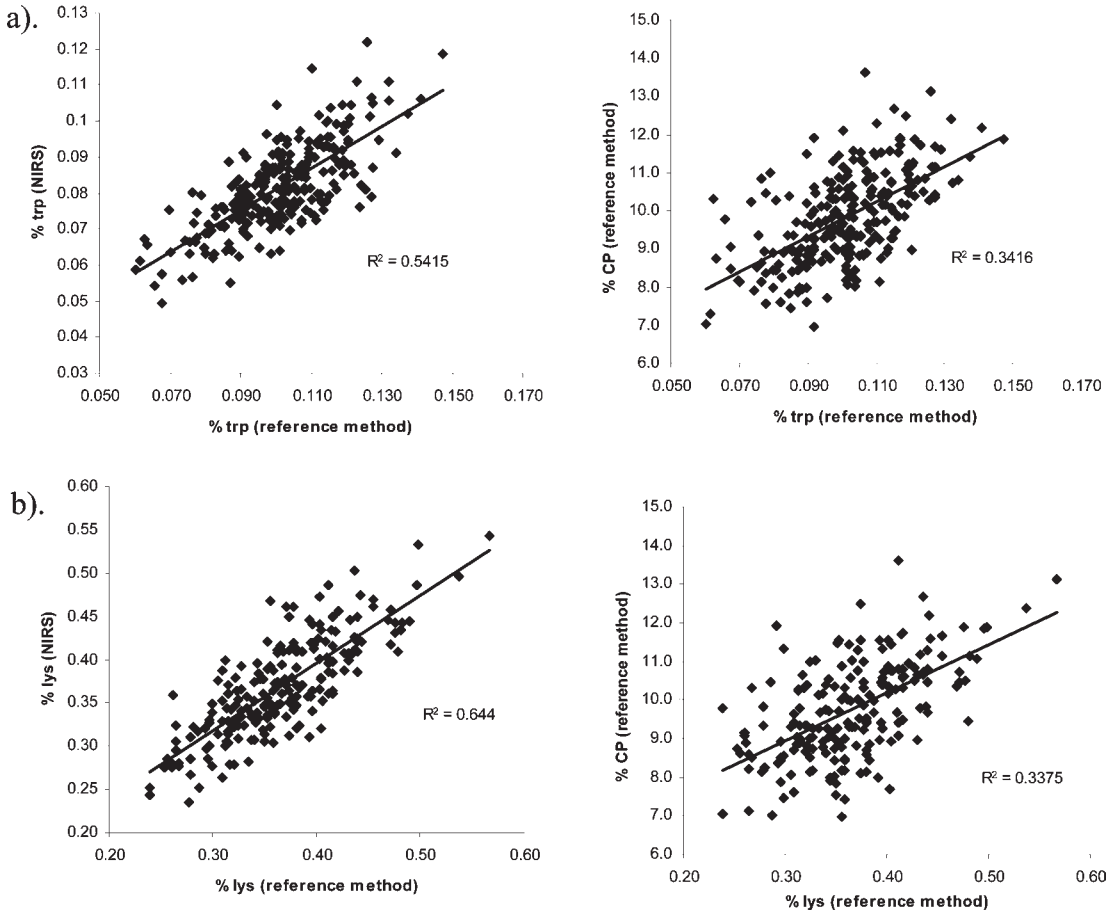

Figure 2. (a) Trp and (b) Lys regressions to NIRS and PC. Data for 266 samples from five different S2 populations from a breeding program.

for Trp or methionine (Met), the RSQpc was about 0.53. In the set of samples used for independent validation in this study, the correlations for PC with Trp and Lys were very small (Table 3).

Although there is generally a high correlation between Trp and Lys concentrations in maize protein, we have observed a substantial range among different populations (R^2 from 0.75 to 0.99; Natalia Palacios-Rojas, unpublished data). However, for QPM breeding strategies based on wet chemistry evaluation of the amino acid content, it has been widely recommended to monitor only Trp and PC during the course of the breeding, not only to save time and resources but also because the colorimetric method for Lys is more laborious, expensive, and time-consuming than the Trp colorimetric method.[14] When thousands of samples have to be screened for PC, Trp, and/or Lys, preferably between harvest and planting or between planting and flowering, to reduce plant numbers and labor for pollinations in breeding nurseries, the time that it takes to analyze these compounds by wet chemistry is a key limitation. Use of NIRS makes selection before planting much more feasible, saving time and resources; screening S2 nurseries before planting could reduce total QPM breeding costs by up to 20% in the lowland tropical QPM breeding program of CIMMYT (Gary Atlin, personal communication).

The PC, Trp, and Lys calibrations presented here have many applications in QPM breeding programs, including conversion of non-QPM germplasm to QPM, pedigree breeding, maintaining QPM populations, and monitoring seed quality during production of QPM seed. Use of NIRS, provided that suitable calibrations are available or can be developed, can efficiently broaden the set of compounds monitored in germplasm improvement activities, including facilitating broader biofortification objectives.[2]

Current QPM breeding schemes at CIMMYT and national programs typically evaluate PC and Trp at S2 and S6 stages of inbreeding, whereas availability of NIRS makes it efficient to screen germplasm at additional breeding stages, thereby reducing the costs of advancing lines with inadequate protein quality. Development of single-seed NIRS methods for screening PC, Trp, and Lys[19,20] would further enhance the efficiency of QPM breeding and remains an objective in our research laboratory at CIMMYT.

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