Published March, 2007

RESEARCH

Quantitative Determination of Phytate and Inorganic Phosphorus for Maize Breeding

Aaron J. Lorenz,* M. Paul Scott, and Kendall R. Lamkey

ABSTRACT

Phytate is the dominant storage form of phosphorus (P) in mature cereal and oil grains. Phosphorus bound in phytate is nutritionally unavailable to monogastric animals and thus contributes to water pollution because it is excreted in the waste. Also, phytate can chelate certain minerals and exacerbate human mineral deficiencies. Our primary objective was to develop a rapid and inexpensive method of measuring phytate and inorganic P (P_i) concentrations in maize (*Zea mays* L.). The procedure reported herein was derived from previously published assays and used to screen 50 inbred lines to determine its potential in a selection program. Grain yield, protein, oil, methionine, lysine, tryptophan, and kernel weight were also measured. Field repeatability values for phytate and P_i (0.78 and 0.91, respectively) suggest that our protocol can be used to make heritable measurements on both traits. Phytate measurements taken with the procedure reported herein matched closely those obtained through ion exchange. The combination of adequate precision and simplicity make this method ideal for breeders interested in improving P_i and phytate levels simultaneously. The positive phytate:protein correlation reported commonly was also detected in this study. A relationship between phytate and kernel weight indicates that selection for low phytate may result in larger kernels.

A.J. Lorenz, Dep. of Agronomy, Univ. of Wisconsin–Madison, Madison, WI 53706; M.P. Scott, USDA-ARS, Dep. of Agronomy, Iowa State Univ., Ames, IA 50011; K.R. Lamkey, Dep. of Agronomy, Iowa State Univ., Ames, IA 50011.This journal paper of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project No. 3755, was supported by the Hatch Act and State of Iowa funds. Part of a dissertation submitted by A.J. Lorenz in partial fulfillment of the requirements for the M.S. degree. Received 17 Mar. 2006. *Corresponding author (alorenz@wisc.edu).

Abbreviations: CV, coefficient of variation; CV_G, genetic coefficient of variation; *lpa*, low phytic acid; NSS, non–stiff stalk; OD, optical density; P_i, inorganic phosphorus; r_F, field repeatability; r_L, lab repeatability; RCBD, randomized complete block design; SS, stiff stalk.

In mature seeds, typically 65 to 80% of total P is in the form of phytic acid (*myo*-inositol-1,2,3,4,5,6-hexakiphosphate), which can make up one to several percent of the seed dry weight (reviewed in Raboy, 2002). Nearly all the phytic acid found in mature seeds is bound to minerals such as K^+, Mg^{2+}, Ca^+, Zn^+, Ba^{2+}, and Fe^{3+} to form a mixed cation salt known as phytate (reviewed in Lott et al., 2000). Inorganic P and cellular P (a component of cellular membranes, DNA, RNA, etc.) are other forms of P in seeds and are generally referred to as "available P" (Raboy, 2001).

Lott et al. (2000) estimated that 65% of the elemental P sold and applied as fertilizer worldwide is sequestered into harvested crop seeds and fruits as phytic acid. Cereal grains and oil seeds have particularly high concentrations of phytate (O'Dell, 1972), which is known to negatively impact the environment as well as human and animal nutrition. Because monogastric animals such as swine and poultry lack the enzyme phytase, most of the P in their maize-based diets passes in their feces onto the landscape, where it contributes to surface water eutrophication (Sharpley et al., 1994). The bioavailability of P in maize-based diets is only 15% (Cromwell and Coffey, 1991), which results in P supplementation to meet

Published in Crop Sci 47:600–606 (2007).
doi: 10.2135/cropsci2006.03.0177

677 S. Segoe Rd., Madison, WI 53711 USA

animal nutritional requirements. Also, as a strong chelator of nutritionally important minerals such as iron and zinc (Erdman, 1981), phytate contributes to human micronutrient deficiencies in developing countries (Raboy, 2002).

Many of the problems associated with P in maize grain are not due to the concentration of total P per se, but rather to the fact that most of the P is bound in phytate (Raboy, 2001). Therefore, it would be desirable to increase the amount of available P and reduce the amount of phytate in maize grain. Low phytic acid (*lpa*) mutant lines that have greatly reduced amounts of phytate, and concomitant increases in P_i have been developed in a variety of crop species, including maize, and have provided valuable information regarding the biochemical, physiological, and nutritional roles of phytate (Raboy et al., 2001; Raboy, 2002). However, slight yield reductions among these *lpa* lines have been reported in maize (Ertl et al., 1998), and germination problems have been observed in other crops (Hulke et al., 2004). Another approach would be to develop a selection program designed to decrease phytate and increase P_i simultaneously. The success of modification through selection depends on the ability to differentiate germplasm by the measurement of traits in a high-throughput, inexpensive, and precise manner that can be incorporated into normal breeding operations. Methods have been developed for the measurement of phytate in plant material, grain samples, and feedstuffs, but most are expensive or time-consuming, requiring heating, cooling, washing, and multiple rounds of centrifuging. Inorganic P assays have been mainly used to qualitatively identify *lpa* mutants, but quantitative determinations of P_i for a large set of normal maize lines have not been reported.

It was our objective to design and report an assay for the simple and quantitative measurement of phytate and P_i in ground maize kernels from field plots. We used this protocol to estimate phytate and P_i concentrations in kernels from 50 inbred lines grown in the summer of 2004. Phytate measurements were validated by selecting a subset of samples and sending them to NP Analytical Laboratories (St. Louis, MO) for phytate determination by ion exchange chromatography. Yield, kernel weight, protein, oil, lysine, tryptophan, and methionine were also measured. The coefficient of variation (CV), genetic coefficient of variation (CV_G), and repeatability for each trait and phenotypic correlations between traits were calculated. This study provides insight into the possibility of simultaneously modifying amounts of phytate and P_i through selection, provides estimates of phenotypic correlations that may be important during selection, and determines differences between inbred backgrounds with respect to the traits of interest. A detailed description of the experimental design, lab protocol, and analysis of phytate and P_i will be given.

MATERIALS AND METHODS

Genetic Material

A set of 50 inbred lines representing the Iowa lines from B73 to B129, 4 North Carolina lines, 1 Nebraska line, Mo17, and 10 unreleased lines were included in this study and are presented in Table 1. Twenty entries in this group represent stiff-stalk (SS) and 30 entries represent non-stiff-stalk (NSS) germplasm.

Field Design and Sampling

The 50 lines were grown in a randomized complete block design (RCBD) with two replications at Ames, IA, during the summer of 2004. Plots consisted of two rows 5.49 m long with 0.76 m between rows. The plots were overplanted and thinned to a uniform plant density of 62,140 plants ha^{-1}. The lines were allowed to open pollinate, and both rows were hand-harvested to estimate yield and collect grain samples. An approximately 454-g grain sample was taken from each field plot and stored. A subsample of 30 whole kernels was taken from each field plot sample and milled so that 70% of the millings passed through an 80-μm mesh screen.

Traits

Yield was estimated by hand-harvesting both rows, drying the ear grain down to 15% moisture, weighing the shelled grain, and converting to Mg ha^{-1}. Protein and oil contents were predicted by near-infrared reflectance of whole grain in the Iowa State Grain Quality Laboratory. Kernel weight was represented as the mass of 250 whole kernels. Amino acids were quantified using a microbial assay (Scott et al., 2004). Protein in 10-mg grain samples was extracted and hydrolyzed in wells of a 96-well plate with 0.2 mg mL^{-1} pepsin in 200 *mM* KCl pH 2.0. This extract was then used to supplement M9 minimal media (Sambrook and Russel, 2001) inoculated with a strain of *Escherichia coli* auxotrophic for the amino acid being measured. For methionine, strain P4X (Jacob and Wollman, 1961) was used, for tryptophan strain CAG18455 (Singer et al., 1989) was used, and for lysine strain KL224 (Birge and Low, 1974) was used. Bacteria were grown until the culture reached a plateau in cell density, which was quantified by the scattering of 595-nm light. Standards of pure amino acids were used to verify that the amount of extract used fell into the linear range of the assay and to convert optical density values into amino acid concentrations. Each sample was measured in triplicate.

Measurement of Phytate and P_i

The colorimetric assays of Vaintraub and Lapteva (1988) and Raboy et al. (2000) were modified and used to quantify relative phytate and P_i concentrations per sample, respectively. Three analytical replications per field block were used where each replication contained all 50 plots in a field block along with 10 standards on a 96-well plate randomized in a row–column lattice design. Ten milligrams (±0.2 mg) ground whole kernels from each subsample was placed in assigned wells, and 200 μL of 0.65 *M* HCl was added to each well. The 96-well plates were shaken at room temperature overnight (~12 h) and then centrifuged at 3000 rpm for 20 min.

Two additional 96-well evaluation plates were used to measure phytate and P_i from the samples in each extraction plate. Thirty microliters of extract was transferred to each evaluation plate,

Table 1. Means for yield, inorganic phosphorus (P_i), phytate, methionine (M), lysine (K), tryptophan (W), protein, oil, and kernel weight of 50 maize lines. Bottom three rows are the stiff stalk (SS), non-stiff stalk (NSS), and experiment means, respectively.

Line	Heterotic group	Yield	P_i	Phytate	M	K	W	Protein	Oil	Kernel weight†
		Mg ha^{-1}				g kg^{-1}				g 250K^{-1}
B73	SS	5.82	0.26	3.27	1.30	1.18	0.77	80.0	38.5	70.0
B84	SS	4.60	0.28	3.40	1.41	1.42	0.72	86.5	33.0	71.9
B90	NSS	2.57	1.06	3.31	1.30	1.32	1.14	113.5	37.5	73.5
B91	NSS	3.81	0.53	3.26	1.05	1.17	0.91	102.5	37.0	61.9
B97	NSS	5.25	0.94	3.38	0.87	1.21	0.75	92.0	36.0	82.2
B99	NSS	3.54	0.67	3.45	1.08	1.02	0.78	102.5	33.5	61.7
B100	NSS	4.36	0.83	3.46	1.24	1.39	0.84	95.0	38.5	65.9
B101	SS	3.30	0.24	3.39	1.65	2.15	0.91	101.5	38.0	56.0
B102	NSS	4.41	0.76	3.51	1.20	1.18	0.76	96.0	39.0	67.5
B103	NSS	3.49	0.51	3.37	1.39	1.64	0.81	96.0	38.0	71.6
B104	SS	6.17	0.86	2.40	1.13	0.86	0.75	78.0	34.0	79.9
B105	SS	5.44	0.18	3.71	1.29	1.72	0.81	95.0	46.0	56.2
B106	NSS	6.30	0.62	3.55	0.86	1.39	0.69	91.5	36.5	60.5
B107	NSS	3.25	0.56	3.40	1.12	1.50	1.06	91.0	37.0	72.4
B108	NSS	5.22	0.60	3.58	1.16	1.00	0.74	100.0	35.5	60.8
B109	SS	6.71	0.32	3.22	1.48	1.23	0.76	87.0	38.0	68.4
B110	SS	6.52	0.34	2.80	1.07	1.13	0.76	85.4	35.5	67.2
B111	SS	5.76	0.23	3.48	1.25	1.24	0.85	84.5	36.5	64.5
B112	NSS	5.52	0.77	2.89	1.08	1.60	0.78	81.5	35.0	65.6
B113	NSS	4.25	0.78	3.27	1.20	1.71	0.64	98.0	34.5	65.4
B114	NSS	5.33	0.73	3.38	1.18	1.60	1.04	87.0	37.0	73.8
B115	NSS	2.87	0.89	3.25	1.31	1.40	0.92	108.5	43.0	76.8
B116	NSS	7.71	0.99	3.16	1.00	1.55	0.75	99.0	35.0	81.1
B117	NSS	4.90	0.66	2.94	1.09	0.96	0.71	105.5	35.5	77.7
B118	NSS	4.90	0.77	3.44	0.95	1.07	0.76	102.5	37.0	78.4
B119	SS	5.66	0.23	3.39	0.86	1.30	0.95	72.6	37.1	70.5
B120	NSS	4.84	0.52	3.23	1.40	1.11	0.87	93.5	34.5	66.8
B121	NSS	7.44	0.38	2.92	1.16	0.84	0.69	78.5	36.0	71.2
B122	NSS	5.41	0.94	2.82	1.19	1.42	0.76	93.5	36.0	65.9
B123	NSS	3.54	0.88	4.09	1.25	1.32	0.82	109.0	39.0	43.2
B124	NSS	5.03	0.87	3.26	1.28	1.08	0.69	102.6	36.0	59.0
B125	NSS	3.63	0.69	3.44	1.50	1.09	0.75	97.0	35.5	65.4
B126	SS	3.90	0.85	3.76	1.34	1.78	0.66	118.5	31.5	73.2
B127	SS	6.01	0.45	3.02	1.17	1.24	0.80	87.5	32.5	73.6
NC394	NSS	1.43	0.86	3.39	1.02	1.20	0.81	93.5	38.5	60.4
B129	SS	6.01	0.63	3.05	1.17	1.42	0.70	89.5	34.5	79.8
N196	SS	3.65	0.53	2.74	1.35	1.28	0.81	91.0	38.5	67.0
Mo17	NSS	5.44	0.55	3.30	0.65	1.73	0.84	96.5	35.0	79.9
B73/B89‡	SS	6.06	0.54	2.94	1.28	0.98	0.72	78.5	38.5	74.0
BS31(*R*)C0‡	SS	4.71	0.44	3.53	1.42	1.02	0.69	96.5	36.0	55.7
BSKRL1(HI)C1‡	SS	4.95	0.58	3.20	1.13	1.20	0.59	81.4	32.9	79.9
BSKRL1(HI)C1‡	SS	7.41	0.34	2.85	0.92	1.31	0.79	70.6	36.1	67.9
BS32(*R*)C0‡	NSS	3.57	0.58	3.15	1.08	1.25	0.67	99.0	46.0	72.2
BSKRL2(H1)C1‡	NSS	4.52	0.51	3.36	1.12	1.12	0.80	102.5	36.0	47.6
NC386	NSS	4.82	0.41	3.16	1.01	0.89	0.66	78.5	42.0	64.1
B97/B99‡	NSS	5.87	0.79	2.80	0.98	1.12	0.69	89.5	36.0	67.3
BS13(S)C8‡	SS	9.14	0.44	2.96	0.97	0.95	0.87	70.0	38.0	65.6
NC376	SS	2.84	0.58	3.63	1.21	1.23	0.74	89.5	34.0	71.6
BSCB1(*R*)C12‡	NSS	3.54	0.39	3.39	1.53	1.29	0.81	116.0	36.0	65.6
NC432	SS	7.12	0.32	2.94	1.38	1.07	0.72	75.5	38.0	65.2
SS§		5.57*	0.43**	3.18	1.24	1.29	0.77	86**	36.0	68.9
NSS§		4.55*	0.70**	3.29	1.14	1.27	0.80	97**	37.0	67.5
Mean		4.98	0.59	3.25	1.18	1.28	0.79	93.0	37.0	68.1

*Significant at the 0.05 probability level.

**Significant at the 0.01 probability level.

†Kernel weight units are grams per 250 whole kernels.

‡Uncoded inbred lines.

§Traits that exhibited significant differences between genetic background means are indicated.

maintaining randomized sample position. Equal volumes of the P_i or phytate quantitative standards were placed in assigned wells. Phytic acid dodecasodium salt from corn (Sigma P-8810) and KH_2PO_4 (Sigma P-5379) were used for the phytate and P_i standards, respectively. Stock solution of the phytate standard was prepared by dissolving 10 mg per 1 mL of 0.65 *M* HCl. Stock solution of P_i was prepared by dissolving 110 mg KH_2PO_4 in 250 mL of 0.65 *M* HCl. For the measurement of P_i, 130 μL distilled deionized H_2O and 100 μL P_i reagent were added to each well. The P_i reagent was made immediately before use and consisted of 2 parts deionized H_2O, 1 part 0.02 *M* ammonium molybdate, 1 part 0.57 *M* ascorbic acid, and 1 part 3 *M* sulfuric acid. A blue color developed after 15 to 20 min at room temperature, at which time the optical density at 820 nm (OD_{820}) was measured using a 96-well spectrophotometer. For the measurement of phytate, 200 μL Wade reagent was added to each well and allowed to react for 15 min at room temperature, at which time OD_{490} was measured. Wade reagent could be stored in a refrigerator for 1 mo and consisted of 2.5 g 5-sulfosalycyclic acid, 0.25 g $FeCl_3 \cdot 6H_2O$, and 150 mL deionized H_2O. The above solution was refrigerated overnight and adjusted to a pH of 3.05 with NaOH the following day. After pH adjustment, deionized H_2O was added for a final volume of 200 mL. This method of phytate measurement will be hereafter referred to as "modified Wade assay." Phytate was converted to phytate P by dividing phytate by 3.55 (Raboy and Dickinson, 1984).

Three groups comprising three entries each were identified as having low, intermediate, and high phytate concentrations according to the modified Wade assay. Both field plot samples of each selected entry were sent to NP Analytical Laboratories (St. Louis, MO) for phytate analysis using their ion exchange method. This method consists of chromatographic separation of phosphorus-containing compounds, which are then quantified by hydrolysis and colorimetric measurement of the resulting phosphorus following reaction with ammonium molybdate. All phytate values reported in this paper actually represent phytate P values. Phytate was converted to phytate P by dividing the measured values of phytate by 3.55 (Raboy and Dickinson, 1984).

Table 2. Phytate values of lines selected to represent low, intermediate, and high levels of phytate concentration based on the modified Wade assay. Pearson's correlation coefficient between the two methods of phytate measurement was 0.95.

Line	Group	Phytate	
		Wade assay†	Ion exchange‡
		g kg^{-1}	
B104	Low	2.40	1.39
N196	Low	2.74	1.80
B110	Low	2.80	1.79
B113	Intermediate	3.27	1.92
B73	Intermediate	3.27	1.94
Mo17	Intermediate	3.30	2.15
B105	High	3.71	2.45
B126	High	3.76	2.61
B123	High	4.07	2.70
LSD (0.05)		0.32	0.30
	Group mean		
	Low	2.65	1.71
	Intermediate	3.28	2.01
	High	3.79	2.59

†Modified assay of Vaintraub and Lapteva (1988).

‡Measurements performed by NP Analytical Laboratories (St. Louis, MO).

Statistical Analysis

The data set from this experiment consisted of two levels, laboratory and field plot data, and outliers were removed at each level according to Anscombe and Tukey (1963). Phytate, P_i, lysine, methionine, and tryptophan data were analyzed statistically at the laboratory level and reanalyzed at the field plot level along with yield, protein, oil, and kernel weight data. Predictions of P_i, phytate, lysine, methionine, and tryptophan concentrations were made using a single standard curve within each field block. Least-squares means of kernel constituent concentrations were estimated for each field plot and subsequently treated as single plot measurements.

To estimate the laboratory repeatability for these traits, a completely random model was fit, and repeatability (r_L) was calculated as $r_L = \sigma_L^2 / [\sigma_L^2 + (\sigma_e^2 / r)]$, where r is the number of lab replications per field block ($r = 3$), σ_L^2 is line variance, and σ_e^2 represents error variance at the laboratory level.

Data on all traits in this experiment at the field plot level were analyzed as a RCBD with both line and replication fit as random effects. Field plot repeatability was estimated as $r_F = \sigma_L^2 / [\sigma_L^2 + (\sigma_e^2 / r)]$, where r is the number of field replications ($r = 2$). Variance components were estimated at the field plot level in this case. Line means were used for the calculation of correlation coefficients between each trait. Contrasts between heterotic groups, SS and NSS, were made for each trait. The CV_G was computed by dividing the standard deviation of the entry means by the experiment mean. The CV was determined by dividing the square root of the mean square error by the experiment mean. CV_G and CV values were expressed as percentages. Phytate values obtained by the modified Wade assay were compared to phytate values measured by ion exchange chromatography (NP Analytical Laboratories) through simple linear regression and correlation of line means. Means for each group (low, intermediate, and high phytate) were calculated for each method of measurement (Table 2).

RESULTS AND DISCUSSION

The standard curves used to predict phytate and P_i in this study were plots of the concentration of known standards versus the standard OD in each assay. These plots were highly linear with R^2 values greater than 0.95 and 0.99 for phytate and P_i, respectively. The strong linear relationship between OD and standard concentration indicate that these standard curves can accurately predict the relative concentration of phytate and P_i in our samples. A nonsignificant Anderson-Darling statistic ($P > 0.10$) and examination of normal probability plots confirmed that the distributions of both phytate and P_i values did not significantly deviate from normality. Coefficients of variation are displayed in Table 3 and ranged from 2.5% for protein to 16.5% for P_i. Line was a highly significant ($P < 0.01$) source of variation for all traits measured. The CV_G can be used to compare degree of variability across traits with different units of

Table 3. Estimated field level variance components (line = σ^2_L, error = σ^2_e); lab and field repeatabilities (r_L and r_F, respectively); coefficient of variation (CV); mean, minimum, and maximum values; and genetic coefficient of variation (CV_G) for each trait.

Trait	Unit	σ^2_L	σ^2_e	r_L†		r_F	CV	Mean	Min	Max	CV_G
				FB 1	FB 2						
Yield	Mg ha^{-1}	1.97	0.49			0.89	14.1	4.98	1.43	9.14	30.0
P_i	g kg^{-1}	0.048	0.0096	0.98	0.99	0.91	16.5	0.59	0.18	1.04	39.0
Phytate	g kg^{-1}	0.86	0.49	0.87	0.89	0.78	6.0	3.25	2.40	4.09	9.4
Methionine	g kg^{-1}	0.034	0.008	0.79	0.94	0.89	7.6	1.18	0.65	1.65	16.4
Lysine	g kg^{-1}	0.061	0.019	0.81	0.81	0.87	11.0	1.28	0.84	2.15	20.8
Tryptophan	g kg^{-1}	0.008	0.006	0.88	0.39	0.73	9.7	0.79	0.59	1.14	13.5
Protein	g kg^{-1}	126.20	5.24			0.98	2.5	92.6	70.0	118.5	12.3
Oil	g kg^{-1}	7.67	1.25			0.92	3.1	36.8	31.5	46.0	7.8
Kernel weight	g 250 K^{-1}‡	66.10	3.92			0.97	2.9	68.1	43.2	82.2	12.1

†Lab repeatability calculated for both field block 1 (FB 1) and field block 2 (FB 2); only one measurement per field plot was taken where r_L is not shown.

‡Grams per 250 whole kernels.

measurement. According to the CV_G values estimated, P_i and yield were highly variable compared to protein, oil, kernel weight, and phytate. An intermediate level of variability was found for the three amino acids (Table 3).

Repeatability values were calculated for each trait (Table 3). We used lab repeatability (r_L) to reflect the reliability of measurements within field plots, and field repeatability (r_F) to reflect the reliability of measurements within inbred lines but from different field plots. Repeatabilities are unitless and, to an extent, can be used to compare the quality of data for individual traits. However, repeatability is also a function of genetic variability and sets an upper limit to heritability (Falconer and Mackay, 1996). The high r_L values for P_i and phytate (>0.87) indicated that multiple measurements within field plots were performed with a good degree of precision. Lower r_F values suggest that kernel sampling, milling, and field variability are potential sources of error for these traits. The r_F of P_i was 0.91, one the highest among the traits included in this study. This result shows that P_i can be measured in a quantitative manner and may be very responsive to selection. The r_F of phytate (0.78) was relatively low, and therefore, phytate may be less heritable when measured using the modified Wade reagent assay. The CV of phytate was 6.0%, an acceptable value. When taken together with the CV_G, the lower repeatability of phytate analysis was likely due to less genetic variability or assay sensitivity.

Phytate measurements using the modified Wade assay were validated by sampling lines with low, intermediate, and high levels of phytate and sending them to NP Analytical Laboratories (St. Louis, MO) for phytate determination by an ion exchange method. Overall, values obtained with our rapid, modified Wade assay agreed very well with the phytate concentrations predicted by ion exchange on a relative basis. The downward bias of the ion exchange method relative to the modified Wade assay may be due to different extraction procedures or other small differences in method. The correlation between the two methods of phytate determination was 0.95. The relationship between the modified Wade assay values and the ion exchange values is displayed in Fig. 1.

The trait means for all 50 inbred lines included in this study are presented in Table 1, along with the SS, NSS, and experiment means. Because this study included only one environment, we can make only limited conclusions on the relative performance of these inbred lines. However, several previous authors investigating phytate and total P in different crops reported either nonsignificant genotype × environment interactions or little to no rank changes among genotypes across environments (Miller et al., 1980; Raboy et al., 1984; Wardyn and Russell, 2004). Therefore, we believe the measurements of these grain P-related traits in one environment should, at least, separate truly superior and inferior lines. Line B90 had a P_i concentration 0.47 g kg^{-1} greater than the study mean. B90 has additional high grain quality characteristics, ranking third in protein content (114 g kg^{-1}), first in tryptophan concentration, and greater than average for methionine and lysine contents. However, B90 ranked 49th among the 50 inbred lines with respect to yield. B104 was determined to have the lowest phytate concentration (2.40 g kg^{-1}), but lower than average protein, tryptophan, lysine, and methionine contents. Because the lines included in this experiment include a nearly equal number from SS and NSS genetic backgrounds, differences between these sources with respect to any of the traits evaluated can be determined and are of relevance to a breeding program designed to alter specific traits. Significant differences were found for yield, P_i, and protein, with the SS heterotic group having a greater yield. Higher values of P_i and protein were found generally among the NSS lines (Table 1).

Correlation coefficients between traits are presented in Table 4. Correlations were calculated to identify trait relationships that should be considered during the process of improving lines or germplasm for phytate or P_i, as well as to compare our results with previously reported correlations to determine if similar relationships exist within this set of public inbred lines. The correlation between phy-

tate and P_i was near zero, which contrasts the phytate:P_i relationship found in *lpa* mutant lines. The *lpa* lines have similar levels of total P relative to normal lines because the drastic reduction in phytate is compensated for by P_i or lower-level *myo*-inositol phosphates (Raboy, 2002). However, a strong, positive relationship between phytate and total P is commonly found among normal lines (Raboy et al., 2001), which indicates that the nonphytate P fraction is generally stable or is positively correlated with phytate. Selection for reduced phytate alone would likely reduce total P rather than alter the form of P like that found in *lpa* mutant lines. Our results show that selection for both reduced phytate and increased P_i is certainly possible. For instance, B104 had the lowest phytate concentration and a P_i concentration 46% greater than the P_i mean. The positive correlation found between phytate and protein (r = 0.51) is undesirable when breeding for superior overall nutritional properties. The positive relationship between phytate and protein detected in the present study is consistent with a secondary selection response reported by Raboy et al. (1989) on analysis of the Illinois low- and high-protein lines in their 83rd generation of divergent selection. Raboy et al. (1984) also found a positive seed phytate:protein correlation among soybean (*Glycine max*) and *G. soja* lines. A negative correlation between yield and phytate indicates that development of high-yielding lines with lower phytate should be an attainable goal. Unfortunately, a negative correlation between P_i and yield was also found. A weak correlation between phytate and lysine (r = 0.31) indicates that lowering phytate via selection may decrease this nutritionally limiting amino acid if attention is not given to this relationship.

An important relationship to consider before selecting for decreased whole kernel phytate is that between phytate and kernel size. Because we measured phytate concentration in ground whole kernels and ~90% of phytate is found within the germ (O'Dell et al., 1972), kernels with larger endosperms should have a diluted concentration of phytate. Raboy et al. (1989) alluded to this when they investigated the relationship between high-oil maize lines and phytate. They speculated that whole kernel phytate and total P concentrations might be contingent on seed size and seed-organ ratio alterations through indirect selection. We found a significant, negative phytate:kernel weight correlation (r = –0.39), in accordance with the idea that whole-kernel phytate concentration decreases as seed size increases. This may be due to a larger endosperm to germ ratio and thus an increased amount of endosperm in the ground subsamples. However, this hypothesis assumes kernel size to be largely a function of endosperm size. This was not necessarily the case in our study (Fig. 2).

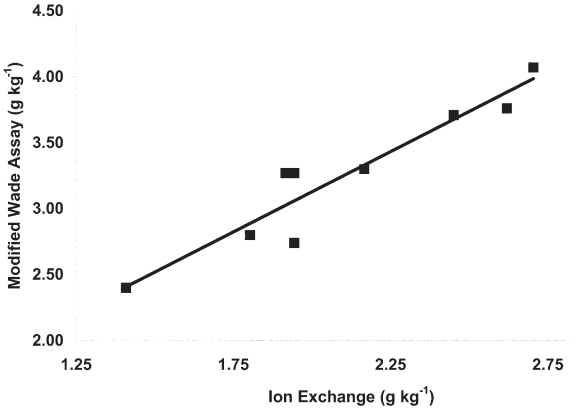

Figure 1. Relationship between phytate measurements obtained through the modified Wade assay and ion exchange (Ralston Analytical Laboratories, St. Louis, MO). Pearson's correlation coefficient between the two phytate measurements was 0.95.

In summary, this study indicates that breeders can make repeatable and thus possibly heritable measurements of P_i and phytate in an inexpensive, high-throughput manner. Using the method described herein, 11 to 12 hours of human labor are needed to analyze 100 samples in triplicate for P_i and phytate. The majority of this labor (9 h) is required for preparation of the extraction plates. Once the extraction plates are prepared, rapid measurements of P_i and phytate can be made simultaneously. To increase sample throughput further, we are evaluating ways of circumventing the weighing of exactly 10 mg of kernel tissue into individual wells. The original assay for P_i that we modified is typically used to qualitatively identify *lpa* mutants (Raboy et al., 2000). Our results suggest that this assay can be used for the quantitative determination of P_i as well, and that P_i may be very responsive to selection, given the range of concentrations detected in this study. According to the CV, phytate measurements with the modified Wade assay were made with an acceptable level of precision. Also, this rapid phytate method produced values that agreed well with values obtained through ion exchange chromatography.

Table 4. Simple correlation coefficients between traits of inbred lines grown in Ames, IA, during the summer of 2004.

Trait	Yield	P_i†	Phytate	Methionine	Lysine	Tryptophan	Protein	Oil
	Mg ha^{-1}				g kg^{-1}			
P_i	–0.29*							
Phytate	–0.47**	0.03						
Methionine	–0.31*	–0.19	0.17					
Lysine	–0.25	0.02	0.31*	0.16				
Tryptophan	–0.25	0.01	0.15	0.05	0.27			
Protein	–0.64**	0.46**	0.51**	0.28	0.30*	0.12		
Oil	–0.15	–0.15	0.11	0.06	0.03	0.18	0.01	
Kernel weight‡	0.19	0.21	–0.39**	–0.27	0.00	–0.03	–0.17	–0.23

*Significant at the 0.05 probability level.

**Significant at the 0.01 probability level.

†P_i, inorganic phosphorus.

‡Units are grams per 250 whole kernels.

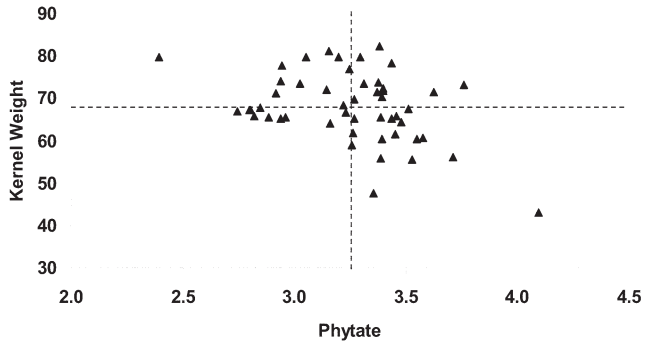

Figure 2. Kernel weight (g per 250 kernels) versus phytate (g kg^{-1}). Horizontal and vertical reference lines indicate the experiment means of each trait.

Therefore, the lower repeatability of phytate concentration was likely due to a lack of genetic variance or assay sensitivity. Refinement of the phytate assay or a search for more natural variation may be necessary to increase repeatability to the levels obtained with P_i measurements. Diluting and pH buffering the extracts may improve results, since phytate recovery is increased when extract pH is closer to 6.0 (Vaintraub and Lapteva, 1988). However, this would add another step, and it should be determined if the increased repeatability justifies the decreased throughput.

The phenotypic correlations detected suggest that a form of multiple trait selection should be employed when developing lines and populations with enhanced yield and nutritional profiles. Selection for decreased whole-kernel phytate may result in lines with larger kernels. Finally, we not only developed rapid phytate and P_i assays, but also combined them with careful experimental design and statistical analysis commonly used by field scientists to improve data quality. Such an approach should be considered when striving for simple, rapid measurements of quality traits not typically measured by breeders to improve repeatability and thus heritability.

Acknowledgments

The authors would like to thank Merinda Struthers, Paul White, and Krystal Kirkpatrick for their technical assistance. Thanks are also due to two referees for their thorough reviews and helpful comments.

References

Anscombe, F.J., and J.W. Tukey. 1963. The examination and analysis of residuals. Technometrics 5:141–159.

Birge, E.A., and K.B. Low. 1974. Detection of transcribable recombination products following conjugation in rec^+, $recb^-$ strains and $recc^-$ strains of *Escherichia coli* k12. J. Mol. Biol. 83:447–457.

Cromwell, G.L., and R.D. Coffey. 1991. Phosphorus: A key essential nutrient, yet a possible major pollutant. Its central role in animal nutrition. p. 133–145. *In* T. P. Lyons (ed.) Biotechnology in the feed industry. Alltech Tech. Publ., Nicholasville, KY.

Erdman, J.W. 1981. Bioavailability of trace minerals from cereals and legumes. Cereal Chem. 58:21–26.

Ertl, D.S., K.A. Young, and V. Raboy. 1998. Plant genetic approaches to phosphorus management in agricultural production. J. Environ. Qual. 27:299–304.

Falconer, D.S., and T.F.C. Mackay. 1996. Introduction to quantitative genetics. 4th ed. Longman Group, Essex, England.

Hulke, B.S., W.R. Fehr, and G.A. Welke. 2004. Agronomic and seed characteristics of soybean with reduced phytate and palmitate. Crop Sci. 44:2027–2031.

Jacob, F., and E. Wollman. 1961. Sexuality and the genetics of bacteria. Academic Press, New York.

Lott, J.N.A., I. Ockenden, V. Raboy, and G.D. Batten. 2000. Phytic acid and phosphorous in crop seeds and fruits: A global estimate. Seed Sci. Res. 10:11–33.

Miller, G.A., V.L. Youngs, and E.S. Oplinger. 1980. Environmental and cultivar effects on oat phytic acid concentration. Cereal Chem. 57:189–191.

O'Dell, B.L. 1972. Dietary factors that affect biological availability of trace elements. Ann. N. Y. Acad. Sci. 199:70–81.

O'Dell, B.L., A.R. de Boland, and S.R. Koirtyohann. 1972. Distribution of phytate and nutritionally important elements among the morphological components of cereal grains. J. Agric. Food Chem. 20:718–721.

Raboy, V. 2001. Seeds for a better future: "Low phytate" grains to help overcome malnutrition and reduce pollution. Trends Plant Sci. 6:458–462.

Raboy, V. 2002. Progress in breeding low phytate crops. J. Nutr. 132:503S–505S.

Raboy, V., F.E. Below, and D.B. Dickinson. 1989. Alteration of maize kernel phytic acid levels by recurrent selection for protein and oil. J. Hered. 80:311–315.

Raboy, V., and D.B. Dickinson. 1984. Effect of phosphorus and zinc nutrition on soybean seed phytic acid and zinc. Plant Physiol. 75:1094–1098.

Raboy, V., D.B. Dickinson, and F.E. Below. 1984. Variation in seed total phosphorus, phytic acid, zinc, calcium, magnesium, and protein among lines of *Glycine max* and *G. soja*. Crop Sci. 24:431–434.

Raboy, V., P.F. Gerbasi, K.A. Young, S.D. Stoneberg, S.G. Pickett, A.T. Bauman, P.P.N. Murthy, W.F. Sheridan, and D.S. Ertl. 2000. Origin and seed phenotype of maize *low phytic acid 1-1* and *low phytic acid 2-1*. Plant Physiol. 124:355–368.

Raboy, V., K.A. Young, J.A. Dorsch, and A. Cook. 2001. Genetics and breeding of seed phosphorus and phytic acid. J. Plant Physiol. 158:489–497.

Sambrook, J., and D.W. Russel. 2001. Molecular cloning: A laboratory manual. 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Scott, M.P., S. Bhatnagar, and J. Betran. 2004. Tryptophan and methionine levels in quality protein maize breeding germplasm. Maydica 49:303–311.

Sharpley, A.N., S.C. Charpa, R. Wedepohl, J.Y. Sims, T.C. Daniel, and K.R. Reddy. 1994. Managing agricultural phosphorus for protection of surface waters: Issues and options. J. Environ. Qual. 23:437–451.

Singer, M., T.A. Baker, G. Schnitzler, S.M. Deischel, M. Goel, W. Dove, K.J. Jaacks, A.D. Grossman, J.W. Erickson, and C.A. Gross. 1989. A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. Microbiol. Rev. 53:1–24.

Vaintraub, I.A., and N.A. Lapteva. 1988. Colorimetric determination of phytate in unpurified extracts of seeds and the products of their processing. Anal. Biochem. 175:227–230.

Wardyn, B.M., and W.K. Russell. 2004. Resource allocation in a breeding program for phosphorus concentration in maize grain. Crop Sci. 44:753–757.