A Modified Colorimetric Method for Phytic Acid Analysis in Soybean


ABSTRACT
A quantitative, reproducible, and efficient phytic acid assay procedure is needed to screen breeding populations and support genetic studies in soybeans [Glycine max (L.) Merr.]. The objective of this study was to modify the colorimetric Wade reagent method and compare the accuracy and applicability of this new method in determining seed phytic acid content in soybean with three well-established phytic acid assay methods: anion exchange column (AEC), high-performance liquid chromatography (HPLC), and $^{31}$P nuclear magnetic resonance (NMR). The CV for repeated measurements of a low phytic acid soybean mutant, CX1834-1-6, ranged from 1.8 to 4.2% ($n = 5$), indicating the results were precise and reproducible. Phytic acid content of 42 soybean genotypes as determined by this method showed a correlation of 93.7 to 96.6% with the measurements by AEC, HPLC, and NMR. According to analysis of covariance, using inorganic P content as a predictor, phytic acid P content in a given sample analyzed by the four assay methods can be estimated with four linear regression models at the $\alpha = 0.01$ level. Compared with HPLC, AEC, and $^{31}$P NMR, this modified colorimetric method is simpler and less expensive for assaying a large number of samples, allowing its effective application in breeding and genetic studies of low phytic acid soybean.

A simple, reproducible, and efficient phytic acid (PA) assay is required to conduct genetic studies and breeding of low PA soybean [Glycine max (L.) Merr.]. Currently, an indirect assay based on the inverse relationship between inorganic phosphorus (Pi) and PA (Chen et al., 1956; Raboy et al., 2000) has been widely used to estimate PA levels in soybean seed samples. Applications of such indirect PA estimations can be found in previous isolation of low PA soybean mutants (Wilcox et al., 2000), in a study of genetic inheritance of the low PA soybean mutant CX1834-1-6 (Oltmans et al., 2004), and in genetic mapping of loci associated with low P A genes in CX1834-1-2 (Walker et al., 2006). This indirect assay has been shown useful for detecting the change in seed PA-P associated with little change in seed total P. However, screening low PA soybean lines from populations with diverse backgrounds and different total P content requires accurate measurements of absolute levels of seed PA.


Abbreviations: AEC, anion exchange column; ANCOVA, analysis of covariance; dd, distilled-deionized; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; ICP, inductively coupled plasma emission spectroscopy; IP, inositol phosphate; NMR, nuclear magnetic resonance; PA, phytic acid; Pi, inorganic phosphorus; QTL, quantitative trait loci.

doi: 10.2135/cropsci2007.03.0122
© Crop Science Society of America
677 S. Segoe Rd., Madison, WI 53711 USA
All rights reserved. No part of this periodical may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopying, recording, or any information storage and retrieval system, without permission in writing from the publisher. Permission for printing and for reprinting the material contained herein has been obtained by the publisher.
Modified Fe(III) precipitation methods (Bowen et al., 2006; Haug and Lantzsch, 1983; Shi et al., 2003) were used to analyze PA in cereals. Because these methods indirectly determine PA content by measuring the excess Fe(III) in solution, the assay requires extra experimental efforts to remove Fe-phytate precipitates before the Fe determination (Vaintraub and Lapteva, 1988). High-performance liquid chromatography (HPLC), anion exchange column (AEC), and $^{31}$P nuclear magnetic resonance (NMR) spectroscopy overcome the low accuracy, acid hydrolysis requirement, and other problems associated with the classic Fe(III) precipitation PA assay method (Reddy et al., 1989; Velickovic et al., 1999; Xu et al., 1992) and have also been used for PA assays. These methods, however, require sophisticated instruments, high maintenance, and specialized staff and are not readily adopted by plant breeders. Both HPLC and AEC methods can only analyze 10 to 30 samples per day, which makes them impractical for routine analysis involving large numbers of samples. By comparison, Latta and Eskin (1980) modified the AEC method (Harland and Oberleas, 1977) by replacing the strong-acid digestion with a simpler colorimetric method for PA-P analysis. The determination of PA content with this colorimetric method is based on the decoloration of the pink Fe$^{3+}$-sulfosalicylate complex (Wade reagent) by PA, which can eliminate the acid hydrolysis and associated laboratory hazards. To remove Pi interference, the Latta and Eskin (1980) procedure still needed to include the AEC step, which is time consuming. Vaintraub and Lapteva (1988) later demonstrated that by adjusting the pH of the crude extract to 2.0 to 2.5, the anion exchange purification step could be omitted by adding Wade reagent directly to acidified extracts. This modification made Vaintraub and Lapteva’s method simpler. Possibly caused by some unknown matrix interferences, Vaintraub and Lapteva’s method (1988) underestimated PA content (Harland and Oberleas, 1977; Latta and Eskin, 1980; Xu et al., 1992). The earlier AEC method also encountered these unknown matrix interferences, but they were ameliorated by pretreating samples with ethylenediaminetetraacetic acid (EDTA) (Harland and Oberleas, 1977; Latta and Eskin, 1980; Xu et al., 1992).

The objectives of this study were (i) to improve the colorimetric (Wade reagent) PA assay (Vaintraub and Lapteva, 1988); (ii) to compare the improved protocol with several well-established methods, including AEC, HPLC, and $^{31}$P NMR; and (iii) to determine the applicability of the modified PA method in screening of low PA soybean.

**MATERIALS AND METHODS**

**Soybean Seed Samples**

A total of 42 soybean lines with a wide range of PA content as previously determined by the modified colorimetric (Wade reagent) method were chosen for this study. The sample set included two commercial soybean cultivars Hutschen and MN 1401, plant introductions PI 87013, PI 407162, low PA mutants CX1834–1-6 and M766 (seeds were kindly provided by J. Wilcox and V. Raboy, USDA-ARS) (Wilcox et al., 2000), four Virginia Tech experimental lines V99-5089, V99-3337, V99-8060, and MFL552, and 32 breeding lines developed from crosses of V99-5089×CX1834–1-6 and CX1834–1-6×V99-3337. These breeding lines were randomly selected with PA levels ranging from 6 to 22 mg g$^{-1}$.

**Phytic Acid and Pi Analyses**

A sample of 50 to 75 random seed from each soybean line were ground with a Cyclone Sample Mill grinder with a 0.5-mm mesh (UDY Corporation, Fort Collins, CO). Samples of 0.50 g of ground powder were thoroughly mixed with 10 mL of 2.4% HCl in 14-mL Falcon tubes. Sample tubes were shaken at 220 rpm for 16 h in a Model 3525 Lab-line incubator shaker (Lab-Line Instruments, Inc., Melrose Park, IL) and centrifuged at 1000 g (Sorvall RT6000B refrigerated centrifuge, Du Pont, Newtown, CT) at 10°C for 20 min. The crude extracts were collected for total P analysis by inductively coupled plasma emission spectroscopy (ICP) and for PA determination by four assay methods.

**Modified Colorimetric (Wade Reagent) Method**

The Vaintraub and Lapteva (1988) method was modified as follows: (i) the extraction time was extended to 16 h, (ii) the temperature of centrifugation was reduced to 10°C, and (iii) a matrix cleaning step was added to increase PA recovery. The details of the protocol were as follows. Crude acid extracts were transferred to 14-mL Falcon tubes containing 1 g NaCl. The contents were shaken at 350 rpm for 20 min to dissolve the salt and were allowed to settle at 4°C for 60 min or at −20°C for 20 min. The mixtures were centrifuged at 1000 g at 10°C for 20 min, and clear supernatants, hereafter referred to as the NaCl-treated supernatant, were collected for color development. This treatment precipitated matrix components that could interfere with the colorimetric reaction. One mL of the clear supernatant was diluted 25 times in a 50-mL Falcon tube by mixing with 24 mL of distilled-deionized (dd) H$_2$O. Three mL of this diluted sample were combined with 1 mL of modified Wade reagent (0.03% FeCl$_3$, 6H$_2$O + 0.3% sulfosalicylic acid) in a 14-mL Falcon tube, thoroughly mixed on a vortex, and centrifuged at 1000 g at 10°C for 10 min. A series of calibration standards containing 0, 1.12, 2.24, 3.36, 5.6, 7.84, or 11.2 mg L$^{-1}$ PA-P were prepared from sodium phytate (Sigma, St. Louis, MO), the P content of which was determined by ICP as 18.38%. Absorbance of color reaction products for both samples and standards was read at 500 nm on a DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA), and calculation of sample PA-P content followed the method described in Latta and Eskin (1980).

**Anion Exchange Column Method**

For the AEC method (Harland and Oberleas, 1986), 1 mL of the seed crude extract without NaCl treatment was diluted by mixing with 23 mL of dd H$_2$O and 1 mL of 0.11 mmol mL$^{-1}$
RESULTS AND DISCUSSION

Modifications of the Colorimetric Method

The colorimetric method of Vaintraub and Lapteva (1988) was found to have poor precision and reproducibility. It also underestimated the PA content when crude soybean seed extracts were used in our study (data not shown), which is in agreement with other reports (Fruhbeck et al., 1995). The method apparently suffered from severe matrix interference with unpurified extracts, although the nature of the interference is unknown. In the earlier development of the AEC method, a NaOH + EDTA solution was used successfully to remove unwanted matrix effects (Harland and Oberleas, 1986). However, EDTA cannot be applied in this colorimetric assay because it interferes with the reaction of the Wade reagent (Tze-Hung Yang, personal communication, 2006). We tested high-speed centrifugation at 11,000 g and precipitating crude soybean extracts in 10% NaCl solution at 4°C for 60 min followed by low-speed centrifugation at 1000 g. The NaCl cleaning step proved to be more effective than a single-step high-speed centrifugation for removing the matrix interference.

The reproducibility and precision of the modified colorimetric method was confirmed through numerous independent assays of the PA-P content of the reference sample, CX1834-1-6. An F test of 10 different runs showed that repeated determinations of CX1834-1-6 PA-P content did not vary significantly (P > 0.38) from one run to another. The CV of five replications within each run was between 1.8 and 4.2% for the 10 independent runs, indicating that the use of NaCl cleaning resulted in an improved analytical precision and reproducibility. However, the centrifugation at 11,000 g did not produce the same precision and reproducibility as the NaCl treatment, suggesting that a much higher centrifugation speed is needed. The NaCl-treated soybean extracts were stable at 4°C up to 24 h. Our modifications are important for those who do not have access to ultra-high-speed centrifugation. With our modified protocol, more than 200 samples can be processed every 2 d, with the only cost being the Wade reagent. The protocol can be converted to a higher throughput procedure using a 96-well microtiter plate reader and single soybean seed samples.

Comparison of the Modified Colorimetric Method with Three Other Methods

Phytic acid P content determined by the modified colorimetric method was highly correlated (P < 0.01) with measurements by the three well-established methods, HPLC, AEC, and 31P NMR (Table 1, Fig. 1). Above 3.0 mg P g−1 level, colorimetric PA-P values were close to those determined by AEC, HPLC, and 31P NMR, except for a few cases measured by 31P NMR (Fig. 1). Below 2.5 mg P g−1, the modified colorimetric method detected PA-P values consistently higher than HPLC and 31P NMR.

**Table 1.** Comparison of Phytic Acid P (mg P g−1) Content Measured Using Three Established Methods and Modified Colorimetric Method.

<table>
<thead>
<tr>
<th>Method</th>
<th>31P NMR</th>
<th>HPLC</th>
<th>AEC</th>
<th>Colorimetric</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 mg P g−1</td>
<td>0.34±0.1</td>
<td>0.36±0.2</td>
<td>0.35±0.1</td>
<td>0.38±0.2</td>
</tr>
<tr>
<td>2.0 mg P g−1</td>
<td>0.54±0.05</td>
<td>0.58±0.06</td>
<td>0.55±0.05</td>
<td>0.60±0.06</td>
</tr>
<tr>
<td>3.0 mg P g−1</td>
<td>0.74±0.04</td>
<td>0.78±0.05</td>
<td>0.75±0.04</td>
<td>0.80±0.05</td>
</tr>
<tr>
<td>4.0 mg P g−1</td>
<td>0.94±0.03</td>
<td>0.98±0.04</td>
<td>0.95±0.03</td>
<td>1.00±0.04</td>
</tr>
<tr>
<td>5.0 mg P g−1</td>
<td>1.14±0.02</td>
<td>1.18±0.03</td>
<td>1.15±0.02</td>
<td>1.20±0.03</td>
</tr>
</tbody>
</table>

Minitab 14 (Minitab, 2004) was used for all statistical and graphical analysis except for analysis of covariance (ANCOVA). SAS version 9.1 (SAS Institute, 2003) was used in ANCOVA to examine the differences in PA across the four assay methods, using Pi determined by the AEC method as a covariate.
The modified colorimetric method overestimated the PA-P content for low PA samples. A possible explanation for this overestimation was that samples with PA-P less than 2.5 mg P g⁻¹ contained higher Pi, which, like PA, precipitated with Fe⁺³ in the Wade reagent and interfered with the colorimetric analysis.

A comparison of the mean and range of seed PA-P content for the 42 soybean lines determined by the four methods is shown in Fig. 2. The modified colorimetric method had the narrowest range with a minimum of 1.91 mg g⁻¹, a maximum of 6.20 mg g⁻¹, and a slightly higher mean of PA-P (3.51 mg g⁻¹), than the other three methods. The higher minimum value could have been due to the Pi interference in the colorimetric analysis. The HPLC and AEC methods can remove Pi interference by species separation, and the ³¹P-NMR method can remove Pi interference by spectroscopic separation because Pi has its distinctive chemical shift. The mean values by HPLC and ³¹P-NMR were almost the same when other inositol phosphate species (IP₁, IP₂, IP₃, IP₄, and IP₅) were not included, but the ³¹P-NMR-P A-P had a wider distribution. The AEC gave a higher mean value than HPLC and ³¹P-NMR-1, which may be because 0.7 mmol mL⁻¹ NaCl eluent of AEC may have contained other inositol phosphates (IP₁, IP₂, IP₃, IP₄, and IP₅). To confirm this, we recalculated the PA-P contents of samples for ³¹P NMR method by including other inositol phosphates. The data distribution (³¹P NMR-2) was similar to that of AEC (Fig. 2), although the ³¹P NMR-2 gave a higher mean value. The HPLC and ³¹P-NMR methods gave more accurate PA-P analysis than either the modified colorimetric or AEC method because neither can separate other inositol phosphates from IP₆.

To explore the differences between the four PA assay methods in determining the PA content as Pi content varies among the 42 soybean samples, the full ANCOVA was specified as follows:

\[ Y_{ij} = \beta_0 + \sum_{j=1}^{4} \beta_j Z_j + \beta_i X_i + \sum_{j=1}^{3} \beta_{ij} X_j Z_j + \varepsilon_{ij} \]

where \( Y_{ij} \) = detected PA-P (mg g⁻¹) from the \( i \)th sample (\( i = 1, 2, \ldots, 42 \)) obtained from the \( j \)th PA assay method; \( j = 1, 2, 3 \) corresponds to the HPLC, AEC, and ³¹P NMR methods; \( \beta_0 \) = modified colorimetric method intercept and \( \beta_4 \) = modified colorimetric method slope; \( X_i \) = inorganic P (mg g⁻¹) content in a given sample; \( Z_j = 1 \), when PA assay method \( j \) was used; otherwise \( Z_j = 0 \); and \( \varepsilon_{ij} \) = random error.

The use of the indicator variables (Z) in the full ANCOVA model allows for the possibility of four distinct linear regression models describing the relationship between PA and Pi. Testing for coincidence between the four models using partial F tests (\( H_0: \beta_1 = \beta_2 = \beta_3 = \beta_6 = 0 \)) was rejected (\( F = 4.46, P < 0.01 \)). Testing for the intercept coefficients (\( H_0: \beta_1 = \beta_2 = \beta_3 = 0 \)) failed to produce significant differences (\( F = 1.42, P > 0.2 \)), whereas testing for interaction between Pi and the assay methods used (\( H_0: \beta_4 = \beta_5 = \beta_6 = 0 \)) gave highly significant differences (\( F = 4.46, P < 0.01 \)). The four assay methods resulted in the same value of PA-P when Pi was approximately 0 mg g⁻¹. But when Pi increased up to 5 mg g⁻¹, the assays significantly differed from each other in the PA-P for a given amount of Pi present in the sample.

Interaction between Pi and assay methods was further analyzed by comparing the HPLC, AEC, and ³¹P NMR against the modified colorimetric assay. For the intercept coefficients \( \beta_0, \beta_1, \beta_2, \) and \( \beta_3, t \) tests showed at the \( \alpha = 0.05 \) level no significant differences either between HPLC and the modified colorimetric method (\( P > 0.4 \)) or between ³¹P NMR and the modified colorimetric method (\( P > 0.18 \) (Table 2). For the slope coefficients \( \beta_4, \beta_5, \beta_6, t \) tests showed significant (\( P < 0.01 \)) interaction between Pi content and PA assay methods. At the \( \alpha = 0.01 \) level, PA-P content can be estimated by the seed Pi in a soybean line with the following linear regression models:

\[ \text{Phytic acid P} \text{ by Colorimetric} = 4.829 - 0.545 \text{ Pi} \]
\[ \text{Phytic acid P by HPLC} = 4.829 - 0.851 \text{ Pi} \]
\[ \text{Phytic acid P by AEC} = 5.485 - 0.892 \text{ Pi} \]
\[ \text{Phytic acid P by NMR} = 4.829 - 0.932 \text{ Pi} \]
Although these models were developed based on only 42 samples, it is possible to estimate PA-P content accurately based on the Pi content in a given sample by developing prediction models in a similar fashion as presented here and using a large set of samples. The models developed in this study include both intercept and slope coefficients accounting for total P variation and are more accurate than the simple inverse estimation of PA-P by Pi used in the indirect PA assay (Chen et al., 1956; Raboy et al., 2000), which considered only the PA-P/Pi ratio.

Applicability of the Modified Colorimetric Method for Breeding Low PA Soybean

The PA-P content of 42 soybean lines by the four methods was plotted against Pi content determined by the AEC method. The results indicate a general inverse relationship between Pi and PA-P; however, closer inspection shows that the relationship broke down at Pi levels below 1.5 mg P g\(^{-1}\). For example, when Pi is about 1 mg P g\(^{-1}\), PA-P ranged from 4 to 7 mg P g\(^{-1}\) (Fig. 3). The results show that the use of Pi content as an indicator of PA content is valid only at certain Pi levels. The assumption for using Pi as a PA indicator is an invariant total P content among samples, and the indirect Pi assay only considers the PA-P/Pi ratio, neglecting the total P variation among samples. According to our assay results, the total P contents of the 42 samples in this study ranged from a minimum of 4.78 mg P g\(^{-1}\) to a maximum of 7.75 mg P g\(^{-1}\), with a mean of 5.91 mg P g\(^{-1}\), and more than 25% of the samples had total PA content 15 to 25% higher or lower than the average. The correlation coefficient between Pi and PA-P was only about 0.70 to 0.88 depending on the PA assay methods used. By comparison, the modified colorimetric method was a direct and quantitative PA assay, which produced correlation coefficients of 0.94 to 0.97 results with the three well-established methods, HPLC, AEC, and \(^{31}\)P NMR.

Compared with the HPLC, AEC, and \(^{31}\)P-NMR methods, the modified colorimetric method is simpler, less expensive, and more practical and useful for screening a large number of soybean samples. Figure 4 shows the correspondence of PA-P levels of 11 soybean genotypes as determined by the modified colorimetric method as well as by the HPLC, AEC, and \(^{31}\)P-NMR methods. The four methods basically give the same rank order for the different soybean genotypes as 1272 (a Virginia Tech breeding line developed from CX1834-1-6 × V99-3337) < CX1834-1-6 < V99-5089 < M766 < Hutcheson < V99-3337 < V99-8060.
\[ \leq \text{PI 87013} \leq \text{MN 1401} \leq \text{MFL 552} < \text{PI 407162}, \]

indicating the effectiveness of the modified colorimetric method for screening purposes.

The modified colorimetric method overestimated the PA-P content in soybean lines containing high Pi content. The Pi level thresholds for high Pi soybean lines are identified in Fig. 5. Below these Pi thresholds, the differences in the analyzed PA-P content by the four methods were less than 1 mg g\(^{-1}\). When Pi \(\leq 3.27\) mg g\(^{-1}\), HPLC and the modified colorimetric determined PA-P with less than 1 mg g\(^{-1}\) difference. When Pi \(\leq 4.78\) mg g\(^{-1}\), AEC and the modified colorimetric method determined PA-P with less than 1 mg g\(^{-1}\) difference, and when Pi \(\leq 2.59\) mg g\(^{-1}\), \(^{31}\)P NMR and the modified colorimetric determined PA-P with less than 1 mg g\(^{-1}\) difference.

With the modified colorimetric method, thousands of soybean breeding lines have been screened for low PA content in the breeding program at Virginia Tech. Based on the PA content, pedigree selections and progeny tests in our breeding program have been efficient and effective. The \(\text{pha1}\) and \(\text{pha2}\) (Oltmans et al., 2004) associated with high Pi level in mapping populations derived from CX1834-1-2 (Walker et al., 2006) also were confirmed to be highly associated with low PA content as determined by the modified colorimetric method in our quantitative trait loci (QTL) mapping population derived from CX1834-1-6, a sister line of CX1834-1-2 (Gao et al., 2006).

Acknowledgments

The study was funded by the United Soybean Board. We thank our statistics consultant, Kevin C. Packard, from the Statistics Department at Virginia Tech for his support with the analysis of covariance.
References