# New Chromophore for Phytic Acid Determination

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**ABSTRACT**

A direct spectrophotometric method was developed to analyze phytic acid without acid digestion. The method was based on the precipitation of phytate as ferric phytate followed by conversion to sodium phytate. On heating, phytate reacted with chromogenic reagent and a blue molybdenum complex was formed. The same reagent reacted with inorganic phosphate and produced a similar blue complex. Both these complexes had maximum absorption at 830 nm and were stable for several hours at room temperature. The color complexes obeyed Beer's Law over a wide range of concentrations of phytic acid and inorganic phosphate. The molar ratio of phosphorus to phytate was determined to be 5.84 by comparing moles of phosphorus in digested phytate to the moles of phytate in undigested phytate. The new chromophore was used to determine phytic acid in eight legume seeds. Values obtained for phytic acid with the new chromophore agreed with those obtained with Bartlett reagent. Among the legumes investigated, soybean had the highest value (23.35 ± 1.60 mg/g of meal), whereas black-eyed pea had the lowest (8.74 ± 0.83 mg/g meal). The advantages of determining phytate from the new chromophore over methods in the literature are that it eliminates the need for acid digestion of phytate to inorganic phosphate; it is easy to prepare and is stable; the chromophore reacts with phytate over a wide range of pH from 1 to 13; and it can be used for monitoring chromatographic separation of inositol phosphates.

Many of the antinutritional factors associated with raw soybean can be eliminated or minimized by proper heat treatment (Hafez et al 1983, 1985). However, poor mineral bioavailability is not directly improved by heating (Erdman 1979). Soybeans, in common with other seeds, contain phytic acid, which influences the bioavailability of trace metals. Several reviews on phytic acid deal with the nutritional implications as well as its chemistry and determination (O'Dell et al 1972, Erdman 1979, Cheryan 1980, Jaffe 1981, Young and Janghorbani 1981).

To evaluate relationships between phytic acid and the availability of trace metals in soybean, one must be able to accurately measure both of them. Several methods are found in the literature for quantitative determination of phytic acid. Of these, most of the chemical methods are based on the determination of phosphorus or iron in isolated ferric phytate (McCance and Widdowson 1935, Schormuller et al 1956, Samotus and Schwimmer 1962, Crean and Haisman 1963, Brooks and Morr 1982, Thompson and Erdman 1982).

Leucena-Conde and Prat (1957) developed a spectrophotometric method for determination of phosphorus. Their reagent gave a blue color with phosphorus and was easy to prepare, stable in the air, yielded a low blank, and did not require the use of a reducing agent. A spray reagent for the detection of phospholipids on chromatograms, based on the reagent of Leucena-Conde and Prat (1957), was prepared by Vaskovsky and Kostetsky (1968). This reagent reacted with phospholipids without heating to give blue spots. Raheja et al (1973) developed a spectrophotometric method for the quantitative determination of phospholipids without acid digestion based on the modified spray reagent formulated by Vaskovsky and Kostetsky (1968).

We have developed a new spectrophotometric method for the quantitative determination of phytic acid without acid digestion, based on the modified reagent of Raheja et al (1973). The method reported here was used to determine phytic acid in eight legume seeds.

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## MATERIALS AND METHODS

All chemicals used were analytical grade and purchased from Sigma Company (St. Louis, MO). Sodium phytate of 97% purity, containing 12 Na per mole from corn (Type V) was used as a standard. The moisture content was determined and found to be 18%. Anhydrous, dibasic sodium phosphate was used as the inorganic phosphorus standard. Soybean, black-eyed pea, lima bean, navy bean, mung bean, winged bean, and black bean were purchased locally.

### Reagents

*Solution A.* Sixteen grams of ammonium molybdate was dissolved in 120 ml of distilled water.

*Solution B.* Forty milliliters of concentrated HCl and 10 ml of elemental mercury were shaken with 80 ml of solution A for 30 min, filtered using Whatman no. 1 filter paper, and the filtrate was used.

*Solution C.* Two hundred milliliters of concentrated $H_2SO_4$ was added carefully to the remainder of solution A and mixed with filtrate of solution B.

*Chromogenic solution.* Forty-five milliliters of methanol was mixed with 25 ml of distilled water and 25 ml of solution C. This solution is stable for at least three months when stored at 5°C (Raheja et al 1973).

*Standard phytate solution.* The phytate solution was prepared by dissolving hydrated sodium phytate in distilled water to contain 11.740 mg of sodium phytate and the volume adjusted to 100 ml.

*Standard inorganic phosphate solution.* Phosphate solution was prepared by dissolving anhydrous $Na_2HPO_4$ in distilled water to contain 1.385 mg $Na_2HPO_4$ and the volume adjusted to 100 ml.

### Preparation of Legume Seeds for Phytate Analysis

The legume seeds were ground in a centrifugal grinding mill (Brinkman, Westberg, NY) and passed through a 0.5-mm pore size screen. The oil was extracted with hexane using Goldfisch apparatus. The defatted samples were dried overnight at room temperature and stored in airtight bottles in the freezer until further experimentation.

### Determination of Moisture Content

The moisture contents of sodium phytate and legume meals were determined according to AOAC methods (1984).

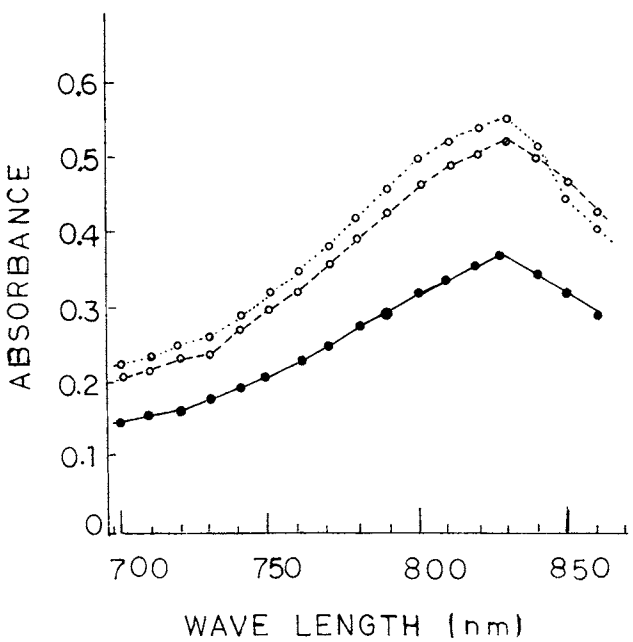


**Fig. 1.** Spectra of colors given by phytic acid (9.24 μg/ml) and $Na_2HPO_4$ (0.66 μg/ml) in the reaction mixture: phytic acid determined by the proposed method (——) and $Na_2HPO_4$ by the new method (- - -) and Bartlett method (·····).

### Extraction of Phytate

Several attempts were made to determine the best conditions for extracting phytate from legumes. Extractions were carried out with 1.2% HCl, 3% trichloroacetic acid (TCA), and 3% $H_2SO_4$ with and without 10% $Na_2SO_4$. The best solvent was found to be TCA. A series of different concentrations of TCA ranging from 1 to 10% were tested for extraction of phytate, and no increase in phytate extractability was observed above the 3% level.

A sample (0.5 g) of legume meal was placed in a 125-ml Erlenmeyer flask and extracted with 25 ml of 3% TCA for 45 min in a shaker of moderate speed at room temperature (23°C). Eight milliliters of the slurry was centrifuged at 20,000 × *g* for 15 min at 23°C. Three milliliters of 1% $FeCl_3 \cdot 6H_2O$ in 1.0*N* HCl was added to 5 ml of supernatant and heated in a boiling water bath for 45 min. It was allowed to cool and centrifuged at 20,000 × *g* for 10 min. The precipitated ferric phytate was suspended in 0.5*N* HCl and incubated at room temperature (23°C) for 2 hr (Ellis et al 1977). The precipitate was washed twice with 0.5*N* HCl allowing a 10-min incubation period between each wash. Three milliliters of 1.5*N* NaOH and 7 ml of distilled water were added to the precipitated ferric phytate and heated in a boiling water bath for 15 min. It was cooled and centrifuged. The supernatant was used for estimation of phytate.

### Determination of Extracted Phytate

Extracted phytate (0.2 ml) was mixed with 4.6 ml of distilled water and 0.2 ml of chromogenic solution and heated in a water bath at 95°C for 30 min. It was allowed to cool, and the color was read at 830 nm against a blank.

Standard phytate solution was used to prepare a series of tubes containing 17.8–177.8 nmol phytic acid per tube. The volumes were adjusted to 5 ml with distilled water and mixed with 3 ml of 1% $FeCl_3 \cdot 6H_2O$ in 1.0*N* HCl. These tubes were treated as described for the legume samples; however, the direct determination of phytate in standard solutions gave the same results as the precipitation method.

### Determination of Phytate Phosphorus

Phytate extract (2.0 ml) was digested using 0.5 ml of 10*N* $H_2SO_4$. It was further treated with three drops of 30% $H_2O_2$ and reheated to complete oxidation. This solution was neutralized with 5*N* NaOH using phenolphthalein as an indicator and adjusted to a known volume; 0.2 ml of the neutralized solution was mixed with 4.6 ml of distilled $H_2O$ and 0.2 ml of chromogenic reagent in a test tube and heated for 15 min in water bath at 95°C. The tube was cooled and read at 830 nm against a blank.

A series of tubes was prepared containing 9.18–97.5 nmol of $Na_2HPO_4$. The volume of each tube was adjusted to 4.8 ml with distilled water, and 0.2 ml of chromogenic reagent was added. These tubes were treated as described above and the color read at 830 nm against a blank.

The Bartlett method (1959) was also used to determine inorganic phosphate in the digested phytate. The amount of phytic acid (mg) was calculated by multiplying milligrams of phytic acid phosphorus by a factor of 3.553, assuming that 1 mol of phytic acid contains 6 mol of phosphorus.

## RESULTS AND DISCUSSION

The new feature of the method we developed is that it does not involve acid digestion of the phytic acid. Instead, the chromogenic reagent reacts directly with the phytic acid phosphorus, and a molybdenum blue color complex is formed. The chromogenic reagent is stable and does not require the use of a reducing agent. When diluted under hot or cold conditions, the reagent itself does not produce a molybdenum blue in the acidic medium (Leucena-Conde and Prat 1957). The stability of the reagent and the final color produced with both phytate and inorganic phosphate makes the method very convenient.

Our data show that the blue color complexes formed with phytate and inorganic phosphate have maximum absorption at 830 nm. This is in agreement with the results of Bartlett (1959),

which gave the same spectral curve with inorganic phosphate (Fig. 1).

The blue complex for phytate obeys Beer's Law in the range 17.8–177.8 nmol of phytic acid (Fig. 2). For inorganic phosphate, this method and the Bartlett method are highly sensitive and could detect 9.8 nmol of phosphorus in 5 ml of final reaction mixture. Further, solutions with high color value could be diluted with distilled $H_2O$ and read in the most accurate range for spectrophotometry without losing proportionality.

In the method reported here, 0.2 ml of chromogenic solution was added directly to the purified sodium phytate or inorganic phosphate, and the mixture heated was at 95° C. A 15-min heating time was selected for inorganic phosphate, the time at which a constant color was obtained (Fig. 3). The heating time of 30 min was selected for phytate, the time at which color was high and hydrolysis of phytate was minimum. Refluxing of phytate with chromogenic solution at 100° C produced a linear increase in color absorbance at 830 nm up to 30 min, and thereafter an exponential increase in absorbance was observed. This indicated the great sensitivity of the chromophore towards high temperature and longer incubation time.

The effect of pH of the phytate solution on color production was studied. When the pH was between 1 and 13 the color production was not affected; however, any change in the pH beyond these limits inhibited the production of color.

To prove that standard sodium phytate used had six atoms of phosphorus per molecule of phytate, the phytate was first precipitated as ferric phytate according to Ellis et al (1977) and then converted to sodium phytate. This phytate was then digested to inorganic phosphate as described in Materials and Methods. The molar ratio of phosphorus to phytate in standard sodium phytate was found to be 5.84. This value is in agreement with those obtained by de Boland et al (1975) for inositol hexaphosphate and is a good indicator of the purity of the phytate standard. The presence of any inorganic phosphate as an impurity in sodium phytate will increase the ratio above 1:6, whereas the presence of inositol phosphates other than hexaphosphate will decrease the ratio below 1:6 (de Boland et al 1975).

As an application of our method, the phytate content of eight legumes, mung bean, black bean, navy bean, red kidney bean, large lima bean, black-eyed pea, soybean, and winged bean were determined using the new chromophore, and the data are shown in Table I. The phytate content of these legumes was also determined after digestion using Bartlett reagent and these data are shown in Table I. The results obtained with chromogenic reagent without digestion of phytate agreed favorably with those obtained with Bartlett reagent. Reported values for phytate in navy beans (Lolas and Markakis 1975), lima beans (Chang et al 1977), and red kidney beans (Iyer et al 1980, Tabekhia and Luhn 1980) agreed with those by the new method. Soybean was found to have the highest phytate content, and this value agreed with those reported by Camire and Clydesdale (1982). However, Erdman (1979), Lolas et al (1976), and de Boland et al (1975) reported lower values. Tabekhia and Luhn (1980) reported a higher phytate content for black-eyed peas and a lower value for mung beans compared to our values. The differences in phytate content between our values and those reported may be attributable to variation in cultivars, varieties, or environmental conditions.

The average value for the major ratio of phosphorus to phytate in the phytate extracts of eight legumes was 5.67 ± 0.09. This ratio

**TABLE I**
**Phytic Acid Content of Legume Meals Determined by the New Chromophore and Compared to the Bartlett Method[a]**

| Legume | mg Phytic Acid/g Defatted Meal[b] | |
|---|---|---|
| | New Chromophore[c] | Bartlett Method |
| Mung bean | 9.48 ± 0.32 | 9.25 ± 0.21 |
| Black bean | 8.85 ± 0.56 | 8.61 ± 0.45 |
| Navy bean | 12.54 ± 0.07 | 11.39 ± 0.78 |
| Red kidney bean | 11.80 ± 0.28 | 11.32 ± 0.43 |
| Large lima bean | 10.77 ± 0.79 | 10.07 ± 0.15 |
| Black-eyed pea | 8.74 ± 0.83 | 8.47 ± 0.52 |
| Soybean | 23.35 ± 1.60 | 21.47 ± 2.03 |
| Winged bean | 14.32 ± 0.16 | 13.94 ± 0.98 |

[a] Dried legume seeds were ground and defatted with hexane as solvent. The solvent was completely evaporated at room temperature. The dried defatted meal was used for assay as described in Materials and Methods.
[b] The analysis was done on nine replicates, and the average is reported ± SEM.
[c] Phytic acid was determined without digestion using chromogenic reagent.

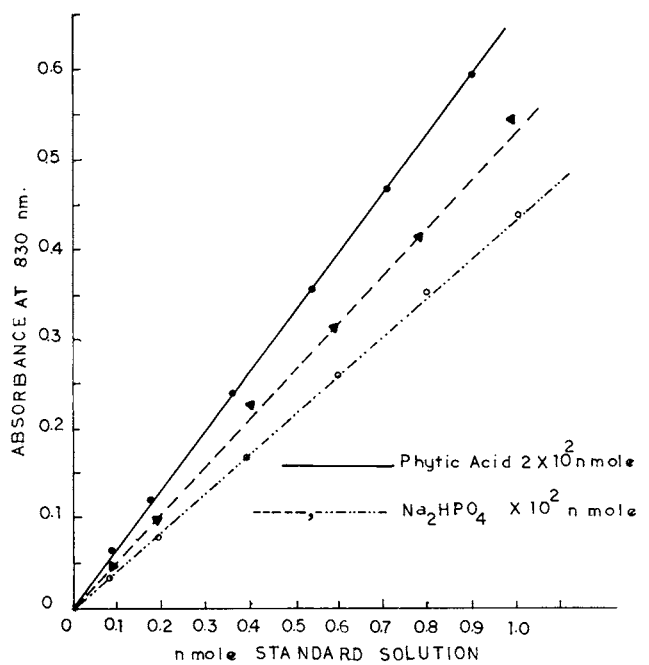


**Fig. 2.** Standard curves of phytic acid by the new method (——) and $Na_2HPO_4$ by the new method (- - -) and Bartlett method (- .. - .. -).

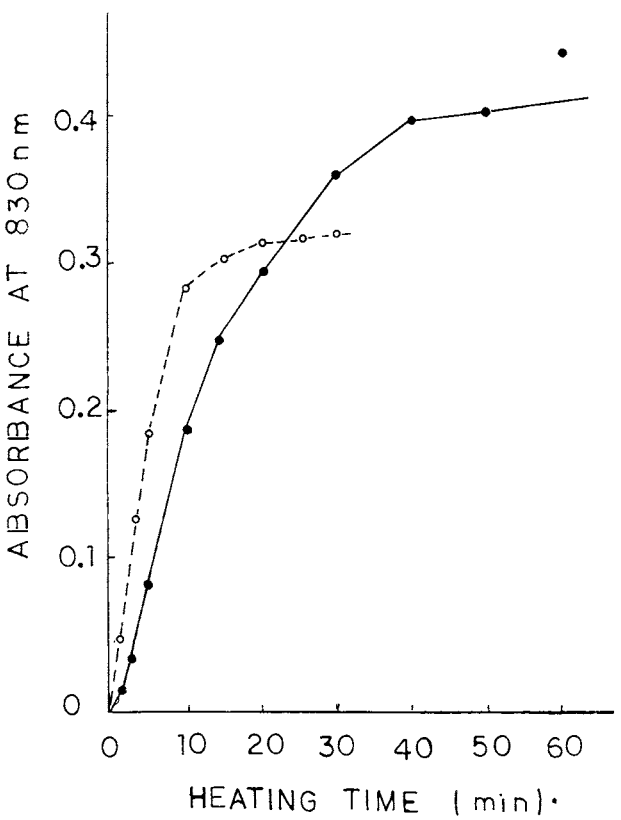


**Fig. 3.** Effect of heating time on color development at 830 nm determined by the new method: (——) phytic acid (117.4 μg/ml) and (- - -) $Na_2HPO_4$ (13.85 μg/ml).

was calculated as described for standard phytate. To investigate the reason for the low value for legume phytate, 2 ml of phytate extract for the legume samples was chromatographed on a Dowex $1 \times 8$ (200–400 mesh, $Cl^-$ form) column $2.0 \times 12$ cm as reported by Nayini and Markakis (1983). Linear gradient elution was performed with 720 ml of 0.0–1.0 *N* HCl at a rate of 2 m/min, and 5-ml fractions were collected. The chromatographic separation was monitored using chromogenic reagent. One milliliter of each of the neutralized fractions was used for the estimation of phytate as mentioned in Materials and Methods. The results indicate the presence of small amounts of di, tri, tetra, and penta phosphates of inositol in addition to phytate, thus explaining the reason for a decrease in the $P_i$/phytate ratio. The chromogenic reagent provided an easy monitoring method for the different types of phosphates of inositol, without any need for digestion of the fractions. The quantitative determination of inositol phosphates, other than phytic acid, is under study.

In conclusion, the greatest advantage of the new chromophore is seen in the determination of the phytic acid without acid digestion, either after precipitation as ferric phytate or after separation by anion-exchange column chromatography. In addition, the new chromophore has been successfully used to monitor the elution of inorganic phosphate and inositol phosphates from ion-exchange columns.

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