

Extraction and Determination of Phytic Acid in Beans (*Phaseolus vulgaris*)¹

RACHEL U. MAKOWER, Western Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Albany, California 94710

ABSTRACT

Analyses of phytic acid by four procedures, all based on precipitation of ferric phytate, were tested on Pinto beans of different maturities and on purified phytic acid. The colorimetric determination of iron in ferric phytate, after initial conversion to ferric hydroxide, has the advantage of greater speed, convenience, and improved separation from interfering substances over the determinations of iron or phosphate in ashed ferric phytate. The indirect determination of iron in the precipitate, by measuring residual iron in solution, was unsatisfactory for analyzing small amounts of phytic acid present in immature beans; only the direct methods gave meaningful results. All four procedures yielded comparable results with mature beans of appreciable phytic acid content. Better extraction of phytic acid from immature beans was obtained with hydrochloric acid than with perchloric or with trichloroacetic acid. Techniques, precautions, and sources of error in extraction are discussed.

Beans, in common with other seeds, contain metal salts of *myo*-inositol-hexaphosphate or phytic acid. Di- and trivalent metal salts of phytic acid are relatively insoluble in water. Ferric phytate is nearly insoluble in acid solution as well as in water. This property has been utilized for the isolation of ferric salts of phytic acid from acid extracts of natural products (1,2,3).

Quantitative determination of phytic acid may be based on the analysis of phosphorus or iron in the isolated ferric phytate (3,4,5) or indirectly, on the determination of the residual iron in solution after precipitation of ferric phytate from a known concentration of ferric salt in acid solution (2). Determination of phytic acid based on phosphorus analysis of ferric phytate has been reviewed by Schormuller et al. (5). Wide variations in values for phytic acid were obtained by several investigators who have used different analytical methods (5,6).

To my knowledge, no measurements of phytic acid have been made on immature beans prior to these studies. Published methods used at the beginning of this work were inadequate to determine the small amounts of phytic acid present in immature beans. It was found necessary to re-examine and modify methods of extraction and methods of determination of phytic acid as applied to Pinto beans of various maturities.

The present paper describes procedures that were developed for the extraction and determination of small amounts of phytic acid in plant material. Some of the problems encountered and the conditions that affect the accuracy and reproducibility of the determinations are discussed. The described procedures were employed in our recently published study on changes in phytic acid content with maturity of Pinto beans (7).

¹Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

MATERIALS AND METHODS

Determination of phytic acid in plant tissues involved the following steps: 1) maceration of tissue in hot alcohol and drying of macerated tissue sample; 2) solubilization and extraction of phytic acid with a strong acid; 3) precipitation of ferric phytate in the extract by adding an excess of ferric chloride; and 4) determination of phytic acid by one or more of four procedures: (a) measuring the amount of ferric ion remaining in solution after the precipitation of ferric phytate (the indirect procedure); (b) conversion of the ferric phytate to ferric hydroxide with alkali and the subsequent colorimetric determination of iron in the separated hydroxide (the $\text{Fe}(\text{OH})_3$ procedure); (c) wet-ashing of the precipitated ferric phytate and measuring the amount of iron or (d) the amount of phosphorus present.

These procedures were tested on purified phytic acid and also on samples of bean tissue containing various amounts of phytic acid. The advantages and shortcomings of the methods or steps are discussed.

Preparation of Purified Phytic Acid

Commercial phytic acid solution was treated with cation-exchange resin (H^+ form) and precipitated alternately with ferric chloride and barium acetate four or five times until the phytic acid appeared homogeneous by paper chromatography. Phytic acid was regenerated from barium phytate by cation-exchange (resin in H^+ form) and from ferric phytate by treatment with sodium hydroxide, removal of ferric hydroxide by centrifugation, and removal of sodium with cation-exchange resin (H^+). Glass-distilled water and chemicals of reagent quality were used. General precautions discussed by Sandell (8) for iron determinations were applied in these studies.

Preparation of Bean Samples for Analysis and Calculations

Locally grown Pinto beans (*Phaseolus vulgaris* var. *Pinto*) were harvested at several levels of maturity and held at -29°C . before preparation of alcohol slurries (7). Frozen bean seeds (or pods) were homogenized to a slurry in a blender in hot ethanol. If necessary, hard components were ground with sand and returned to the tissue slurry. The slurries were heated 15 min. (steam), cooled, and made up to volume and to 80% ethanol.

Sampled slurries were dried in vacuo to constant weight for subsequent analysis. Reagent blanks and reference standards were carried through all steps of extraction and analysis and used in calculations. Standard deviations were calculated by the method of Dean and Dixon (9). All values for phytic acid were calculated on the assumption that four ferric atoms combine with one molecule of phytic acid ($\text{Fe}_4\text{P}_6\text{C}_6\text{H}_6\text{O}_{24}$) (10,11). Calculations were on a dry weight basis.

Extraction of Phytic Acid from Bean Tissue

The efficiency of extraction by the commonly used acids was evaluated by analysis of phytic acid content in the extracts in which the ferric hydroxide procedure was used. Extraction of phytic acid with 0.5N HCl gave the most satisfactory results with both immature and mature beans of low and high phytic acid content, respectively. Extraction with 0.5N HClO_4 was satisfactory only for

mature and nearly mature beans where the phytic acid content was relatively high. As shown in Table I, extraction of immature beans with 0.5N HClO₄ was less complete than extraction with 0.5N HCl. Stirring and mild warming (to 60°C.) aided extraction. Extraction of immature beans with 0.3N CCl₃COOH gave no ferric phytate precipitate on treatment with ferric chloride.

Contrary to an earlier report (3), multiple extractions with small volumes of acid (with stirring and mild heating) were more efficient in extracting phytic acid from beans than a single extraction with the same total volume of acid for the same total period of time. The gain in phytic acid extracted by multiple extraction over single extraction was most evident with very immature bean seeds.

The adopted conditions were to extract the dry, powdered tissue sample three or four times with small volumes of 0.5N HCl (about 3 ml. HCl for each extraction of about 0.2 g. of dry sample) with continuous stirring. The mixture was heated to nearly 60°C. during the first few min. of each extraction step, but stirring was continued for 40 min. longer at room temperature. After each extraction, the creamy mixture was centrifuged at 17,300 × g for 30 min. The acidity of the combined supernatants was reduced with alkali to a pH value near 2, and the extract was made up to volume and refrigerated.

Bean pods contained only a small amount of phytic acid. Therefore, they were extracted only with 0.5N HCl. Because of the bulky nature of the pods, larger volumes of acid solution were used for each step in the extraction, and the final volume, after neutralization, was reduced by concentration in vacuo. Reproducible and consistent results were obtained even though the phytic acid content of pods was very low (Table I).

Precipitation of Ferric Phytate

Phytic acid was precipitated with standard ferric chloride solution (about 1 mg. Fe per ml. in 0.375N HCl). An excess of iron in the reaction mixture was necessary for quantitative precipitation of ferric phytate (10,11). Satisfactory precipitation

TABLE I. EXTRACTION OF PHYTIC ACID FROM PINTO BEAN SEEDS AND PODS WITH PERCHLORIC AND HYDROCHLORIC ACIDS (Averages of 4 to 6 determinations)^a

Maturity	Seeds or Pods		Phytic Acid ^b in mg. per g. Solids Extracted with:			
	Total solids	%	0.5N HClO ₄		0.5N HCl	
			Phytic acid mg.	Fe/P ^c	Phytic acid mg.	Fe/P ^c
A, immature seeds	16.3		0	...	1.31 ± 0.07	1.24
B, immature seeds	24.8		0.17 ± 0.02	...	3.45 ± 0.26	1.26
Partly mature seeds	47.1		8.84 ± 0.14	1.34	7.95 ± 0.12	1.18
Fully mature seeds	85.1		9.36 ± 0.13	1.33	9.62 ± 0.13	1.19
A, immature pods	10.3		0.44 ± 0.05	...
C, immature pods	13.3		0.31 ± 0.07	...
Somewhat immature pods	19.4		0.19 ± 0.08	...

^aStandard deviations were calculated by the method of Dean and Dixon (see ref. 9).

^bThe phytic acid was determined by the ferric hydroxide procedure.

^cFe/P = weight ratio of iron to phosphorus in the ferric phytate precipitate. Theoretical ratio for 4 Fe: 6P in phytic acid = 1.20.

was obtained with a mole ratio of added Fe to total P (Fe/P) of 4/3 or higher, and with a final acid concentration of 0.15N HCl. Similar conditions were found by Crean and Haisman working with peas (4). Thus, it was first necessary to determine the total phosphorus content from which the required quantities of standard ferric chloride and HCl to be used were calculated. A small total volume was maintained in order to keep the concentration of phytic acid in the final reaction mixture above 0.07 mg. per ml. The reaction was carried out in centrifuge tubes which were placed in boiling water for 30 min. or longer, until the ferric phytate was coagulated. Concentrations of phytic acid below 0.07 mg. per ml. required up to 1 hr. of heating for precipitation of ferric phytate. After cooling, the tubes were centrifuged 30 min. at $17,300 \times g$ and decanted. The ferric phytate was washed twice with 0.1N HCl and centrifuged as above. The collected supernatants were made up to volume for the determination of phytic acid based on residual iron (2), while the washed ferric phytate precipitate was used for all other determinations.

Precipitation of Ferric Hydroxide from Ferric Phytate and Determination of Iron

The ferric phytate precipitate in the centrifuge tubes was dispersed in 0.5 ml. water and was treated with 0.5 ml. of 0.6N NaOH. After the contents had been mixed, the tubes were heated for 30 to 50 min. in a boiling-water bath to coagulate the $\text{Fe}(\text{OH})_3$ precipitate, cooled, centrifuged 10 min. at $17,300 \times g$, decanted, washed with water, recentrifuged as above, and decanted again. The washed ferric hydroxide precipitate was dissolved in 0.5 ml. of 0.5N HCl with heating in boiling water (10 min.), transferred to a 10- or 25-ml. volumetric flask with several portions of 0.1N HCl, and made up to volume and to 0.1N HCl.

The iron was determined colorimetrically with *o*-phenanthroline, after reduction of Fe^{+3} to Fe^{+2} with hydroxylamine, and measured at 515 or 520 $m\mu$ (8,12). The precipitation of ferric phytate was made in quadruplicate; two samples of ferric phytate were used for precipitation of ferric hydroxide and colorimetric determination of iron, as described above, while the other two samples of ferric phytate were ashed with perchloric acid and H_2O_2 for determination of phosphorus on the same material (13). Iron was also determined in the ashed samples with *o*-phenanthroline, as above. Blanks and known ferric chloride standards, as controls, were included with all determinations.

The supernatants and washings from the precipitation of ferric phytate were diluted to 50 or 100 ml. The iron remaining from precipitation of phytate was determined colorimetrically as above, and the result was used for the calculation of the phytic acid content by difference from the iron originally added (indirect procedure).

Notes on Analytical Procedures

The recovery of total Fe^{+3} added to the reaction mixture, i.e., the sum of Fe^{+3} in ferric phytate and Fe^{+3} in supernatants, added up (within experimental error) to the expected value of Fe^{+3} introduced as ferric chloride. This indicates little or no loss of iron in the analytical procedures employed.

High-speed centrifugation of ferric phytate minimized losses in the decanting and washing steps. Washing of ferric phytate precipitate with hydrochloric acid is necessary to remove ferric phosphate, ferric chloride, and other salts which would

interfere in later analytical steps. Long heating and high-speed centrifugation also helped to coagulate and compact small quantities of ferric hydroxide formed from small amounts of ferric phytate on treatment with alkali. The use of the same centrifuge tube for many of the necessary steps helped to minimize transfer errors.

Colorimetric determination of iron with *o*-phenanthroline was adopted because of its high sensitivity and color stability (8,12). Colorimetric determination of iron directly on the suspended ferric phytate (after centrifugation and washing as described in "Methods") under several sets of conditions was unsuccessful; the development of color with *o*-phenanthroline was too slow and too incomplete for quantitative determination. Evidently, the iron phytate combination is stronger than those formed with the colorimetric reagents for iron. Neither is iron removed from ferric phytate by ethylenediamine tetraacetate. Either ashing or separation from phytic acid must be carried out before determination of the iron.

The amount of inorganic iron present in the acid extract of beans was very small; hence, corrections were not necessary.

Adding hydrogen peroxide, to avoid interference by reducing substances (14), was found unnecessary in the determinations of phytic acid in bean seeds and pods.

In extracts from very immature beans and from bean pods, a precipitate with ferric chloride was obtained which is not recoverable as ferric phytate; i.e., other compounds in the reaction mixture were precipitated with ferric iron. However, this precipitate does not react in the subsequent step with alkali; it is not converted to ferric hydroxide, nor is it dissolved in the later treatment with acid, but remains behind as a white solid. It does not appear to be ferric phytate, as shown by very low phosphorus content on ashing. It may be separated by centrifugation after the addition of acid to dissolve the ferric hydroxide. This solid can, however, become a source of error in determinations of phytic acid by the other procedures, i.e., from the measurement of iron in the wet-ashed ferric phytate precipitate or in determinations from residual iron in the reaction mixture.

RESULTS AND DISCUSSION

Table II presents the comparative results of four procedures used to analyze a purified solution of phytic acid at three levels of concentration. The concentration of the undiluted phytic acid solution was 9.5 mg. per ml. This value is based on 17 determinations of iron released by alkali from the precipitated ferric phytate. It agrees within 2 to 3% with concentrations of phytic acid calculated from the analysis of organic phosphate of the same solutions. The purity of this phytic acid preparation was confirmed by ascending (15) and descending (16) paper chromatography.

The identity of phytic acid extracted from Pinto beans of several maturities and precipitated by iron was confirmed by similar chromatographic tests as mentioned above. The phytic acid recovered from the iron phytate and chromatographed in three different solvent systems migrated at the same rate as the purified phytic acid. As a further confirmation, the hydrolyzed phytic acid derived from the beans gave a color test characteristic for *myo*-inositol (17), which is a part of the phytic acid molecule.

Results obtained by all four procedures are not grossly different from each other (Table II). The best reproducibility, i.e., the lowest standard deviation, was

TABLE II. COMPARISON OF ANALYSES BY FOUR PROCEDURES ON SOLUTIONS OF PURIFIED PHYTIC ACID^a

Phytic Acid in Sample ^b			Phytic Acid per ml. Original Solution by Four Methods				
Diluted Sample ml.	mg.	In Reaction Mixture mg./ml.	From Fe Phytate via Fe(OH) ₃ mg.	From Residual Fe in Soln. mg.	From Wet-Ashed Ferric Phytate		Fe/P wt. ratio
					From Fe mg.	From P mg.	
1	0.19	70	9.16 ± 0.16	8.86 ± 0.26	9.37 ± 0.05	8.49 ± 0.09	1.33
2	0.38	115	9.48 ± 0.12	8.96 ± 0.63	9.42 ± 0.10	8.77 ± 0.15	1.32
4	0.76	133	9.38 ± 0.16	8.53 ± 0.26	10.04 ± 0.15	9.05 ± 0.05	1.28
Average of above			9.34 ± 0.19	8.78 ± 0.25	9.61 ± 0.39	8.77 ± 0.33	1.31
Group average ^c			9.50 ± 0.30	8.88 ± 0.32

^aThe original solution contained 9.5 mg. phytic acid per ml. as determined from data in column 4. The figures are averages of 3 to 17 determinations. Standard deviations were determined by the method of Dean and Dixon (see ref. 9).

^bVolume of purified phytic acid (diluted 50-fold) contained 0.19 mg. phytic acid per ml. before dilution by reagents in reaction mixture.

^cPooled group average of 17 determinations for the method based on Fe(OH)₃ from ferric phytate and 8 determinations for the residual iron in the reaction solution.

TABLE III. DETERMINATION OF PHYTIC ACID IN PINTO BEANS
OF DIFFERENT MATURITIES
(Averages of 3 to 6 determinations)^a

Description of Sample	Phytic Acid per g. Solids by Four Procedures					
	Solids %	From Fe Phytate via Fe(OH) ₃ mg.	Indirect, from Residual Fe in Soln. mg.	From Ashed Ferric Phytate ^b		
				From Fe mg.	From P mg.	Fe/P mg.
A, immature	16.3	1.31 ± 0.07	0.044 ± 0.02	1.33 ± 0.07	1.29 ± 0.10	1.24
B, immature	24.8	3.45 ± 0.26	0.108 ± 0.08	3.38 ± 0.78	3.53 ± 0.63	1.21
F, partly mature	47.7	9.45 ± 0.28	9.33 ± 0.66	9.71 ± 0.25	9.20 ± 0.63	1.26
J, mature	85.1	9.36 ± 0.13	10.37 ± 0.42	9.75 ± 0.24	9.55 ± 0.05	1.23
K, mature	87.6	11.10 ± 0.24	11.89 ± 1.13	11.08 ± 0.24	10.20 ± 0.46	1.30

^aStandard deviations were estimated by the method of Dean and Dixon (see ref. 9).

^bFe/P = weight ratio of iron to phosphorus in ashed ferric phytate; theoretical = 1.20 for mole of 4 Fe/6 P.

obtained with the Fe(OH)₃ procedure. The average results by the direct iron procedures (Fe(OH)₃ and ashed Fe⁺³) agree within 3%, whereas the results by the indirect, residual iron procedure differ up to 9% from the other two iron methods.

Far greater differences between the above procedures were observed in the results with Pinto beans given in Table III. All three iron-based procedures agree reasonably well in mature beans of relatively high phytic acid content. But in immature beans of low phytic acid content, the differences in results become very large. The amount of phytic acid determined by the direct iron procedures was of a different order of magnitude (30 times as great) from that determined by the indirect iron procedure (Table III, samples A and B). These differences must be ascribed to the inherent inaccuracy of the latter procedure. At low phytic acid levels, the indirect procedure is subject to large errors because the results are based on small differences of two relatively large numbers. The indirect procedure is clearly not suitable for determinations of small amounts of phytic acid in immature beans.

It may be noted that the two procedures based on the direct measurement of iron in ferric phytate (via Fe(OH)₃ and via ashing) agree within 4% at all stages of bean maturity and all phytic acid levels tested. This confirms the quantitative nature of the conversion of ferric phytate to ferric hydroxide by the employed procedures.

The phytic acid values based on phosphorus in Tables II and III are lower than those based on iron. The Fe/P weight ratios in the last columns of both tables thus are higher than the theoretical ratio of 1.20 (assuming 4 Fe/6P in ferric phytate). This is true for measurements on purified phytic acid as well as for much of the phytic acid extracted from beans. Occlusion of iron to ferric phytate or possible formation of complexes might explain the high Fe/P values (11). Nevertheless, the differences between phytic acid values based on the determination of phosphate vs. those based on iron (in Tables II and III) do not exceed 10%.

GENERAL CONCLUSIONS

When dealing with small amounts of phytic acid in limited quantities of

material, as in bean ovules, the selection of methods is important and the conditions of extraction and determination must be controlled to obtain meaningful results.

A comparison of the four procedures for determining phytic acid described above leads to the conclusion that the determination of iron in ferric phytate via ferric hydroxide gives results comparable to those obtained in determinations via phosphorus or iron, or both, from wet-ashed ferric phytate. The method based on residual iron in the reaction mixture is more variable and subject to error, especially in material with small amounts of phytic acid. Yet it may be useful for a rapid analysis of material with high levels of phytic acid, if cautiously applied. The advantages of the determination of iron in ferric phytate via ferric hydroxide are: 1) it is faster and more convenient than the ashing methods; 2) it permits the removal of interfering, iron-containing material that coprecipitates with ferric phytate in immature beans.

Acknowledgments

The author wishes to thank L. M. White, Mrs. M. C. Long, and Mrs. B. A. Ricci for some of the phosphorus analyses.

Literature Cited

1. HEUBNER, W., and STADLER, H. Über eine Titrationsmethode zur Bestimmung des Phytins. *Biochem. Z.* 64: 422 (1914).
2. YOUNG, L. The determination of phytic acid. *Biochem. J.* 30: 252 (1936).
3. McCANCE, R. A., and WIDDOWSON, ELSIE M. Phytin in human nutrition. *Biochem. J.* 29B: 2694 (1935).
4. CREAN, D. E. C., and HAISMAN, D. R. The interaction between phytic acid and divalent cations during the cooking of dried peas. *J. Sci. Food Agr.* 14: 824 (1963).
5. SCHORMÜLLER, J., HOHNE, R., and WÜRDIG, G. Untersuchungen zur Bestimmung des Phytins. *Deut. Lebensm.-Rundschau* 52: 213 (1956).
6. MARRESE, R. J., DUELL, R. W., and SPRAGUE, M. A. A comparison of three current methods for the analysis of phytin phosphorus. *Crop. Sci.* 1(1): 80 (1961).
7. MOKOWER, RACHEL, J. Changes in phytic acid and acid-soluble phosphorus in maturing Pinto beans. *J. Sci. Food Agr.* 20: 82 (1969).
8. SANDELL, E. B. *Colorimetric determination of traces of metals* (3rd ed.). Interscience: New York (1959).
9. DEAN, R. B., and DIXON, W. J. Simplified statistics for small numbers of observations. *Anal. Chem.* 23: 636 (1951).
10. ANDERSON, G. Effect of iron/phosphorus ratio and acid concentration on the precipitation of ferric inositol hexaphosphate. *J. Sci. Food Agr.* 14: 352 (1963).
11. EARLEY, E. B. Determining phytin phosphorus. *Ind. Eng. Chem. (Anal. Ed.)* 16: 389 (1944).
12. ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS. *Official methods of analysis* (10th ed.). The Association: Washington, D.C. (1965).
13. ALLEN, R. J. L. The estimation of phosphorus. *Biochem. J.* 34: 858 (1940).
14. SAMOTUS, B., and SCHWIMMER, S. Indirect method for determination of phytic acid in plant extracts containing reducing substances. *Biochim. Biophys. Acta* 27: 389 (1962).
15. SCHORMÜLLER, J., and WÜRDIG, G. Über die Trennung und Identifizierung der Inositolphosphorsäureester durch Papierchromatographie. *Z. Lebensm.-Untersuch. Forsch.* 105: 397 (1957).
16. DesJOBERT, A., and PETEK, F. Chromatographie sur papier des esters phosphoriques de l'inositol. *Bull. Soc. Chim. Biol.* 38: 871 (1956).
17. SALKOWSKI, E. Über eine Verbesserung der Schererschen Reaction auf Inosit. *Z. Physiol. Chem.* 69: 478 (1910).

[Received September 9, 1969. Accepted November 17, 1969]