

Structural Model for Ferric Phytate: Implications for Phytic Acid Analysis

D. B. THOMPSON and J. W. ERDMAN, JR., Department of Food Science, University of Illinois, Urbana 61801

ABSTRACT

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Numerous phytic acid assays rely on the precipitation of phytate with ferric ion. Work with the iron-phosphorus molar ratio of the ferric phytate precipitated from soybean extracts indicated that this ratio was subject to variation. Further investigation regarding the precipitate led to a molecular

hypothesis that may help to explain the variable nature of the molar ratio of this precipitate. Phytic acid assays that rely on analysis of iron from the ferric phytate precipitate are not recommended.

The various methods for the analysis of phytic acid (*myo*-inositol hexaphosphate) were recently reviewed (Thompson and Erdman 1982). Many of these methods employ ferric chloride for precipitation of phytate from extracts from plant foods. Analysis of the resultant ferric phytate precipitate for either phosphorus or iron has been used to calculate the original amount of phytic acid in the extract.

Three assumptions are made when precipitation of ferric phytate is employed as an analytical tool: precipitation of phytate is quantitative; contamination of the precipitate with lower phosphate esters of inositol does not occur; and contamination of the precipitate with inorganic phosphorus does not result in false elevation in apparent phytate content. When phytic acid is calculated by analysis of iron from the precipitate, a fourth assumption is made—that the molar ratio of iron-phytate phosphorus is definite.

Using HPLC, Graf and Dintzis¹ showed that the addition of ferric chloride to wheat bran extract resulted in the complete precipitation of phytate. Clearly, under appropriate conditions, precipitation of phytate can be quantitative. De Boland et al (1975) examined the ferric phytate precipitate from extracts of corn germ, wheat, rice, soybean flakes, and sesame meal. Only the hexaphosphate of *myo*-inositol was found in the precipitate of these diverse samples. Therefore, for these materials, contamination with lower phosphate esters should not be a problem. Ellis et al (1977) noted that the presence of high inorganic phosphate in an extract can lead to contamination of the precipitate. However, they showed no significant effect due to inorganic phosphorus in wheat bran, which was reported to contain only 4.5% of total phosphorus as inorganic phosphorus. Earle and Milner (1938) reported the inorganic phosphorus in soybeans to be 4.5% of total phosphorus; thus, one would not expect significant contamination of the phosphorus in the ferric phytate precipitate from soybean extract.

A lingering concern that has not been adequately investigated is the reliability of the iron-phosphorus molar ratio. Our objective was to examine the variable nature of ferric phytate and to propose a hypothesis regarding the structure of the complex.

MATERIALS AND METHODS

Materials

Soybeans used throughout these studies were of the Bonus 75 variety grown in Champaign County, IL. Beans were stored at 1°C until processing. Whole soybeans were heated at 200°F for 20 min in a Variable Air Dryer (Proctor and Schwartz, Inc., Philadelphia, PA) to harden the hulls. Hulls were loosened with the aid of a spinning drum-plate apparatus. Spacing between the drum and plate was adjusted to split the cotyledon and to loosen the hulls. Hulls and split cotyledons were partitioned using a forced-air conveyor belt system. Remaining hulls were removed by hand. Soy meal was prepared using a Grain Mill attachment to a model K5-A

Hobart Mixer (Hobart Corporation, Troy, OH). Soy meal was passed through a 60-mesh screen and stored in plastic pouches at 4°C until analysis. Moisture was determined to be 8.3% by overnight drying at 105°C in a convection oven (Blue Line, Model OV500, Chicago, IL). All subsequent analyses of this soy meal were on an "as-is" moisture basis.

Phytate Assays

Phytate assays employing analysis of the precipitate for iron were done by a modification of the method of Earley and deTurk (1944) made by Forbes² and further modified by Thompson and Erdman (1982).

A 2-g sample of soy meal was placed in an Erlenmeyer flask, and 100.0 ml of 1.2% HCl + 10% Na₂SO₄ was added. The flask was stoppered and shaken for 2 hr on a mechanical shaker at room temperature. The extract was then vacuum filtered through #4 Whatman paper. The filtrate (10.0 ml) was pipetted into a 50-ml centrifuge tube. Deionized water (10 ml) was added, followed by 12 ml of FeCl₃ solution (2.0 g FeCl₃ · 6H₂O + 16.3 ml of concentrated HCl). The contents were stirred, heated 75 min in boiling water, covered, and cooled for 1 hr at room temperature. The tube was centrifuged at 1,000 × g for 15 min in a model SBV International centrifuge (International Equipment Co., Boston, MA). The supernatant was decanted and discarded, and the pellet was thoroughly washed three times with a solution of 0.6% HCl and 2.5% Na₂SO₄. After each wash, the contents were centrifuged at 1,000 × g for 10 min and the supernatant discarded.

Ten milliliters of concentrated HNO₃ were added to the resulting pellet. The contents were transferred quantitatively to a 400-ml beaker with several small portions of deionized water. Four drops of concentrated H₂SO₄ were added, and the contents were heated approximately 30 min on a hot plate until only the H₂SO₄ remained. Approximately 4–5 ml of 30% H₂O₂ was added, and the mixture was returned to the hot plate at low heat until bubbling ceased. The residue was then dissolved in 15 ml of 3*N* HCl and heated for 10–15 min. The resulting solution was made up to 100.0 ml volume, diluted 1:5, and then analyzed for iron.

This procedure differed from that of Earley and deTurk (1944) in the following ways: sugars were not removed by alcohol extraction; the amount of ferric ion added was 3.6 times the theoretical amount, according to the method of Earley (1944); heating was employed to shorten the 4-hr time for precipitation; and the precipitate was digested and analyzed for iron.

Iron was analyzed by atomic absorption spectrophotometry. A model 306 Perkin-Elmer spectrophotometer with digital ppm readout (with a linear range of 1–3 ppm) was used.

Where indicated, phosphorus in the digested ferric phytate precipitate was analyzed by the method of Bartlett (1959), which is a modification of the method of Fiske and Subbarow (1925).

RESULTS AND DISCUSSION

The modified Earley and deTurk (1944) method, which relies on analysis of the ferric phytate precipitate for iron, was performed on different days on 13 different triplicate samples of soy meal with

¹Graf and Dintzis. 1981. Personal communication.

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²R. M. Forbes. 1978. Personal communication.

1.2% HCl and 10% Na₂SO₄ in the extraction step and 0.6% HCl and 2.5% Na₂SO₄ in the wash steps. A phytate value of 1.40 ± 0.13% (mean ± S.D.) was found, with a range of 1.12–1.60%. Analysis of the ferric phytate precipitate for phosphorus was performed on the same number of triplicate samples under the same extraction and wash conditions. These were not always the same samples that were analyzed for iron. The value obtained was 1.38 ± 0.07%, with a range of 1.29–1.53%.

The higher variability resulting from measurement of iron was further investigated. Calculation of phytic acid by measuring iron depends on a definite and constant ratio of iron to phytic acid phosphorus. To make use of iron analysis of the precipitate, a reproducible iron-phosphorus molar ratio must be achieved in the precipitate. According to Earley (1944), this molar ratio is 4:6. Because this ratio is so important, we examined it for those triplicate samples for which both iron and phosphorus of the digested precipitate were analyzed. For those seven different triplicate samples analyzed on seven different occasions, the iron-phosphorus ratio varied from 3.48:6 to 4.39:6. Although the mean of these seven samples was 4.04:6, very close to the theoretical value, a standard deviation of 0.33 was high.

Other workers have been unable to consistently achieve the ideal 4:6 ratio. Morris and Ellis (1976) found that ferric phytate prepared from wheat bran according to the method of Earley and deTurk (1944) had an iron-phosphorus ratio varying from 3.2:6 to 3.5:6. In her analysis of the ferric phytate precipitate prepared from purified phytate, Makower (1970) found a ratio of 4.38:6, whereas the iron-phosphorus ratio found from mature pinto beans was 4.22:6.

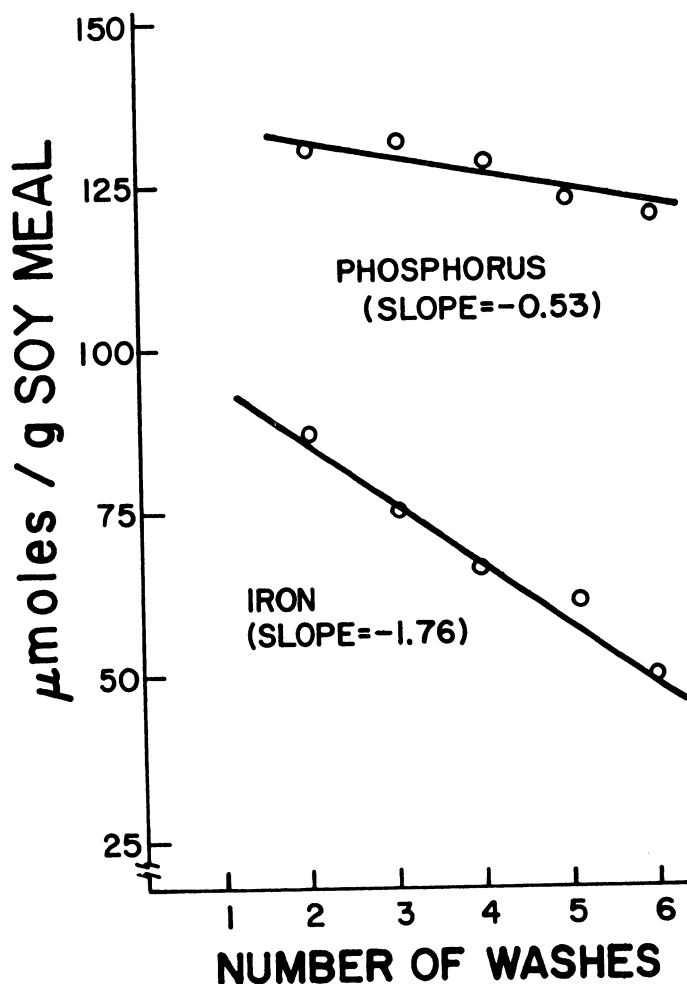


Fig. 1. Effect of washing the ferric phytate precipitate. Ferric phytate was precipitated from soy meal extracted with 1.2% HCl + 10% Na₂SO₄. The wash solution was 0.6% HCl + 2.5% Na₂SO₄. Each point represents the mean of three determinations.

Effect of Washing

The ferric phytate precipitate forms in an aqueous ferric chloride medium. The washing steps are crucial for analysis of iron in the precipitate because incomplete washing will lead to contamination of a precipitate with soluble ferric ion. An experiment was performed to test the effect of the washing procedure on the amount of iron measured in the precipitate. Five identical samples of soy meal were extracted with 1.2% HCl + 10% Na₂SO₄. These five samples were treated identically through the precipitation, centrifugation, and second washing steps. One sample was centrifuged and digested after the second wash, and the rest of the samples were subjected to additional wash steps. Subsequent samples were digested after a third, fourth, fifth, and sixth wash step. Iron and phosphorus analyses were performed on each sample. The effect of washing on iron and phosphorus content of the precipitate is shown in Fig. 1. The effect of washing on the iron-phosphorus ratio is shown in Table I. The change in this ratio with additional washing indicates that the composition of the precipitate is changing and that the loss of iron is not simply due to precipitate solubilization. Clearly, the iron-phosphorus ratio obtained in the ferric phytate precipitate is a function of the number of washes. This seemingly innocuous step may account for much of the variation encountered in the iron-phosphorus ratio. Because the slope of the iron line in Fig. 1 is steeper than the phosphorus line, slight day-to-day variation in wash conditions could well account for the higher standard deviation of the iron analysis procedure as compared to the phosphorus analysis procedure.

Effect of Salts

Further investigations revealed that the iron-phosphorus ratio varied with other conditions as well. The presence of sulfate ion in the extraction and wash steps elevated the iron-phosphorus ratio when compared to extraction and precipitation without sulfate. Paired experiments performed on three different days produced the results found in Table II. The results obtained without sulfate varied considerably. The iron-phosphorus ratio was lower without sulfate in each experiment. Thus, achievement of a 4:6 ratio of iron-phosphorus appears to depend heavily on the presence of sulfate ion.

Paired experiments were also done to compare the effect of chloride and sulfate ions, as the sodium salts. Chloride ion lowered the iron-phosphorus ratio when it was substituted at the same ionic strength for sulfate ion in extraction and wash steps: a value of 3.50:6 was found when chloride was present, considerably lower than the 4.22:6 obtained when sulfate was used. These results

TABLE I
Effect of Washing on Fe: P Ratio of Precipitate^a

Number of Washes	Fe: P Molar Ratio ^b
2	3.96 ^c
3	3.49
4	3.16
5	2.98
6	2.46

^a Precipitated after extraction with 1.2% HCl + 10% Na₂SO₄. Washed with 0.6% HCl + 2.5% Na₂SO₄.

^b Ratio of moles iron:6 moles phosphorus.

^c Mean of three determinations.

TABLE II
Effect of Na₂SO₄ on the Fe: P Molar Ratio in Ferric Phytate

Day	Experimental Conditions	
	1.2% HCl Extract, 0.6% HCl Wash	1.2% HCl + 10% Na ₂ SO ₄ Extract, 0.6% HCl + 2.5% Na ₂ SO ₄ Wash
1	3.29 ^a	4.33
2	3.93	4.39
3	3.04	4.06

^a Ratio of moles iron:6 moles phosphorus. Each value is the mean of three determinations.

suggest that the higher iron-phosphorus ratio when sulfate is used may be due to a specific effect of sulfate rather than simply to ionic strength.

In addition to the influence of sulfate on the iron-phosphorus ratio of the precipitate, other effects of sulfate were observed. When sulfate was present in extraction and precipitation steps, precipitation occurred almost immediately upon contact of the centrifuge tube with the boiling water bath. Large (approximately 5 mm in diameter) clumps of white precipitate formed quickly and moved by convection, and the remaining solution became quite clear within a 15-min heating period. However, without sulfate, no precipitation occurred after 15 min of heating; instead the solution remained cloudy. Thirty to 45 minutes were usually enough to cause visible flocculation, not in the form of large clumps but of finer (≤ 1 mm in diameter), more translucent particles that often interacted delicately with each other to form an easily dispersed spherical network of about the diameter of the 50-ml centrifuge tube.

Another observation regarding sulfate is that a precipitate formed in its presence required sulfate in the wash solution for stability. Without sulfate in the wash solution, the standard three washes led to a loss greater than 80% of iron and phosphorus in the precipitate. The precipitate pellet was markedly smaller after washing. The main means of loss appeared to be the precipitate dispersing into such fine particles that centrifugation was ineffective; no gross loss of precipitate occurred when decanting the wash solution.

When the precipitate formed in the absence of sulfate, this dispersive behavior was not observed upon washing without sulfate.

Varied Levels of Ferric Ion

Investigation of the proper iron concentration for precipitation of the material in the 1.2% HCl and 10% Na₂SO₄ extraction supported the work of Earley (1944), which showed that a lower concentration of iron leads to a lower-than-ideal iron-phosphorus ratio (Fig. 2). Based on these results, he suggested using a 3.6-fold excess of ferric ion over what would give a 4:6 iron-phosphorus molar ratio in the extract before precipitation.

An experiment was performed in which three different iron concentrations were used for precipitation: the standard 3.6-fold excess iron recommended by Earley (1944), 2.4-fold excess iron, and 1.2-fold excess iron. The iron-phosphorus ratio decreased from 4.22:6 from the 3.6-fold excess to 3.69:6 for 1.2-fold excess Fe. Although the values did not agree precisely with those obtained by Earley, the ratio clearly had decreased.

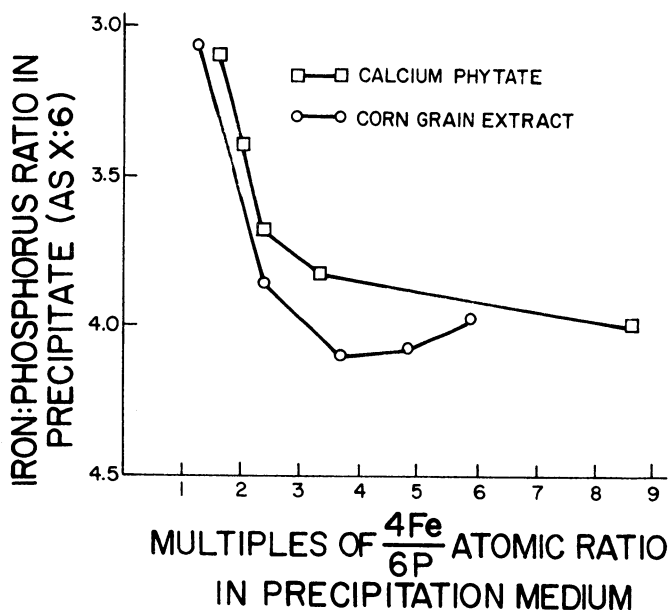


Fig. 2. Effect of ferric ion concentration of the Fe:P ratio in the ferric phytate precipitate (modified from Earley 1944).

Heating Time

The length of heating of the precipitates had a variable influence on the iron-phosphorus ratio, depending on the presence of sulfate. Heating with sulfate for 15 min produced an iron-phosphorus ratio of 3.77:6, whereas heating for 75 min gave a similar iron-phosphorus ratio of 3.79:6. Without sulfate, heating for 75 min gave an iron-phosphorus ratio of 2.5:6, and heating for 180 min increased the iron-phosphorus molar ratio to 2.9:6.

Nature of Ferric Phytate

Variation in the iron-phosphorus ratio is not surprising when one considers that the often-cited 4:6 ratio is probably not based on some identifiable structure with a definite stoichiometric relationship; rather, this 4:6 ratio may be approximated under carefully controlled experimental conditions designed to select for it from the continuum of possible iron-phosphorus ratios.

The experimental data in this paper provide some information regarding the nature of the iron-phosphorus ratio of the precipitate, and this information has led to a hypothesis regarding the structure of the ferric phytate precipitate. Eight ferric ions can be intimately connected with one phytate anion (Figs. 3 and 4A) if a network structure for the precipitate is assumed. In this model each ferric ion would be shared between two phytates. Such a model would account for a 4:6 iron-phosphorus molar ratio in an ideal system. Atomic models predict that the planes of adjacent phytate rings would not be the same, and one may imagine a repeating "pleated-sheet" structure for the precipitate. In an ideal situation then, a 4:6 ratio may be explained. However, the resulting structure would be considerably constrained sterically. It would be exceedingly difficult for the phytate anions to become arranged in exactly the proper configuration to allow maximum ferric ion

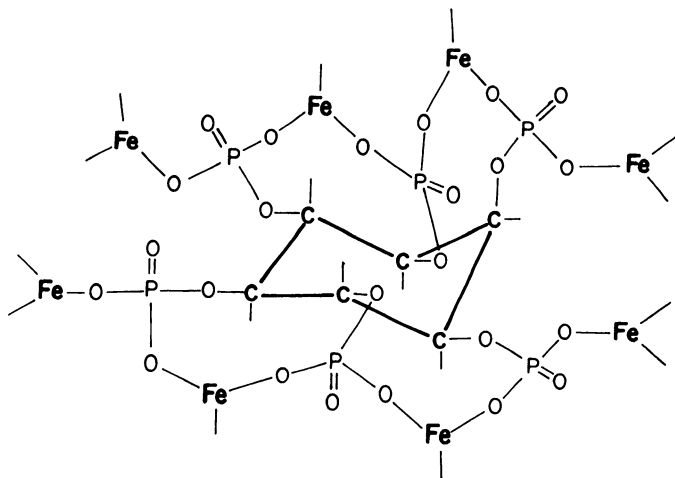


Fig. 3. Idealized structure for ferric phytate with a Fe:P ratio of 4:6. This ratio results because each Fe atom is coordinated to two phytate anions, only one of which is shown.

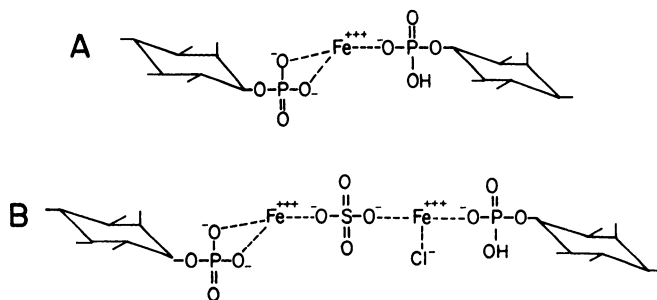


Fig. 4. A, a ferric ion link between phosphate groups of adjoining phytate anions. For clarity, only one phosphate group is shown on each phytate anion; B, a sulfate bridge between two ferric ions coordinated to two phytate anions.

linking. Our results show that heating increases the iron-phosphorus ratio when sulfate is not present in extraction and precipitation steps. Due to the steric constraints of the precipitate structure, it is likely that even with long heating, not all of the possible positions for ferric ion would be filled. Our results show that, without sulfate ion, the ratio is well below 4:6.

The presence of the sulfate group can cause a change in the ligands in coordination with the ferric ion. Sulfate bridges are known in several transition metal complexes that are stable in aqueous solution (Jones 1964). If sulfate is postulated as an occasional bridge between two ferric ions (Fig. 4B), the basis for the 4:6 stoichiometry changes. Ferric ions would no longer be shared between phytate anions in every case, and thus the iron-phosphorus ratio would increase. Steric constraints would be considerably lessened due to the increased distance between phytate anions, allowing the precipitate to form more easily.

This model is supported by the higher iron-phosphorus ratio with sulfate present and also by the observation that considerable heating is necessary for precipitate formation without sulfate. The integral nature of sulfate in the precipitate structure is also supported by the observed dispersal of the precipitate formed in the presence of sulfate when it is washed in a solution without sulfate.

The model may be used to explain Anderson's (1963) observation that excess iron may lead to solubilization of ferric phytate. As the ferric-ion concentration increases relative to phytate, a decreasing tendency for ferric ions to link phytate anions would be expected. Each phytate anion might become saturated with ferric ion, thus decreasing the chance that a single ferric ion would link two phytate anions and increasing the chance that a single phytate anion-multiple ferric ion soluble complex would form. Figure 3 would approximate this case, except that the attached ferric ions would be coordinated to additional nonphytate ligands instead of to other phytate anions, as in the precipitate structure.

The decreased iron-phosphorus ratio with washing of the precipitate may also be explained with reference to the structure for the ferric phytate precipitate. In such a complex system, in which the large polyvalent anions link up through metal ions, steric considerations make it very unlikely that the ideal state would be reached; consequently all ferric ion would not be equally firmly held. Some would be an integral part of the precipitate network, and some would be more peripheral. Washing would tend to remove the more peripherally bound ferric ions but would have less effect on the phytate phosphorus because each phytate anion would be bound to the precipitate network by several ferric ions.

Ellis and Morris (1979) found that the iron in tetraferrous phytate

is more available to the rat than the iron of diferric phytate. This observation might well be a result of the relative lability of the fourth mole of iron in tetraferrous phytate. Such lability is not surprising in a structure in which the fourth mole of iron would be difficult to fix into the precipitate, as is true even in the presence of sulfate (Earley 1944).

CONCLUSION

Given the fluctuating nature of the iron-phosphorus ratio seen even when a standardized method is used, the analysis of iron from the ferric phytate precipitate is not recommended. Reliance on the arbitrary and variable ratio should be avoided.

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