Improved Ion-Exchange Phytate Method

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ABSTRACT

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A modified ion-exchange method was developed for phytate analysis. Data from the modified method indicate that the apparent phytate levels previously determined in some materials by ion-exchange methods were lower than actual levels because some phytate in the acid extract is bound to metal, to protein, or to both. The effect of apparent binding was eliminated when ethylenediamine tetraacetate was added to the acid extract and the pH adjusted to 6. The modified procedure recovers phytate quantitatively from wheat products, human composite diets, and fecal samples. In contrast to data from other ion-exchange methods, data from the modified ion-exchange method agreed closely with that from the iron-precipitation method when the phytate content of the product was above 0.2%. The iron-precipitation procedure is not sensitive to low concentrations of phytate. Concentration is not a factor with the modified ion-exchange procedure.

Iron precipitation and ion exchange are the two most widely used methods for the assay of phytate. Cosgrove (1980) indicated in his review that phytate values from the iron-precipitation method were much higher than those from the ion-exchange method. Harland and Oberleas (1977), who analyzed several textured vegetable protein samples by both methods, reported that in every case but one, phytate values were higher from iron precipitation than from ion exchange. When Ellis and Morris (1982) compared the two methods, phytate values for 12 of 14 products were higher from the iron precipitation than from ion exchange. However, when we precipitated the phytate with iron and converted it to sodium phytate before applying the sample to the ion-exchange column, the phytate values agreed closely with those from the iron-precipitation method. Our results suggested that the phytate values from the ion-exchange method of Harland and Oberleas (1977) were low because of interfering substances in the acid extract. Our results also indicated that the interfering substances were eliminated when phytate was precipitated and converted to sodium phytate. That procedure, however, was too time consuming, and the iron-precipitation method does not give quantitative results at low concentrations. We, therefore, undertook to develop a rapid, quantitative, ion-exchange method for analysis of phytate.

MATERIALS AND METHODS

Materials

White bread, wheat bran, and soy isolate were obtained from the Division of Nutrition, Food and Drug Administration, Washington, DC. Dephytinized wheat bran, wheat-bran muffins, and composite diets were prepared as described by Morris et al (1980). Human fecal samples were from the study by Morris et al (1980). The bran-muffin mix was purchased locally. Ion-exchange columns were glass-barrel Econo-columns (Bio-Rad Laboratories, Richmond, CA) 0.7 × 15 cm. The anion-exchange resin was 200–400 mesh AG1-X8 chloride form (Bio-Rad Laboratories). Sodium phytate was purchased from Sigma Chemical Co. (St. Louis, MO).

Reagents

Disodium ethylenediamine tetraacetate (EDTA) - NaOH solution (0.11*M* EDTA - 0.75*M* NaOH): 10.25 g of Na_2 (EDTA) and 7.5 g of NaOH were added to a 250-ml volumetric flask and made to volume with deionized water. A molybdate solution of 12.5 g of ammonium molybdate was added to a 500-ml volumetric flask, dissolved in deionized water, and then made to volume. The molybdate solution was made fresh weekly and filtered through Whatman no. 41 filter paper on the day of use. Sulfonic acid reagent, 0.2 g 1-amino-2-naphthol-4-sulfonic acid, 2.4 g of sodium sulfite, and 12 g of sodium bisulfite were dissolved in 90 ml of deionized water and then made to 125 ml. The sulfonic acid reagent was prepared fresh weekly and stored at 4°C.

Methods

Samples (2 g) of dried material were extracted for 2 hr at room temperature with either 40 ml of 2.4% HCl or with 1.2% HCl containing 10% sodium sulfate when the extracts were to be precipitated with iron. The mixture was centrifuged and then filtered through Whatman no. 41 filter paper. The filtrate could be

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refrigerated up to five days without loss of phytate. The iron-precipitation method of Ellis et al (1977) was used as a basis for evaluating the present ion-exchange method.

The present ion-exchange method is a modification of the procedure by Harland and Oberleas (1977). The 0.7 × 15.0-cm tubes were clamped vertically and packed with 0.5 g of anion-exchange resin in a deionized water slurry. Before use, the columns were washed with 10 ml of 0.7 *M* NaCl to assure chloride saturation of the resin and then with two column volumes of deionized water. One milliliter of the sample extract was mixed with 1 ml of EDTA-NaOH solution, and then diluted to about 30 ml with deionized water and applied to the column. The sample container was rinsed, and the column was washed with 15 ml of deionized water to elute most of the inorganic phosphate. The remaining inorganic phosphate was eluted with 15 ml of 0.1 *M* NaCl. The phytate was then eluted from the column with 15 ml of 0.7 *M* NaCl and collected in a 100-ml micro-Kjeldahl flask. Concentrated H_2SO_4 (0.5 ml), concentrated HNO_3 (3.0 ml), and two glass beads were added to the digestion flask. The mixture was digested on medium heat until active boiling ceased, and a cloud of thick yellow vapors appeared in the neck of the flask. The flasks were heated for 5 min more on medium heat and for 5 min on low heat, and then allowed to cool. Deionized water (15 ml) was added, and the flasks were placed in a boiling water bath for 15 min. The content of the digestion flask was quantitatively transferred to a volumetric flask (50 ml when the phytic acid concentration of the sample was less than 20 mg/g and 100 ml when the phytic acid concentration was greater than 20 mg/g). The phosphorus in the digest was determined by the Fiske-Subbarow (1925) method. When the 50-ml volumetric flask was used, for example, the content was diluted to about 35 ml with deionized water, 3 ml of molybdate solution was added, and the content mixed; 1 ml of sulfonic acid reagent was added, and the flask was made to volume with deionized water. After standing for 10–15 min, the color was read at 640 nm on a Bausch and Lomb colorimeter. When the 100-ml volumetric flask was used, 0.5 ml of concentrated H_2SO_4 was added, and all other reagents were doubled. The phytic acid values were calculated from the organic phosphorus by assuming that one molecule of phytic acid contained six molecules of phosphorus. The percent recovery of added phytic acid was calculated as follows:

$$\text{Percent Recovery} = \frac{\text{mg of phytic acid found in spiked sample} - \text{mg of phytic acid found in unspiked sample}}{\text{mg of added phytic acid}} \times 100.$$

TABLE I
Effect of EDTA[a] on Recovery of Phytate by Ion-Exchange Chromatography

Material[b]	Percent Recovery[c]	
	Without EDTA	With EDTA
White bread	97.7±1.1	99.4±0.9
Human composite diet	90.5±1.0	99.6±0.8
Wheat bran	83.2±0.7	97.4±1.3
Wheat-bran muffin	74.0±0.3	98.9±1.8
Human feces	78.4±0.8	98.9±1.7
Swine feces	79.4±0.7	99.8±1.5

[a] Ethylenediamine tetraacetate.
[b] Nineteen milligrams of phytic acid as sodium phytate was added per gram of material.
[c] Mean ± standard deviation of three determinations.

RESULTS AND DISCUSSION

In the preliminary work, we used the method of Harland and Oberleas (1977) based on ion-exchange methodology. We found that adjusting the pH of an acid extract of wheat-bran muffin from 2 to 6 before adding the sample to the column increased the recovery of phytic acid from 65% at pH 2 to 90% at pH 6. In contrast, the recovery of phytate from fecal samples decreased from 70% at pH 2 to 30% at pH 6. Apparently, recovery from fecal samples decreased at pH 6 because phytate complexed with metal; metal concentration was about 10 times as high in fecal as in muffin samples. Some divalent metals combine readily with phytate at pH 6 (Oberleas 1973). We added calcium chloride, magnesium acetate, and sodium phytate to an aqueous medium. The divalent metals did not affect the recovery of phytate at pH 2 when we used the method of Harland and Oberleas (1977). Recovery, however, decreased to 70% when the pH of the medium was adjusted to 6. On the other hand, the recovery of phytate from wheat-bran muffins may have increased because pH 6 is above the isoelectric point of the proteins. Okubo et al (1976) showed that at pH levels above the isoelectric point of glycinin (pH 4.9), no binding could be detected between glycinin and phytic acid. Between pH 5.0 and 2.5, however, binding produced insoluble complexes. To minimize the effect of both metals and protein, we added EDTA to the sample extract and adjusted the pH to 6 with NaOH before applying the sample to the ion-exchange column. The effect of EDTA-NaOH on recovery of phytate added to various materials is shown in Table I. In the absence of EDTA-NaOH, recoveries were poor from all products except white bread. When EDTA-NaOH was added to the samples, recoveries were 97–100%.

Dilution of the sample extract before its introduction into the ion-exchange column apparently is flexible. Increasing the concentrations of the composite diet extract from 1:30 to 2:30 did

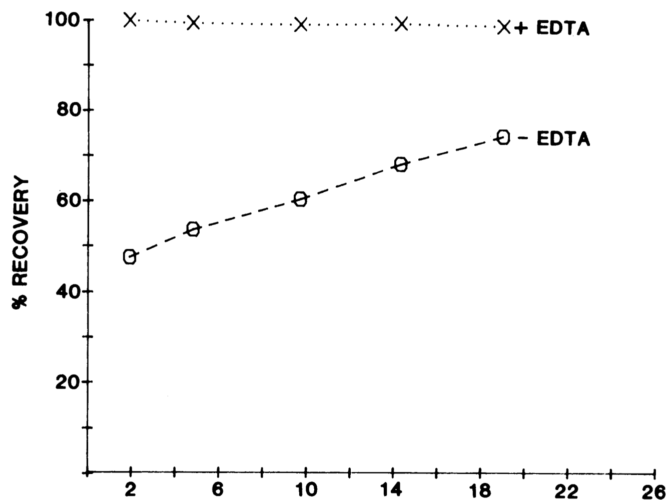

Fig. 1. Influence of phytate concentration on recovery from dephytinized wheat-bran muffins.

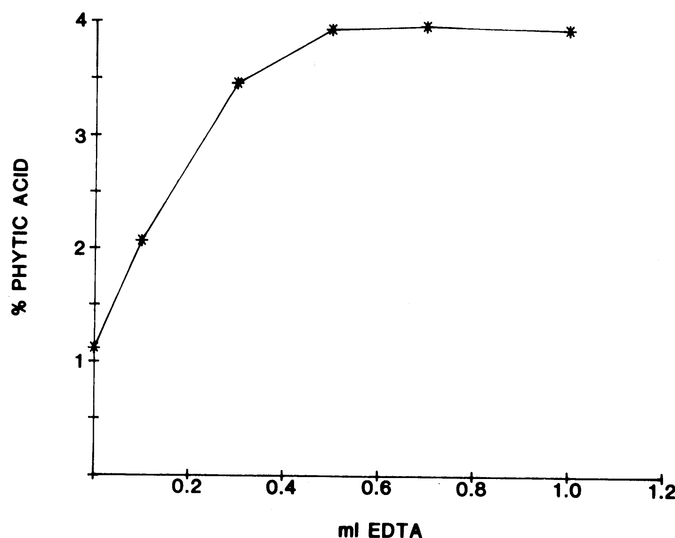

Fig. 2. Influence of ethylenediamine tetraacetate (EDTA) concentration on the phytate values of fecal samples. The EDTA solution contains 100 μmol of EDTA per milliliter.

TABLE II
Effect of Metals and Proteins on Ion-Exchange Phytate Method

Materials[a]	Percent Recovery[b] Without EDTA[c]	With EDTA
Ca (2 mg), Mg (1 mg)[d]	99.2±1.3	99.2±1.2
Casein (1 g)	96.3±0.5	98.8±0.7
Casein (1 g) Ca (2 mg), Mg (1 mg)	85.7±0.8	98.0±0.4
Egg white (1 g)	92.6±1.0	97.9±0.7
Egg white (1 g) Ca (2 mg), Mg (1 mg)	73.8±1.1	98.8±1.4

[a] Nineteen milligrams of phytic acid as sodium phytate was added per gram of material. All materials were extracted with 20 ml of 2.4% HCl.
[b] Mean ± standard deviation of three determinations.
[c] Ethylenediamine tetraacetate.
[d] Calcium as calcium chloride; magnesium as magnesium acetate.

not affect phytate value. At the dilution of 2:30, however, 2 ml of EDTA-NaOH solution was added per sample.

Figure 1 shows the effect of phytate concentration on recovery from dephytinized wheat-bran muffins. In the absence of EDTA-NaOH, percent recovery increased linearly with concentration of added phytate. In the presence of EDTA-NaOH, recovery was excellent at all concentrations.

The effect of EDTA concentration on determining endogenous phytate of fecal samples is shown in Fig. 2. The pH was adjusted to 6 for all levels of EDTA concentrations including zero. In the new modified method, 1 ml of EDTA-NaOH solution was added per milliliter of acid extract. The phytate concentration of the fecal samples, however, reached a plateau at 0.5 ml of EDTA-NaOH. The results, therefore, suggest that 1 ml of EDTA-NaOH solution in the modified method is a fairly large excess because fecal samples had the highest metal content of all samples tested.

The effects of purified proteins and metals on recovery of phytate are shown in Table II. No detectable phytate appeared in casein or egg white. Those proteins, however, are used in animal diets, and we were concerned about their effect on the phytate analysis of a composite diet. In the absence of EDTA-NaOH, calcium and magnesium apparently did not affect recovery. The proteins alone had only a slight effect. When the metals and proteins were combined, however, the effect was synergistic, and recovery was depressed. In contrast, lactalbumin (not shown) did not affect recovery of phytate with or without metals.

To determine whether the unrecovered phytate in extracts of bran muffins remains on the ion-exchange column or elutes with the nonphytate phosphorus, we measured the distribution of the phosphorus in the eluates (Table III). The results indicate that in the presence of EDTA-NaOH, all phosphorus in the extract was recovered with H_2O, 0.1*M* NaCl, and 0.7*M* NaCl. Without EDTA-NaOH, residual phosphorus remained on the ion-exchange column and was eluted with 2*N* HCl. Analysis of the 2*N* HCl eluate for inositol indicated that the phosphorus was indeed phytate phosphorus. Apparently, most of the unrecovered phytate remains on the column when no EDTA-NaOH is added to the extract. These data (Table III) also indicate that without EDTA, about 20% of the unrecovered phytate elutes with the nonphytate phosphorus. The results further suggest that 2*N* HCl, as the eluting solvent for phytate, gives higher phytate values than 0.7*M* NaCl when no EDTA-NaOH is added. We found, however, that the phytate values were still 5–10% lower without than with EDTA-NaOH. Graf and Dintzis (1982) eluted the phytate from the ion-exchange column with 2*N* HCl instead of 0.7*M* NaCl because HCl is volatile and can therefore be removed before the sample is applied to the HPLC.

The iron-precipitation procedure and the modified ion-exchange methods are compared in Table IV. In contrast to data from published ion-exchange methods (Cosgrove 1980, Harland and Oberleas 1977), data agree closely between our modified ion-exchange procedure and the iron-precipitation method. The iron-precipitation method of Ellis et al (1977) was designed to eliminate any inorganic phosphate that might precipitate with the ferric

TABLE III
Effect of EDTA[a] on the Distribution of Phosphorus in Eluates[b]

Eluate	μg Phosphorus[c] Without EDTA	With EDTA
H_2O and 0.1*M* NaCl	1,346±19	1,094±24
0.7*M* NaCl	4,355±63[d]	5,596±47[d]
2*N* HCl	841±19[d]	NDP
Total P found	6,543±97	6,675±19
Percent recovery	99.0±1.5	100.7±0.5

[a] Ethylenediamine tetraacetate.
[b] An acid extract of bran muffins; total phosphorus in extract was 6,611±55 μg.
[c] Mean ± standard deviation for four determinations.
[d] Phytate phosphorus.

TABLE IV
Comparison of Iron-Precipitation and Modified Ion-Exchange Phytate Methods

Product	Method and Percent of Phytic Acid[a] Iron Precipitation	Modified Ion Exchange
Soy isolate	1.58±0.02	1.49±0.08
Wheat-bran muffin	1.27±0.03	1.23±0.06
Wheat bran	2.86±0.07	2.83±0.06
Human feces	3.98±0.10	3.91±0.09

[a] Mean ± standard deviation for four determinations of endogenous phytic acid.

phytate. In analyses of samples with low (<0.2%) phytic acid contents, however, iron precipitation of the phytic acid in the extract was not quantitative. Graf and Dintzis (1982) reported similar findings.

Our present data confirm our published data (Ellis and Morris 1982), showing that interfering substances in the acid extract result in lower phytate values by the ion-exchange method. The addition of EDTA-NaOH to the acid extract to eliminate the interference does not lengthen the time required for the ion-exchange method. One analyst can analyze about 18 samples per day.

In summary, our new modified anion-exchange method for analysis of phytate gives quantitative results for wheat products, human composite diets, and feces. Preliminary results suggest that it might also be used for other products. Our results indicate that interfering substances in the acid extract explain the difference in levels of phytate determined by iron-precipitation and published ion-exchange methods. Phytate values of previous ion-exchange methods are low because of interfering substances in the acid extract. The addition of EDTA to the sample extract and adjustment of the pH to 6 eliminated that interference such that levels of phytate agreed closely between our new ion-exchange method and the iron-precipitation method.

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