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Determination of Phytic Acid in Foods by High-Performance Liquid Chromatography

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Our recently reported high-performance liquid chromatographic method for the determination of phytic acid [*myo*-inositol 1,2,3,5/4,6-hexakis(dihydrogen phosphate)] has been examined in greater detail and adapted to the analysis of foods. This slightly modified procedure consists of extraction of sample with 0.5 N HCl, filtration, dilution with water, passage of extract over anion-exchange resin AG 1-X8, and elution of phytic acid with 2 N HCl. An aliquot is taken to dryness, redissolved in 5 mM sodium acetate, and analyzed by reverse-phase liquid chromatography on a μBondapak C_{18} column. As little as 0.003% phytic acid in foods may be quantitated with a coefficient of variation of less than 5%. The high sensitivity and specificity, superior accuracy, reliability, and mechanical ease of the procedure and the lack of interference from high concentrations of protein and cations appear to make this method suitable for routine analyses of most food samples.

Much of the world's supply of food protein is derived from cereals and soybeans. With a continually expanding protein need, increasing emphasis is placed on developing the necessary food technology to use other plant proteins directly for human consumption. Concomitantly, in Western diets there is now a trend toward substituting meat with plant foods. Despite the caloric adequacy of these plant foods, they do have a lower mineral content and may interfere with the bioavailability of minerals. Reports in the literature suggest use of such foods may lead to human imbalances of Ca^{2+} (Mellanby, 1949), Mg^{2+} (Seelig, 1964), Fe^{3+} (Davies and Nightingale, 1975), and Zn^{2+} (Oberleas and Harland, 1981). Well-documented examples include Zn^{2+} deficiencies in some Iranian tribes (Reinhold, 1972) and in low-income preschool children in the United States (Hambidge et al., 1976).

The putative antinutritional agent present in plant foods that lowers the mineral bioavailability is phytic acid [*myo*-insoitol 1,2,3,5/4,6-hexakis(dihydrogen phosphate)]. It constitutes 1–6% by weight of most nutritionally important legume, cereal, and oilseeds (Cheryan, 1980), where it serves as a store of phosphate that becomes available during germination (Hall and Hodges, 1966). It forms insoluble complexes with di- and trivalent cations at neutral pH (Vohra et al., 1965), potentially rendering these minerals unavailable for intestinal absorption. Several recent review articles discuss the chemistry and nutritional implications of phytic acid (Maga, 1982; Cheryan, 1980; Erdman, 1979; O'Dell, 1979).

Most commonly used methods for the determination of phytic acid are modifications of the procedure developed by Heubner and Stadler (1914). Such methods involve the precipitation of an iron complex at low pH and the subsequent quantitation of phosphorus, iron, or inositol in the precipitate or analysis for residual iron in the supernatant (Oberleas, 1971). Valid criticism generated by numerous assumptions and uncertainties inherent in these methods has instigated the development of new analytical methods that are independent of the formation of an iron phytate complex. A high-performance liquid chromatographic method for the determination of phytic acid was recently reported (Graf and Dintzis, 1982) that combines an ion-exchange procedure (Harland and Oberleas, 1977) with HPLC on a μBondapak C_{18} column (Tangendjaja et al., 1980).

This paper describes a modification and application of our previously reported HPLC method for the determination of phytic acid in a variety of foods.

MATERIALS AND METHODS

Materials. Sodium phytate and boving serum albumin (BSA) were purchased from Sigma Chemical Co. All other chemicals were of reagent grade. Anion-exchange resin AG 1-X8 mesh 200–400 (Cl^- form) was obtained from Bio-Rad Laboratories. Disposable polypropylene minicolumns were purchased from Kontes Scientific Glassware. The HPLC equipment was from Waters Associates and consisted of the following modules: solvent delivery system, Model M-45; automated sample introduction system, Model 710B WISP; μBondapak C_{18} column (0.4 × 30 cm); absorbance detector, Model 440; differential refractometer, Model R401. UV absorption and refractive index were monitored on a Beckman two-channel potentiometric recorder; the refractive index peak areas were obtained through integration by a Modcomp Computer Model Classic 7870. All solvents were prepared with distilled, deionized water, filtered through a 0.45-μm Millipore filter, and degassed by sonication for 1 min. The liquid chromatography column was stored in 0.02% sodium azide.

HPLC Method for Phytic Acid Determination. After being lyophilized (Virtis lyophilizer), pulverized in a cyclone mill (Udy Analyzer Co., Boulder, CO) and defatted with petroleum ether for 4 h in a Soxhlet extractor,

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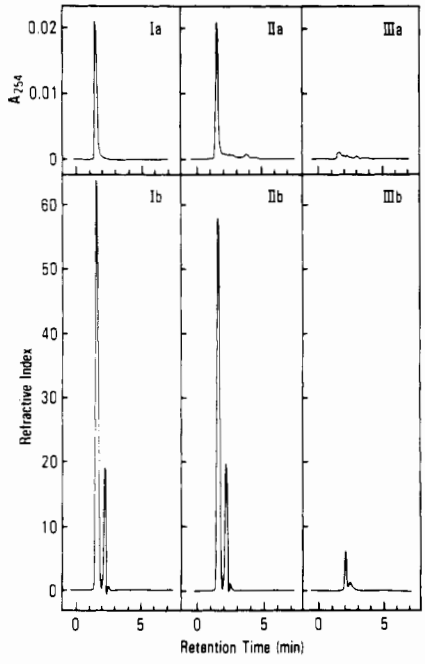

Figure 1. High-performance liquid chromatograms of phytic acid. A solution of 7 mg of phytic acid in 20 mL of 0.5 N HCl was shaken for 2 h and treated by our procedure. Absorbance at 254 nm (panel Ia) and refractive index (panel Ib) were recorded simultaneously. In panels IIa and IIb are shown UV and RI chromatograms of 7 mL of peanut extract, respectively. Treatment of the peanut extract with $FeCl_3$ eliminated the phytic acid peak (panels IIIa and IIIb).

the samples were extracted with 0.5 N HCl (1 g in 20 mL) by vigorous mechanical agitation for 2 h at room temperature. The slurry was centrifuged at 2000*g* for 10 min, filtered through Whatman No. 1 paper, and further clarified by filtration through a 5-μm Millipore filter. The clear filtrate was diluted with 4 volumes of water and percolated over 0.65 mL of AG 1-X8 resin packed in disposable polypropylene minicolumns as previously described (Latta and Eskin, 1980). This dilution with water instead of with 0.125 N NaOH as originally proposed (Graf and Dintzis, 1982) greatly improved the method as discussed in full detail below. The resin column was washed with two aliquots, 10 and 5 mL, of 0.1 N HCl. Phytic acid was eluted with 10 mL of 2 N HCl applied in 1-mL aliquots to reduce the average rate of elution to approximately 0.4 mL/min. A 2-mL aliquot of the eluate was taken to dryness overnight in a vacuum desiccator, and the residue was redissolved in 2 mL of 5 mM sodium acetate. Then 100 μL was applied to the μBondapak C_{18} column and developed with 5 mM NaOAc at a flow rate of 1.5 mL/min. UV absorption at 254 nm and refractive index were recorded on a strip chart recorder.

Colorimetric Method for Phytic Acid Determination. Phytic acid in the clear 0.5 N HCl filtrate was precipitated with $FeCl_3$ and determined by measuring inorganic phosphate of the wet-ashed pellet as described previously (Graf and Dintzis, 1982).

RESULTS AND DISCUSSION

A typical high-performance liquid chromatogram of phytic acid is shown in Figure 1. Phytic acid elutes immediately after the void volume, which corresponds to 1.60 ± 0.03 min under the described conditions. It is well separated from a second peak at 2.18 ± 0.03 min. This minor refractive index peak is seen in all chromatograms and may be caused by minor inorganic impurities, since all common salts tested have a retention time of 2.2 min.

Table I. Effect of Dilution on Recovery of Phytic Acid from AG 1-X8 Column

volume of 0.1 N HCl, mL[a]	% recovery of phytic acid[b]
10	95.8 ± 3.5
50	96.0 ± 1.6
100	94.8 ± 0.8
200	95.0 ± 0.5

[a] Sodium phytate (12.0 mg) was dissolved in different volumes of 0.1 N HCl and applied to 0.65 mL of anion exchange resin. The column was washed with 15 mL of 0.1 N HCl, and phytic acid was eluted with 10 mL of 2 N HCl as described under Materials and Methods. [b] Mean and standard deviation of three samples.

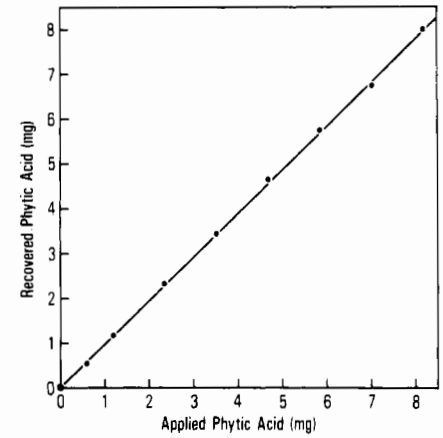

Figure 2. Standard curve for phytic acid in presence of Ca^{2+} and BSA. Different amounts of phytic acid (as indicated on the *X* axis) were dissolved in 10 mL of 0.5 N HCl containing 0.05% Ca^{2+} and 0.625% BSA, and the solutions were agitated at room temperature for 2 h. The simulated extracts were analyzed for phytic acid as described under Materials and Methods. Each value represents the mean of three determinations.

Recoveries of phytic acid ranged between 94 and 96% under our conditions. Much lower values were obtained when phytic acid was eluted with 1 N HCl instead of 2 N HCl or when the rate of elution was increased by eluting with one 10-mL aliquot. Conversely, reducing the rate of elution further by eluting with 20 0.5-mL aliquots instead of 10 1-mL aliquots only increased the recovery from 95.3 ± 0.7% (n = 4) to 97.8 ± 1.7% (n = 4). This change would not increase significantly the overall accuracy and precision of the results obtained from food samples, because the standards are subjected to the identical treatment.

Recoveries are independent of the volume in which phytic acid is applied, as shown in Table I. Also, they are independent of the amount of phytic acid applied, as is demonstrated by the linearity in Figure 2. The capacity of the AG 1-X8 resin was determined to be approximately 20 mg of phytic acid/column. However, we never applied more than 10 mg/column because larger amounts may cause deviation from linearity in Figure 2. The linearity in Figure 2, combined with the volume independence of recoveries as demonstrated in Table I, confer to our assay a sensitivity of 0.3 mg of phytic acid or 0.015% phytic acid/sample and excellent accuracy and linearity over the entire range of phytic acid contents. The detection limit could be increased at least 5-fold if necessary by evaporating a larger aliquot of the eluate and redissolving the residue in a smaller volume. This degree of sensitivity and linearity is unlikely with any method based on the precipitation of ferric phytate, mainly because low concentrations of ferric phytate fail to precipitate and because excess $FeCl_3$ tends to solubilize the complex (Cheryan,

Table II. Effect of Minerals on Recovery of Phytic Acid

cation present	mineral content of sample, ppm	% recovery of phytic acid[a]	mineral content of foods, ppm: mean ± SD[b]	highest value	reference
none		100			
Ca^{2+}	10000	102 ± 6	618 ± 576 (142)	3442	Kirschmann (1975)
	20000	94 ± 7			
Mg^{2+}	5000	103 ± 1	704 ± 836 (103)	3167	Kirschmann (1975)
	10000	98 ± 5			
Cu^{2+}	100	101 ± 1	4.1 ± 4.9 (84)	19	Kirschmann (1975)
	500	104 ± 2			
Zn^{2+}	20	98 ± 1	17 ± 24 (96)	154	Murphy et al. (1975)
	200	99 ± 1			
	2000	99 ± 1			
	20000	98 ± 1			
Fe^{3+}	20	99 ± 1	33 ± 56 (143)	351	Kirschmann (1975)
	200	102 ± 1			
	1000	66 ± 2			
	2000	48 ± 1			
	10000	0			

[a] Mean and standard deviation of four samples. Values have been standardized to relative recoveries, the control being 100%. [b] We calculated the mean and standard deviation of published mineral contents of vegetables, seeds, nut products, and grain products; the number of food items is shown in parentheses.

Table III. Phytic Acid Content of a Range of Foods

food sample	phytic acid, % (w/w): HPLC method[a]	colorimetric method[a]	other methods[b]
dehulled sesame seeds[c]	5.36 ± 0.09	5.24 ± 0.14	5.2 (deBoland et al., 1975)
wheat germ[c]	2.40 ± 0.07	2.40 ± 0.03	3.90 (O'Dell et al., 1972)
Spanish peanuts[c]	1.88 ± 0.05	1.82 ± 0.02	1.76 (Harland and Prosky, 1979)
soy flakes[c]	1.84 ± 0.03	1.78 ± 0.04	1.52 (deBoland et al., 1975)
chicken[d]	0		
ground beef[d]	0		
enriched white rice[c]	0.23 ± 0.01		0.21 (McCance and Widdowson, 1960)
dehydrated pinto beans[c]	1.40 ± 0.03		0.54-1.58 (Lolas and Markakis, 1975)
dehydrated green split peas[c]	0.85 ± 0.02		0.86 (McCance and Widdowson, 1960)
Kellogg's 40% bran flakes cereal[c]	1.17 ± 0.03		
Roman Meal wheat bread[e]	0.38 ± 0.01		0.33 (O'Neill et al., 1980)
human diet[f]	0.17		

[a] See detailed description under Materials and Methods. Mean and standard deviation of three samples; not corrected for defatting. [b] Reference shown in parentheses. [c] Pulverized and extracted with petroleum ether. [d] Lyophilized, pulverized, and extracted with petroleum ether. [e] Air-dried, pulverized, and extracted with petroleum ehter. [f] Lyophilized, pulverized, extracted with petroleum ether and desugared with warm EtOH-H_2O (4:1 v/v). Phytic acid value is corrected to original lyophilized diet sample. This high-fat, high-carbohydrate diet was obtained from the Human Nutrition Center, Grand Forks, ND.

1980). Consequently, 0.12% phytic acid in soy hulls was detected only by HPLC and not by a precipitative method (Graf and Dintzis, 1982).

Though strong anion exchange resins can interact with and catalyze reactions with certain sugars (Buhler et al., 1955; Turton and Pacsu, 1955), our results indicate that AG 1-X8 resin is inert to phytic acid. The unusual stability of phytic acid to hydrolysis is demonstrated by the fact that no loss occurs when the 0.5 N HCl filtered extract is placed in a boiling water bath for 5 min.

We also examined the effect of cations on phytic acid recovery. The cations most prevalent in foods suspected to contain phytic acid have no effect on recoveries (Table II). Trace minerals not listed in Table II should have no effect either, both because of their lower concentration and because their interaction with phytic acid is weaker than that between Cu^{2+} and phytic acid (Vohra et al., 1965). Recoveries are also independent of the concentration of monovalent cations, since they fail to complex with phytic acid. Ferric ions interfere with the assay when present in concentrations exceeding 200 ppm. Samples containing more than 200 ppm of iron should thus be extracted with a larger volume of 0.5 N HCl in order to avoid this interference. However, of 143 food items tested, only 3 contained more than 200 ppm of iron. Similar interference by iron would be expected with all traditional methods, and the analytical chemist should be aware of this potential problem when determining phytic acid in iron-fortified foods.

Finally, we investigated the effect of protein on our assay, since 0.5 N HCl extracts considerable amounts of protein from most oilseeds. The addition of up to 125 mg of bovine serum albumin (BSA) to 7 mg of phytic acid dissolved in 10 mL of 0.5 N HCl had no detectable effect on the recovery of phytic acid or on the UV chromatogram. These results demonstrate that BSA elutes from AG 1-X8 at 0.1 N HCl without interacting with phytic acid. Similarly, clean UV chromatograms were obtained from peanut (Figure 1) and soy flake extracts (not shown), both of which contained substantial quantities of protein. In view of the report that phytic acid interacts with BSA and plant proteins very similarly (Cheryan, 1980), most proteins will probably not interfere with our method.

The recovery of phytic acid from food samples depends not only on the recovery of spiked phytic acid but also on its extractability from foods. There is no consensus on the quality of extracting solvents, perhaps because it varies partially with different substrates. We chose HCl both because of its volatility and because it has been demonstrated rather convincingly to possess excellent extractive

properties under the conditions we used (Makower, 1970; Latta and Eskin, 1980). The screening of different extractants was not within the scope of this study, and the optimum conditions for extracting a particular set of samples and the compatibility of other extractants, such as 3% trichloroacetic acid or Na_2SO_4 in HCl, with the proposed method remain to be determined.

The application of our method to the determination of phytic acid in peanuts resulted in clean chromatograms, as shown in Figure 1. Three lines of evidence indicate that the first refractive index peak in panel IIb of Figure 1 is due solely to phytic acid: (1) the ratio of refractive index peak to UV peak is approximately equal to that of pure phytic acid standard in Figure 1; (2) treatment of the extract with $FeCl_3$ completely eliminates the refractive index peak (Figure 1); (3) values for phytic acid content of peanuts determined by the HPLC method and by a colorimetric method are identical within experimental error. We determined the phytic acid concentration in a wide range of foods, and in each case we obtained chromatograms similar to those shown in Figure 1. Table III summarizes the results of these determinations and juxtaposes them with values obtained by the colorimetric method and with previously published values. The excellent agreement observed in most cases further substantiates the validity of the proposed method.

The major modification of our original method (Graf and Dintzis, 1982) consists of diluting the 0.5 N HCl extract with water instead of 4 volumes of 0.125 N NaOH. This change features three improvements that greatly extend the applicability of the method: (1) proteins acquire a net positive charge and thus do not adsorb to the AG 1-X8 resin, as is demonstrated by the extremely low UV absorbance in Figure 1; (2) high concentrations of divalent cations do not precipitate phytic acid as they would during neutralization; (3) high concentrations of cations are not retained by phytic acid on AG 1-X8 resin, thus minimizing the salt peak. With this modification the method should apply to most legumes, grains, seeds, and human diets.

Our improved method for determining phytic acid is sensitive, selective, reproducible, convenient, and reliable to very low phytic acid concentrations. It lacks interference from other biological compounds, and it is easily adaptable to routine analysis for phytic acid in foods.

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