HPLC Separation and Quantitation of Phytic Acid and Some Inositol Phosphates in Foods: Problems and Solutions

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A macroporous polymer column was used to resolve a mixture of inositol hexakis-, pentakis-, tetrakis-, and trisphosphates within 5-9 min. Optimized eluting systems based on acetonitrile or methanol had improved resolution and reduced chromatographic run times. The silica-based anion exchange column used in the analysis of cereal samples was used to prepare small amounts of pure inositol pentakis-, tetrakis-, and trisphosphates for calibration standards. A number of potential analytical problems are discussed, and solutions are proposed.

Keywords: *HPLC; phytic acid; inositol phosphates; ion-pair chromatography*

INTRODUCTION

Phytic acid (inositol hexakisphosphate, IP6) and some partially phosphorylated inositols are common components in many grain- and legume-based food products. A number of studies have shown that some of these inositol phosphates can interfere with the bioavailability of iron, calcium, and zinc (Morris, 1986). Because of the numerous health benefits of dietary fiber, the consumption of brans from various grains and legumes is increasing. Some brans can contain over 5% phytic acid. Consequently, the ingestion of large amounts of dietary fiber as bran-containing foods will concomitantly result in the ingestion of large amounts of phytic acid. If the consumer is eating a diet marginal in essential minerals, this phytic acid may lead to a nutritional deficiency.

Ion-pair HPLC is a rapid, accurate, and simple method to measure the amount of phytic acid in food products. It permits the food processor to expeditiously monitor the manufacturing process so as to minimize phytic acid carry-through. The 5'-nucleotides, present in some foods and commonly used as flavor enhancers, do not interfere with this assay.

An analytical procedure for phytic acid analysis was recently developed (Lehrfeld, 1989). It used ultrasonication for extraction, a commercially available silica-based anion exchange (SAX) column for concentration and purification, and a high-performance liquid chromatographic (HPLC) method for analysis. The method used the macroporous polymer column PRP-1 and an eluting solvent composed of methanol, formic acid, sulfuric acid, and tetrabutylammonium hydroxide. A typical chromatographic run took 14 min. Herein is reported a modified protocol utilizing acetonitrile in place of methanol. The new methodology permits a reduction in the time required for a chromatographic run to 5 min with no loss in resolution. Also reported is an optimized methanol-based system for which a typical run time is 9.5 min.

Milligram quantities of inositol pentakis-, tetrakis-, and trisphosphates (IP5, IP4, and IP3, respectively) were needed as HPLC reference standards. They were prepared from a phytic acid hydrolysate. The hydrolysate was placed on a SAX column, and the isomers were separated by elution with increasing concentrations of HCl.

Slight deviations from the protocol can lead to unsatisfactory results. A number of potential problems are discussed, and solutions are proposed.

MATERIALS AND METHODS

Materials. Sodium phytate (dodecasodium salt hydrate), phytic acid (40% w/w solution in water), and tetrabutylammonium hydroxide (TBNOH, 40% w/w solution in water) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Phytic acid (50% in water) was purchsed from TCI America (Portland, OR). Silica-based anion exchange columns (SAX, size 3cc) were purchased from Varian (Harbor City, CA). Cation exchange resin AG 50W-X4 50-100 mesh H^+ was purchased from Bio-Rad (Richmond, CA). HCl must be at least trace element grade. Water for HPLC was purified by double distillation or by a Barnstead NANOpure II apparatus and stored in clear glass containers. All other reagents used were analytical grade obtained from various vendors. Pressure tubes used to hydrolyze phytic acid were obtained from ACE Glass (East Brunswick, NJ).

Sample Extraction. The amount of phytic acid extracted from a sample is the same whether by sonication (Lehrfeld, 1989) for 1.5 min, stirring for 2 h, or using a rotator for 2 h. The ratio of sample (g) to 0.5 M HCl (mL, trace element grade) is 0.1-0.5/5. The absolute sample size is dependent on the estimated concentration of phytic acid in the sample. For example, an appropriate sample size for brans with 4% phytic acid is 0.1 g.

Sample Cleanup. The aliquot from the extract (2.5 mL) was diluted with water (1/10) and placed onto the SAX column. The column was washed with 2 mL of water, and then the inositol phosphates were eluted with 2 mL of 2 M HCl (trace element grade). The eluate was evaporated to dryness with a Savant Speedvac concentrator at 40 °C or a vortex evaporator at 40 °C or by placing it in a vacuum desiccator (mmHg <0.5) containing sodium hydroxide and Drierite at room temperature for several hours. The residue was dissolved (ultrasonic bath for 6 min) in a vacuum-filtered buffer solution prepared by adding 1.6 mL of TBNOH, 0.2 mL of 5 M sulfuric acid, and 0.1 mL of formic acid (91%) to 100 mL of a methanol-water solution (51.5%). The solution was centrifuged at 14 000 rpm for 6 min to remove any suspended material prior to injection into the HPLC. The absolute amount of buffer used to reconstitute the residue is dependent on the estimated concentration of phytic acid in the sample being analyzed. Some suggestions are offered in Table 1.

Sample Analysis. HPLC analysis was performed using a Waters pump Model M6000 (Milford, MA) equipped with a 20 or 100 μL fixed-loop injector Model C6W, Valco Instrument Co. (Houston, TX) or a Spectra-Physics Model 8100 pump and

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Table 1. Suggested Sample Size and Reagent Quantities for the Quantitation of Samples Containing Variable Amounts of Phytic Acid

phytic acid[a] (%)	sample size[b] (mg)	extract aliquot[c] (mL)	buffer[d] (mL)	concn[e] (mg/mL)
0–0.1	500	2.5	0.5	0–0.5
0.1–1.0	300	2.5	1.5	0.1–1.0
1.0–5.0	100	2.5	3.0	0.17–0.83

[a] Estimated concentration of phytic acid in sample being analyzed. [b] Approximate weight of sample to be used for analysis. [c] Volume of extract to be diluted with water and placed on SAX column. [d] Volume of buffer solution used to reconstitute residue for injection into the HPLC. [e] Concentration of phytic acid in sample injected into HPLC.

autosampler (San Jose, CA); a macroporous polymer HPLC column PRP-1 5 μm (150 × 4.1 mm), Hamilton Co. (Reno, NV); and a refractive index detector Model 156, Altex (Berkeley, CA) or a Waters Model 410. Detector signals were processed by a ModComp computer system Model 32/85, Modular Computer Systems (Ft. Lauderdale, FL).

Three solvent systems were compared: the initially used methanol-based solvent system A, an acetonitrile-based system B, and an optimized methanol-based system C. The optimized methanol-based system was developed because of the lower toxicity of methanol compared with acetonitrile. It is much easier to maintain a stable baseline with methanol than with acetonitrile.

Mobile phase A was prepared by mixing 560 mL of methanol and 440 mL of 0.035 M formic acid in water. Ten milliliters of TBNOH solution was added, and the pH was adjusted to 4.3 by the addition of 72% (w/w) sulfuric acid. Solvent was pumped through a heated (40 °C) PRP-1 column at a rate of 0.6 mL/min. Injection volume was 20 μL (Figure 1).

Mobile phase B was prepared by mixing 430 mL of acetonitrile, 570 mL of 0.035 M formic acid, and 10 mL of TBNOH and adjusting the pH to 4.3 with 72% sulfuric acid. Solvent was pumped through a PRP-1 column at room temperature at a rate of 1.0 mL/min. Injection volume was 20 μL (Figure 2).

Mobile phase C was prepared by mixing 515 mL of methanol and 485 mL of water. Eight milliliters of TBNOH, 1 mL of 5 M sulfuric acid, 0.5 mL of formic acid (ACS reagent, 91%), and 0.2 mL of a phytic acid solution (5 mg/mL) were added to the methanol–water mixture. The pH was 4.10. The mobile phase was pumped through a PRP-1 column heated at 45 °C at a rate of 1 mL/min. Injection volume was 20 μL.

The phytic acid solution was prepared by dissolving 83 mg of sodium phytate in 5 mL of water, washing the solution through a minicolumn containing 2.2 mL of Dowex 50W-X8 H^+, and adjusting the volume of the eluent to 10 mL.

Solvent Concentration. A 1:1 methanol–water mixture shrinks in volume by about 4% from that of pure water. Consequently, adding-to-a-volume water to methanol will give a different composition than adding-to-a-volume methanol to water. Addition of a fixed volume of methanol to a fixed volume of water gave consistent results. Before the mixed solvent is added to the HPLC reservoir bottle, it is filtered. During vacuum filtration methanol preferentially will be lost. The amount lost will vary with the temperature in the room, the tightness (pore size) of the filter membrane, and the length of time the solvent is kept under vacuum. Adding methanol to make up lost volume gives fairly consistent results.

Measured (Apparent) pH. The pH of a methanol–water solution will vary with the concentration of methanol. For example, a precise amount of TBNOH, formic acid, and sulfuric acid will have a pH of 3.3 in water, and apparent pH values of 5.7 in methanol and 4.1–4.35 in 51–56%, respectively, methanol–water mixtures. The pH reading from different probes can vary by as much as 0.25 unit, even after calibration with the same buffers. Because of variability in the response time of various electrodes, it is easy to overshoot the 4.3 target pH when using a slow response electrode during addition of sulfuric acid. For example, a Beckmann general purpose pH electrode when first dipped into the eluting solvent gave a pH reading of 3.88. After 75 min, the pH reading finally reached a plateau at 4.21. In contrast to this, an Aldrich electrode with a narrow thin wall gave a stable reading of 4.30 within 2 min. The probe should be carefully washed after calibration; the phthalate used in pH 4 buffer elutes in the same region as IP5 and can cause a phantom peak.

Samples Analyzed. Phytic acid standards, cereal grains, soy concentrate, oat cereal, toasted wheat cereal, toasted corn cereal, and the sample of wheat bran were prepared as previously described (Lehrfeld, 1989). The rice brans were a group treated in various ways. All samples of rice bran were extracted with hexane before analysis by HPLC to remove the oil. Injection of samples containing large amounts of oil can damage the column (change the chromatographic behavior of the column).

The effect of column dormancy on the quantitation was negligible. Two sets ($n = 3$) of wheat bran were extracted and analyzed, one during a period of continuous column usage and the other after the column had been dormant for a week. The values for IP6 had an RSD of 3.6.

Primary Standard. Phytic acid, dodecasodium salt hydrate, was used as the calibration standard. The water content was calculated from the percent phosphorus and the percent carbon. On this basis the water content of the current standard is 19%. Calculated: P, 17.03%; C, 6.55%; Na, 25.1. Found: P, 17.0%; C, 6.51%; Na, 24.6. HPLC analysis on an HPX 87H column indicated the presence of 0.4% phosphoric acid.

Phytic Acid Hydrolysis. Sodium phytate (4.3 g) was dissolved in 26 mL of water and poured through a cation exchange column (2.5 × 18 cm, AG 50W-X4) to exchange hydrogen for sodium. The eluent was evaporated to a dry syrup (2.79 g). A sample of the colorless syrup was analyzed for phytic acid by HPLC and was found to contain 97.5% IP6 and 2.5% IP5. The syrup was dissolved in 50 mL of water, and 5 mL aliquots were added to a series of glass pressure tubes and heated at 148 °C for 40, 75, 95, 120, and 180 min in an aluminum heating block. The hydrolysates were analyzed by HPLC (Lehrfeld, 1989). A similar experiment was performed at 97 °C (Figure 3).

Fractionation of Inositol Phosphates. A phytic acid hydrolysate or the commercial phytic acid solution was diluted to contain about 8–20 mg/mL solids. One milliliter of this solution was added to 10 mL of water and poured onto the SAX column (new or previously used) attached to the vacuum manifold. Ten milliliters of wash water was poured through the column. The vacuum was maintained at 50–75 mmHg. The retained inositol phosphates were desorbed from the column by the sequential addition (5–10 mL portions) of hydrochloric acid (0.10, 0.15, 0.20, 0.25, 0.30, 0.35, and 2 mL of 2 N HCl). The individual eluents were collected in weighed 20 mL scintillation vials, and the hydrochloric acid was removed by evaporation at 35 °C on a Savant Speedvac concentrator. The dried fractions were weighed and analyzed by ion-pair HPLC (Figure 4).

RESULTS AND DISCUSSION

A new PRP-1 column with mobile phase A readily resolves IP3, IP4, IP5, and IP6 (Figure 1). The column gradually degrades as the number of injections increases. Resolution can be maintained by decreasing the eluotropicity of the solvent and adjusting for the broadened peaks by increasing elution rate and column temperature. After around 3000+ injections, the column efficiency decreased to such a degree that IP3 now appeared on the tail of the solvent peak. It was no longer possible to balance a decrease in eluotropicity (Robinson et al., 1980) with an increase in elution rate because the back pressure was approaching the column limit (5000 psi). Acetonitrile (cP20° = 0.37, $E°$ = 0.072) has a lower viscosity and a higher eluotropic index than methanol (cP20° = 0.60, $E°$ = 0). With the acetonitrile system (mobile phase B), an excellent balance between

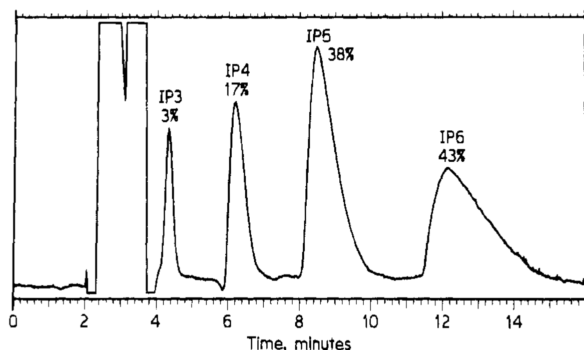

Figure 1. PRP-1 column with methanol-based eluent (mobile phase A) results of reversed-phase high-performance liquid chromatography of a commercial phytic acid solution by ion-pair chromatography. Sample was eluted from a Hamilton PRP-1 column with a 0.015 M formic acid solution containing 56% methanol and 0.4% tetrabutylammonium hydroxide. The pH of the eluent was adjusted to 4.3 with sulfuric acid. The column temperature was 40 °C, and the elution rate was 0.6 mL/min.

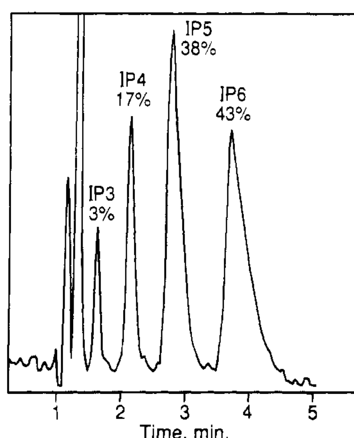

Figure 2. PRP-1 column with acetonitrile-based eluent (mobile phase B) results of reversed-phase HPLC of a commercial phytic acid solution by ion-pair chromatography. The sample was eluted from a Hamilton PRP-1 column with a solvent composed of 0.02 M formic acid containing 43% acetonitrile, 0.4% TBNOH, and sufficient sulfuric acid to adjust the pH to 4.3. The column was kept at room temperature, and the elution rate was 1.0 mL/min.

eluotropicity and elution rate was found that gave better resolution of IP3, IP4, IP5, and IP6 with the old column (Figure 2) than was obtained with a new column with the methanol-based system. In addition, the elution rate could be increased to 2 mL/min to give a comparable chromatogram in 2.5 min. Routine use at 2 mL/min is not recommended because of high back pressure (>4000 psi). Back pressure can be reduced by raising column temperature to 45 °C.

A standard calibration curve for IP6 was prepared by utilizing mobile phase B, the acetonitrile-based system. The detector response is 0.9 as large as that obtained with the methanol system. Nevertheless, it is linear between 0.5 and 10 mg/mL. The correlation coefficient was calculated to be 0.999.

A sample of wheat bran from the previous study was treated, as previously described, and quantitated by utilizing the acetonitrile-based system. The sample was found to contain 5.34% IP6 vs 5.44 ± 0.16% previously reported.

As previously reported, a negative air peak can occur under IP4. The position of the air peak depends on the concentration of acetonitrile. Generally, it is small relative to the sample peaks. The vacancy peak approximates the size of a phylate peak containing 0.019 mg/mL. The sample can be partially deaerated by utilizing a 10 mL syringe. However, some autosamplers with variable injection capabilities inject an air bubble with the sample. The vacancy peak created by the air bubble can distort or eliminate the IP4 peak in the sample.

It is important that the reagents be pure, and the water must be free of organic and inorganic impurities. Trace element grade HCl must be used for sample extractions and elution of inositol phosphates from the SAX column. The concentration of heavy metals in analytical grade HCl is sufficiently high to cause the formation of an insoluble phytate–metal complex after evaporation of the SAX eluate. This can result in the complete loss of a phytate peak in the chromatogram. Samples containing large amounts of iron may have to be pretreated with a cation exchange resin. Ion-pair chromatography has been described as a double-layer dynamic ion exchange process (Iskandarani and Pietrzyk, 1982). The addition of any organic or inorganic impurities can severely distort the complex equilibria and change the chromatography. The presence of small amounts of K^+ completely destroyed the resolution in the methanol system.

A column employing the previously used methanol system gave satisfactory (not excellent) resolution of IP4, IP5, and IP6 for 5000 injections. The acetonitrile system, described herein, gives excellent and very rapid resolution of IP3, IP4, IP5, and IP6. This system may be as durable as the methanol-based system. Subsequent columns used with the optimized methanol system gave excellent resolution for more than 5000 injections. The column degraded due to excessive pressure buildup caused by incomplete removal of small particles in the injected sample. Centrifugation of all samples at 14 000 rpm solved this problem.

It appeared that the simplest source for "pure" IP3, IP4, and IP5 would be an acid hydrolysate of phytic acid. Reported methods for hydrolyzing sodium phytate varied from adjustment of pH to about 4 and heating at 100 °C for 4–8 h to the addition of HCl and heating at 100 °C for several hours. Each of these procedures required a cleanup step at the end of the reaction to remove some anion or cation.

The approach taken here was initially to remove the sodium ion by ion exchange chromatography and then let phytic acid act as its own acidic catalyst. To determine which reaction conditions would give a suitable mix of isomers, a series of hydrolysis experiments was carried out at 148 °C and the hydrolysates examined by ion-pair HPLC. The results are summarized in Table 2. As expected, the shorter hydrolysis times provide solutions with higher concentrations of IP6, IP5, and IP4 relative to IP3. The solution hydrolyzed for 75

Table 2. Isomer Distribution as a Function of Hydrolysis Time

	hydrolysis time			
isomer group[a]	40 min	75 min	95 min	120 min
IP3	0.90[b]	3.7	4.4	5.5
IP4	7.0[b]	14.0	10.5	8.9
IP5	13.6[b]	17.0	9.9	5.9
IP6	19.6[b]	7.1	3.6	1.7

[a] Analyzed by ion-pair chromatography. Hydrolysis temperature 148 °C. [b] Concentration of isomer group in mg/mL. Starting concentration of phytic acid was 56 mg/mL.

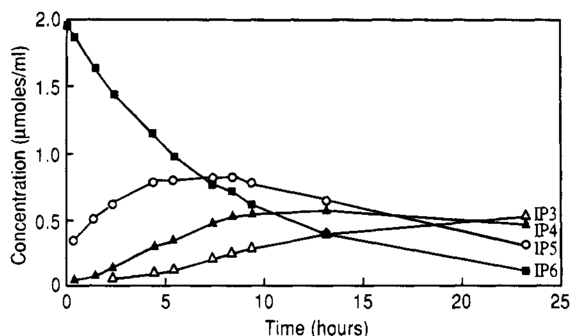

Figure 3. Hydrolysis of phytic acid at 97 °C. The formation of IP3, IP4, and IP5 was followed by HPLC.

Table 3. Analyses of SAX Eluents

eluting solvent (HCl)	isomer distribution[a] (%)			
	IP3	IP4	IP5	IP6
0.10	93.7[b] (100)[c,d]	6.3 (0)	0	0
0.15	10.7 (94.2)	84.4 (5.8)	6.0 (0)	0
0.20	0 (4.1)	16.6 (93.1)	82.7 (2.8)	0
0.25	0 (1.1)	0.7 (14.7)	80.5 (84.2)	17.6 (0)
0.30	0 (0.7)	0 (2.6)	9.6 (96.1)	89.2 (0.6)
0.35	0	0	2.0 (14.8)	96.2 (87.2)

[a] Analyzed by ion-pair chromatography. Commercial 40% phytic acid was stock solution. [b] Previously used column. [c] Figures in parentheses represent isomer distribution found when new SAX columns were used. [d] Very small amount of IP3 and probably contaminated with IP2 and IP.

min was deemed the most useful since it furnished the highest yield of IP4 and IP5. At 97 °C the rate of hydrolysis was much slower. Ten hours was required to get a good isomer mix (Figure 3). A commercial 40% w/w phytic acid solution was found to contain a suitable mixture of isomeric inositol phosphates. The solution contained IP6 (36.7%), IP5 (38.2%), IP4 (20.5%), IP3 (4.6%), and 15% unknown materials in addition to an appreciable amount of iron. If larger quantities of IP3 are desired, a more suitable approach might be a synthesis from *myo*-inositol and phosphoric acid. Initially, the inositol phosphate mixtures were fractionated on the SAX minicolumns, individually evaporated, weighed, and analyzed by ion-pair chromatography. In Table 3 is a summary of a typical run. After marginal differences between columns were noted, the eluents were combined and flash evaporated. A second run through the columns using these enriched fractions gave isomers of 93+% purity (Figure 4).

The SAX columns can be used multiple times for analysis and preparation of fractions. The retentivity of the columns decrease slightly with use. When used in the preparative mode, the concentration of acid may have to be reduced to optimize the yield of the various fractions (Table 3). After each use, they must be washed immediately with water to neutrality, then washed with methanol, and dried. Some columns have been used in excess of 10 times.

The SAX column described here offers a larger loading capacity than the ACCELL QMA column suggested by Wreggett and Irvine (1987). If positional isomers are of concern, this method using HCl as the eluent is probably not suitable. LiCl could be a suitable substitute, inasmuch as pH would not become too low and the LiCl could be removed by solvent extraction.

Small changes in mobile phase composition can change retention times, resolution of peaks, and peak shape. Seemingly, similar recipes can give different chromatograms. These shifts in peak position are

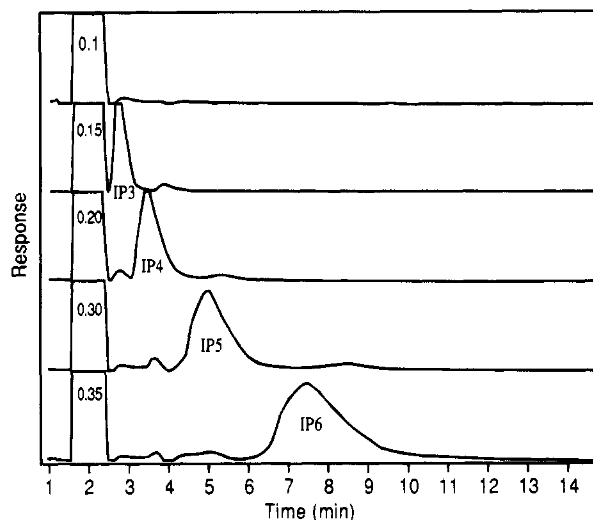

Figure 4. HPLC of SAX purified inositol phosphates: combined eluates of 0.1, 0.15, 0.20, 0.30, and 0.35 N HCl fractions (n = 12).

commonly caused by methanol evaporation, preparing solutions by adding-to-volume, and pH variations. Accuracy and precision are marginally impacted by these deviations. However, neglecting to add phytic acid to the eluting solvent or using a less than trace-element grade of HCl can cause a substantial loss of accuracy and precision.

Phytate Addition to Solvent. Sample adsorption onto metal in a chromatography system has been reported (Dolan, 1991). We have found that nonspecific adsorption of phytate occurs with stainless steel columns. Both silica-based C_{18} columns and polymeric-based PRP-1 columns exhibit this phenomenon. Consequently, a first injection of a sample containing phytate may show no peaks. Subsequent injections will show peaks of increasing size until a plateau is reached when most of the active sites have been blocked by phytate. Therefore, several trial injections of phytic acid must precede any analysis. Blocking of the active sites is an equilibrium phenomenon. Continued washing of the chromatography column with eluting solvent not containing phytic acid will slowly open up the active sites, resulting in subsequent losses of phytate. Sequential injections of a sample containing 0.1 mg/mL phytate (1 injection/h) showed diminishing peak sizes over a period of 15 h. Addition of phytic acid to the eluting solvent (1–3 mg/L) effectively blocks all of the active sites. The exact amount needed is a function of the system. Commercial phytic acid (a mixture of IP3, IP4, IP5, and IP6) can be used, but it should be cleaned up by washing the solution through a C_{18} and a cation SPE column. Another alternative is to substitute a plastic column for the metal column. A PRP-1 PEEK column does not require the addition of any phytic acid to the eluting solvent.

The method is robust in that a number of changes can be made in the protocol without impairing the analysis. For example, the acid and quaternary amine used to prepare the buffer can be changed as well as the column itself.

Acids. Phosphoric, nitric, sulfuric, and acetic acids can be substituted for the sulfuric–formic acid mixture. Resolution is not as good as with the optimized sulfuric–formic acid mixture. Phosphoric acid, however, offers the advantage that nonspecific adsorption of phytate does not occur with metal columns.

Quaternary Amines. Other amines can be used, for example benzyltrimethylammonium hydroxide (BTMNOH) and tetraethylammonium hydroxide (TENOH). Resolution is not improved. However, they offer the advantage that less organic solvent is needed. A direct relationship exists between the carbon content of the amine and the percent organic solvent required. With BTMNOH a methanol–water mixture of 21.5% can be used, while with TENOH only 4.5% methanol is needed. The reduction of methanol from 51.5% to 4.5% may be of special interest for economic and environmental reasons.

Other Columns. Sandberg and Ahderinne (1986) reported the use of C_{18} reversed-phase columns. In our experience, they do not last as long as the polymer-based column. However, the theoretical plates are considerably higher; consequently, the peaks are sharper. We have found a number of commercially available, inexpensive C_{18} columns from Rainin and Alltech to be satisfactory. Other suppliers certainly have suitable columns. Baseline separations of IP6, IP5, IP4, and IP3 can be obtained in 8–15 min with a solvent composition similar to mobile phase C. However, the methanol–water mixture must be modified. For example, C_{18} columns of 50 × 4.6 mm, 150 × 4.6 mm, 250 × 4.6 mm, 250 × 2.1 mm required methanol–water concentrations of 44%, 47%, 54.5%, and 46%, respectively. The solvent elution rate for all columns was 1 mL/min except the 250 × 2.1 mm column, which was 0.4 mL/min. With the PRP column, the eluting solvent can be left in the column for weeks. When the pump is restarted, one is able to run analyses within 15–30 min. The C_{18} column has to be washed after use and then equilibrated before analyses are resumed.

Two vacancy peaks can occur in the chromatogram. One can be found to precede IP3. This negative peak is caused by the presence of phosphoric acid and carbon dioxide in the injected sample and the depletion of TBNOH in the solvent plug preceding the sample. The second negative peak can coincide with IP4 and is caused predominantly by air (nitrogen and oxygen are partially separated). If IP4 is present, detector response will be the algebraic sum of the positive IP4 peak and the negative air peak. Consequently, the amount of IP4 will be underreported. With injected samples containing more than 0.5 mg/mL, the vacancy peak is small relative to the size of the IP4 peak, and thus the reduction in peak size is negligible. The size of the vacancy peak is proportional to injection size. The size of the vacancy peak from a 100 μL injection is equivalent to a sample containing 19 μg/mL of IP4. Thus, concentrating the sample and injecting a smaller volume would also minimize the problem. The vacancy peak can be reduced, not eliminated, by deaerating the water in which the sample is dissolved or by dissolving the sample in the deaerated eluting solvent containing twice the normal buffer concentration. The vacancy peak from a freshly prepared deaerated buffer solution is negligible. Two other remedies are available for samples containing small amounts of IP4. The position of IP4 can be shifted so that the vacancy peak will occur between IP3 and IP4. Thus, a change in the methanol concentration from 51.5% to 50% and a longer run time (14 vs 9 min) will provide this shift. Alternatively, because the size of the vacancy peak is constant for a given injection size, one can measure the area of the vacancy peak in a solvent run and add it to the area of IP4 or let the computer subtract a solvent run from a sample run. The sample in Figure 5 was dissolved in water. The shoulder on IP4 demonstrates the effect of the vacancy peak.

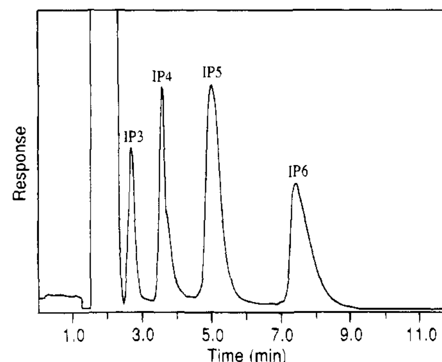

Figure 5. PRP-1 column with optimized methanol-based eluent (mobile phase C) results of reversed-phase HPLC of a phytic acid hydrolysate by ion-pair chromatography. The sample was eluted from a Hamilton PRP-1 column with a solution composed of 0.012 M formic acid, 0.005 M sulfuric acid, and 0.32% TBNOH in a 51.5% methanol–water mixture. The column was kept at 45 °C, and the elution rate was 1 mL/min.

The sample size for food or cereals containing less than 0.1% phytate should be increased to 2 g and extracted with 20 mL of 0.5 M HCl. Ten milliliters of the extract is diluted with 100 mL of water and poured through the SAX column. At low levels (20 μg) adsorption losses onto the SAX column and the glass vial can occur. Small additional losses can occur due to traces of heavy metals. These can be reduced by adding EDTA to the reconstituting solvent. Quantitation at low levels requires a separate calibration curve because of a slight shift in the intercept. The general rule "always bracket your samples with standards" certainly applies.

Over a short period of time solutions of inositol phosphates in water are stable. A sample of a phytic acid hydrolysate was stored at room temperature for 2 weeks. The absolute and relative values for IP3, IP4, IP5, and IP6 from the two runs were within ±2% of each other. The inositol phosphates are also stable in the 0.5 M HCl solution used for the extraction. A 1 mL aliquot was removed from one of the rice bran samples and analyzed on day 1. A second aliquot was removed on day 14 and analyzed. A less than 2% reduction in IP6 and a corresponding increase in IP5 were noted in the second sample.

A series of samples containing 0.008–10.7% phytic acid were analyzed using the optimized methanol methodology. The oil was removed from the treated rice brans with hexane before the phytic acid was extracted with HCl. The results are found in Table 4. These values when compared to values found in the literature (Lehrfeld, 1989; Reddy et al., 1982) indicate that how a sample is stored and dried can have an appreciable effect on the repeatability of an analysis. Freshly milled corn was found to have appreciably more phytic acid than corn milled a month earlier and stored at room temperature (0.78% vs 1.05%). The percent of phytic acid is low, while the percent of IP5 and IP4 is unusually high in the listed grain samples. These samples were specially treated to determine the effect of heat on the phytic acid content of these grains. It is readily apparent that partial hydrolysis of phytic acid can occur at 85 °C. Continued heating after removal of water from the grain sample can cause additional losses of phytic acid by ester or anhydride formation. The bean samples

Table 4. Phytic Acid Analysis of Food, Legume, and Grain Samples by HPLC

	phytic acid in sample[a] (%)	rel %			
		IP6	IP5	IP4	IP3
barley (Betzes)[b]	0.56	69	22	6	3
beans, Great Northern	1.12	93	5	2	0
beans, lima	0.84	87	9	4	0
beans, navy	1.09	91	6	4	0
beans, pinto	0.93	90	5	5	0
buckwheat[b]	1.08	91	8	0.6	0.4
corn, steeped	0.08	74	20	3	3
corn (dent)[b]	0.72	82	16	2	0
corn cereal, toasted	0.07	60	22	10	8
corn flour	0.1	26	38	25	11
millet[b]	0.81	87	11	1	1
oat cereal, toasted	0.38	51	32	13	5
oats (Terra)[b]	0.62	81	16	2	1
rice bran (0.55% oil)	8.70	93	7	NM[c]	NM
rice bran (22% oil)	6.55	92	8	NM	NM
rye[b]	0.57	54	31	11	4
sorghum[b]	0.81	76	19	4	1
soy concentrate	10.7	88	12	0	0
soy flakes, defatted[b]	1.64	92	7	0.5	0.5
spinach	0.008[d]	100	0	0	0
wheat (HRW)[b]	0.77	88	11	1	0
wheat (SWW)[b]	0.65	83	15	2	0
wheat cereal toasted	0.76	70	24	5	1
wild rice	.42	60	26	10	4

[a] Average of three determinations and a RSD less than ±4.1 unless otherwise stated. [b] Samples were heated at 85 °C for 24 h. [c] NM, not measured. [d] A single determination from a 2 g sample.

were not heated. The phytic acid contents of the bean samples are in the ranges reported in the literature. The value for wild rice is much lower than previously reported (0.42 vs 2.2). This is not surprising inasmuch as wild rice is normally cured (naturally fermented) before marketing. Consequently, the phytic acid content may vary from batch to batch.

The method is versatile in that modifications can easily be made and large numbers of samples can be processed in a short period of time. Foods containing small amounts of phytic acid can be analyzed by increasing the size of the sample. The SAX columns can easily handle 100 mL of diluted extract and concentrate the analyte. The small volume of HCl (2 mL) used to remove the inositol phosphates from the SAX column permits 12−24 samples to be handled simultaneously. The vacuum manifold and the evaporation device determine the number of samples. The chromatography column can be of varied types. We prefer the Hamilton PRP-1 column because of its long life and the ability to store the column with the buffer for short periods of time. Removal of the column from the HPLC and storage in a ready-to-go condition improves efficiency by allowing the HPLC to be used for other analyses. After 1 week of being down, the column can be equilibrated and ready to do an analysis within 15−30 min. This is especially useful if small numbers of samples need to be analyzed at irregular intervals. The samples can be extracted in a few minutes by ultrasonication and rapidly run on the equilibrated column. The ability to change buffer systems from one requiring 51.5% methanol (TBNOH) to one using only 4.5% methanol (TENOH) offers economic and environmental advantages.

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