High-Performance Liquid Chromatography Analysis of Phytic Acid on a pH-Stable, Macroporous Polymer Column

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ABSTRACT

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A procedure was developed that enables up to 60 samples containing phytic acid to be analyzed in one day. Extraction time was reduced to 1–3 min per sample by using ultrasonic irradiation. Concentration of the extract was simplified by using a commercially available silica-based, anion-exchange column. High-performance liquid chromatography analysis of the concentrated sample on a macroporous polymer column was accomplished in 8 min. The column easily resolved mixtures containing inositol hexaphosphate, inositol pentaphosphate, and inositol tetraphosphate. Samples of wheat bran as small as 50 mg were analyzed by this system.

Phytic acid (myo-inositol 1,2,3,5/4,6-hexakis [dihydrogenphosphate]) is a common constituent of most mature cereal grains and some vegetables and fruits (Oberleas 1973). In grains it often occurs as phytin (a mixed calcium-magnesium salt of phytic acid) and can represent 60–90% of total phosphorous (Lolas and Markakis 1975). Levels can vary with growing conditions, maturity (Makower 1970), type, variety, and mill fraction of the grain. For example, whole wheat can contain 0.32% phytate, the germ fraction 1.1% (O'Dell et al 1972), and the bran fraction 5%. Sesame seed contains 5.18% phytate (deBoland et al 1975), whereas beans (*Phaseolus vulgaris*), depending on variety, contain 0.54–1.58% (Lolas and Markakis 1975).

Excessive amounts of phytic acid in the diet can have a negative effect on mineral balance because phytic acid forms insoluble complexes with Cu^{2+}, Zn^{2+}, Fe^{3+}, and Ca^{2+} at physiological pH values (Graf 1986, Nolan et al 1987) and, consequently, reduces the bioavailability of these minerals (Morris 1986). Nutritional deficiencies can (Oberleas 1973) but need not necessarily result (Lee et al 1988). Feeding studies utilizing quail (Tao et al 1986) showed that the antinutritive effect can be eliminated by the partial dephosphorylation of phytic acid (IP6) to myo-inositol tetrakisphosphate (IP4) or the lower esters. Additionally, processing (Han and Wilfred 1988) and fermentation during dough making (Nayini and Markakis 1983) can dephosphorylate phytic acid to the lower phosphate esters. However, most of the current convenient analytical methods (Graf and Dintzis 1982a,b; Harland and Oberleas 1986) do not differentiate phytic acid from partially dephosphorylated phytic acids; i.e., the methods will not differentiate grain products that can reduce mineral bioavailability from grain products that cannot.

The teleological role of phytic acid also directs our attention to the lower inositol phosphate esters. Currently, phytic acid is considered to be a reserve of phosphorous and cations in plants (Williams 1970), a ballast (i.e., a system to control the level of free phosphate), and possibly an antioxidant to preserve seed viability. Recently it was shown that signal transduction affecting contraction, secretion, proliferation, and photoresponse in animal cells is moderated by myo-inositol (1,4,5) trisphosphate (IP3), myo-inositol (1,3,4,5) tetrakisphosphate (IP4), and calcium (Neher 1987). A similar system appears to exist in plant systems (McMurray and Irvine 1988). A rapid and simple procedure to isolate, identify, and quantify the lower phosphate esters of inositol would facilitate work in this new area.

Oberleas and Harland (1986) recently reviewed the various methods of phytic acid analysis. In general these methods require three steps: extraction, concentration and/or purification, and analysis. Most current procedures use a 1.5–3 hr extraction with 2.4% HCl (Harland and Oberleas 1986). Subsequent concentration and purification may be by anion exchange on a resin-based column or by precipitation as ferric phytate. Phosphorous analysis of the digested eluent from the anion-exchange column or the digested ferric phytate precipitate permits calculation of the amount of phytic acid in the original sample. In both cases the presumption is that the only source of phosphorous is phytic acid. In ferric phytate precipitation, an additional assumption is that complete precipitation of only IP6 occurs. Unfortunately, the anion-exchange column also retains the lower phosphate esters. The "pure" ferric phytate precipitate can also contain some of these partially dephosphorylated phytic acids. Because these methods do not differentiate IP6, IP5, IP4, etc., calculations can be in error. The indirect method of phytic acid analysis in which phytic acid is added to a standard solution of ferric ions has similar deficiencies. After precipitation of ferric phytate is complete, the remaining ferric ion concentration is determined spectrophotometrically (chromophore varies with investigator). The relationship between Fe^{3+} and phytic acid in the precipitate is assumed to be stoichiometric (Fe:P, 4:6, Wheeler and Ferrell 1971) as is complete precipitation (Ellis et al 1977, Kikunaga et al 1985). Partially phosphorylated inositols, if present, are incorrectly included in these phytic acid determinations.

Partially phosphorylated inositols have been identified and quantified by liquid chromatography on an anion-exchange column utilizing formate, NaCl, or HCl gradients. Dagher et al (1987) followed the sequential hydrolysis of phytic acid in high-bran bread using a Dowex 1-X8 column and a linear HCl gradient. This procedure requires large volumes of eluent, long elution times, and large numbers of individual analyses (Bartlett 1982, Phillipy et al 1987, Minear et al 1988, Shayman and BeMent 1988). The solvent gradient precludes the use of ultraviolet (UV) or refractive index (RI) detectors. However a recently described postcolumn detection system circumvents these problems (Mayr 1988, Phillippy 1988). A mass detector or a moving wire flame-ionization high-performance liquid chromatographic (HPLC) detector might be a suitable alternative to the postcolumn detection system.

Previous applications of HPLC to the analysis of phytic acid (Tangendjaja et al 1980; Camire and Clydesdale 1982; Graf and Dintzis 1982a,b; Knuckles et al 1982) used reversed-phase silica columns (C-18 and refractive index detectors). Unfortunately, the solvent front coincided with the phytic acid peak and made quantitation difficult. Lee and Abendroth (1983) recognized the problems associated with quantitating peaks that coincide with solvent fronts and made the quantum leap to the concept of ion pair in order to separate the solvent front from analyte. Sandberg and Ahderinne (1986) and Sandberg et al (1987) extended this application by demonstrating that IP6, IP5, IP4, and IP3 could be separated by adjusting the pH from 7.1 to 4.3.

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The official method for phytate in foods (Harland and Oberleas 1986) is elegant but rather lengthy and lacks the ability to identify and measure the lower phosphate esters of myo-inositol. I wanted a procedure that would address these deficiencies and allow samples to be completely analyzed in one day. The method reported herein shortens the extraction and concentration procedures and uses a durable polymer column for HPLC analysis. The method can identify and quantify IP6, IP5, IP4, and IP3. A comparison between the customary phytic acid analytical methodology (method A) and the method presented in this communication (method B) is summarized in Table I.

MATERIALS AND METHODS

Figure 1 describes the methodology developed for the quantitation of phytic acid in grains and legumes using samples as small as 50 mg.

Samples analyzed (A, Fig. 1) were in the form of flakes or were milled to pass through a 40-mesh screen. Some samples were of indeterminant age. Oats (Dal, a high-protein variety) were from a crop harvested ca. 1973, sorghum and barley from ca. 1977. The whole corn, soy, and wheat brans were from recent harvests and had been stored in a freezer. Moisture determinations were made on samples heated at 100°C in a vacuum oven to constant weight (4 hr). A sample (0.5 g) was added to a 50-ml centrifuge tube (28.5 × 104 mm polyallomer) containing 10 ml of 0.5*M* HCl and stirred to ensure removal of air pockets. The tip of the ultrasonic microprobe (Ultrasonic liquid processor, model W-385, equipped with a 1/8-in. standard tapered microtip probe; Heat Systems-Ultrasonics, Inc., Farmingdale, NY) was inserted halfway into the liquid, and the sample was sonicated for 1–1.5 min (1-sec cycle, 50% duty at energy level 5.0). The suspension (B, Fig. 1) was centrifuged (Sorvall SS-3 automatic centrifuge; Dupont Inst., Newtown, CT) at 15,000 rpm for 15 min. An aliquot (1–5 ml) of supernatant (C, Fig. 1) was removed and diluted with 20 ml of H_2O and poured onto an Analytichem silica-based, anion-exchange (SAX) column (quaternary amine Bond Elut column, 500 mg; Analytichem International, Harbor City, CA) that was connected to a vacuum manifold (Visiprep; Supelco, Bellefonte, PA) set at 50–75 mmHg. The loaded SAX column (D, Fig. 1) was washed with 10 ml of 0.05*M* HCl, and the resin-bound inositol polyphosphates then were eluted with 2 ml of 2*M* HCl into 16 × 125 mm test tubes (E, Fig. 1). Eluted samples were dried by vortex evaporation (vortex evaporator equipped with an aluminum heating block for 16-mm test tubes; Buchler Instrument, Fort Lee, NJ) in vacuo at 40°C. The residue (F, Fig. 1) was resuspended with 1.0 ml (0.5 ml if sample contained less than 1% phytic acid) of water containing 15 μl of tetrabutylammonium hydroxide and sonicated in an ultrasonic bath (model T9BH, L & R Manufacturing, Kearney, NJ) for 5 min. If a precipitate remained after this 5-min solubilization step, it was removed by centrifugation for 5 min at 14,000 rpm in the microcentrifuge (Eppendorf centrifuge model 5415; Brinkmann, Westbury, NY). Twenty microliters of the clear supernatant (G, Fig. 1) was injected into the HPLC unit. The system consisted of a Spectra-Physics (San Jose, CA) model 8100 HPLC, equipped with an autosampler model 8110, capable of injecting 80 samples unattended, a 20-μl fixed loop autoinjector, a model SP8440 variable-wavelength UV-Vis detector and an Altex (Beckman Instruments; Fullerton, CA) model 156 RI detector. For phytic acid analysis, a PRP-1 5-μm (150 × 4.1 mm) reversed-phase analytical column (Hamilton Co., Reno, NE) was used. Mobile phase was prepared by mixing 560 ml of methanol and 440 ml of 0.035*M* formic acid. Ten milliliters of tetrabutylammonium hydroxide (40%, w/w, solution in water) was added, and the pH was adjusted to 4.3 by the addition of 72% (w/w) sulfuric acid (0.3–0.35 ml). Solvent was pumped through a heated (40°C) PRP-1 column at a rate of 0.9 ml/min. Injection volume was 20 μL. A typical chromatogram of a sample containing IP6, IP5, IP4, and IP3 is shown in Figure 2. Peak areas were integrated by a mainframe ModComp computer system (model 32/85; Modular Computer Systems, Ft. Lauderdale, FL). Six standards were run with each series, covering a range from 0.33 to 6.0 mg/ml phytic acid (Fig. 3). Sodium phytate was used as a standard and showed only one peak by HPLC analysis. Concentrations were adjusted by correction for moisture (1 mg of sodium phytate is equivalent to 0.625 mg of phytic acid). Phosphorous analysis of sodium phytate (Aldrich Chemical Co., Milwaukee, WI) was 19.8% (theor. 20.1%).

Smaller samples (0.05–0.1 g) were analyzed as above, except that 15-ml polystyrene centrifuge tubes were used, extraction was with 5 ml of 0.5*M* HCl, sonication was at energy level 4 for 1 min, centrifugation of suspension B (Fig. 1) was at 1,800 rpm for 4 min, and 2 ml of supernatent C-1 (Fig. 1) was diluted with

A. Sample (0.5 g grain or legume specimen in 10 mL, 0.5 M HCl)
↓ Sonicate 1-3 min
B. Suspension
↓ Centrifuge 15000 rpm 15 min
C. Supernatent (Discard Residue)
1. Dilute 1-5 mL with 20 mL water
2. Pour onto SAX column
3. Wash with 10 mL 0.05 M HCl
↓
D. SAX Column (Loaded with IP6,IP5,IP4,IP3)
↓ Elute column with 2 mL 2.0 M HCl
E. Eluate (Contains IP6,IP5,IP4,IP3)
↓ Vortex evaporate at 40°C
F. Residue (Contains IP6,IP5,IP4,IP3)
1. Solubilize (1 mL)
2. Centrifuge 14000 rpm 3 min
↓
G. Supernatent (Sample for HPLC; Contains IP6,IP5,IP4,IP3)

Fig. 1. Scheme for the extraction, purification, and analysis of phytic acid in grain and legume samples.

TABLE I
Comparison of Phytic Acid Analysis Procedures

Method	Extraction	Concentration	Evaporation	Chromatography
Method A	2-hr stirring, 0.5*N* HCl	AG-1×8 column (gravity), 1.5–3 hr	vacuum desiccator, overnite	reversed-phase C-18 silica based
Method B	1–2.5 min sonication, 0.5*N* HCl	SAX[a] commercial column, vacuum manifold, 15–20 min, 12 samples	vortex evaporator, 1.5 hr	reversed-phase macroporous polymer

[a]Silica-based, anion-exchange.

15 ml of water (See Fig. 1). The sensitivity of the method can be increased by solubilizing residue F (Fig. 1) with 0.5 ml of water instead of 1 ml or by increasing the injection size of G (Fig. 1).

Phytic acid (6.2 min) and phosphoric acid (9.3 min) were easily resolved on a Bio-Rad HPX 87H column (7.8 × 300 mm) using $0.01N$ H_2SO_4 as the eluting solvent at 0.6 ml/min. Detection can be by RI or UV at wavelength 190.

RESULTS AND DISCUSSION

The method outlined in Figure 1 is suitable for the quantitation of inositol triphosphate (IP3), inositol tetraphosphate (IP4), inositol pentaphosphate (IP5), and inositol hexaphosphate (IP6, phytic acid) because the extraction and purification procedures used transfer the inositol phosphates from the grain sample to supernatent G (Fig. 1). Because the current report covers applications with an old PRP-1 column, i.e., subsequent to more than 3,000 injections, reproducible quantitation of IP3 was variable and is thus not reported. The variability was due to the difficulty in choosing a suitably reproducible tangent on the rapidly descending solvent peak (Fig. 2). A new column gave baseline separations of all four peaks. During the following discussion, inositol phosphates should be understood to mean IP6, IP5, and IP4, unless otherwise specified, and phytic acid to mean IP6 only.

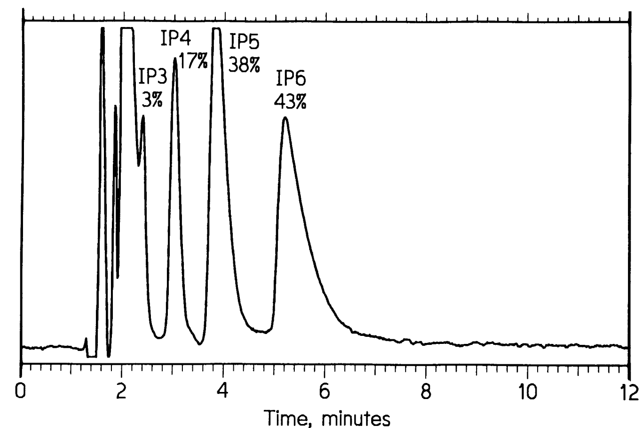

Fig. 2. Reversed-phase high-performance liquid chromatography of a commercial phytic acid solution by ion-pair chromatography. Sample was eluted from a Hamilton PRP-1 column with $0.015M$ formic acid solution containing 56% methanol and 0.4% tetrabutylammonium hydoxide. The pH of the eluent was adjusted to 4.3 with sulfuric acid. The column temperature was 40°C, and the elution rate was 0.9 ml/min. **IP3** = inositol triphosphate, **IP4** = inositol tetraphosphate, **IP5** = inositol pentaphosphate, **IP6** = inositol hexaphosphate, phytic acid.

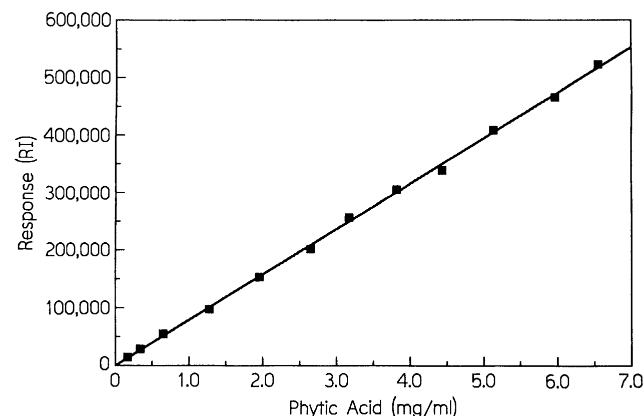

Fig. 3. Standard calibration curve. Correlation between integrated values of refractive index (**RI**) detector response and concentration of phytic acid.

Effects of Extraction Conditions

Treating the sample suspended in $0.5M$ HCl with ultrasonic irradiation reduced the extraction time required for most samples to about 1–3 min. A few problems were noted. Sesame seed, if irradiated for 5–6 min, formed a gel-like suspension. Other samples foamed excessively and formed air pockets that caused incomplete extraction. This problem did not occur if ultrasonication was reduced to 1–1.5 min and pulsed energy was used instead of continuous energy.

Wheat bran and sesame seed samples sonicated for 1, 3, and 5 min did not differ in the amount of inositol phosphates extracted from each sample type. Because the amount of inositol phosphates extracted from peas was variable at 1 min sonication, sonication time was extended to 1.5 min.

Sample Concentration

Concentration of phytic acid is generally accomplished on a Dowex 1 column. However, recovery of phytic acid from some anionic columns can be variable (Ellis and Morris 1986). In addition, the earlier protocols required stepwise addition of 10 ml of $2M$ HCl (Graf and Dintzis 1982b), a tedious, time-consuming procedure. In other words, two factors have to be considered: 1) ability of column to retain the inositol phosphates, and 2) the ease with which the inositol phosphates can be washed off the column. The Analytichem SAX column was superior on both counts. The columns retained 96% of a mixture (40 mg total) of IP6, IP5, IP4, and IP3. At the 20-mg level the columns retained 100% of the mixture. Elution with 2 ml of $2M$ HCl recovered 99.5–100.5% of these inositol phosphates. Columns with three different lot numbers were used and found to be satisfactory; i.e., their adsorption and elution characteristics were similar. If the columns were immediately washed with water until eluent

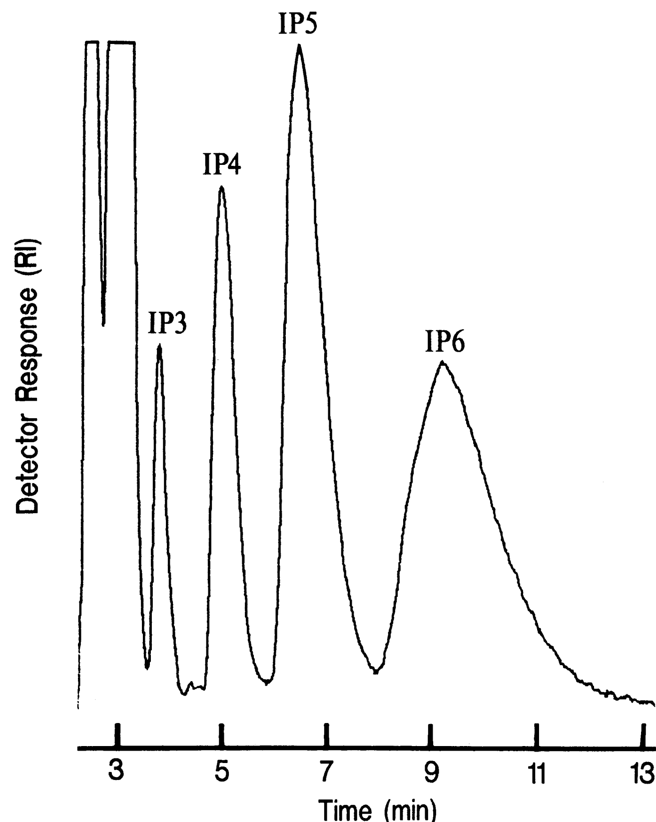

Fig. 4. Reversed-phase high performance liquid chromatography of a commercial phytic acid solution by ion-pair chromatography. Sample was eluted from a Hamilton PRP-1 column with a $0.015M$ formic acid solution containing 60% methanol and 0.4% tetrabutylammonium hydoxide. The pH of the eluent was adjusted to 4.3 with sulfuric acid. The column temperature was 28°C, and the elution rate was 0.5 ml/min. **RI** = refractive index, **IP3** = inositol triphosphate, **IP4** = inositol tetraphosphate, **IP5** = inositol pentaphosphate, **IP6** = inositol hexaphosphate, phytic acid.

pH is 7, they could be reused without affecting results. Some columns clogged after several uses with plant extract samples, whereas others were used 10 times. Bakerbond S.P.E. columns can be used but have a lower capacity (no more than 10 mg of a mixture of inositol phosphates). Extract-clean columns by Alltech have a lower capacity, and elution of phytic acid required more than 2 ml of HCl, thus they were unsatisfactory. The column retained only 75% of a mixture (40 mg total) of IP6, IP5, IP4, and IP3. Elution with 2 ml of 2*M* HCl removed only 9% of the phytic acid held on the column. The corrosive nature of the HCl solutions required an all-glass and plastic vacuum manifold.

We verified Sandberg's (1986) observation that the solutions containing phytic acid can be safely vacuum evaporated at <40° C. Solutions containing 4 mg of sodium phytate in 2 ml of 2*M* HCl were vortex evaporated at 40° C; the residue was redissolved in water, and the solution was chromatographed as described in the experimental section. No new peaks were found and quantitation gave recoveries of 99±3%. These samples run on an HPX 87 H column with 0.01*N* H_2SO_4 showed no phosphoric acid peak.

HPLC

The Hamilton PRP-1 column was selected because it has a usable pH range of 1-14, is stable to a wide range of solvents, and is suitable for ion-pair chromatography. Initially, I planned to eliminate the evaporation step and inject the 2*M* HCl eluate (E, Fig. 1) directly onto the PRP-1 column, since the PRP-1 column is stable to acids. Unfortunately, the detector response of a solution containing 2 mg/ml phytic acid in water was different from the response of 2 mg/ml phytic acid in 2*N* HCl. Apparently, a large excess of hydrogen and chloride ions interfered with the quantitation. When percent recovery of phytic acid (the peak area of an HCl solution containing 2 mg of sodium phytate per milliliter divided by the peak area of the same concentration in water × 100) was compared with HCl concentration, results showed 100% recovery only for 0 and 0.125*M* HCl. Recovery was 76% with 0.25*M* HCl, 53% at 0.5*M*, and 17% at 1.0*M*. This required that the solution from the SAX column be taken to dryness by vortex evaporation, which removes HCl, and reconstituted as previously described, prior to chromatography.

The PRP-1 column was surprisingly durable. After 500 injections, baseline resolution of IP6, IP5, and IP4 was still obtained within 9 min by a mobile-phase mixture containing 56% methanol (Fig. 2). When the column was new, baseline resolution of IP6, IP5, IP4, and IP3 was readily obtained in 13 min by a solvent mixture containing 60% methanol (Fig. 4). As the column aged, peaks broadened and column selectivity decreased. Column selectivity (ability to resolve peaks) was increased by reducing the concentration of methanol to 56%, and peak width was reduced by increasing column temperature from 30 to 40° C and increasing flow rate from 0.5 to 0.9 ml/min. Some cereal grain samples have a tendency to clog the column, as shown by an increase in pressure from 2,600 to over 3,200 psi. Eluting with 0.01-0.05*N* H_2SO_4 at 0.9 ml/min for 1 hr readily clears the column. Another 2 hr was required to reequilibrate the column. The solvent can be conserved by recirculation; however, after 2-3 cycles some small phantom peaks started to appear. Since their peak area represented less than 1% of the peak area found with a typical wheat bran sample, their presence would not compromise the analysis. Their presence was detected by a blank injection.

HPLC Adjustments with Column Age

As the column aged, experimental parameters had to be changed to maintain good resolution. Because knowledge of these relationships can be useful for maximizing analytical efficiency, a summary of some of them follows. The experimental parameters considered were: age of column, sample size, solvent composition, pH, and column temperature.

As expected, column efficiency decreased with column usage. The number of theoretical plates (*N*) is one measure of column efficiency. After 3,000 injections *N* decreased by 60%. When the column was overloaded by increasing the amount of IP6 injected from 10 to 100 μg, the peak width broadened and the peak

TABLE II
Analysis of Phytic Acid by High-Performance Liquid Chromatography

Grain	Relative Percent[a]			Phytic Acid (IP6)[b] Found (%)	Phytic Acid Literature Value (%)	Reference
	IP6	IP5	IP4			
Barley, Hiproly (P)[c]	84	13	3	1.18	0.97-1.08	Maga 1982
Beans, pinto (P)	48	40	12	0.59	0.54-1.58	Maga 1982
Corn						
bran (P)	100	···	···	0.05	0	···
bran wet milled Bestbran-90	100	···	···	0.07	···	···
whole (P)[d]	72	22	6	0.78	0.89	deBoland et al 1975
whole (P)[e]	95	5	···	1.05	0.89	deBoland et al 1975
Oat bran, Quaker Cereal (P)	76	19	5	1.40	···	···
Oat groats, Otee (P)	82	16	2	1.37	···	···
Oat, whole, Dal (P)	78	19	3	0.75	0.79-1.01	Reddy et al 1982
Peas, green split (P)	70	25	5	0.40	0.85	Graf & Dintzis 1982a
Rye, Balbo (P)	78	19	3	1.01	0.88-0.94	Bartnik & Szafranska 1987
Sesame seed, dehulled (P)	83	16	1	5.36	5.36	Graf & Dintzis 1982a
Sorghum, TE77 (P)	84	16	···	0.47	0.57	Reddy et al 1982
Soy flakes, Amsoy (P)	82	14	4	1.33	1.84	Graf & Dintzis 1982a
Soy hull (P)	90	10	···	0.12	0.10	Graf & Dintzis 1982a
Sugar beets, Duofiber	100	···	···	0.01	···	···
Triticale, FAS GRO (P)	83	15	2	1.00	0.5-1.89	Reddy et al 1982
Wheat bran						
Eagle (F)[f]	92	8	···	5.57	3.5	Graf & Dintzis 1982b
Waldron (F)	91	8	1	5.44	4.8	Graf & Dintzis 1982b
WWW AACC (F)[g]	93	6	1	3.95	3.8	Graf & Dintzis 1982b
WWW AACC (P)	91	7	2	5.38	3.8	Graf & Dintzis 1982b
Wheat germ (P)	76	21	3	1.99	2.40	Graf & Dintzis 1982a
Wheat, whole cereal shredded	84	14	2	0.95	···	···
Wheat, whole, soft Arthur (P)	82	16	2	1.08	1.15	deBoland et al 1975

[a] Weight percent.
[b] Corrected for moisture.
[c] P = powder through 40-mesh screen.
[d] 30 days since grinding.
[e] Freshly ground.
[f] F = flake.
[g] Sonicator probe tip 1/8-in from bottom of tube.

retention time decreased by 25%. Nevertheless, the regression plot of concentration versus peak area still had a correlation coefficient of 0.999. Methanol concentration was limited to a useful range of 50–70%. As the methanol concentration was increased, elution time decreased, as did resolution. For example, a 10% increase in methanol concentration (59 to 65%) caused a 21% reduction in elution time of IP6. A 50% reduction in ionic strength reduced elution time by 33%; however, resolution and column capacity were markedly reduced.

Age of solvent has an effect on retention times. Elution time of IP6 was increased by 9% when a week-old rather than a fresh elution solvent was used. The change in pH is probably the dominant factor. A fresh solution adjusted to pH 4.3 reached a plateau at pH 4.6 within a week. Resolution of all peaks remained satisfactory. Partial esterification of formic and sulfuric acids with methanol is the probable cause of the increase in pH. The eluting solvent can have a pH between 4.2 and 5.2 and still give satisfactory resolution. As pH decreased from 4.3, peak retention times decreased until they coalesced. As mobil phase pH increased, peak elution times increased, plateaued at pH 5.5–7, and then coalesced as the pH increased further.

Since the above experiment (effect of pH on resolution) was performed starting with a standard solution and adjusting the pH by adding sulfuric acid or tetrabutylammonium hydroxide, the effects noted are in reality the sum of the effects due to changes in pH and ionic strength. The cation of the base is important. Adjustment of the pH to 5.2 with potassium hydroxide resulted in the complete loss of resolution; four peaks became two poorly separated peaks. Ion-pair chromatography is dependent upon a complex series of equilibria (Iskandarani and Pietrzyk 1982). The addition of K^+ displaces the equilibrium point in an unfavorable way. An increase of 10°C in column temperature (31 to 41°C) reduced the retention time, i.e., resulted in faster chromatographic runs (IP6 25%, IP5 18%, IP4 14%, IP3 9%), and only marginally reduced resolution. Increasing the column temperature has the additional advantage of reducing column pressure (3,200 to 2,400 psi).

A negative peak due to air dissolved in the injected sample occured before IP6 when 65% methanol was the solvent and between IP4 and IP5 with 56% methanol. The peak is of little significance for most samples since the area is comparable to a phytic acid peak area containing 0.17 mg/ml. With samples containing only trace amounts of IP6, IP5, and IP4, this overlapping peak could distort the quantitation. The sample can be deaerated by drawing up 1 ml of sample in a disposable 10-ml syringe, capping the tip, pulling the plunger out to the 5-ml mark (creating a partial vacuum), and shaking for 15 sec. Injection of this deaerated sample generally gave a negative peak only 10% the size of the original peak.

Several observations can be made at this point that might be the basis for some application studies. In a heterogeneous sample, the phytic acid content of two different sieve fractions was found to be different (5.2 vs. 3%). Freshly milled corn had appreciably more phytic acid than corn milled a month earlier (0.78 vs. 1.05%; Table II); the milling process apparently allowed the endogenous phytase to come in contact with the phytic acid. Several samples contained appreciable amounts (relative percent) of partially phosphorylated myo-inositols (IP5 5–40%; Table II). Are these artifacts due to storage or do some samples naturally contain appreciable amounts? This method, with its ability to rapidly screen large numbers of samples, could facilitate such investigations.

The modified extraction and concentration procedures combined with the use of the Hamilton PRP-1 column permit the rapid screening of cereal grains and legumes for phytic acid and other partially phosphorylated inositols (Table II). One technician, alone, has handled 48 samples, and with some assistance for ultrasonication could handle over 60 samples in a day, i.e., go from step A to G (Fig. 1), place G in sample vials, and then let the autosampler inject the samples with the computer collecting and tabulating the data. Samples containing more than 0.5% phytic acid had coefficients of variation less than 3%, whereas samples containing less than 0.1% had coefficients of variation as high as 20%. The coefficient of variation for samples containing such small amounts could be reduced by using a larger sample and a more stable and sensitive detector. The standard curve (Fig. 3) had a correlation coefficient of 0.999. Homogeneous samples were easily and reproducibly run using 100 mg. If the sample is heterogeneous, it would be best to increase the sample size to at least 500 mg.

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