Improved Determination of Phytate by Ion-Exchange Chromatography

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An improved method for the ion chromatographic determination of phytic acid is presented. The method is based on a recently published chromatographic method combined with a treatment of the samples with EDTA. Separation and quantitation of phytic acid were performed on an anion-exchange column. Phytic acid was detected at 300 nm after reaction with a ferric salt in an in-line postcolumn derivatization. The improved method described here was compared with a recently published HPLC method without EDTA treatment and with a method using small single-use ion-exchange columns. It was found that the HPLC method without EDTA treatment of the samples results in values too low for phytic acid, whereas the method using small single-use ion-exchange columns gives values too high. The differences between the results of the three methods were statistically significant ($p < 0.01$).

INTRODUCTION

Phytate is the trivial name for inositol hexaphosphate and is present in many grains and seeds. It is a potential source of phosphorus which can be liberated during the germinating stage of the seeds. Phytate is a polydentate ligand capable of binding metal ions; divalent and trivalent ions especially are firmly bound. These bound metal ions are nutritionally only partly available. Moreover, the analytical determination of phytate may be disturbed by these strong metal-ligand interactions. These interactions render the determination of phytate complicated in many samples.

An early method for the determination of phytate was based on the precipitation of a phytate-iron complex (Oberleas, 1971). This method has been the standard for more than a decade, yielding satisfactory results for grains and seeds with higher phytate contents. The procedure is less suited for products having a low phytate content. An improved method, using ion-exchange column separation, was published more recently (Harland and Oberleas, 1977). This method includes the following steps. An acidic extract of the sample is applied to a small column filled with an anion-exchange resin, and the column is rinsed with a buffer solution of low ionic strength. The phytate is then eluted from the column with a salt solution of higher ionic strength and, finally, the phytate content of the eluate is determined colorimetrically.

This procedure was further improved by Ellis and Morris (1983), who treated the extracts with EDTA to eliminate the interference of metal ions with the phytate determination. This method was recently tested in a collaborative study (Harland and Oberleas, 1986). Unfortunately, optimal results are obtained only with special batches of the ion-exchange resin. Another disadvantage of the procedure of Ellis and Morris is that it is rather laborious. Automated procedures with high-performance liquid chromatography (HPLC) are much more attractive.

Phillippy and Johnston (1985) recently described a promising HPLC method. The phytate content in a series of samples was determined, and the results were compared with those obtained with the procedure of Ellis and Morris (1983). The phytate values obtained with the HPLC method were lower than those obtained with the Ellis and Morris procedure. The cause of this difference might be that the procedure of Phillippy and Johnston does not include an EDTA treatment of the extracts.

Recently, Cilliers and van Niekerk (1986) also published a liquid chromatographic procedure for the determination of phytic acid. Some aspects of their method have been incorporated in our improved analytical procedure. This new method is essentially the chromatographic procedure described by Phillippy and Johnston (1985) and by Cilliers and van Niekerk (1986), combined with a treatment of the sample extracts with EDTA to eliminate the interference of metal ions.

In this study three analytical procedures for the determination of phytate are compared and the differences discussed.

A different approach was published by Sandberg and Ahderinne (1986). They developed an HPLC method using a reversed-phase silica column.

MATERIALS AND METHODS

Materials. Phytic acid was obtained from Sigma (St. Louis, MO). The batch was found to contain 15% moisture. Phytase from wheat was obtained from Sigma. The other reagents used were of analytical grade and were obtained from Merck (Darmstadt, FRG).

Food samples were purchased in food stores in Zeist. The grains and the chyme sample were obtained from the Institute for Livestock Feeding and Nutrition Research (Lelystad, The Netherlands).

The small prepacked columns with ion-exchange resin were of the type Baker 10, No. 7091 (Baker, Phillipsburg, NJ). They were used with the Baker 10 extraction system, No. 7018-0. Membrane filters with 0.45-μm pore size were supplied by Millipore Corp. (Bedford, U.K.).

Liquid Chromatography System. The chromatograph consisted of two modified LDC-Milton Roy minipumps from Biotronik (Munich, FRG). One pump was used for the eluent and one for the reagent. The single-headed pumps were provided with pulse damping devices as are commonly used in automated amino acid analyzers. The anion-exchange column used was a Dionex HPIC AS-3 (0.50 cm i.d. × 25 cm). A Dionex HPIC AG-3 guard column was used. The injector was from Pharmacia (Uppsala, Sweden). The injection volume was 50 μL. The reaction coil was of poly(tetrafluoroethylene) tubing with an internal diameter of 0.3 mm and a length of 3 m. It was kept in an oil bath at 50 °C. A variable UV-vis detector (300 nm) (ABI Analytical, Kratos Division, Ramsey, NJ) was used.

Methods. *Extraction Procedure.* About 2 g of each sample was accurately weighed and shaken with 20 mL of 0.8 M HCl for 2 h at room temperature. The slurry was centrifuged at 1800*g* for 10 min. The supernatant was separated by decantation and filtered over a paper filter. The phytate content of this filtrate was determined by HPLC (method A). For methods B and C part of the filtrate was mixed with a solution of EDTA and NaOH (see below). The mixture was stored at 4 °C until analyzed.

Method A. The sample extract (1.0 mL) was diluted with water (10 mL). After filtration through a Millipore filter (0.45

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μm), 50 μL of this mixture was injected onto the column of the liquid chromatography system. Only eluent C was used.

Method B (the New Method). In a 10-mL volumetric flask 1.0 mL of the sample extract was mixed with 0.25 mL of 2.8 M NaOH, 0.75 mL of of a solution of sodium acetate (167 g/L), and 1.0 mL of EDTA solution (40 g/L, adjusted to pH 6.0 with 2.8 M NaOH). The mixture was allowed to stand at room temperature for 15 min. The volumetric flask was made up to volume with water. The pH of the resulting mixture was about 6. If necessary, this solution can be diluted further with a solution of sodium acetate (15 g/L, pH 6). After filtration through a Millipore filter (0.45 μm), 50 μL of the solution was injected onto the column of the liquid chromatograph system.

Method C. The Baker 10 anion-exchange columns were rinsed sequentially before use with 3 mL of methanol, 3 mL of ammonia (25%), 3 mL of water, 3 mL of 0.3 M HCl, and 3 mL of water. The sample extract (1.0 mL) was mixed with 1 mL of a solution containing 0.11 M EDTA and 0.75 M NaOH. The resulting mixture had a pH of about 6. Of the mixture 1.0 mL was diluted with 30 mL of water and applied to the Baker 10 column. The column was rinsed twice with 5 mL water and twice with 5 mL of 0.3 M NaCl. Then the column was eluted twice with 2 mL of 0.8 M NaCl. The eluent obtained was transferred to a 10-mL volumetric flask, and the flask was made up to volume with deionized water. The phytic acid was determined by a colorimetric method as described by Latta and Eskin (1980). One milliliter of the reagent—a solution of 0.03% iron(III) chloride hexahydrate and 0.3% sulfosalicylic acid in water—was transferred into a disposable cuvette. Depending on the concentration of the phytic acid solution, a volume between 0.1 and 2.0 mL of the sample solution was added to the reagent in the cuvette. Water was added to make a total volume of 3.0 mL, and the liquid was mixed. After the mixture had stood for about 10 min, the color was read at 500 nm on a Beckman spectrophotometer. The phytic acid concentration was calculated by using a standard curve.

Liquid Chromatography Conditions. Eluent A is an aqueous solution of sodium nitrate and EDTA; 2.5 g of sodium nitrate is dissolved in 1 L of water containing 0.5 mL of an EDTA solution (40 g/L) and 0.2 mL of a pentachlorophenol solution (500 mg/100 mL of ethanol). The pH is adjusted to 6.0 with 2.8 M NaOH.

Eluent B is an aqueous solution of 0.1 M sodium nitrate containing 0.2 mL of pentachlorophenol solution (500 mg/100 mL of ethanol)/L. The pH is adjusted to 3.5 with eluent C.

Eluent C is an aqueous solution of nitric acid (9 mL/L).

The elution program is eluent A for 5 min, eluent B for 5 min, eluent C for 13 min, and eluent A for 7 min. The flow of the eluent was 1 mL/min.

The postcolumn reagent is a solution of 2.2 g of iron(III) perchlorate nonahydrate and 12.8 mL of 70% perchloric acid in 1.0 L of water. The reagent was stored in a brown flask. A flow of 0.5 mL/min was applied.

RESULTS AND DISCUSSION

The phytate contents in several samples of foodstuffs and feeds were analyzed by the following three methods: a chromatographic method very similar to the method of Phillippy and Johnston (1985) and to that of Cilliers and van Niekerk (1986) (method A), a new method which is a chromatographic method combined with an EDTA treatment of the extracts (method B), and a colorimetric determination after concentration of the phytic acid on a small ion-exchange column, a procedure very analogous to that of Ellis and Morris (1983) (method C).

The results are summarized in Table I. The results of the three methods do not differ significantly for every sample tested. Overall, however, the results of the three methods were found to be significantly different ($p < 0.01$).

Table I. Phytic Acid Content of Samples Determined with Methods A–C (Mean Values and SEM of Four Analyses)

sample	phytic acid, g/100 g of dry matter					
	method A		method B		method C	
	mean	SEM	mean	SEM	mean	SEM
soybean meal	0.965[a]	0.029	1.442[b]	0.005	1.590[c]	0.012
maize	0.583[a]	0.014	0.735[b]	0.008	0.651[c]	0.011
wheat	0.667[a]	0.013	0.785[b]	0.004	0.824[b]	0.006
barley	0.693[a]	0.014	0.822[b]	0.007	0.845[b]	0.019
chyme	0.527[a]	0.004	0.693[b]	0.001	0.837[c]	0.007
wheat bran	3.963[a]	0.016	4.315[b]	0.016	3.978[a]	0.042
roasted coffee	0.099[a]	0.003	0.108[a]	0.003	0.179[b]	0.009

[a] Mean values with a different superscript differ significantly ($p < 0.01$).

The procedure described by Phillippy and Johnston (1985) (method A) resulted in lower phytate values than did the procedure of Ellis and Morris (1983) (method C). The procedure of Phillippy and Johnston (1985) may result in values too low for phytate because the extracts have not been treated with EDTA. EDTA functions as a metal scavenger, eliminating the interference of metal ions with the phytate determination as was shown by Ellis and Morris (1983). On the other hand, the procedure of Ellis and Morris (1983) may result in values too high for phytate because in their procedure lower inositol phosphates are only partly separated from inositol hexaphosphate and raise the phytate values obtained. Seeds and grains usually contain few if any lower inositol phosphates, but in bread or in chyme samples high levels of these lower inositol phosphates may be present (Nayini and Markakis, 1983). Polycarboxylic acids may also interfere with the phytate determination according to the method of Ellis and Morris (1983) (see below). Our improved chromatographic method (method B) generally results in higher phytate values than the procedure of Phillippy and Johnston (1985) (method A). Our values are lower than the values obtained with the method of Ellis and Morris (1983) (method C).

Extraction Procedure. The samples were extracted with 0.8 M HCl (Ellis and Morris, 1982). For comparison, some extractions with a 3% solution of trichloroacetic acid (TCA) were performed (Cilliers and van Niekerk, 1986). However, analysis of these extracts resulted in lower phytate values. This may be explained as follows. TCA is often used for the denaturation of proteins. Phytate has a high affinity to proteins with a high polarity. Denaturation of proteins during the extraction with TCA will probably result in occlusion of phytic acid, which will be lost in the filtration step.

The HCl extracts were treated with EDTA at a pH of about 6. At this pH the metal ions are more strongly bound by EDTA than they are by phytate. The processing of some samples, with a high content of calcium carbonate, resulted in a pH much higher than 6. This problem was solved by adding only 0.1 mL instead of 0.25 mL of 2.8 M NaOH to the acidic sample extract.

Colorimetric Method (Method C). Ellis and Morris (1983) used the anion-exchange resin AG 1-X8 and obtained satisfactory results when properly selected batches of resin were used (Ellis and Morris, 1985). We found that columns for single use gave invariably good results. The ion-exchange columns were first rinsed with a solution of 0.3 M NaCl to remove free phosphate and other interfering substances. The inositol hexaphosphate was eluted from the column with a solution of 0.8 M NaCl. The phytate in the eluate was determined according to the method of Latta and Eskin (1980) by using the Wade reagent, a solution of ferric sulfosalicylate.

The linearity of the method was tested with solutions of pure phytic acid. A linear response was observed for a quantity of phytic acid between 5 and 5000 μg. A linear calibration curve of $y = 0.713 - 0.311x$ with a correlation coefficient of 0.998 ($n = 6$) was obtained for extinction at 500 nm against the phytic acid concentration applied. Experiments have shown that gallic acid, chlorogenic acid, and inositol pentaphosphate interfere with phytic acid in method C. One milligram of gallic acid, chlorogenic acid, and inositol pentaphosphate gave a response comparable

Figure 1. Chromatogram of the analysis of phytic acid in wheat with method A.

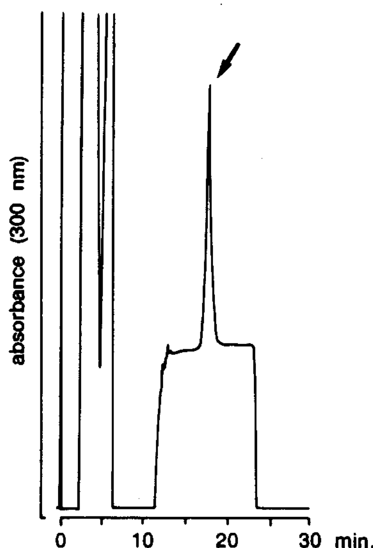

Figure 2. Chromatogram of the analysis of phytic acid in barley with method B.

with 0.07, 0.04, and 0.27 mg of phytic acid, respectively. Many other substances such as oxalic acid or salicylic acid appeared not to interfere with phytic acid in method C.

Liquid Chromatographic Method (Methods A and B). For a study on the degradation of phytic acid by phytase in our laboratory, many determinations of phytic acid were necessary. The work published by Cilliers and van Niekerk (1986), who used a Waters IC-Pak A column, looked very promising. We adopted their procedure and obtained very good results with the first two columns of that type. Several thousands of analyses were carried out successfully. Unfortunately, the third and fourth IC-Pak A columns from Waters had different properties. With these columns the analytical procedure of Cilliers and van Niekerk gave irreproducible results. Then we adopted the procedure of Phillippy and Johnston, who used a Dionex HPIC AS-3 column. This type of column has now been in use in our laboratory for more than 3 years. Until now all of the columns of this type have shown good comparable results. A chromatogram of the analysis of phytic acid with method A is presented in Figure 1.

The improved HPLC method (method B) is a specific method for the determination of phytic acid. A chromatogram is presented in Figure 2. Only very acidic substances will elute together with phytic acid from the anion-exchange column. Only acids with strong coordinative properties will give complexes with the ferric ions of the reagent at this low pH, resulting in an increase of the absorption at 300 nm. Several compounds that might interfere with the phytic acid peak were injected. These include salicylic acid, gallic acid, chlorogenic acid, ferulic acid, citric acid, and oxalic acid. None of these compounds gave a peak in the chromatogram, and so they do not interfere with phytic acid.

The anion-exchange column used (Dionex AS-3) has a low capacity and is easily overloaded. A linear response was observed when the injected quantity of phytic acid was between 0.25 and 15 μg. A linear calibration curve of $y = -1.909 + 41.68x$ and a correlation coefficient of 0.9994 ($n = 12$) were obtained for the peak area against the phytic acid concentration injected. The life span of the Dionex AS-3 column, protected by a guard column, is more than 2000 runs.

The acidic sample extracts were treated with EDTA. A concentration of 4000 mg of EDTA/L in the resulting mixture was found to be optimal. Concentrations of 2000 mg of EDTA/L or less resulted in too low phytate peaks. Probably these concentrations of EDTA were too low to inhibit the interference of metal ions with the phytate. Concentrations of 6000 mg of EDTA/L or higher disturbed the chromatography as a result of too high an ion concentration.

The use of a heated reaction coil resulted in higher peaks in the chromatogram. Maximum peak heights were obtained when a 3 m long coil (i.d. 0.3 mm) heated to 50 °C was used.

The improved HPLC method can easily be modified for the determination of lower inositol phosphates. The pH of the eluting buffer must be higher and the ionic strength must be lower than was used for the analysis of phytic acid.

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