A Simple and Rapid Procedure for Phytate Determination in Soybeans and Soy Products

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ABSTRACT: A rapid and simple analytical procedure to determine the phytate (myo-inositol hexaphosphate) content in soybeans and soy products is described. Minimal sample preparation and automated HPLC analysis were achieved for large numbers of samples. Determination of the phytate content in a variety of soybean samples, including animal feeds, demonstrated the simplicity, reliability, and economy of the procedure compared with other time-consuming, complicated, and laborious methods.

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KEY WORDS: Myo-inositol hexaphosphate, phytate determination, phytic acid, soybeans.

Phytic acid, myo-inositol hexaphosphate, is the principal storage form of phosphorus in soybeans (1) and is known to associate with the β-conglycinin (7S) fraction of the storage proteins (2–4). Phytic acid is also known to have a strong affinity for zinc, iron, and trace minerals in foods and feeds. Its association with storage proteins results in relatively high concentrations of phytic acid in commercial soy protein products, such as soy meal, that are mainly used for swine and poultry feeds (1,2,5). The presence of phytic acid has a definite effect on the economic value and nutritional quality of the animal feeds. Presently, an intensive collaborative effort in soybean breeding programs, the Better Bean Initiative of the United Soybean Board, is under way to develop low-phytate soybean cultivars. Existing soybean populations are being screened for low-phytate genotypes using a colorimetric assay of total inorganic phosphorus. Such an assay is indirect. Although inorganic phosphorus is an indicator of phytic acid content, particularly in distinguishing very low phytate mutant seed from normal seed, the correlation between inorganic phosphorus and phytic acid concentration from our three studies has been very low (with *r* values ranging from 0.30 to 0.72; Burton, J.W., D.W. Israel, and P. Kwanyuen, unpublished data) in standard soybean materials. Direct measurement of phytic acid in soybeans has been reported (6–11). However, the procedure is time-consuming, complicated, laborious, and expensive. Therefore, a rapid and simple procedure for the quantitative assay of phytic acid is needed to facilitate and accelerate the ongoing development of low-phytate soybeans.

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In this paper, we present a rapid and simple procedure for determining the phytate content in soybean seeds and other soy products. It requires minimal sample preparation, and the HPLC analysis can easily be set up for automation with commercially available equipment for large numbers of samples. The procedure also can be developed to a standard protocol as a reference methodology for determining the phytate content in soybeans and soybean products.

MATERIALS AND METHODS

Chemicals. 1-Methylpiperazine (99%) was from Aldrich Chemical Co. (Milwaukee, WI). D-Myo-inositol hexaphosphate dipotassium salt (+95%), 5-sulfosalicylic acid dihydrate, $FeCl_3$, and $NaNO_3$ were from Sigma (St. Louis, MO). HCl was from Fisher Scientific (Fair Lawn, NJ). All other chemicals were of analytical grade. Purified Milli-Q water of 18 mΩ-cm resistivity was used for the preparation of all reagents and buffers. All were filtered through 0.2-μm membranes.

Sample collection. A total of 340 samples were received for this study from several cooperators in the Better Bean Initiative, sponsored by the United Soybean Board. These samples included both developing and mature soybean seeds, defatted soybean meals, and a variety of commercial poultry feeds (wheat, corn, and soybeans).

Sample preparation. Unless otherwise stated, all experiments and procedures were performed at room temperature. Soybean seeds and unground samples were ground in a centrifugal grinding mill equipped with a 24-tooth rotor and 1.0-mm stainless-steel ring sieve with the motor speed set at 15,000 rpm. This setting produced ground samples with a uniform particle size of less than 0.5 mm. For convenience, extraction was done for 1 h in a 20-mL vial with 0.5 N HCl in a ratio of 1:20 (wt/vol) while stirring. In our experiments, 0.5 g of sample and 10 mL of 0.5 N HCl were used throughout. Approximately 2 mL of crude extract from each sample was centrifuged at $18,000 \times g$ for 10 min in a microcentrifuge. An aliquot of 1 mL of supernatant containing phytate was then filtered with a 1-mL tuberculin syringe and a 13-mm/0.45-μm syringe filter. Filtered samples could be stored at 4°C for several days prior to HPLC analysis.

Instrumentation. The HPLC system used for eluting phytate consisted of a binary pump, a vacuum micro-degasser for buffers, and an autosampler. An isocratic pump was used for the delivery of Wade's color reagent (12) in a postcolumn reaction

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with phytate. A variable wavelength detector was used for post-column detection of phytate at 500 nm with the signal polarity setting on negative. The system was also equipped with software to control the operation and data acquisition.

Sample analysis. Chromatography was performed on a binary HPLC system with a 50 × 4.6 mm PL-SAX strong anion-exchange column (Polymer Laboratories, Amherst, MA) equipped with a 7.5 × 4.6 mm guard column. Elution of phytate was achieved with a 30-min linear gradient of 0.01 M 1-methylpiperazine, pH 4.0, to 0.5 M $NaNO_3$ in 0.01 M 1-methylpiperazine, pH 4.0, at a flow rate of 1 mL/min as described by Rounds and Nielsen (9) with modifications. Wade's color reagent (12) consisting of 0.015% (wt/vol) $FeCl_3$ and 0.15% (wt/vol) 5-sulfosalicylic acid (also at a flow rate of 1 mL/min) and phytate eluted from the column were mixed in a mixing tee with inline check valves for both eluents installed prior to the mixing tee to prevent backflow. The postcolumn reaction was allowed to take place in a 0.05 × 210 cm polyetheretherketone tubing at the combined flow rate of 2 mL/min. The absorbance was monitored at 500 nm, and the detector signals and/or phytate peaks were processed and integrated by the chromatographic data acquisition system.

RESULTS AND DISCUSSION

HCl concentration and extraction. Extraction of phytate at room temperature for 2 h with HCl has been reported at various concentrations, ranging from 0.14 to 0.5 N (6,8,9,13). Although Latta and Eskin (6) reported that extraction time could be reduced to 1 h with 0.28 N HCl, and Graf and Dintzis (8) achieved maximal recovery of phytate in 2 h with 0.2 N HCl, all extractions were performed with a mechanical agitator or shaker. When using a magnetic stirrer, however, we found that the optimal concentration of HCl for maximal recovery of phytate was 0.5 N, and phytate was readily extracted in 1 h with 0.5 N HCl, as shown in Table 1.

HPLC analysis. In our earlier experiments, the phytate analyses were performed using an ISCO V^4 absorbance detector (Lincoln, NE), and the phytate peaks were manually integrated using a planimeter for determination of the peak areas instead of autointegration as described in the Materials and Methods section. These included crude phytate extracts and phytic acid standards used for preparing a standard curve. No significant difference was found between these two methods of detection and determination. Phytic acid standards with concentrations ranging from 10 to 100 μg can be determined conveniently and with precision by this procedure. The absorbance of the standards at 500 nm is linearly proportional to the concentration throughout, with r^2 of 0.9998, as shown in Figure 1.

Large numbers of samples were used to test the automated HPLC analysis, and they were all extracted in 0.5 N HCl (pH 1.7). However, most HPLC systems and equipment are manufactured to operate, and are recommended for operation, at pH values above 2. To avoid corrosion of the autosampler injector seat caused by residual acid on the outer surface of the needle and, more important, cross-contamination among samples, it was necessary to wash the injector needle prior to injection. With this parameter, we found that the CV was <1 with 50 μL of sample injected, whereas a CV of nearly 10 was found with 10 μL of sample injected, although the latter is commonly used and has been reported. Therefore, a 50-μL volume of sample was used for the analysis throughout our experiments.

TABLE 1
Effect of Extraction Time on the Recovery of Phytate in 0.5 N HCl

Time (h)	Phytate extracted[a] (mg/g dry wt)	Time (h)	Phytate extracted[a] (mg/g dry wt)
0.25	10.67 ± 0.02	2	11.28 ± 0.14
0.5	11.00 ± 0.21	4	11.31 ± 0.22
1	11.33 ± 0.11	8	11.49 ± 0.39

[a]Values are means ± SE of three determinations.

To demonstrate the reproducibility of the procedure, eight analyses of the same sample were performed each day for a period of 5 d. We found that the CV of these analyses was 0.8 within a day, whereas the value of 1.6 was seen after 5 d with the total of 40 analyses from the same sample.

Figure 2 shows typical chromatographic profiles of the phytic acid standard and phytate extracted from a soybean seed sample with a prominent peak marked as IP_6. In all 340 samples obtained from various sources, including processed meals and feeds, mature seeds, and developing seeds, we discovered that a majority of phytate was in the myo-inositol hexaphosphate species, with a negligible amount of possibly myo-inositol pentaphosphate, IP_5, as seen in the chromatograms. Since most of the phytate (+95%) in soybeans is in the form of inositol hexaphosphate, there is no need for discrimination or separation of other forms of phytate in the analysis. Thus, the cycle time for the HPLC analysis can be substantially reduced.

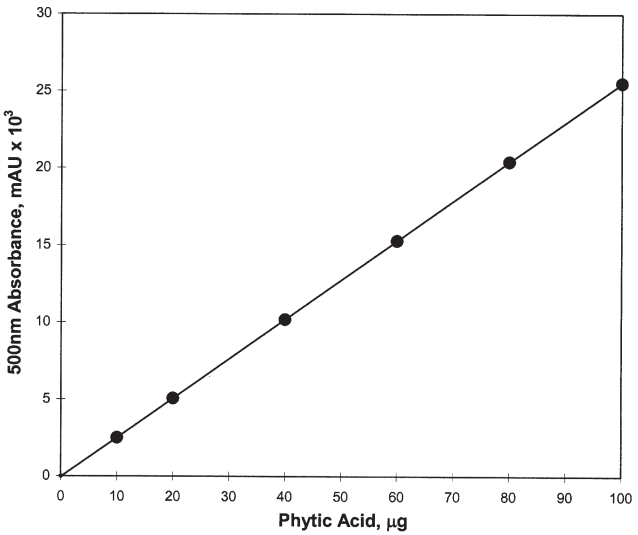

FIG. 1. Standard curve for phytic acid. Various concentrations of phytic acid (as indicated in micrograms) were dissolved in 0.5 N HCl and were determined by HPLC, as described in the Materials and Methods section. r^2 = 0.9998.

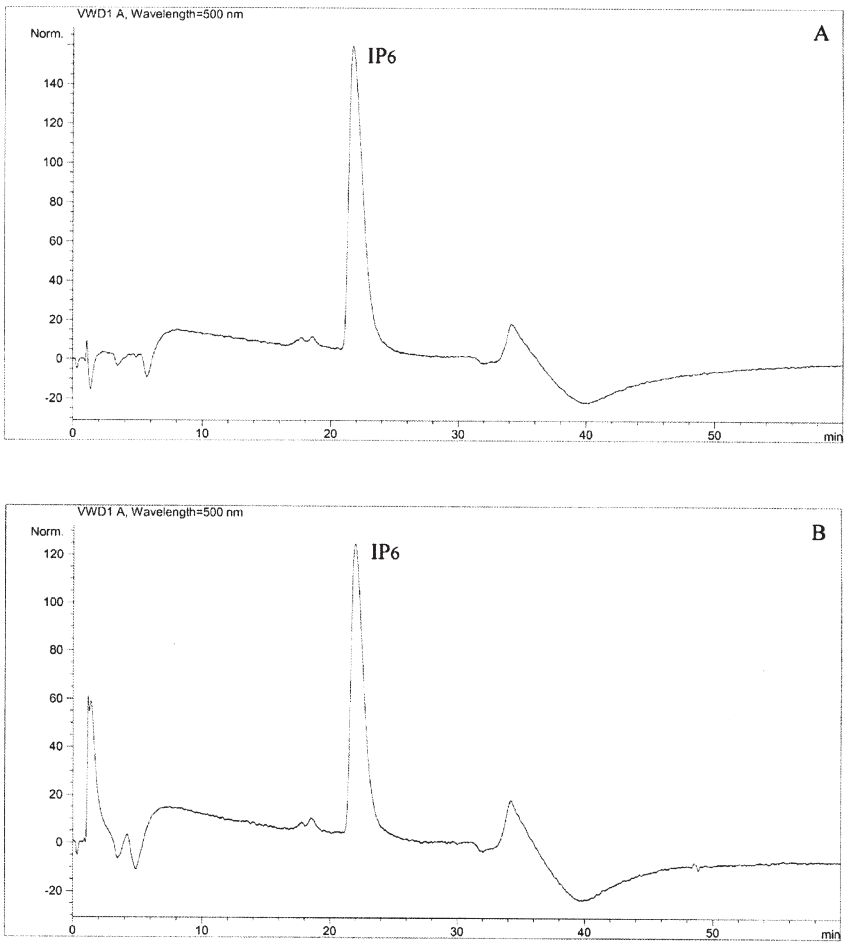

FIG. 2. Typical chromatographic profiles of phytic acid (myo-inositol hexaphosphate, IP_6) standard (A) and phytate extracted from a soybean sample (B). The profile includes a 30-min gradient of $NaNO_3$ and 30-min column equilibration.

Table 2 shows the phytate content of selected samples from different sources. Of the 340 samples analyzed, we found that the phytate in most mature soybean seeds and their processed meals ranged from 14.5 to 25.4 mg per gram dry weight except for that of a low-phytate soybean mutant (CX1834A-1-4) and its processed meal.

Figure 3 shows a chromatographic profile of an extracted phytate sample after the HPLC analysis had been run on automated mode for more than 48 h. The profile shows several negative sharp peaks caused by the appearance and accumulation of protein precipitates in the detector flow cell. These peaks randomly appear on the chromatograms and can cause possible error in the autointegration and subsequent phytate determination if they are included in the peaks of interest. The crude acid extract consisted not only of phytate but also of other compounds, including soybean lipids and proteins. Soybean proteins are known to precipitate at a mild acidic pH (or pH 4.0), which is the optimal pH for the mobile phases of the HPLC analysis. Although a very small volume of sample was injected in each analysis cycle in comparison with the whole HPLC mobile phase, this minute amount of protein contained in such a sample can be precipitated and accumulated over time at pH 4.

Maintenance of the column and detector flow cell. To eliminate the aforementioned problem with the HPLC analysis, we recommend that proper cleanup and maintenance of the column and the detector flow cell be performed after 48 h of analysis. After 48 h of continuous HPLC analysis, the column should be flushed in a reverse-flow manner with 60 mL of 0.1 N NaOH with the detector flow cell connected in tandem. This should be followed by 60 mL of water wash. Residual hydrophobic components from lipid contamination should also be removed from the column by 60 mL of isopropanol wash, followed by 60 mL of water. The column should then be equilibrated with 60 mL of 1.0 M NaCl and reconditioned with the HPLC mobile phase prior to use.

In conclusion, the procedure for phytate determination presented here can be readily used for soybean and soybean products, including other animal feeds. The procedure requires minimal sample preparation and is cost effective. The HPLC analysis can easily be set up for automation to accommodate large

TABLE 2
Phytate Content of Selected Samples from Different Sources

Sample	Phytate (mg/g dry wt)	Sample	Phytate (mg/g dry wt)
Developing seeds		Processed soy meals	
CX1834A-1-1[a]	19.3	Standard soy meal	14.5
CX1834A-1-4[a]	7.3	Low-phytate soy meal	3.4
Mature seeds		Soy 48 Cargill[b]	22.7
N93-2164[b]	25.4	Poultry feeds	
NC-Roy[b]	14.8	Wheat bran 13.5%	64.4
Prolina[b]	21.9	Wheat middlings 12.5%	55.7
Vernal[b]	17.1	Corn load 4 #1[c]	10.4
Young[b]	18.8	Gluten 60[d]	22.7
Balbuena[b]	19.1		

[a]From Purdue University (West Lafayette, IN).
[b]From USDA-ARS (Raleigh, NC).
[c]From Wilder Brothers Farm Supply (Franklinton, NC).
[d]From Gold Kist Bonnlee Feedmill (Siler City, NC).

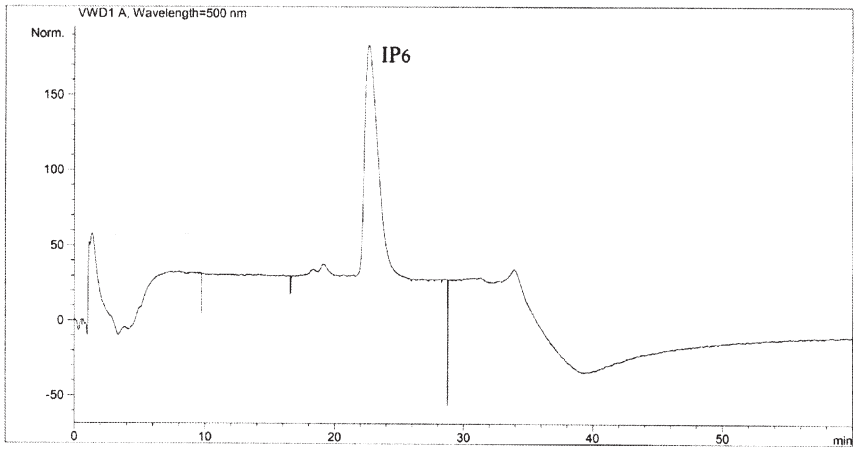

FIG. 3. Chromatographic profile of a phytate sample extracted from soybeans after the HPLC analysis had been run continuously for more than 48 h. This 61st sample profile shows several negative sharp peaks caused by protein precipitates that were passing through the detector flow cell.

numbers of samples. It also provides a simple and rapid method for the direct assay of phytate in soybean seeds, which is currently needed to develop low-phytate soybean cultivars in breeding programs. Because of its simplicity, the procedure can potentially be adopted and used to identify and confirm low-phytate genotypes of soybeans from the breeding populations. This procedure also can be used as a standard protocol and reference methodology for the determination of phytate.

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