FOOD COMPOSITION AND ADDITIVES

Optimization of the Treatment of Wheat Samples for the Determination of Phytic Acid by HPLC with Refractive Index Detection

ROSA AMARO, MIGUEL MURILLO, ZURIMA GONZÁLEZ, ANDRÉS ESCALONA, and LUÍS HERNÁNDEZ
Universidad Central de Venezuela, Facultad de Ciencias, Escuela de Química, Centro de Química Analítica, Laboratorio de Métodos Cromatográficos, Apartado 47102, Caracas 1041-A, Venezuela

The treatment of wheat samples was optimized before the determination of phytic acid by high-performance liquid chromatography with refractive index detection. Drying by lyophilization and oven drying were studied; drying by lyophilization gave better results, confirming that this step is critical in preventing significant loss of analyte. In the extraction step, washing of the residue and collection of this water before retention of the phytates in the NH2 Sep-Pak cartridge were important. The retention of phytates in the NH2 Sep-Pak cartridge and elimination of the HCl did not produce significant loss ($P = 0.05$) in the phytic acid content of the sample. Recoveries of phytic acid averaged 91%, which is a substantial improvement with respect to values reported by others using this methodology.

Phytic acid (inositol hexakisphosphate, $InsP_6$) is widely distributed in commonly consumed foods. It is found in high concentrations in the seeds of grains, pulses, and oleaginous products, and in lesser amounts in tubers and garden produce. The amount of phytate in food has been of interest to nutritionists for many years. Originally perceived as an antinutrient for its ability to precipitate and decrease the bioavailability of minerals such as calcium, iron, and zinc, phytate is now also credited with positive attributes related to the prevention of oxidation, cancer, atherosclerosis, and kidney stones (1, 2). The determination of phytic acid has constituted an analytical problem for a long time. Until 1980, phytic acid was exclusively determined through nonspecific precipitation methods (3). Currently, procedures in which HPLC is included are more and more common (4). The problem with the classic methods of analysis is that they cannot differentiate between $InsP_6$ and its lower phosphate forms, which can overestimate $InsP_6$ content (5, 6). One of the chromatographic techniques used to determine $InsP_6$ in food is HPLC using ion-pairing with refractive index (RI) detection (7–16). Nevertheless, recent studies have reported low recoveries (<72%) of $InsP_6$ determined in cereals by this technique (16–18). In the work described in this paper, each of the wheat treatment steps for the extraction of phytic acid and its subsequent determination by HPLC with RI detection were studied, with the purpose of improving the recoveries achieved by this technique.

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Corresponding author's e-mail: rosa.amaro@ciens.ucv.ve

Experimental

Materials

Sodium phytate, 79%, w/w (dodecasodium salt hydrate), determined by elemental inductively coupled plasma-optical emission spectrometry phosphorus analysis; phytic acid, dipotassium salt, 95%, w/w; tetraethylammonium hydroxide (TENOH; 40%, w/w, in aqueous solution); tetrabutylammonium hydroxide (TBNOH; 1.0 M, in methanol solution); and trace select ultra HCl, 30%, w/w, were supplied by Aldrich Chemical Co. (Milwaukee, WI). NH2 Sep-Pak cartridges were supplied by Millipore Corp. (Milford, MA). Ion-exchange resin (AG 50W-X4 of H^+, 50–100 mesh) was from Bio-Rad Laboratories (Richmond, CA); 99.8% high-purity methanol from Riedel-de Haën AG Co. (Seelze, Hannover, Germany); 90%, w/w, purity formic acid from BDH Laboratory Reagents Co. (Poole, UK), and 18 MΩ/cm deionized water.

Instrumentation

The HPLC system consisted of a Hewlett-Packard (Waldbronn, Germany) 1100 solvent delivery system, a Rheodyne 9125 6-port injection valve (Rheodyne, Cotati, CA) with a 20 μL PEEK (poly-ether-ether-ketone) injection loop, a Hamilton PRP-1 PEEK column (Hamilton, Reno, NV; 5 μm, 150 × 4.6 mm id), and an RI detector Model 1047 A (Hewlett-Packard). The analog output from the detector was connected to A/D converter Model SS420, and the data were processed with EZCHROM software, provided by Scientific Software, Inc. (Pleasanton, CA). Connections between injection valves, injection loop, and pump were made with 0.250 mm id, PEEK material pipe. Solvent was pumped at a flow rate of 0.6 mL/min. The mobile phase was 0.015 M formic acid solution containing 5%, w/w, methanol and 0.28%, w/w, TENOH. The column temperature was kept at

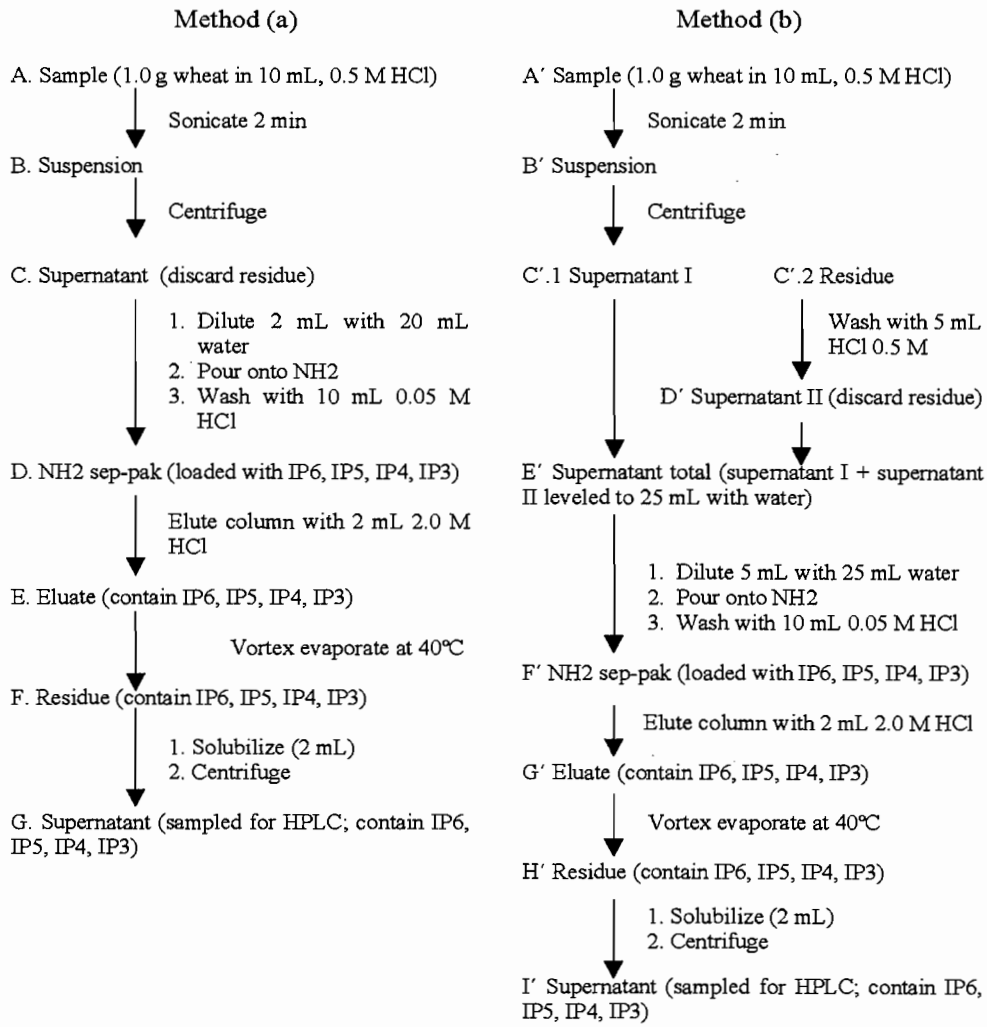

Figure 1. Scheme of methods (a) and (b) for extraction, purification, and determination of phytic acid in wheat.

30°C. An ultrasonic homogenizer 4710 Series, equipped with a 1/8 in. standard tapered microchip probe (Cole-Parmer Instrument Co., Chicago, IL) and an IEC Centra CL2 centrifuge (Thermo Electron Corp., Milford, MA), was used.

Phytic Acid Hydrolysis

The mixture of the inositol phosphates from tri- to hexaphosphate was obtained through hydrolysis of phytic acid. Sodium phytate (0.5316 g) was dissolved in 20 mL water, and the solution was poured through a cation-exchange column (3 × 10 cm, AG 50W-X4). This solution was transferred to a 100 mL volumetric flask and diluted to volume with pure water. A 20 mL aliquot of the solution was heated under reflux to 95°C for 10 h. The hydrolyzed products were placed in a 100 mL volumetric flask, and the contents of the flask were diluted to volume.

Sample Extraction

Figure 1 is a schematic for the extraction of phytic acid in wheat samples.

Method (a).—A wheat sample (1 g) was added to a 10 mL centrifuge tube containing 10 mL 0.5 M HCl, and the contents were stirred to ensure removal of air pockets. The tip of the ultrasonic microprobe was inserted halfway into the liquid, and the sample was sonicated for 2 min (40% output and 25 W tune). The suspension (B) was centrifuged at 3400 rpm for 15 min. The supernatant (C) was separated, and the residue was discarded. An aliquot (2 mL) of supernatant (C) was diluted with 20 mL water and poured onto an NH2 Sep-Pak

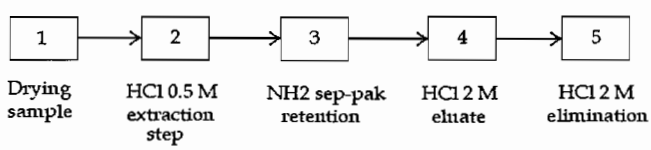

Figure 2. Block diagram of the sample treatment.

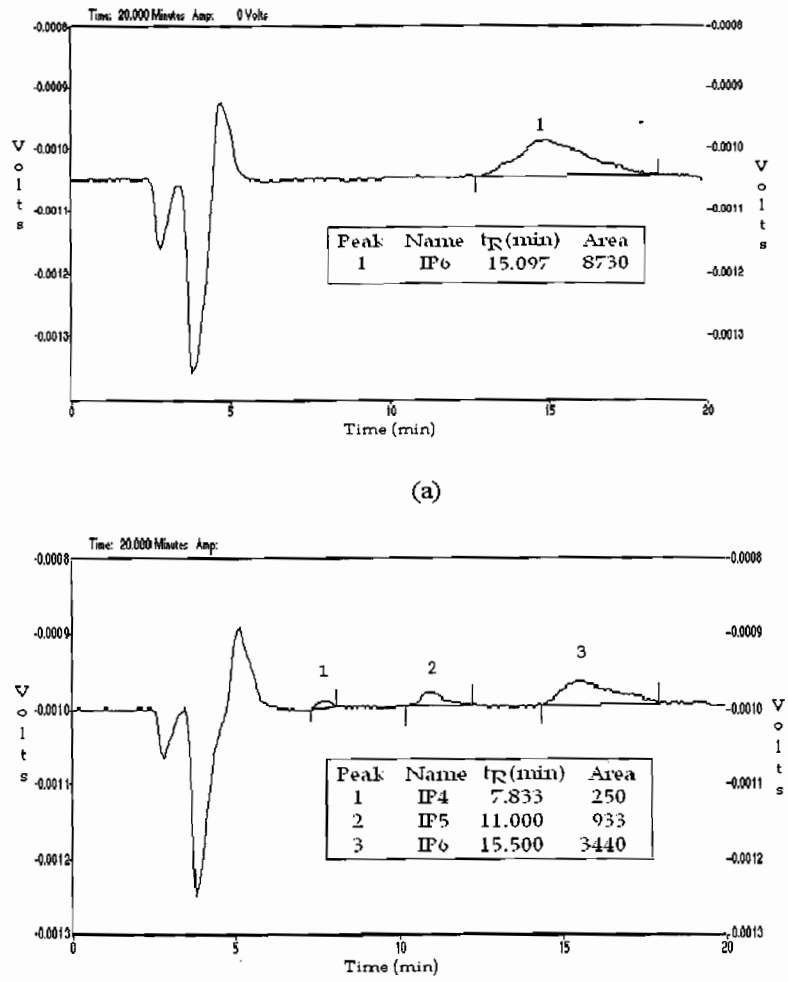

Figure 3. Chromatograms of (a) phytic acid and (b) hydrolyzate of phytic acid hydrolysis at 95°C for 10 h. Chromatographic conditions: column type PRP-1 Hamilton, 150 × 4.6 mm id, 5 μm particle size. Mobile phase: 0.28%, w/w, tetraethylammonium hydroxide and 5%, w/w, methanol in sufficient formic acid to adjust the pH to 4.3; the elution rate was 0.6 mL/min.

Table 1. Statistical data for Standards 1–3 with *F*- and *t*-test values for the evaluation of the effect of the elimination of HCl on the PEEK area and the retention of phytates in the NH2 Sep-Pak cartridges[a]

Sample	Average	S[b]	F_{exp}[c]	t_{exp}[d]
Standard 1	7851	202	5.09	1.49
Standard 3	8243	456	2.67	1.57
Standard 2	7563	331		

[a] $P = 0.05$; $n = 4$.
[b] S = standard deviation.
[c] F-value estimated from experimental data ($F_{theoretical} = 15.44$).
[d] t-Value estimated from experimental data ($t_{theoretical} = 2.45$).

cartridge, aided by the use of a disposable syringe. The loaded NH2 Sep-Pak (D) was washed with 10 mL 0.05 M HCl, and the inositol polyphosphates were eluted with 2 mL 2 M HCl into 10 mL brand recovery flasks. Eluted samples (E) were dried by vortex-evaporation in a vacuum at 40°C. The residue (F) was resuspended with 2 mL mobile phase, and centrifuged for 5 min at 3400 rpm. A 20 μL aliquot of clear supernatant (G) was injected into the HPLC unit.

Method (b).—In this method, steps "A" and "B" were identical to those in *Method (a)*. After the suspension was centrifuged, the supernatant (C′.1) was decanted into a 25 mL volumetric flask, and the residue (C′.2) washed with 5 mL 0.5 M HCl. This second supernatant (D′) was centrifuged and combined with supernatant (C′.1), and the volume was diluted to 25 mL with water (E′). At this point, the residue was

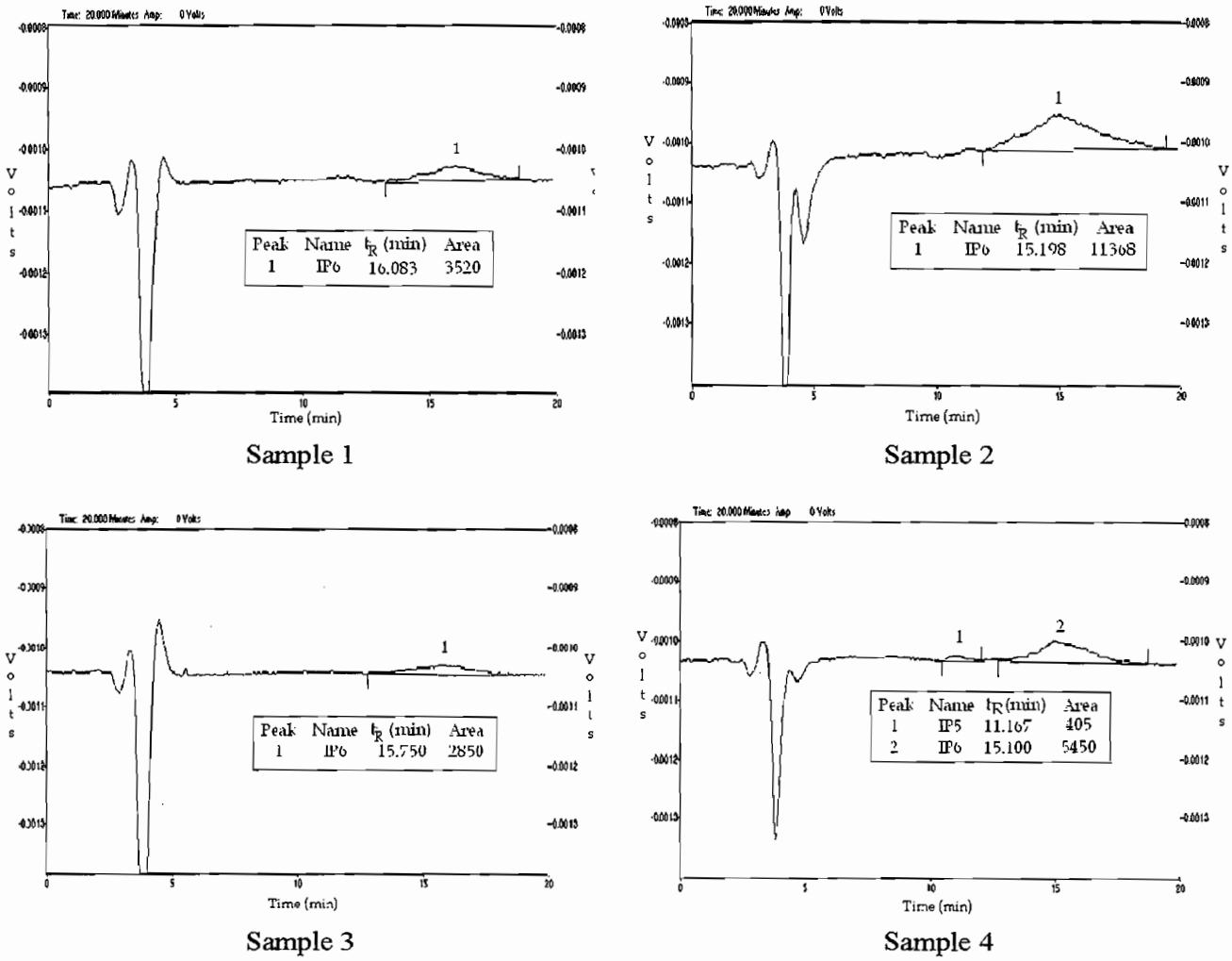

Figure 4. Chromatograms of Sample 1, drying by lyophilization and extraction by *Method (a)*; Sample 2, drying by lyophilization and extraction by *Method (b)*; Sample 3, oven drying and extraction by *Method (a)*; and Sample 4, oven drying and extraction by *Method (b)*.

discarded. A 5 mL aliquot of the total supernatant (E′) was diluted with 25 mL water, and the solution was passed through an NH2 Sep-Pak cartridge, aided by a disposable syringe. The procedure was continued in the same manner as in steps E–H of *Method (a)*.

Drying

To evaluate the drying of the sample, drying by lyophilization and oven drying were studied, and the one that provided the greater phytic acid recovery was selected. To evaluate each drying process, 2 samples of wheat were dried by either lyophilization (samples 1 and 2) or oven drying (samples 3 and 4). Then 1.0000 ± 0.0001 g of each dried sample was extracted; 2 extractions were by *Method (a)* (samples 1 and 3), and 2 were by *Method (b)* (samples 2 and 4).

Evaluation of Sample Treatment

Figure 2 shows a diagram of the most important processes in the treatment of the wheat. In order to evaluate each one of these processes, it is important to consider that in stages 1 and 2, the phytic acid is in the wheat, and in stages 3–5, it is in a solution already separated from the wheat. In this manner, processes 3 through 5 were evaluated with standards that simulate the characteristics of the solutions obtained during the extraction of phytic acid from the wheat, whereas processes 1 and 2 were evaluated directly in the wheat. Three standards were required for this evaluation. Standard 1 was used only through the HCl elimination process (stage 5), Standard 2 was used through processes 3–5, and Standard 3, which does not go through any of the processes, was used as a reference standard for the statistical calculation.

Table 2. Statistical data obtained by the paired *t*-test[a] for evaluation of drying by liophilization and oven drying

Sample	Area	d[b]	$d_{average}$[c]	S_d[d]	t_{exp}[e]
Lyophilized	2850				
	2710				
	2785				
	3520	1440			
	3770	1460			
	3650	1097			
Oven-dried	1410	920	1184	249	11.65
	1250	1285			
	1688	900			
	2600				
	2485				
	2750				

[a] $P = 0.05$; $n = 6$; $t_{theoretical} = 2.5$.
[b] Difference between the paired values.
[c] Average of the differences between the paired values.
[d] Standard deviation of differences between the paired values.
[e] *t*-value estimated from experimental data.

Table 3. Statistical data obtained by the paired *t*-test[a] for evaluation of extraction of phytates with and without residue washing

Sample	Area	d[b]	$d_{average}$[c]	S_d[d]	t_{exp}[e]
With residue washing	3520				
	3770				
	3650				
	2600	670			
	2485	1060			
	2750	865			
Without residue washing	2850	1190	1014	212	11.71
	2710	1235			
	2785	1062			
	1410				
	1250				
	1688				

[a] $P = 0.05$; $n = 6$; $t_{theoretical} = 2.5$.
[b] Difference between the paired values.
[c] Average of the differences between the paired values.
[d] Standard deviation of differences between the paired values.
[e] *t*-Value estimated from experimental data.

Standards

The following standards required for the evaluation of the sample treatment were prepared from phytic acid dipotassium salt, 95%, w/w: Standard 1, phytic acid at 560 mg/L in 2.0 M HCl; Standard 2, phytic acid at 560 mg/L in 0.05 M HCl; and Standard 3, phytic acid at 560 mg/L in mobile phase.

The HCl used for the preparation of the standards and the extraction of the phytic acid in the wheat was hyperpure to avoid possible loss of the phytate (12).

Results and Discussion

Figure 3 shows the chromatograms of (a) phytic acid and (b) the hydrolyzate of phytic acid. It is possible that IP6 is the peak with the longer retention time (Figure 3a), and that under thermal conditions the lower inositol phosphate forms (Figure 3b), such as IP5 and IP4, are produced (9, 10, 12).

Evaluation of Elimination of HCl

Table 1 shows the results obtained for Standards 1 and 3. The statistical *F* value indicates that the variances of both methods are equal. The 2-tailed *t*-test for equal variances demonstrates that a statistically significant difference ($P = 0.05$) between the evaluated areas does not exist. These results indicate that there is no significant loss of analyte in the rotoevaporation process.

The cold drying method was also evaluated to eliminate the HCl, as Park et al. (16) suggested, but the analyte losses were significant as a result of the use of the rotoevaporator at a temperature of ≤40°C as the method for the elimination of the HCl suggested.

Evaluation of Retention and Elution of Phytates in NH2 Sep-Pak Cartridge

In order to perform this study, the areas obtained for Standard 3 were statistically compared with those obtained for Standard 2. Table 1 shows that, for the *t*-value of 2 tails with equal variances ($P = 0.05$), the difference between the 2 measurements of the 2 processes is insignificant. Therefore, it is possible to conclude that no analyte loss occurs in the amino cartridge.

Evaluation of Drying and Extraction of Phytic Acid Sample

Figure 4 shows the chromatograms obtained for samples 1–4 (*see Drying* methodology). The areas of phytic acid peaks in the chromatograms were larger for samples dried by lyophilization than for those dried in an oven, and recoveries of phytic acid were better when the extraction process included an additional step of washing the residue. The chromatogram corresponding to sample 4 contains another peak produced by the decomposition of phytic acid as a result of oven drying.

In order to determine if the differences in the peak areas obtained for oven-dried and lyophilized samples are statistically significant, a statistical *t*-test was used to compare the matched data. Table 2 shows the results of the statistical process. It may be noted that there is a significant difference of 95% in reliability between these 2 results, with drying by

lyophilization having higher reliability. When the step of washing was considered in the statistical study, it was found that washing the residue significantly increased the PEEK area (Table 3).

This finding demonstrates the need to wash the residue, probably because of the inhomogeneity of the phytic acid in the extracted supernatant. When the residue is washed, all the phytic acid is collected in a single solution.

Lehrfeld (12) reported phytic acid loss with oven drying; nevertheless, it is possible to find reports of methods that use oven drying in their methodology. This study demonstrated that the most appropriate method for drying the sample is lyophilization. Under these conditions, recoveries of phytic acid from wheat were between 90 and 92%.

Conclusions

Oven drying cereal samples generates phytic acid loss; therefore, drying by lyophilization is recommended because phytic acid easily decomposes at temperatures of >40°C. In the extraction of phytic acid, an additional step in which the residue is washed with 0.5 mL 0.5 M HCl does not generate significant losses when rotoevaporation is performed at temperatures of ≤40°C. The recoveries of phytic acid from wheat were between 90 and 92%.

Acknowledgments

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