FOOD COMPOSITION AND ADDITIVES

A Novel and Rapid Colorimetric Method for Measuring Total Phosphorus and Phytic Acid in Foods and Animal Feeds

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Phytic acid, or *myo*-inositol hexakisphosphate, is the primary source of inositol and storage phosphorus in plant seeds and has considerable nutritional importance. In this form, phosphorus is unavailable for absorption by monogastric animals, and the strong chelating characteristic of phytic acid reduces the bioavailability of multivalent minerals such as iron, zinc, and calcium. Currently, there is no simple quantitative method for phytic acid; existing methods are complex, and the most commonly accepted method, AOAC *Official Method*SM 986.11, has limitations. The aim of this work was to develop and validate a simple, high-throughput method for the measurement of total phosphorus and phytic acid in foods and animal feeds. The method described here involves acid extraction of phytic acid, followed by dephosphorylation with phytase and alkaline phosphatase. The phosphate released from phytic acid is measured using a modified colorimetric molybdenum blue assay and calculated as total phosphorus or phytic acid content of the original sample. The method was validated to a maximum linearity of 3.0 g phytic acid/100 g sample. Accuracy ranged from 98 to 105% using pure phytic acid and from 97 to 115% for spiked samples. Repeatability ranged from 0.81 to 2.32%, and intermediate precision was 2.27%.

Phytic acid (phytate; *myo*-inositol 1,2,3,4,5,6-hexakisphosphate; $InsP_6$) is the primary source of inositol and storage phosphorus in plant seeds, contributing as much as 90% of total phosphorus (1, 2). The abundance of phytic acid in legumes and cereal grains coupled with its antinutritional characteristics highlight its nutritional importance in the foods and animal feeds industries. Phosphorus in this form is unavailable for absorption by monogastric animals such as pigs and poultry due to a lack of endogenous phytases that are specific for the dephosphorylation of phytic acid. In addition, the strong chelating characteristic of phytic acid reduces the bioavailability of other essential dietary nutrients such as minerals, trace elements (e.g., Ca^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, and $Fe^{2+/3+}$), proteins, and amino acids (3–5). In human nutrition, phytic acid is reported to inhibit absorption of iron, zinc, and calcium and can cause nutritional deficiencies in populations in developing countries (6–8). Animal feeds with a high phytic acid content are generally supplemented with inorganic phosphate; however, this causes increased fecal phosphate levels and subsequent eutrophication of waterways. Supplementation of animal feeds with commercial phytases is an increasingly popular alternative and reduces the associated environmental issues caused by inorganic phosphate supplementation (9). Phytase hydrolyzes the phosphate esters in phytic acid, releasing phosphorus and, therefore, making it available for absorption by monogastric animals. In addition, the antinutritional chelating effects of phytic acid are also removed. The ability to measure phytic acid levels of animal feeds permits the correct dosing levels of commercial phytase preparations, providing a significant financial advantage by removing the requirement of feeds supplementation with the more expensive inorganic phosphates.

At the onset of this study, there was no simple method for measuring phytic acid that was amenable to high-throughput analyses. Existing methods included ferric chloride titration, HPLC, anion exchange chromatography (AEC), and NMR spectroscopy; however, each of these methods has associated disadvantages (10–12). The ferric chloride titration method is arduous, in that it requires removal of precipitate before colorimetric determination of iron and is an indirect measure of phytic acid. HPLC and AEC are time-consuming low-throughput methods and, therefore, impractical for routine analysis of large numbers of samples. HPLC and NMR spectroscopy methods require sophisticated and expensive instruments and highly skilled analysts. Even the generally accepted AOAC *Official Method*SM **986.11** (13) has obvious limitations: each individual sample analysis requires arduous, time-consuming AEC, requiring a separate AEC column for each sample, followed by acid hydrolysis of phytic acid and colorimetric determination of phosphorus. A major assumption is that only phytic acid is purified by the AEC, and though this is viable for nonprocessed grains or legumes in which phytic acid ($InsP_6$) can make up >90% of the total *myo*-inositol phosphates, it is not viable for processed samples (14, 15). Processed samples can contain higher proportions of lower *myo*-inositol phosphate forms (e.g., $InsP_3$, $InsP_4$, $InsP_5$) that will coelute with phytic acid and, therefore, contribute to overestimation of the phytic acid content in the sample (16).

This study describes the successful development and validation of a simple, high-throughput method for the measurement of total phosphorus and phytic acid in nonprocessed foods and feeds. The principle of this method (Figure 1) involves acid extraction of phytic acid and *myo*-inositol phosphates from a food or feed sample, followed by enzymatic dephosphorylation by phytase that is specific for phytic acid ($InsP_6$) and the lower *myo*-inositol phosphate forms (i.e., $InsP_2$, $InsP_3$, $InsP_4$, $InsP_5$). Subsequent

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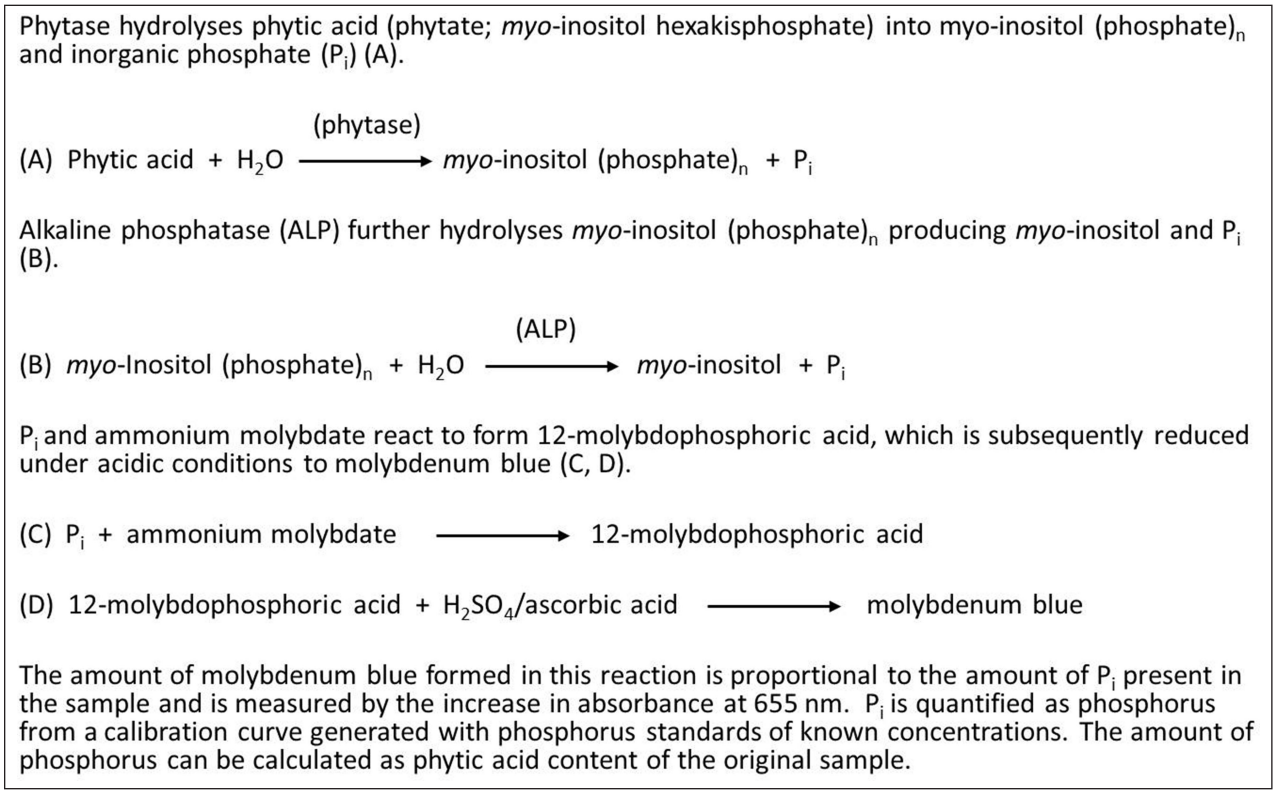

Figure 1. Reaction scheme of the phytic acid assay.

enzymatic dephosphorylation by alkaline phosphatase (ALP) ensures the release of the final phosphate moiety from *myo*-inositol monophosphate ($InsP_1$) which is relatively resistant to the action of the phytase. The phosphate released from phytic acid is measured using a modified colorimetric molybdenum blue assay and calculated as total phosphorus or phytic acid content of the original sample (17, 18).

Materials and Methods

Materials

ALP (Cat. No. E-ALPEC; EC 3.1.3.1; 80 U/mL), phytase (EC 3.1.3.26; 12 000 U/mL), ALP assay buffer (400 mM glycine, 4 mM magnesium chloride, and 0.4 mM zinc sulfate, pH 10.4), and phytase assay buffer (200 mM sodium acetate, pH 5.5) were supplied as part of the Total Phosphorus and Phytic Acid Assay Kit (Cat. No. K-PHYT) and obtained from Megazyme International (Bray, County Wicklow, Ireland). Barley flour, malt flour, oat flour, wheat flour, and American long grain rice were samples within Megazyme International. Odlums Cream Plain Flour, Kellogg's All-Bran Original cereal, pinto beans, and red lentils were obtained from Tesco Ltd (Greystones County Wicklow, Ireland).

Ammonium molybdate (Cat. No. 31402), ascorbic acid (Cat. No. 95210), glycine (Cat. No. G7403), magnesium chloride hexahydrate (Cat. No. M9272), phosphate standard solution (Cat. No. 38364), phytic acid dipotassium salt (Cat. No. P5681), sodium acetate trihydrate (Cat. No. S8625), sulfuric acid (Cat. No. 84718), trichloroacetic acid (Cat. No. 33731), and zinc sulfate heptahydrate (Cat. No. Z0251) were obtained from Sigma Aldrich, (Dublin, Ireland). Hydrochloric acid (Cat. No. 1.00317.2500) and sodium hydroxide pellets (Cat. No. 1.06482.5000) were obtained from Merck Millipore (Cork, Ireland).

Methods

(a) *Preparation of color reagent for phosphorus determination.*—

(*1*) *Solution A.*—Ascorbic acid (10 g) was dissolved with stirring in approximately 90 mL distilled water, followed by the addition of 5.35 mL concentrated sulfuric acid. The final volume was adjusted to 100 mL with distilled water.

(*2*) *Solution B.*—Ammonium molybdate (1.25 g) was dissolved with stirring in approximately 20 mL distilled water, and then the final volume was adjusted to 25 mL with distilled water.

(*3*) *Color reagent.*—One part Solution B was added to five parts Solution A (e.g., 25 mL Solution B was added to 100 mL Solution A). Color reagent was prepared on the day of use.

(b) *Preparation of phytase and ALP assay buffers.*—For the phytase assay buffer (200 mM sodium acetate, pH 5.5), sodium acetate trihydrate acid (27.2 g) was dissolved with stirring in distilled water (0.9 L), and the pH was adjusted to 5.5 by drop-wise addition of concentrated hydrochloric acid. The final volume was adjusted to 1 L with distilled water.

For the ALP assay buffer (400 mM glycine, 4 mM magnesium chloride, and 0.4 mM zinc sulfate, pH 10.4), glycine (30 g), magnesium chloride hexahydrate (813 mg), and zinc sulfate heptahydrate (115 mg) were dissolved with stirring in distilled water (0.9 L), and the pH was adjusted to 10.4 by addition of

sodium hydroxide pellets. The final volume was adjusted to 1 L with distilled water.

(c) *Extraction of phytic acid from food or feed samples.*—Dried food or feed samples were milled to pass a 0.5 mm screen in a Fritsch Pulverisette 14 mill. Milled sample (1 g) was added to 20 mL hydrochloric acid (0.66 M) and mixed with vigorous stirring for a minimum of 3 h and up to 24 h at ambient temperature. The extract (1 mL) was transferred to a 1.5 mL microfuge tube and centrifuged at 11 000 × *g* for 10 min. The supernatant (0.5 mL) was transferred to a fresh microfuge tube and neutralized by addition of 0.5 mL sodium hydroxide (0.75 M) and used in the enzymatic dephosphorylation of phytic acid reaction.

(d) *Enzymatic dephosphorylation of phytic acid by phytase and ALP.*—Each single sample extract, as prepared in the *Extraction of Phytic Acid from Food or Feed Samples* section, was applied to a "Total Phosphorus" reaction and a "Free Phosphorus" reaction. The Total Phosphorus reaction contained distilled water (0.60 mL), phytase assay buffer (0.20 mL), sample extract (0.05 mL), and phytase at 12 000 U/mL (0.02 mL). The Free Phosphorus reaction contained distilled water (0.62 mL), phytase assay buffer (0.20 mL), and sample extract (0.05 mL). Reaction solutions were mixed thoroughly and incubated at 40°C for 10 min. After incubation, ALP assay buffer (0.20 mL) and ALP at 80 U/mL (0.02 mL) were added to the Total Phosphorus reaction. Distilled water (0.02 mL) and ALP assay buffer (0.20 mL) were added to the Free Phosphorus reaction. Reaction solutions were mixed thoroughly and incubated at 40°C for 15 min. All reactions were terminated by the addition of 0.3 mL trichloroacetic acid (50%, w/v) and thorough mixing using a vortex mixer, followed by centrifugation at 11 000 × *g* for 10 min. The supernatant (1 mL) was transferred to a fresh 1.5 mL microfuge tube for use in the colorimetric determination of phosphorus assay.

(e) *Colorimetric determination of phosphorus.*—Supernatants obtained from the enzymatic dephosphorylation of phytic acid procedure (*see* above) were applied to the colorimetric determination of phosphorus assay. Color reagent (0.5 mL) was added to 1 mL supernatant in a 1.5 mL microfuge tube, mixed thoroughly using a vortex mixer, and incubated in a water bath set at 40°C for 1 h. After incubation, all reaction solutions were mixed thoroughly using a vortex mixer. Approximately 1 mL was transferred to a 1 cm path-length microcuvette, and the absorbance at 655 nm of each solution was recorded. The absorbance values of samples and phosphorus standard solutions were used in the calculation of total phosphorus and phytic acid.

(f) *Preparation of a phosphorus calibration curve.*—Phosphorus standard solutions STD0, STD1, STD2, STD3, and STD4 at concentrations of 0, 0.1, 0.5, 1, and 1.5 mg phosphorus/L, respectively, were prepared in distilled water using a traceable phosphorus standard solution. Each standard solution was applied to the colorimetric determination of phosphorus assay, and the absorbance values at 655 nm were used in the calculation of total phosphorus and phytic acid.

(g) *Linearity of the phytic acid assay.*—The linearity of the enzymatic dephosphorylation of phytic acid reaction was assessed using phytic acid solutions prepared in distilled water up to 30 mg/mL. Each phytic acid solution (1 mL) was applied to the phytic acid assay.

The linearity of the colorimetric determination of phosphorus assay was assessed using phosphorus standard solutions prepared in distilled water up to 7.5 μg/mL. Each standard solution was applied to the assay, and the absorbance values at 655 nm were recorded.

(h) *Accuracy of the phytic acid assay.*—The accuracy of the phytic acid assay was assessed using a pure phytic acid sample. Solutions of phytic acid at four concentrations (3.75, 7.5, 15, and 25 mg/mL in distilled water) were prepared in duplicate, and 1 mL of each sample was applied to the phytic acid assay in triplicate.

(i) *Precision, LOD, and lower LOQ of the phytic acid assay.*—Repeatability (intra-assay precision) and intermediate precision (interassay precision) of the phytic acid assay were assessed using a milled oat flour sample. Five replicate samples of the milled oat flour were applied to the phytic acid assay in four separate experiments.

(j) *Sample analysis, recovery, and specificity of the phytic acid assay.*—A variety of nine milled food or feed samples were applied to the phytic acid assay in duplicate, with and without phytic acid spiking. The samples used were barley flour, malt flour, oat flour, wheat flour, American long grain rice, Odlums Cream Plain Flour, Kellogg's All-Bran Original cereal, pinto beans, and red lentils. Spiked samples were supplemented with 7.5 mg phytic acid, which was added before the extraction of phytic acid procedure.

(k) *Calculation of total phosphorus and phytic acid.*—The absorbance value of STD0 obtained from the colorimetric determination of phosphorus assay was subtracted from the absorbance values of the other phosphorus standard solutions (STD1–STD4) to obtain $\Delta A_{phosphorus}$ for each standard.

The slope, M ($\mu g/\Delta A_{phosphorus}$), for each individual phosphorus standard solution and the mean M were calculated as follows:

$$M\left(\mu g/\Delta A_{phosphorus}\right) = \frac{P(\mu g)}{\Delta A_{phosphorus}}$$

$$\text{mean } M\left(\mu g/\Delta A_{phosphorus}\right) = \frac{M_{STD1} + M_{STD2} + M_{STD3} + M_{STD4}}{4}$$

The total phosphorus content of a sample was calculated as follows:

$$\text{Total phosphorous}\left(g/100g\right) = \frac{mean\ M \times V \times F}{10\,000 \times w \times v} \times \Delta A_{phosphorus}$$

where *mean M* is the average M value of phosphorous standards ($\mu g/\Delta A_{phosphorus}$), *V* is the original sample extract volume (mL), *F* is the dilution factor of the sample in the extraction and the dephosphorylation reaction, $\Delta A_{phosphorus}$ is the absorbance change of sample, 10 000 is the conversion from μg/g to g/100 g, *w* is the original weight of sample material used in the extraction (g), and *v* is the sample volume used in the colorimetric determination of phosphorus assay (mL).

It follows for phosphorus:

$$\text{Total phosphorous}\left(g/100g\right) = \frac{\text{mean M} \times 20 \times 55.6}{10000 \times 1.0 \times 1.0} \times \Delta A_{phosphorus}$$

The phytic acid content of a sample was calculated as follows:

$$\text{Phytic acid } (g/100g) = \frac{\text{phosphorus } (g/100g)}{0.282}$$

where 0.282 is the factor used to convert the measured phosphorus content to phytic acid content because phytic acid comprises 28.2% phosphorus. The calculation of phytic acid assumes that the amount of phosphorus measured is specifically released from phytic acid ($InsP_6$) and not from any other phosphate esters including the lower *myo*-inositol phosphates ($InsP_{1-5}$).

These calculations can be simplified using the Mega-Calc® Excel calculator found on the Megazyme website and in the Supplemental Information to this paper.

Results and Discussion

Phytic Acid Assay Validation

Linearity.—The linearity of the phytic acid assay comprising the extraction of phytic acid, the enzymatic dephosphorylation of phytic acid reaction, and the colorimetric determination of phosphorus assay is dictated by the dynamic ranges of the enzymatic dephosphorylation of phytic acid reaction and the colorimetric determination of phosphorus assay. The linearity of the colorimetric determination of phosphorus assay is also affected by the limitation of the spectrophotometer used to read the absorbance values.

The linearity of the enzymatic dephosphorylation of phytic acid reaction was observed up to 30 mg/mL (Figure 2A). In the standard phytic acid assay procedure, this equates to a maximum phytic acid content of 3.0 g phytic acid/100 g when 1 g of solid sample is applied to the assay.

The linearity of the colorimetric determination of phosphorus assay was observed up to a phosphorus concentration of 7.5 µg/mL (Figure 2B).

The eventual limitation to the dynamic range of the phytic acid assay was the accuracy of the spectrophotometer above an absorbance of approximately 1.5 absorbance units. Using a maximum absorbance threshold of 1.5 in the colorimetric determination of phosphorus assay, as generated by the 7.5 µg/mL phosphorus standard solution, equates to a maximum phytic acid content of approximately 3.1 g/100 g when 1 g of solid sample is applied to the phytic acid assay.

Accuracy.—A high level of accuracy was displayed by the phytic acid assay across all three phytic acid concentrations, with the percentage recovery being within 5% (Table 1). Within this experiment, the repeatability (intra-assay precision) of the triplicate determinations at each phytic acid concentration was extremely high, with CVs ranging from 0.24 to 2.53%.

Precision, LOD, and lower LOQ.—The repeatability (intra-assay precision) of the phytic acid assay was extremely high, with CVs ranging from 0.81 to 2.32% for the individual experiments that were performed. The intermediate precision (interassay precision) was also extremely high, displayed by a CV of 2.27% across all of the experiments (Table 2). This level of precision indicates that the reliability and repeatability of the phytic acid assay is suitable for measuring phytic acid in food and feed samples.

Within this experiment, the LOD and lower LOQ were estimated to be 0.09 and 0.31 g of phytic acid/100 g, respectively. LOD and lower LOQ were calculated as 3 × σ of the blank sample solution absorbance and 10 × σ of the blank sample solution absorbance, respectively, using absorbance values from 20 replicates.

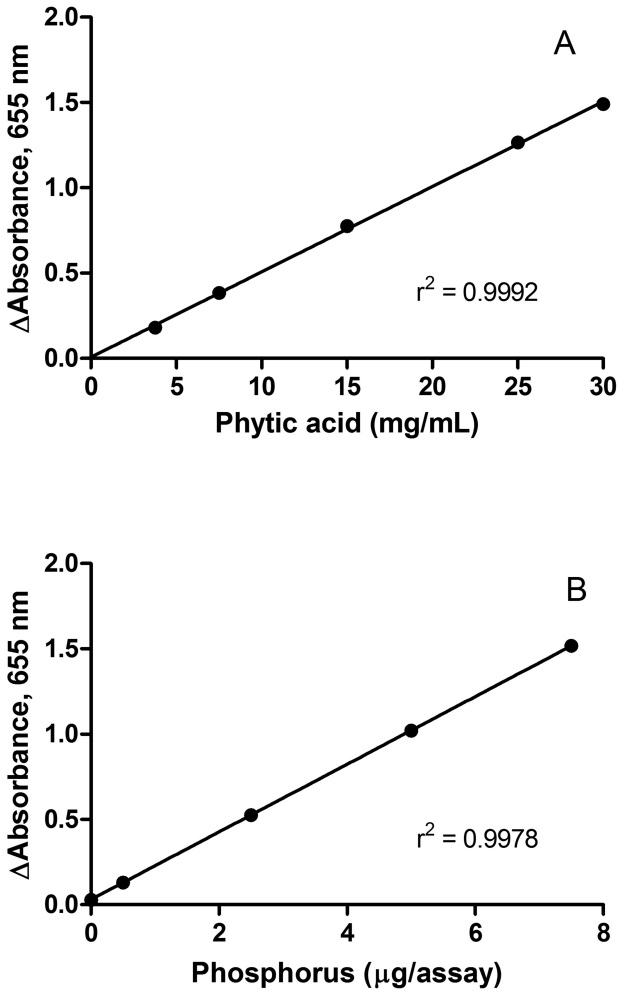

Figure 2. Linearity of the phytic acid assay. The linearity of (A) the enzymatic dephosphorylation reaction and (B) the colorimetric phosphorus determination assay were assessed using pure samples of phytic acid and phosphorus, respectively.

Sample analysis, recovery, and specificity.—The recovery rates of the phytic acid spike for each sample ranged from 97 to 115% (Table 3). This provides further confirmation that the

Table 1. Accuracy and repeatability of the phytic acid assay[a]

Prepared concn, mg/mL	Measured concn, mg/mL[b]	Accuracy: recovery, %	Repeatability: CV, %
3.75	3.69	98	1.68
3.75	3.67	98	2.53
7.50	7.81	104	1.45
7.50	7.79	104	0.69
15.00	15.71	105	1.42
15.00	15.76	105	0.34
25.00	25.71	103	0.43
25.00	25.64	103	0.24

[a] Solutions of phytic acid at four concentrations (3.75, 7.5, 15, and 25 mg/mL in distilled water) were prepared in duplicate, and 1 mL of each sample was applied to the phytic acid assay in triplicate.

[b] Values were obtained from triplicate determinations.

Table 2. Repeatability and precision of the phytic acid assay[a]

Replicate	Phytic acid, g/100 g				Intermediate precision
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	
1	1.916	1.965	1.932	1.824	
2	1.915	1.961	1.931	1.935	
3	1.959	1.930	1.905	1.839	
4	1.945	1.935	1.925	1.861	
5	1.916	1.942	1.844	1.851	
Mean	1.930	1.947	1.908	1.862	1.91
SD	0.020	0.016	0.037	0.043	0.04
Repeatability: CV, %	1.06	0.81	1.94	2.32	2.27

[a] Five replicate samples of milled oat flour were applied to the phytic acid assay in four separate experiments.

accuracy and specificity displayed by the phytic acid assay are suitable for the measurement of phytic acid in food and feed samples.

Conclusions

Phytic acid has gained considerable nutritional importance due to its negative effects on the bioavailability of various minerals in foods and animal feeds. The measurement of phytic acid is crucial for some industrial processes and research programs, e.g., to determine dosing rates of supplemental phytase to animal feeds and for plant breeding programs in the development of low-phytate crops.

In this study, we have successfully developed and validated a simple, high-throughput method for the measurement of total phosphorus and phytic acid in foods and feeds. This method addresses some constraints of other existing methods used for the measurement of phytic acid. It does not require expensive equipment such as HPLC or NMR, nor does it involve time-consuming or throughput-limiting steps such as AEC, as used in AOAC *Official Method* **986.11**. The validation of this phytic acid assay highlights excellent assay performance, with levels of accuracy, repeatability, precision, and specificity ideally suited for the intended application. The basic principle of this assay is that phosphate from phytic acid and lower *myo*-inositol phosphate forms is released by the action of phytase and ALP. The enzymatically released phosphate is measured using a modified colorimetric molybdenum blue assay and calculated as total phosphorus or phytic acid content in the original sample. However, this calculation requires the very same assumption that is required for AOAC *Official Method* **986.11**: that all of the phosphorus being measured is specifically released from phytic acid and not from any other phosphate esters, including the lower *myo*-inositol phosphates ($InsP_{1-5}$). This assumption holds true for nonprocessed samples, but not for processed samples, which can contain higher proportions of lower *myo*-inositol phosphates that will contribute to the overestimation of phytic acid.

In summary, the phytic acid assay developed in this study is ideally suited for the measurement of phytic acid in nonprocessed foods and animal feeds.

Table 3. Sample analysis and recovery[a]

Sample	Phytic acid, mg		Accuracy: recovery, %
	Sample only	Spiked sample	
Barley flour	4.3	11.8	100
Malt flour	5.3	12.7	99
Oat flour	18.2	26.7	112
Wheat flour	1.1	8.4	97
American long grain rice	1.9	9.6	102
Odlums Cream Plain Flour	2.6	11.2	115
Kellogg's All-Bran Original	15.6	23.7	107
Pinto beans	8.9	17.3	112
Red lentils	6.4	14.6	109
Phytic acid	7.5		

[a] Milled food and feed samples were applied to the phytic acid assay in duplicate, with and without phytic acid spiking at 7.5 mg, and recovery rates of the phytic acid spike were assessed.

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