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PHYTIC ACID (PHYTATE)/ TOTAL PHOSPHORUS

Measured as phosphorus released by phytase and alkaline phosphatase

ASSAY PROCEDURE

K-PHYT 05/19

(50 Assays per Kit)

INTRODUCTION:

Phytic acid (phytate; *myo*-inositol 1,2,3,4,5,6-hexakisphosphate) is the primary source of inositol and storage phosphorus in plant seeds contributing ~ 70% of total phosphorus. The abundance of phytic acid in cereal grains is a concern in the foods and animal feeds industries because the phosphorus in this form is unavailable to monogastric animals due to a lack of endogenous phytases; enzymes 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 (e.g. Ca^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, $Fe^{2+/3+}$), proteins and amino acids.[2] High phytic acid content feeds are generally supplemented with inorganic phosphate, however this causes increased faecal phosphate levels and subsequent eutrophication of waterways. Alternatively, supplementation with commercial phytases is becoming increasingly popular and reduces the requirement for inorganic phosphate supplementation as well as the associated environmental issues.

Currently, there is no commercially available, simple, quantitative method for phytic acid and, while such measurement is relatively complex, the generally accepted AOAC Method 986.11 has limitations.[3] For each individual analysis the method requires cumbersome anion-exchange purification and a major inherent assumption here is that only phytic acid is purified. While this assumption is viable for non-processed grains for which phytic acid comprises at least 97% of total inositol phosphates, it is not viable for processed foods and feeds which can contain higher levels of some lower *myo*-inositol phosphate forms (i.e. IP_3, IP_4, IP_5) that would co-elute with phytic acid and contribute to overestimation of the phytic acid content.[4,5]

Given the complexities of the purification and measurement of phytic acid separate from lower *myo*-inositol phosphate forms, Megazyme has developed a simple, quantitative method (**K-PHYT**) to measure total "available phosphorus" released from food and feed samples that is amenable to high numbers of samples and does not require tedious anion-exchange purification.[1] This method involves acid extraction of inositol phosphates followed by treatment with a phytase that is specific for phytic acid (IP_6) and the lower *myo*-inositol phosphate forms (i.e. IP_2, IP_3, IP_4, IP_5). Subsequent treatment with alkaline phosphatase ensures the release of the final phosphate from *myo*-inositol phosphate (IP_1) which is relatively resistant to the action of phytase. The total phosphate released is measured using a modified colourimetric method and given as grams of phosphorus per 100 g of sample material.[6,7]

Alkaline phosphatase will also release phosphate from monophosphate esters other than *myo*-inositol phosphate, however analyses of a broad spectrum of samples using **K-PHYT** indicated that phosphorus released from monophosphate esters other than *myo*-inositol phosphate is small.

PRINCIPLE:

Phytase hydrolyses phytic acid (phytate; *myo*-inositol hexakisphosphate) into *myo*-inositol (phosphate)$_n$ and inorganic phosphate (P_i) (1).

$$\text{(1)}\quad \text{Phytic acid} + H_2O \xrightarrow{\text{(phytase)}} \textit{myo}\text{-inositol (phosphate)}_n + P_i$$

Alkaline phosphatase (ALP) further hydrolyses *myo*-inositol (phosphate)$_n$ producing *myo*-inositol and P_i (2).

$$\text{(2)}\quad \textit{myo}\text{-Inositol (phosphate)}_n + H_2O \xrightarrow{\text{(ALP)}} \textit{myo}\text{-inositol} + P_i$$

P_i and ammonium molybdate react to form 12-molybdophosphoric acid, which is subsequently reduced under acidic conditions to molybdenum blue (3, 4).

$$\text{(3)}\quad P_i + \text{ammonium molybdate} \longrightarrow \text{12-molybdophosphoric acid}$$

$$\text{(4)}\quad \text{12-molybdophosphoric acid} + H_2SO_4 \text{ / ascorbic acid} \longrightarrow \text{molybdenum blue}$$

The amount of molybdenum blue formed in this reaction is proportional to the amount of P_i present in the sample and is measured by the increase in absorbance at 655 nm. P_i is quantified as phosphorus from a calibration curve generated using standards of known phosphorus concentration (see pages 9 & 10).

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay is specific for the measurement of phosphorus released as "available phosphorus" from phytic acid, *myo*-inositol (phosphate)$_n$ and monophosphate esters by phytase and alkaline phosphatase.

This kit does not measure *myo*-inositol in either its free or phytase/alkaline phosphatase released forms. However, if such analyses are required please refer to the *myo*-inositol assay kit (**K-INOSL**) in the assay kits section of the Megazyme website (**www.megazyme.com**).

The smallest differentiating absorbance for the assay is 0.005 absorbance units. This corresponds to a phosphorus concentration of ~ 2.82 mg/100 g of original sample (or phytic acid concentration of ~ 10 mg/100 g). Under the conditions of the standard assay procedure the detection limit is ~ 11.29 mg phosphorus/100 g of original sample (or ~ 40 mg phytic acid/100 g), which is derived from an absorbance difference of 0.020.

The assay is linear over the range of ~ 0.5 to ~ 7.5 µg of phosphorus per assay. In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.020 may occur. Under the conditions of the standard assay procedure this corresponds to a phosphorus concentration of ~ 2.82 mg to ~ 11.29 mg/100 g (or phytic acid concentration of ~ 10 mg to ~ 40 mg/100 g) of original sample material.

Typical values obtained for a range of food samples using **K-PHYT** are given in Table 2, page 12.

INTERFERENCE:

Interfering substances in the sample being analysed can be identified by including an internal standard. Quantitative recovery of this standard would be expected. Losses in sample handling and extraction are identified by performing recovery experiments, i.e. by adding phytic acid to the sample at the initial extraction step.

SAFETY:

The general safety measures that apply to all chemical substances should be adhered to.

For more information regarding the safe usage and handling of this product please refer to the associated SDS that is available from the Megazyme website.

KITS:

Kits suitable for performing 50 assays are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (25 mL, pH 5.5) and sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 2: Phytase suspension (1.2 mL).
Stable for > 2 years at 4°C.

Bottle 3: Buffer (25 mL, pH 10.4), plus $MgCl_2$, $ZnSO_4$ and sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 4: Alkaline phosphatase suspension (1.2 mL).
Stable for > 2 years at 4°C.

Bottle 5: Phosphorus standard solution (24 mL, 50 µg/mL) and sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 6: Oat flour control powder (5 g; Phosphorus content, see bottle).
Stable for > 5 years at room temperature.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Use the contents of bottle 1 as supplied.
Stable for > 2 years at 4°C.

2. Use the contents of bottle 2 as supplied. Before opening for the first time, shake the bottles to remove any enzyme that may have settled on the rubber stopper. Subsequently, store the bottles in an upright position. **Swirl the bottle to mix contents before use.**
Stable for > 2 years at 4°C.

3. Use the contents of bottle 3 as supplied.
Stable for > 2 years at 4°C.

4. Use the contents of bottle 4 as supplied. Before opening for the first time, shake the bottle to remove any enzyme that may have settled on the rubber stopper. Subsequently, store the bottle in an upright position. **Swirl the bottle to mix contents before use.**
Stable for > 2 years at 4°C.

5. Use the contents of bottle 5 as supplied.
Stable for > 2 years at 4°C.

6. To use the oat flour control in the test, extract 1 g in 20 mL of hydrochloric acid (0.66 M) during the sample extraction process of the standard assay procedure (see section A, page 7). Stable for 1 week at 4°C.

NOTE: The oat flour control extract is expected to generate values within 10% of the value provided on the bottle. The oat flour control extract can also be used directly as a spike where it is suspected that inhibition is being caused by substances in the sample. In such cases, the concentration of phosphorus released from the oat flour control must be accounted for in the calculation of total phosphorus [g/100 g] (see page 10).

PREPARATION OF REAGENT SOLUTIONS (NOT SUPPLIED):

1. Colour reagent:

Solution A. Ascorbic acid (10% w/v)/Sulphuric acid (1 M): 100 mL

- Add 10 g ascorbic acid (Sigma cat. no. 95210) to 90 mL of distilled water.
- Add 5.35 mL of concentrated sulphuric acid (Sigma cat. no. 258105 or equivalent) and dissolve the ascorbic acid. Make to volume (100 mL) with distilled water. Stable for 1 week at 4°C.

Solution B. Ammonium molybdate (5% w/v): 25 mL

Add 1.25 g ammonium molybdate (Sigma cat. no. 277908 or equivalent) to 20 mL of distilled water and dissolve. Make to volume (25 mL) with distilled water. Stable for 1 month at 4°C.

Solution B must be added to Solution A. Mix 1 part Solution B with 5 parts Solution A (e.g. add 1 mL Solution B to 5 mL Solution A). Prepare 0.6 mL per sample to be analysed (including blanks and standards). **Prepare fresh on day of use.**

Amounts required:

- Solution A (mL) = No. of samples x 0.5 mL
- Solution B (mL) = No. of samples x 0.1 mL

2. Trichloroacetic acid (50% w/v): 100 mL

Add 50 g of trichloroacetic acid (Sigma cat. no. T6399) to 60 mL of distilled water and dissolve. Make to volume (100 mL) with distilled water. Stable for > 6 months at 4°C.

3. **Hydrochloric acid (0.66 M): 1 L**
   Add 54.5 mL of hydrochloric acid (Sigma cat. no. 258148 or equivalent) to 945.5 mL of distilled water and mix. Store at room temperature.

4. **Sodium hydroxide (0.75 M): 200 mL**
   Add 6 g of sodium hydroxide pellets (Sigma cat. no. 795429 or equivalent) to 180 mL of distilled water and dissolve. Make to volume (200 mL) with distilled water. Store at room temperature.

5. **Phytic acid**
   Where a pure phytic acid control sample may be required, use of the following product is recommended: Phytic acid dipotassium salt (Sigma cat. no. P5681).

EQUIPMENT (RECOMMENDED):

1. Glass beakers (75 mL).
2. Disposable 1.5 mL polypropylene microfuge tubes, e.g. Sarstedt cat. no. 72.690.550 (www.sarstedt.com).
3. Disposable 13 mL polypropylene tubes, e.g. Sarstedt cat. no. 60.541.685 PP (www.sarstedt.com).
4. Disposable plastic micro-cuvettes (1 cm light path, 1.5 mL), e.g. Plastibrand®, semi-micro, PMMA; Brand cat. no. 759115 (www.brand.de).
5. Micro-pipettors, e.g. Gilson Pipetman® (100 µL and 1 mL).
6. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® [to dispense 0.5 mL aliquots of buffer (solution 1), buffer (solution 2) and colour reagent; to dispense 0.3 mL aliquots of trichloroacetic acid].
7. Stop clock.
8. Analytical balance.
9. Spectrophotometer set at 655 nm.
10. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
11. Heated water bath (set at 40°C).
12. Microfuge (required speed 13,000 rpm).

STANDARD ASSAY PROCEDURE:

A. SAMPLE EXTRACTION:

1. Accurately weigh approx. **1 g** of sample material into a 75 mL glass beaker. Add **20 mL** of hydrochloric acid (0.66 M), cover the beaker with foil and stir vigorously for **a minimum of 3 h at room temperature (preferably overnight for convenience).**

2. Transfer 1 mL of extract to a 1.5 mL microfuge tube and centrifuge at 13,000 rpm for 10 min. Immediately transfer 0.5 mL of the resulting extract supernatant to a fresh 1.5 mL microfuge tube and neutralise by addition of 0.5 mL of sodium hydroxide solution (0.75 M). Use the neutralised sample extract in the enzymatic dephosphorylation reaction procedure described below.

B. ENZYMATIC DEPHOSPHORYLATION REACTION:

Reaction tube: microfuge tube (1.5 mL)
Temperature: ~ 40°C
Final volume: 1.39 mL

Pipette into 1.5 mL microfuge tubes	Free Phosphorus	Total Phosphorus
distilled water	0.62 mL	0.60 mL
solution 1 (buffer)	0.20 mL	0.20 mL
sample extract	0.05 mL	0.05 mL
suspension 2 (phytase)	-	0.02 mL
Mix by vortex and incubate in a water bath set at 40°C for 10 min. After 10 min, start the next reaction by addition of:		
distilled water	0.02 mL	-
solution 3 (buffer)	0.20 mL	0.20 mL
suspension 4 (ALP)	-	0.02 mL
Mix by vortex and incubate in a water bath set at 40°C for 15 min. After 15 min, stop the reaction by addition of:		
trichloroacetic acid (50% w/v)	0.30 mL	0.30 mL
Centrifuge the terminated reaction at 13,000 rpm for 10 min. **Do not mix the tube after centrifugation.** Carefully pipette the supernatant for colourimetric determination of phosphorus (see section C, page 8).		

C. COLOURIMETRIC DETERMINATION OF PHOSPHORUS: MANUAL FORMAT

NOTE: For each batch of samples that is applied to the colourimetric determination of phosphorus, **a phosphorus calibration curve must be performed concurrently** using the same batch of colour reagent (see section D below).

Wavelength: 655 nm
Cuvette: 1 cm light path (glass or plastic; 1.5 mL semi-micro)
Temperature: ~ 40°C
Final volume: 1.5 mL
Sample solution: 0.5-7.5 µg of phosphorus (in a 1.0 mL sample volume)

Read against air (without a cuvette in the light path) or against water

Pipette into a 1.5 mL microfuge tube	Sample
sample or phosphorus standard colour reagent	1.00 mL 0.50 mL
Mix by vortex and incubate in a water bath set at 40°C for 1 h. After 1 h, mix by vortex and then transfer **1 mL** to a semi-micro cuvette and read the absorbance at 655 nm (A_{655}) **within 3 h.**	

D. PREPARATION OF PHOSPHORUS CALIBRATION CURVE:

Prepare the standard phosphorus solutions as described in the table below and treat as samples for the colourimetric determination of phosphorus (see section C above). Stable for 1 week at 4°C.

Pipette into 13 mL polypropylene tubes	STD 0 (0 µg)	STD 1 (0.5 µg)	STD 2 (2.5 µg)	STD 3 (5 µg)	STD 4 (7.5 µg)
distilled water phosphorus standard soln. 5 total volume	5.00 mL - 5.00 mL	4.95 mL 0.05 mL 5.00 mL	4.75 mL 0.25 mL 5.00 mL	4.50 mL 0.50 mL 5.00 mL	4.25 mL 0.75 mL 5.00 mL

CALCULATION:

A. PHOSPHORUS CALIBRATION CURVE:

1. Determine the absorbance (A_{655}) of each phosphorus standard (STD 0-4). Subtract the absorbance of STD 0 from the absorbance of the other standards (STD 1-4), thereby obtaining $\Delta A_{phosphorus}$ (for an example see Table 1 below).

2. Calculate M as follows, for each standard (STD 1-4):

$$\mathbf{M} = \frac{P\ (\mu g)}{\Delta A_{phosphorus}} \qquad [\mu g/\Delta A_{phosphorus}]$$

3. Calculate the ***mean M*** as follows:

$$\textbf{\textit{mean M}} = \frac{(M_{STD1} + M_{STD2} + M_{STD3} + M_{STD4})}{4} \qquad [\mu g/\Delta A_{phosphorus}]$$

Use "mean M" to calculate the phosphorus content of the test samples in section B.

Example:

Examples of the phosphorus calibration curve calculations are given in Table 1 along with a graphical representation of a typical phosphorus calibration curve (Figure 1).

Table 1. Calculations for a typical phosphorus calibration curve

Phosphorus standard	P [µg]	A_{655}	$\Delta A_{phosphorus}$	$[\mu g/\Delta A_{phosphorus}]$
STD 0	0	0.046	0.000	-
STD 1	0.5	0.144	0.098	5.102
STD 2 -	2.5	0.543	0.497	5.030
STD 3	5.0	1.040	0.994	5.030
STD 4	7.5	1.537	1.491	5.030
mean M	-	-	-	**5.075**

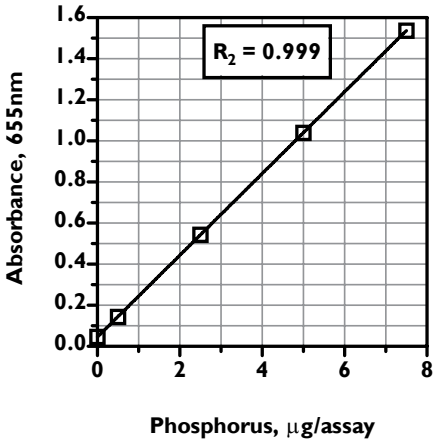

Figure 1. Calibration curve demonstrating the linearity of the colourimetric phosphorus determination as described in the **K-PHYT** procedure. The phosphorus standards were analysed in a cuvette with a 1.0 cm light path.

B. PHOSPHORUS/PHYTIC ACID CONTENT:

Determine the absorbance (A_{655}) for both the "Free Phosphorus" sample and the "Total Phosphorus" sample. Subtract the absorbance of the "Free Phosphorus" sample from the absorbance of the "Total Phosphorus" sample, thereby obtaining $\Delta A_{phosphorus}$.

The concentration of phosphorus can be calculated as follows:

$$c = \frac{\text{mean M} \times 20 \times F}{10{,}000 \times 1.0 \times v} \times \Delta A_{phosphorus} \quad [g/100\ g]$$

where:

mean M = mean value of phosphorus standards [µg/$\Delta A_{phosphorus}$]
20 = original sample extract volume [mL]
F = dilution factor
ΔA = absorbance change of sample
10,000 = conversion from µg/g to g/100 g
1.0 = weight of original sample material [g]
v = sample volume (used in the colourimetric determination step)

It follows for phosphorus:

$$c = \frac{\text{mean M} \times 20 \times 55.6}{10{,}000 \times 1.0 \times 1.0} \times \Delta A_{phosphorus} \quad [g/100\ g]$$

$$= \text{mean M} \times 0.1112 \times \Delta A_{phosphorus} \quad [g/100\ g]$$

It follows for phytic acid:

$$c = \frac{\text{phosphorus [g/100 g]}}{0.282} \quad [g/100\ g]$$

NOTE:

1. Where the absorbance (A_{655}) obtained for a sample is below 0.100 absorbance units or above that of STD 4 refer to "Modified Sample Extraction", below.
2. The calculation of phytic acid content assumes that the amount of phosphorus measured is exclusively released from phytic acid and that this comprises 28.2% of phytic acid.
3. These calculations can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears on the Megazyme website (www.megazyme.com).

CONSIDERATIONS:

MODIFIED SAMPLE EXTRACTION:

a) Samples with A_{655} above that of STD 4: Where the absorbance (A_{655}) obtained for a sample is above that of STD 4, repeat the standard assay procedure using **1 g** of sample material in **100 mL** of hydrochloric acid (0.66 M). In this instance the original sample extract volume [mL] becomes **100**.

Hence, it follows for phosphorus:

$$c = \frac{\text{mean M} \times \mathbf{100} \times 55.6}{10{,}000 \times 1.0 \times 1.0} \times \Delta A_{phosphorus} \quad [g/100\ g]$$

Conversion to phytic acid content [g/100 g] is the same as described above.

b) Samples with A_{655} below 0.100: Where the absorbance (A_{655}) obtained for a sample is below 0.100 absorbance units repeat the standard assay procedure using an appropriate amount of sample material [g] in the sample extraction step as recommended in the extraction table on page 12:

Extraction Table

A_{655} generated phytic acid (g/L)	Recommended amount of sample material (g)
0.03-0.05	4.0
0.05-0.10	2.5

Example:

For a sample that has generated A_{655} of 0.07 using the standard assay procedure, it is recommended that the sample is re-analysed using **2.5 g** of material in the extraction step. In this instance the sample weight in the calculation for phosphorus [g/100 g] becomes **2.5**.

Hence, it follows for phosphorus:

$$c = \frac{\text{mean M} \times 20 \times 55.6}{10{,}000 \times \mathbf{2.5} \times 1.0} \times \Delta A_{\text{phosphorus}} \quad [\text{g}/100\ \text{g}]$$

Conversion to phytic acid content [g/100 g] is the same as described on page 11.

Table 2. Typical values obtained for a range of food samples using **K-PHYT**.

Sample	**Total Phosphorus [g/100 g]**	**Phytic acid [g/100 g]**
Barley Flour (Lot 60301)	0.105	0.371
Malt Flour (Lot 61001)	0.131	0.465
Oat Flour (Lot 00101)	0.499	1.771
Wheat Flour (Lot 70201)	0.029	0.103
American Long Grain Rice	0.049	0.174
Kellogg's Original "All Bran"	0.453	1.608
Pinto Beans Milled	0.214	0.758
Red Lentils Milled	0.181	0.643
Odlum's Baker's Special Bran	1.008	3.575
Soybean Meal (Lot 51101)	0.512	1.815

All samples were analysed using the standard assay procedure of **K-PHYT** with the colourimetric determination of phosphorus performed in cuvettes with a 1 cm light path.

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