4.10.06

AOAC Official Method 2000.12
Phytase Activity in Feed
Colorimetric Enzymatic Method
First Action 2000

(Applicable to the determination of phytase activity in animal feed in the range of 200–400 phytase units [FTU]/kg.)

Caution: See Appendix B, "Enzyme Preparations."

See Table **2000.12A** for the results of the interlaboratory study supporting acceptance of the method.

A. Principle

Phytase is incubated with sodium phytate, liberating inorganic phosphate at 37 ± 0.1°C and pH 5.5. Incubation is stopped by adding acid molybdate–vanadate reagent which also produces a colored complex with the phosphate produced. The color of the yellow vanadomolybdophosphor complex, which is a measure for the amount of phosphate, is measured at 415 nm.

B. Apparatus

(**a**) *Water bath*.—Thermostatically controlled to 37.0 ± 0.1°C with circulating water.

(**b**) *Diluter*.—Provided with 0.500 and 5.000 mL cylinders, Hamilton Microlab 1000 (Hamilton Bonaduz AG; Bonaduz, Switzerland), or equivalent.

(**c**) *Dispensers*.—Adjusted to 4.00, 50.0, and 100 mL.

(**d**) *Spectrophotometer*.—Operating at 415 nm, with a spectral bandwidth 8 nm, with 10.00 mm continuous-flow cuvette with debubbler system, Pye Unicam PU 8600 (Helma GmbH & Co. KG, Postbox 1163, D-79371, Müllheim, Germany), or equivalent.

(**e**) *Centrifuge*.—Relative centrifugal force of 3000 × *g*, provided with rotor with inserts for 11 centrifuge tubes of 15 mL each.

(**f**) *Vortex mixer*.—2500 rpm.

(**g**) *Laboratory mill*.—Provided with 1 mm sieve and 6-tooth rotor, Ultra centrifugal mill ZM 100 (Retsch GmbH & Co. KG, Postbox 1554, D42759 Haan, Germany), or equivalent.

(**h**) *Filter*.—Paper filters, grade 595, 0.16 mm (Schleicher & Schuell; Dassel, Germany), or equivalent.

C. Reagents

Note: Prepare all solutions containing phytase in glassware. Plastic (disposable) material interferes with the assay. Do not use phosphoric acid or detergents that contain phosphate to wash glassware.

Use ultra high purity water, 18 MW-cm resistivity, for the preparation of all reagents and test solutions.

(**a**) *Phytase enzyme*.—A highly concentrated phytase enzyme preparation can be obtained from DSM Food Specialties, R&D Analysis, 010-0585, PO Box 1, 2600 MA Delft, The Netherlands, or equivalent. Use activity value certified by manufacturer.

(**b**) *Tween 20 solution*.—Quantitatively transfer 10.0 g Tween 20 (polyoxyethylene sorbitan monolaureate, for synthesis grade; Merck KgaA, Postbox 64271 Darmstadt, Germany) into 100 mL volumetric flask, dissolve in 80 mL H_2O, dilute to volume with H_2O, and mix well. Prepare fresh before use.

(**c**) *Dilution buffer*.—Transfer 1.76 g 4M acetic acid (24 mL diluted to 100 mL), 30.0 g Na acetate·$3H_2O$, and 0.147 g $CaCl_2 \cdot 2H_2O$ into 900 mL H_2O in 1 L volumetric flask. Adjust to pH 5.5 by dropwise addition of acetic acid. Add 1 mL Tween 20 solution, (**b**), and dilute to volume with H_2O. Prepare fresh before use.

(**d**) *Feed buffer*.—Transfer 30.0 g Na acetate·$3H_2O$ and 10.05 g $CaCl_2 \cdot 2H_2O$ into 1 L volumetric flask, dissolve in 900 mL H_2O, and add 0.1 g Tween 20. Adjust to pH 5.5 by dropwise addition of acetic acid and dilute to volume with H_2O. Prepare fresh before use.

(**e**) *Phytic acid*.—Dodecasodium salt, from rice (P3168, Sigma Chemical Co., PO Box 14508, St. Louis, MO 63178, USA, or equivalent).

(**f**) *Phytic acid substrate*.—Quantitatively transfer 8.40 g phytic acid, (**e**), into 1 L volumetric flask and dissolve in 900 mL dilution buffer, (**c**). Adjust to pH 5.5 by dropwise addition of 4M acetic acid (24 mL diluted to 100 mL), and dilute to volume with H_2O. Prepare fresh before use. If other than $\cdot 12H_2O$ product supplied, adjust weight accordingly.

(**g**) *Ammonium heptamolybdate stock solution*.—Transfer 100 g ammonium heptamolybdate ($H_{24}Mo_7N_6O_{24} \cdot 4H_2O$) into 1 L volumetric flask and dissolve in ca 900 mL H_2O. Add 10 mL NH_4OH and dilute to volume with H_2O. Solution is stable up to 1 month when stored at ambient temperature (20–25°C) shielded from light.

(**h**) *Ammonium vanadate stock solution*.—Transfer 2.35 g ammonium vanadate (NH_4VO_3) into 1 L volumetric flask and completely dissolve in ca 400 mL H_2O at 60°C. Add slowly while swirling 20 mL HNO_3 (7 + 13), cool to room temperature, and dilute to volume with H_2O. Solution is stable up to 1 month when stored at ambient temperature (20–25°C) shielded from light.

(**i**) *Color stop mixture*.—Mix 250 mL heptamolybdate solution and 250 mL vanadate solution and add slowly, while swirling,

Table 2000.12A. Interlaboratory study results for the determination of phytase activity in feed using a colorimetric enzymatic method

Feed[a]	FTU/kg	No. labs [b(c)]	r	s_r	% RSD_r	R	s_R	% RSD_R
Piglet	269	10 (1)	48.2	17.2	43.2	325.4	116.2	6.4
Layer	367	10 (1)	66.4	23.7	33.6	345.0	123.2	6.5
Fattening pig	299	10 (1)	72.2	25.8	27.6	231.6	82.7	8.6
Broiler	317	8 (0)	70.0	25.0	20.5	182.0	65.0	7.9
Turkey	346	8 (0)	53.5	19.1	22.4	216.4	77.3	5.5
Layer breeders	208	8 (0)	33.9	12.1	27.6	160.7	57.4	5.8

[a] Blind duplicate test sample pairs.

[b(c)] Where, b = number of laboratories retained after eliminating outliers; (c) = number of laboratoriess removed as outliers.

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Table 2000.12B. Serial dilutions for calibration line solutions

Stock solution	Stock solution, mL	Dilution buffer, **C**(**c**), to be added, mL	Diluted calibration line solution, mL	Dilution buffer, **C**(**c**), to be added, mL	Solution to be incubated, mL	Dilution buffer, **C**(**c**), to be added, mL	Activity
A	0.250	4.750	0.080	9.920	0.100	1.900	0.007
B	0.250	4.750	0.150	9.850	0.100	1.900	0.014
A	0.250	4.750	0.100	4.900	0.200	1.800	0.036
B	0.250	4.750	0.150	4.850	0.200	1.800	0.054
A	0.250	4.750	0.200	4.800	0.200	1.800	0.072

165 mL HNO_3 (7 + 13). Cool to room temperature, and dilute to 1 L with H_2O. Prepare fresh before use.

D. Determination

Note: Some metal ions, such as Cu, Fe, Zn, etc., may inhibit the enzyme reaction when present in millimolar range.

(**a**) *Test sample pretreatment*.—(*1*) *Feeds*.—Grind ca 100–150 g laboratory sample with mill, **B**(**g**). Grind until all material has passed through 1 mm sieve. Mix test sample thoroughly and in 125 mL glass beakers, weigh, in duplicate, ca 5 g test portion to the nearest 1 mg. Add 50 mL feed buffer, **C**(**d**), and stir with magnetic stirrer 60 min. Filter the top layer through paper. Dilute filtered solution with dilution buffer, **C**(**c**), to phytase activity within the range of 0.01–0.07 FTU/2 mL solution, and store in melting ice until incubation. Perform final dilution of test portion and in duplicate (test sample and test sample blank).

(*2*) *Preparation of calibration line solutions*.—Before use, let phytase standard, **C**(**a**), attain ambient temperature (20–25°C). In duplicate, in 200 mL volumetric flasks (stock solutions A and B), weigh to nearest 1 mg amounts of phytase standard, corresponding to 36 000 FTU. Dissolve in dilution buffer, bring to volume with dilution buffer, **C**(**c**), and mix. Perform the following serial dilutions of phytase standard solutions A and B. Prepare final dilutions in duplicate (standard and standard blank). *See* Table **2000.12B**.

Prepare fresh phytase standard solutions and dilute calibration line solutions before use. Store calibration line solutions in melting ice until incubation and analyze as described below.

(**b**) *Incubation*.—(*1*) *Calibration line solutions and test portions*.—Monitor time exactly using a stopwatch. Starting at time = 0 min, in the order of the series and at regular time intervals, place one of the tubes to be incubated into water bath at 37.0 ± 0.1°C. Starting at time = 5.0 min, in the same order of the series and at the same regular time intervals, add 4.00 mL phytic acid substrate, **C**(**f**), at 37.0 ± 0.1°C with a dispenser and mix with vortex mixer, **B**(**f**). Place tubes into water bath maintained at 37.0 ± 0.1°C. At time = 65.0 min, in the same order and with the same time intervals, terminate incubation by adding 4.00 mL color stop mixture, **C**(**i**), with a dispenser, and mix.

(*2*) *Blanks*.—Starting at time = 0 min (stopwatch), in the order of the series and with regular time intervals, place tubes containing blank in water bath maintained at 37.0 ± 0.1°C to equilibrate. At time = 5.0 min, in the same order of the series and with the same time intervals, add 4.00 mL color stop mixture, **C**(**i**), with a dispenser, mix, and place tubes on work bench. Next, add 4.00 mL phytic acid substrate, **C**(**f**), to all blank tubes, and mix.

Centrifuge the calibration line, test portion, and blank tubes for 5 min at 3000 × *g*. Measure absorbance at 415 nm with spectrophotometer, zeroing the instrument with H_2O. Repeat the analysis with a more diluted solution whenever the activity found is > 0.07 FTU/2 mL solution to be incubated or if absorbance of incubated solution is >1.000.

E. Expression of Phytase Activity

One phytase unit is defined as that quantity of enzyme that will liberate 1 mol inorganic *ortho*-phosphate per minute under the conditions of the assay.

Calculation of enzyme activity.—Calculate results with a calculator or computer program that can perform parabolic regression. If a calculator or computer program is not available, then calculate enzyme activity manually as follows:

(*1*) Calculate absorbance (A) of calibration line solutions and test sample solutions (A = absorbance test sample – absorbance blank).

(*2*) On linear graph paper, plot exactly calculated activities of calibration line solutions (FTU/2 mL) against corresponding absorbances.

(*3*) Draw best fitting polynomial curve not forcing it through the origin (each calibration line point should deviate no more than 5% from the curve). From this curve, read the activity of test sample solutions in FTU/2 mL.

(*4*) Calculate phytase activity of test samples as follows:

$$\text{Phytase activity, FTU/kg} = (\text{FTU/2 mL to be analyzed}) \times (f)/W$$

where f = total dilution factor of test sample based on test portion weight of 1.000 g; W = weight of test portion, g.

Report final activity of enzyme preparation as average of duplicate analysis to 2 significant figures.

Reference: *J. AOAC Int*. **84**, 629(2001).

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