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A Comparison of the Test Tube and the Dialysis Tubing in Vitro Methods for Estimating the Bioavailability of Phosphorus in Feed Ingredients for Swine

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The validity of a simplified in vitro test tube (TT) method was compared with a more complicated dialysis tubing (DT) method to estimate the percentage of available phosphorus (P) in 41 plant origin and five animal origin feed ingredients for swine. The TT method using 1.0 or 0.25 g samples was compared with the DT method using 1.0 g samples at two pancreatic incubation times (2 vs 4 h) in a 3 × 2 factorial arrangement of treatments. Each DT and TT method treatment was replicated three and six times, respectively. Both methods utilize three enzymatic digestions: (i) predigestion with endoxylanase and β-glucanase for 1 h, (ii) pepsin digestion for 2 h, and (iii) pancreatin digestion for 2 or 4 h. For the TT method, the entire procedure was conducted in a 50 mL conical centrifuge tube and replicated six times. For the DT method, the first two digestions were conducted in a 10 mL plastic syringe before the contents were quantitatively transferred into a segment of DT for the pancreatic digestion. The percentages of hydrolyzed P for plant origin ingredients measured by the DT method using 1.0 g samples and the TT method using 0.25 g samples were highly correlated (r = 0.94−0.97, $P < 0.001$) with each other and with published in vivo available P values for swine. Repeatabilities for these two methods ranged from 99.64 to 99.86%. The TT method using 1.0 g samples, however, did not provide valid estimates of P availability for all ingredients. For animal origin ingredients, neither method was significantly correlated (r = 0.1−0.6, $P \geq 0.4$) with published in vivo available P values. In conclusion, the accuracy and validity of the TT method using 0.25 g samples with a 2 h pancreatic digestion time was equal to or superior to the DT method using 1.0 g samples with a 4 h pancreatic digestion time for estimating P availability in plant origin feed ingredients.

KEYWORDS: In vitro; swine; feed ingredients; phosphorus; bioavailability

INTRODUCTION

The majority of the phosphorus (P) in cereal grains and oil seeds is bound in the form of phytic acid or phytate (myo-inositol hexakis-dihydrogen phosphate) (*1*−*3*) and is poorly digested by monogastric animals because these animals produce little to no intestinal phytase, the enzyme required to hydrolyze P from the inositol ring (*4*, *5*). Therefore, about 70−85% of the natural P in feeds fed to swine and poultry is not digested and is excreted in their waste (*6*−*9*). When adequate cropland is not available for manure application, excess P will accumulate in the soil and may become an environmental problem (*10*). The addition of a phytase enzyme premix to cereal grain-oil seed meal diets fed to poultry and swine increases P utilization and reduces P excretion in manure (*11*−*13*). The commercial development of low phytic acid grains (*6*−*9*) and low phytic acid soybean meal (*14*) will also reduce P excretion in manure because of their high mineral digestibilities.

Because the percentage of available P (aP) in plant origin feed ingredients varies substantially, a valid estimate of the aP in these feedstuffs is essential to avoid oversupplementation with P that increases the excretion of P in manure (*15*). The most valid way to determine the availability of P in feed ingredients is by in vivo testing using animals, and the apparent digestibility of P is the most valid evaluation criteria for swine (*16*). However, P availability determined by conducting animal experiments is expensive and time consuming. Therefore, a valid in vitro test to estimate aP in feed ingredients will be faster and lower cost than in vivo testing. An in vitro procedure using dialysis tubing (DT) was developed to estimate P availability in plant origin ingredients for swine and poultry (*17*−*19*). However, the DT method requires time for the preparation of the DT, the dialysis buffer, and the complicated and time-consuming transfer of digesta into the DT. Therefore, our

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laboratory developed a simple in vitro test tube (TT) method that is easy to use, fast, inexpensive, and valid for the estimation of aP in plant origin feed ingredients for swine (*20*). The objectives of this experiment were (i) to test our simplified and faster in vitro TT method for estimating P availability on a variety of feed ingredients used in swine feeds as compared with our in vitro DT method and published in vivo P availability values for swine, (ii) to evaluate a 2 vs 4 h pancreatic incubation time for the DT and TT methods, and (iii) to evaluate 0.25 and 1.0 g sample sizes for the TT method.

MATERIALS AND METHODS

A companion paper by Bollinger et al. (*20*) describes in detail our TT in vitro method for estimating the availability of P in feedstuffs used in swine diets and the modifications made in our original DT method. That experiment found that the accuracy and validity of the TT method was equal to the DT method for estimating the availability of P in corn, barley grains, and soybean products.

Feed Ingredient Sample Procurement and Preparation. Forty-one plant origin feed ingredients and five animal origin feed ingredients were used in this study. Ingredient samples were procured as follows: Five barley cultivars (Harrington check, hulled and hull-less low phytic acid 422, low phytic acid 635, and low phytic acid 955) were obtained from Victor Raboy (U.S. Department of Agriculture—Agricultural Research Service, Aberdeen, ID). Six wheat cultivars and two wheat byproducts (soft white winter with high or normal amylopectin, hard red winter with high or low protein, hard red spring with high or low protein, wheat bran, and wheat middlings) were obtained from Edward Souza (University of Idaho, Moscow, ID). Field peas, sunflower seeds, and canola seeds were obtained from Robert Harrold (North Dakota State University, Fargo, ND). High oil corn, high oleic high oil corn, and corn 98B were obtained from Pioneer Hi-Bred Int. (Johnston, IA). Two rice cultivars (cocodrie and cypress) were obtained from Martin Farms (Bernie, MO). Corn bran was obtained from J. M. Swank (North Liberty, IA). Grain sorghum was obtained from the Missouri Seed Foundation (Columbia, MO). Dried skim milk, lentils, and rye were purchased from a local grocery. Alfalfa meal, spray-dried blood plasma, canola meal, corn 98A, oats A and B, spray-dried whey, and the remaining ingredients (**Table 1**) were obtained from the University of Missouri Feed Mill. All ingredient samples were ground in a laboratory mill to pass a 1 mm mesh screen. Then, subsamples were ground in a Tecator 1093 sample mill to pass a 0.5 mm mesh screen. All samples were kept in sealed plastic bags at 4 °C until analyzed.

Chemicals. All chemicals used were reagent quality or better. Water was 16−18 MΩ.

Enzymes. Natugrain containing endoxylanase (8250 units/g EC 3.2.1.8) and endo-β-glucanase (6000 units/g EC 3.2.1.6) was provided by BASF Corporation (Mount Olive, NJ). Porcine pepsin (P-6887; EC 3.4.23.1) and pancreatin (P-7545; activity = 8 × USP) were purchased from Sigma Chemical Co. (St. Louis, MO).

In Vitro Procedures. The simplified TT in vitro method developed by Bollinger et al. (*20*) is a modification of our original DT in vitro method (*17*). Both the TT and the DT methods have three consecutive enzymatic digestions: (i) predigestion, (ii) pepsin digestion, and (iii) pancreatin digestion. These steps are described as follows.

Modified DT Method. Each ingredient sample was analyzed in triplicate.

Predigestion. One gram of finely ground diet ingredient (0.5 mm screen) was mixed with 3 mL of 0.04% sodium azide solution containing 5.3 mg of Natugrain (endoxylanase 8250 units/g and endo-β-glucanase 6000 units/g, BASF Corporation) per mL in a 10 mL plastic syringe. The sample and enzyme solution were vortexed and incubated in a water bath with shaking at 120 rpm and 39 °C for 60 min.

Pepsin Digestion. The digesta was mixed with a 1.0 mL of a 0.85 N HCl solution containing 24000 U of porcine pepsin per mL. After the digesta and enzyme solution were vortexed, the digesta was incubated in the same water bath at 39 °C for 120 min.

Pancreatin Digestion. At the end of the 120 min pepsin digestion, samples were quantitatively transferred to DT segments (18 cm long), and 1.3 mL of a 0.8 M $NaHCO_3$ solution containing 22.60 mg porcine pancreatin/mL (8 × USP) was added to the peptic digesta. After the digesta and enzyme solution were well-mixed, the DT was sealed on each end with clamps. The DT (molecular weight cut off 12000−14000, 1.6 cm in diameter, Sigma Chemical Co.) was placed in a 250 mL flask containing 100 mL of 0.05 M succinate buffer. Samples were incubated at 39 °C with shaking at 120 cycles per min for 2 or 4 h as required for this experiment. After the pancreatic incubation phase,

Table 1. Analyzed Values for tP, Phytate P, and Calculated aP for Plant and Animal Origin Feed Ingredients

ingredients	analyzed values: total P (%)[a]	analyzed values: phytate P (%)[b]	calcd P availability (%)[c]
plant origin feed ingredients			
alfalfa meal, 17% CP	0.215	0.032	85.1
barley cultivars:			
Harrington check	0.354	0.242	31.6
low phytic acid 422	0.338	0.134	60.4
low phytic acid 635	0.296	0.083	72.0
low phytic acid 955	0.292	0.022	92.5
naked low phytic acid 422	0.293	0.124	57.7
corn hybrids:			
corn 94	0.269	0.234	13.0
extruded corn 94	0.280	0.238	15.0
corn 97	0.277	0.225	18.8
corn 97 low phytic acid	0.288	0.096	66.7
corn 98A	0.274	0.237	13.5
corn 98B	0.249	0.221	11.2
high oil corn	0.313	0.241	23.0
high oleic acid corn	0.349	0.264	24.4
lentils	0.352	0.177	49.7
oat cultivars:			
oats A	0.338	0.241	28.7
oats B	0.397	0.267	32.7
field peas	0.477	0.297	37.7
rice (with hulls) cultivars:			
rice, cocodrie	0.284	0.211	25.7
rice, cypress	0.281	0.237	15.7
rice, 2001	0.317	0.248	21.8
rye	0.305	0.231	24.3
sorghum grain	0.290	0.238	17.9
wheat cultivars:			
hard red spring, low protein 2077	0.392	0.318	18.9
hard red spring, high protein 2076	0.362	0.288	20.4
hard red winter, low protein 2074	0.279	0.213	23.7
hard red winter, high protein 2075	0.279	0.208	25.4
soft red winter MO980405	0.423	0.355	16.1
soft white winter 2073	0.328	0.257	21.6
soft white winter, high amylopectin 2070	0.356	0.280	21.3
cereal byproducts:			
corn bran	0.068	0.027	60.3
rice bran	2.093	1.745	16.6
wheat bran	1.149	1.051	8.5
wheat middlings	1.154	1.030	10.7
oil seeds and byproduct meals:			
canola meal	1.199	0.932	22.3
canola seed, full fat	0.712	0.568	20.2
whole soybeans	0.514	0.369	28.2
whole soybeans, extruded	0.561	0.397	29.2
soybean meal	0.715	0.512	28.4
soybean meal, extruded	0.549	0.387	29.5
sunflower seed with hulls, full fat	0.503	0.434	13.7
animal origin feed ingredients			
blood plasma, spray dried	1.416		
fish meal, Menhaden	3.961		
meat and bone meal, pork	4.208		
dried skim milk	1.037		
dried whole whey	0.726		

[a] tP was analyzed in triplicate by an AOAC method (1990). [b] Phytic acid content was analyzed in triplicate by an AOAC method (1990) on plant ingredients. Animal origin feed ingredients do not contain phytate. [c] Calculated (estimated) availability of P (%) = [(tP − phytic acid P)/tP] × 100.

hydrolyzed P (hP) in the succinate buffer was determined colorimetrically on a spectrophotometer at 415 nm as described by Engelen et al. (*21*). A blank with the same enzyme additions was subtracted from the gross hP values obtained for individual samples.

Two TT Methods. Two ingredient sample sizes of 1.0 and 0.25 g were tested, using six replications for each sample size. The two sample sizes were considered as two TT methods for statistical analysis of the data. The procedures described below were the same for both sample sizes.

Predigestion and Pepsin Digestion. For the TT methods, the (i) predigestion and (ii) pepsin digestion were the same as that for the DT method except that the sample was placed in a 50 mL polypropylene, conical centrifuge tube with a plug−seal closure (Fisherbrand, Fisher Scientific, Pittsburgh, PA).

Pancreatin Digestion. At the end of the 120 m pepsin digestion, 1.3 mL of a 0.8 M $NaHCO_3$ solution containing 22.6 mg of porcine pancreatin/mL (8 × USP) was added to the peptic digesta. After it was mixed, the digesta was incubated at 39 °C with shaking at 120 cycles per min for 2 or 4 h. The digesta pH after pancreatin addition was about 7.06 in blanks. At the end of the pancreatic digestion phase, the TTs were placed in a 0 °C ice bath to halt enzymatic activity in the digesta. A 2 N HCl solution was added to the digesta and mixed thoroughly to stop enzyme activity and dilute the digesta. The volume of 2 N HCl added (10−40 mL) was dependent on the estimated amount of hP in digesta. After 2 N HCl additions, tubes were centrifuged at 1000*g* for 20 min to remove particulates from supernatants. The clear sample supernatant was analyzed for hP as described above for the DT method.

Digesta pH. Digesta pH of each ingredient was measured at the end of the 2 and 4 h pancreatic incubations using the TT method with 1.0 g samples. After an individual digesta sample was vortexed, six pH measurements (Corning pH meter model 120, Corning Inc., Corning, NY; general purpose pH electrode, Cole Parmer, Chicago, IL) were taken at 0 °C to measure repeatability.

Total P (tP) and Phytic Acid P Determinations. tP was determined in triplicate by wet ashing (using nitric + perchloric acid digestion) and running the molybdenum−vanadate (MoVan) method for inorganic P (*22*). Phytic acid P was determined in triplicate by column chromatography (*22*).

Statistical Analysis. The in vitro comparison data for the effect of method (DT method with 1.0 g samples and TT method with 1.0 or 0.25 g samples) and pancreatic incubation (digestion) time (2 or 4 h) on hP were analyzed as a completely random design analysis of variance (ANOVA) (*23*) using SAS (SAS Institute Inc., Cary, NC). Treatments were arranged as a 3 × 2 factorial according to the model $X_{ijk} = \mu_{ijk} + a_i + \beta_j + a\,\beta_{ij} + e_{ijk}$, where μ_{ijk} = overall mean, a_i = method, β_j = time, $a\,\beta_{ij}$ = interaction of method × time, e = error contribution with average 0 and variance d2, $i = 1...a$, $j = 1...b$, and $k = 1...n$. Pancreatic digestion pH was determined at the 2 and 4 h incubation times for the TT method as a completely random design ANOVA using SAS. Repeatability (*R*, intraclass correlation) values for the DT method with 1.0 g samples (three replications) and the TT method with 0.25 g samples (six replications) were calculated as a random effect ANOVA using the Proc Nested procedure of SAS according to the model $R = 100 \times [\sigma s^2/(\sigma e^2 + \sigma s^2)]$, where σs^2 = variance among samples and σe^2 = variance within samples. The significance of *R* was tested using the *F* statistic. Coefficients of regression and correlation were determined (*23*) between the hP values from the DT method using 1.0 g samples, the TT method using 0.25 g samples (both methods with 2 and 4 h incubations), calculated aP values [(phytic acid P subtracted from tP) × 100], and published in vivo aP values for swine (*29*). Regression analysis ($Y = a + bX$) was conducted using model II least squares methodology of SAS where both variables are random (both variables are independent and subject to measurement error). Different notations are used for the model $Y = a + bX$ (i.e., $X_2 = a + bX_1$), because method comparisons are true correlations.

RESULTS AND DISCUSSION

tP, Phytic Acid P, and Calculated aP Values for Feed Ingredients. The analyzed values for tP and phytic acid P for the ingredients used in this experiment are shown in **Table 1**. The analyzed values for tP and phytic acid P are within the range of published values for these ingredients (*2*, *24*−*27*). For plant origin feed ingredients, calculated aP was estimated by subtracting the percentage of phytic acid P from the percentage of tP (**Table 1**). Veum et al. (*8*, *9*) found that the calculated aP value was a good estimate of the availability of P in normal (wild type) and low phytic acid hybrid corn and barley cultivars.

hP Release by in Vitro Method and Pancreatic Incubation Time. *Plant Origin Feed Ingredients.* There were significant ($P < 0.05$) in vitro method × incubation time interactions for 34 of the 41 plant origin feed ingredients, with a trend ($P \leq 0.10$) for three ingredients (corn 94, corn 97, and corn bran), as shown in **Table 2**. There was no method × time interaction ($P \geq 0.14$) for four ingredients (Harrington check barley, low phytic acid barley 955, higher oleic acid corn, and sorghum grain) because there was less difference between the methods and (or) the hydrolysis time for those ingredients.

For the DT method, the percentages of hP for the 4 h incubation were higher ($P \leq 0.05$) and closer to published in vivo aP values (*28*, *29*) as compared with the 2 h incubation. This may be attributed to the use of DT in the DT method, because the hP must pass through the DT (MW cut off of 12000−14000) into the succinate buffer before it can be measured as hP.

For the TT method, the 0.25 g sample size and the 2 h pancreatic incubation time provided the most valid estimates of aP in these plant-based ingredients as compared with published in vivo data for swine (*28*, *29*). There was excellent agreement between the percentage hP obtained by the TT method using 0.25 g samples with a 2 h pancreatic hydrolysis and the published in vivo aP values for the barley cultivars, canola meal, the corn hybrids, the soybean products, and the wheat cultivars. Increasing the incubation time to 4 h for the TT method increased ($P \leq 0.05$) the percentage of hP measured for seven ingredients (extruded corn 94, corn 98B, high oil corn, corn bran, oats B, sunflower seed, and wheat middlings) and reduced ($P \leq 0.05$) the percentage of hP measured for 17 ingredients (all of the soybean products, all of the wheat cultivars, rice, corn bran, corn 98A, and canola seed). The decline in hP with the 4 h as compared with the 2 h incubation for the TT method indicates that the longer incubation time resulted in some recomplexing of hP, which reduced the validity of the 4 h incubation values for the TT method.

For the TT method, the 1.0 g sample size with 2 or 4 h incubations did not provide valid estimates for the percentage of hP for 23 ingredients (all of the barley and wheat cultivars, two rice cultivars, all the soybean products, canola seed, lentils, sunflower seed, wheat bran, and wheat middlings). However, the hP values obtained using 1.0 g samples were within an acceptable range for the remaining ingredients. The poor results obtained when using 1.0 g samples to estimate P availability in cereal grains and soybean products were corrected by reducing the sample size to 0.25 g as described in our companion paper on the development of the TT method (*20*). Reducing the sample size from 1.0 to 0.25 g without reducing the amount of enzymes added during the predigestion, peptic, and pancreatic incubations had the beneficial effect of increasing the fluid volume and enzyme concentrations 4-fold relative to sample dry matter. This improved sample mixing and increased the effectiveness of the enzymes during the three successive incubation periods.

However, in situations where a sample size of 0.25 may be too small to provide a homogeneous and representative sample, it may be prudent to increase the sample size to 0.5 or 1.0 g.

Table 2. Pancreatic Incubation Time X in Vitro Method Interaction Means for the Percentage of hP in Plant and Animal Origin Feed Ingredients

	DT method, 1.0 g sample			TT method, 1.0 g sample			TT method, 0.25 g sample			*P* value		
	pancreatic incubation time			pancreatic incubation time			pancreatic incubation time					
ingredient	2 h	4 h	SEM[a]	2 h	4 h	SEM[a]	2 h	4 h	SEM[a]	pancreatic incubation time	in vitro method	time × method
plant origin feed ingredients												
alfalfa meal	55.3^{b}	71.8^{c}	2.8	89.4^{d}	92.6^{d}	2.0	90.8^{d}	93.4^{d}	2.0	<0.001	<0.001	0.017
barley cultivars:												
Harrington check	25.7^{b}	32.0^{c}	1.4	27.0^{b}	28.1^{b}	1.0	45.2^{d}	48.1^{d}	1.0	0.001	<0.001	0.141
low phytic acid 422	41.8^{b}	50.6^{c}	1.1	42.9^{b}	43.9^{b}	0.8	65.3^{d}	65.4^{d}	0.8	<0.001	<0.001	<0.001
low phytic acid 635	51.7^{d}	60.1^{e}	0.8	45.9^{b}	48.5^{c}	0.5	68.5^{d}	69.1^{d}	0.5	<0.001	<0.001	0.001
low phytic acid 955	64.8^{bc}	76.0^{d}	2.4	62.0^{b}	67.8^{c}	1.7	92.1^{e}	95.1^{e}	1.7	<0.001	<0.001	0.161
naked low phytic acid 422	48.2^{c}	55.9^{d}	1.1	39.0^{b}	40.5^{b}	0.8	65.9^{e}	66.2^{e}	0.8	<0.001	<0.001	<0.001
canola meal	13.2^{b}	21.5^{d}	1.0	17.9^{c}	19.2^{cd}	0.7	20.9^{d}	20.9^{d}	0.7	<0.001	<0.001	<0.001
canola seed (full fat)	13.5^{d}	20.8^{e}	0.6	5.1^{b}	6.4^{c}	0.4	26.6^{g}	25.0^{f}	0.4	<0.001	<0.001	<0.001
corn hybrids:												
corn 94	7.7^{b}	9.2^{c}	0.4	14.8^{d}	16.8^{e}	0.3	9.4^{c}	10.1^{c}	0.3	<0.001	<0.001	0.094
extruded corn 94	6.8^{b}	9.1^{c}	0.5	16.6^{f}	16.4^{f}	0.3	10.4^{d}	13.5^{e}	0.3	<0.001	<0.001	<0.001
corn 97	7.4^{b}	10.0^{d}	0.5	14.5^{e}	15.1^{e}	0.4	8.6^{bc}	9.5^{cd}	0.4	<0.001	<0.001	0.099
corn 98A	7.0^{b}	8.9^{b}	0.8	11.4^{c}	12.7^{c}	0.5	22.8^{e}	20.5^{d}	0.5	0.572	<0.001	0.003
corn 98B	8.0^{b}	10.3^{c}	0.3	16.5^{e}	18.7^{f}	0.2	10.2^{c}	11.2^{d}	0.2	<0.001	<0.001	0.017
high oil corn	5.2^{b}	7.3^{c}	0.4	13.7^{f}	14.1^{f}	0.3	9.3^{d}	10.8^{e}	0.3	<0.001	<0.001	0.050
high oleic acid corn	4.5^{b}	6.3^{c}	0.4	12.2^{e}	12.6^{e}	0.3	9.7^{d}	10.1^{d}	0.3	0.004	<0.001	0.135
low phytic acid corn	37.4^{b}	44.1^{c}	0.8	45.5^{c}	47.0^{d}	0.5	45.9^{cd}	45.9^{cd}	0.5	<0.001	<0.001	<0.001
corn bran	27.4^{b}	35.7^{c}	2.8	49.8^{de}	50.0^{de}	1.6	48.3^{d}	53.5^{e}	1.6	0.005	<0.001	0.096
lentils	11.6^{b}	15.8^{c}	0.6	20.7^{d}	20.6^{d}	0.5	32.2^{f}	30.7^{e}	0.5	0.048	<0.001	<0.001
oat cultivars:												
oats A	14.2^{c}	17.4^{d}	0.4	13.7^{bc}	13.4^{bc}	0.3	13.3^{bc}	13.0^{b}	0.3	0.002	<0.001	<0.001
oats B	12.1^{d}	14.3^{e}	0.2	10.3^{b}	10.8^{c}	0.2	10.7^{bc}	12.1^{d}	0.2	<0.001	<0.001	<0.001
field peas	9.1^{b}	11.3^{c}	0.5	17.0^{e}	14.8^{d}	0.4	23.8^{f}	22.8^{f}	0.4	0.327	<0.001	<0.001
rice (with hulls) cultivars:												
rice, cocodrie	6.3^{b}	9.1^{de}	0.5	7.9^{cd}	7.8^{c}	0.3	7.6^{c}	9.3^{e}	0.3	<0.001	0.133	0.004
rice, cypress	6.9^{b}	8.6^{c}	0.5	8.2^{c}	8.5^{c}	0.3	15.7^{e}	13.1^{d}	0.3	0.556	<0.001	<0.001
rice, 2001	8.4^{c}	11.4^{d}	0.4	6.6^{b}	7.2^{b}	0.3	15.0^{f}	12.6^{e}	0.3	0.186	<0.001	<0.001
rice bran	3.4^{b}	4.6^{d}	0.1	3.5^{b}	3.5^{b}	0.1	4.2^{c}	4.2^{c}	0.1	<0.001	<0.001	<0.001
rye	42.6^{b}	49.6^{d}	0.7	46.2^{c}	48.0^{d}	0.5	77.4^{f}	74.9^{e}	0.5	<0.001	<0.001	<0.001
sorghum grain	4.4^{b}	5.6^{b}	0.6	8.4^{c}	9.0^{c}	0.4	8.8^{c}	8.6^{c}	0.4	0.170	<0.001	0.420
soybean products:												
whole soybeans	13.4^{c}	16.8^{d}	0.8	0.0^{b}	0.0^{b}	0.5	29.4^{f}	22.7^{e}	0.5	0.038	<0.001	<0.001
whole soybeans, extruded	14.8^{c}	20.2^{d}	0.4	2.3^{b}	2.3^{b}	0.3	30.6^{f}	27.2^{e}	0.3	0.016	<0.001	<0.001
soybean meal, 48%	12.4^{c}	17.6^{d}	0.4	0.7^{b}	1.2^{b}	0.3	22.0^{f}	19.6^{e}	0.3	<0.001	<0.001	<0.001
soybean meal, extruded	13.4^{d}	17.4^{e}	0.5	2.8^{b}	6.3^{c}	0.3	30.4^{g}	26.1^{f}	0.3	0.003	<0.001	<0.001
sunflower seed with hulls, full fat	15.7^{b}	20.9^{d}	0.5	15.9^{b}	16.3^{b}	0.4	18.1^{c}	19.7^{d}	0.4	<0.001	<0.001	<0.001
wheat cultivars:												
hard red spring, low protein 2077	24.4^{c}	27.4^{d}	0.8	22.2^{b}	23.3^{bc}	0.6	41.1^{f}	36.9^{e}	0.6	0.926	<0.001	<0.001
hard red spring, high protein 2076	21.2^{c}	26.3^{d}	1.0	18.7^{b}	21.3^{c}	0.7	42.1^{f}	36.5^{e}	0.7	0.322	<0.001	<0.001
hard red winter, low protein 2074	21.5^{b}	28.1^{c}	1.7	19.0^{b}	20.9^{b}	1.2	45.0^{e}	37.2^{d}	1.2	0.842	<0.001	<0.001
hard red winter, high protein 2075	26.2^{c}	31.5^{d}	1.1	22.4^{b}	25.3^{c}	0.8	47.9^{f}	42.7^{e}	0.8	0.186	<0.001	<0.001
soft red winter M0980405	30.3^{c}	34.1^{d}	0.7	28.5^{b}	28.8^{bc}	0.5	45.9^{f}	41.0^{e}	0.5	0.564	<0.001	<0.001
soft white winter 2073	19.8^{c}	24.6^{d}	0.8	17.0^{b}	18.3^{bc}	0.5	30.5^{e}	25.7^{d}	0.5	0.414	<0.001	<0.001
soft white winter, high amylopectin	23.5^{c}	27.4^{d}	0.6	19.6^{b}	20.5^{b}	0.4	39.6^{f}	36.4^{e}	0.4	0.202	<0.001	<0.001
wheat bran	18.9^{b}	22.7^{c}	0.4	22.5^{c}	22.8^{c}	0.3	34.0^{d}	34.0^{d}	0.3	<0.001	<0.001	<0.001
wheat middlings	18.7^{bc}	21.8^{d}	0.4	18.4^{b}	19.5^{c}	0.3	31.1^{e}	32.1^{f}	0.3	<0.001	<0.001	0.025
animal origin feed ingredients												
blood plasma	42.2^{d}	45.6^{e}	0.6	19.2^{b}	24.7^{c}	0.4	80.6^{f}	83.1^{g}	0.4	<0.001	<0.001	0.003
fish meal, Menhaden	9.2^{b}	11.3^{b}	1.2	56.7^{c}	61.9^{d}	0.8	85.7^{e}	85.9^{e}	0.8	0.004	<0.001	0.022
meat and bone meal (pork)	4.9^{b}	6.9^{b}	1.6	50.7^{c}	57.2^{d}	1.1	88.1^{e}	85.8^{e}	1.1	0.063	<0.001	0.003
dried skim milk	45.9^{b}	62.1^{f}	0.6	45.8^{b}	48.1^{c}	0.4	54.7^{d}	56.3^{e}	0.4	<0.001	<0.001	<0.001
dried whole whey	60.9^{b}	69.1^{d}	1.0	66.4^{c}	66.8^{cd}	0.7	74.6^{e}	75.2^{e}	0.7	<0.001	<0.001	<0.001

[a] The SEM for the DT method with 1.0 g samples is about 150% of the SEM for the TT method with 0.25 g samples because replication per sample was three for the DT method and six for the TT method. Means in the same row with no common superscript differ ($P \leq 0.05$).

Then, the volume of solution (enzymes, acids, etc.) used in each of the three consecutive enzymatic digestions will need to be increased (2- or 4-fold, respectively) relative to the volumes used for the 0.25 g sample, and the 50 mL conical centrifuge tube with closure will need to be replaced with a larger volume (100 or 200 mL, respectively) centrifuge bottle with closure. Therefore, using a larger sample size will increase the solution cost per sample and reduce the number of samples that can be run daily unless additional shaker bath and centrifuge capacities are available.

The main effects of method and time are presented in **Table 3** for the ingredients that did not have a significant ($P \geq 0.14$) method × pancreatic hydrolysis time interaction. For the Harrington check and the low phytic acid barley grains, the percentage of hP was higher ($P \leq 0.05$) for the TT method using 0.25 g samples as compared with the other two methods. For high oleic acid corn, both TT methods (1.0 and 0.25 g samples) had acceptable hP values that were higher ($P \leq 0.05$) than the hP value obtained with the DT method. For sorghum, both TT methods had the same percentages of hP that were

Table 3. Main Effect Means of in Vitro Method and Pancreatin Incubation Time for Percentage of hP Released from Feed Ingredients

	in vitro method						pancreatic incubation time		
	DT, 1.0 g samples		TT, 1.0 g samples		TT, 0.25 g samples				
ingredient[a]	means	SEM	means	SEM	means	SEM	2 h	4 h	SEM
barley, Harrington check	28.8[b]	1.0	27.6[b]	0.7	46.6[c]	0.7	32.6[b]	36.1[c]	0.7
barley, low phytic acid 955	70.4[c]	1.7	64.9[b]	1.2	93.6[d]	1.2	73.0[b]	79.6[c]	1.8
high oleic acid corn	5.4[b]	0.3	12.4[d]	0.2	9.9[c]	0.2	8.8[b]	9.7[c]	0.2
sorghum grain	5.0[b]	0.4	8.7[c]	0.3	8.7[c]	0.3	7.2	7.8	0.3

[a] No significant ($P \geq 0.14$) method × pancreatic hydrolysis time interactions for these ingredients. The SEM for the DT method with 1.0 g samples is about 150% of the SEM for the TT method with 0.25 g samples because replication per ingredient sample was three for the DT method and six for the TT method. The main effect means in the same row with no common superscript differ ($P \leq 0.05$).

Table 4. Endogenous Phytase and Acid Phosphatase Activities Reported for Common Feed Ingredients[a]

ingredient	phytase (units/kg)[b]	acid phosphatase (units/kg)[c]
alfalfa meal	0–60	NA
barley	582–1016	3.82
canola meal	41	4.99
corn	15–70	1.64
field peas	86–116	5.41
sorghum grain	24	NA
Oats	84	2.31
rye	5147	21.96
soybean meal	8	NA
whole soybean	32–45	1.88
wheat	1193–1637	10.25
wheat bran	2957–4624	14.11

[a] Adopted from Ravindran et al. (*1*) and Viveros et al. (*27*). [b] One unit of phytase activity was defined as the amount of phytase that liberated inorganic P from a 0.0015 M sodium phytate solution at the rate of 1 μmol/min at pH 5.5 and 37 °C (ref *26*). [c] One unit of acid phosphatase activity was defined as the amount of acid phosphatase that liberated 1 μmol of *p*-nitrophenol/min from 1 mL of 10 mM disodium *p*-nitrophenyl at pH 4.5 and 37 °C (ref *31*). NA, data not available.

higher ($P \leq 0.05$) than the value obtained with the DT method. For the main effect pancreatic hydrolysis time (**Table 3**), a 4 h incubation time produced higher ($P \leq 0.05$) hP values than 2 h except for sorghum where incubation time was not significant ($P = 0.30$).

The presence of endogenous phytase and acid phosphatase enzymes in cereal grains and cereal byproduct feeds will increase the bioavailability of P in those feed ingredients as compared with feed ingredients that contain little to no phytase enzyme activity (*1*, *27*, *30*, *31*). Barley, rye, wheat, and wheat bran contain higher concentrations (activity/kg) of phytase and acid phosphatase than other feed ingredients (**Table 4**). Those feed ingredients also had higher in vitro hP values than other cereal grains and cereal byproduct feed ingredients (**Table 2**).

The storage location of phytic acid in plant seeds also affects the P bioavailability of the feed ingredient. Corn is unique because most of the phytic acid is located in the germ portion of the kernel, whereas barley, wheat, and rice contain phytate in the germ and the hull (aleurone/ pericarp) portions of the seed (*24*, *28*). Soybeans contain phytate in protein bodies distributed throughout the seed, whereas most other oil seeds contain phytate primarily as crystalloid and globular structures (*24*, *28*).

Animal Origin Ingredients. There were significant ($P \leq 0.05$) interactions between in vitro method and pancreatic incubation time for all five animal origin feed ingredients (**Table 2**). The TT method using 0.25 g samples with a 2 h incubation time produced higher hP values than the other methods. However, the only valid in vitro estimate of hP for the animal origin ingredients was for meat and bone meal that had an in vitro value of 88% as compared with published in vivo aP values for swine that ranged from 70 to 90% (*15*, *28*, *29*). Therefore, in general, these in vitro methods are not valid for estimating aP in animal origin ingredients with the exception of meat and bone meal. Liu et al. (*18*) have reported that the in vitro DT method was not valid for estimating P availability in animal origin ingredients for swine. Because the availability of P in animal origin feed ingredients is high (91−97%) (*29*) and the aP values are close to those obtained for feed-grade phosphates (*16*), a valid in vitro procedure to estimate the availability of P in animal origin ingredients is of considerably less importance as compared with plant origin ingredients.

Repeatability of the in Vitro Methods. Both the DT and the TT in vitro methods had very high and consistent percentage repeatability values ($P < 0.001$) based on three and six replicates per feed ingredient, respectively. For the pancreatic incubation times of 2 and 4 h and both times pooled, respectively, the repeatabilities (%) were 99.64, 99.86, and 96.50 for the DT method and 99.80, 99.84, and 98.88 for the TT method. Repeatability was slightly lower when both incubation times were pooled.

Measurement of Digesta pH. Digesta pH was measured at the completion of the 2 and 4 h pancreatic incubations for all ingredients using the TT method with 1.0 g samples (**Table 5**). Digesta pH was lower ($P \leq 0.05$) after the 4 h incubation as compared with the 2 h incubation for 17 ingredients, with a trend ($P = 0.08$) for a lower pH at 4 h for one ingredient. There were trends ($P \leq 0.10$) for increases in digesta pH at 4 h as compared with 2 h for three ingredients, and one ingredient had a higher ($P \leq 0.05$) digesta pH at 4 h than at 2 h. However, for 24 of the ingredients, there were no differences ($P > 0.14$) between the 2 or the 4 h incubation times in digesta pH. Therefore, the longer incubation did not have a consistent effect on digesta pH, with digesta pH tending to either decline with the longer incubation or remain statistically unchanged. However, the overall 4 h digesta pH mean (6.75) was statistically lower ($P = 0.01$) than the overall 2 h pH mean (6.77) because of the small range in pH between ingredients as indicated by the small standard error (0.02) for the pH mean. The six consecutive pH measurements on each feed ingredient were highly repeatable ($P < 0.001$), with *R* values of 91.1 and 93.7% for the 2 and 4 h pancreatic incubations, respectively. Argenzio and Southworth (*32*) found that intestinal pH in growing swine gradually declined slightly over time as the digesta passed through the small intestine, cecum, and colon. Our digesta pH values at the end of the 2 and 4 h incubations are within the normal range of in vivo small intestine pH values for swine (*32*, *33*).

Table 5. Digesta pH Values of Plant and Animal Origin Feed Ingredients at the End of the 2 or 4 h Pancreatic Incubation Using the 1.0 g TT Method

ingredient	pancreatic incubation time 2 h	4 h	SEM	*P* value[a]
plant origin ingredients				
alfalfa	6.87	6.83	0.02	0.04
barley cultivars:				
Harrington check	6.88	6.85	0.01	0.05
low phytic acid 422	6.87	6.85	0.01	0.02
low phytic acid 635	6.88	6.84	0.02	0.01
low phytic acid 955	6.86	6.84	0.01	0.14
naked low phytic acid 422	6.84	6.86	0.01	0.10
canola meal	6.69	6.65	0.02	0.02
canola seed, full fat	6.59	6.44	0.27	0.01
corn hybrids:				
corn 94	6.80	6.76	0.02	0.01
extruded corn 94	6.84	6.80	0.02	0.21
corn 97	6.81	6.79	0.01	0.25
corn 98A	6.81	6.79	0.01	0.14
corn 98B	6.79	6.76	0.02	0.03
high oil	6.81	6.78	0.02	0.20
high oleic acid	6.80	6.79	0.01	0.20
low phytic acid	6.79	6.76	0.02	0.24
corn bran	6.56	6.64	0.04	0.08
lentils	6.75	6.72	0.02	0.36
oats cultivars:				
oats A	6.92	6.90	0.01	0.18
oats B	6.82	6.76	0.03	0.01
field peas	6.76	6.74	0.01	0.65
rice (with hulls) cultivars:				
rice, cocodrie	6.90	6.92	0.01	0.51
rice, cypress	6.87	6.91	0.02	0.51
rice, 2001	6.98	7.09	0.06	0.01
rice bran	6.64	6.58	0.03	0.01
rye	6.87	6.85	0.01	0.55
sorghum grain	6.85	6.87	0.01	0.14
soybean products:				
whole soybeans	6.92	6.88	0.07	0.31
whole soybeans, extruded	6.85	6.82	0.02	0.22
soybean meal	6.96	6.98	0.01	0.14
soybean meal, extruded	6.94	6.95	0.01	0.51
sunflower seed with hulls, full fat	6.55	6.37	0.09	0.01
wheat cultivars:				
hard red spring, low protein 2077	6.85	6.79	0.01	0.08
hard red spring, high protein 2076	6.84	6.79	0.03	0.03
hard red winter, low protein 2074	6.80	6.80	0.01	0.94
hard red winter, high protein 2075	6.90	6.85	0.03	0.03
soft red winter M0980405	6.88	6.86	0.03	0.36
soft white winter 2073	6.80	6.83	0.01	0.49
soft white winter, high amylopectin 2070	6.82	6.84	0.01	0.46
wheat bran	6.79	6.75	0.02	0.01
wheat middlings	6.68	6.64	0.02	0.01
animal origin feed ingredients				
blood plasma	6.90	6.95	0.02	0.08
fish meal, Menhaden	6.65	6.59	0.03	0.01
meat and bone meal (pork)	6.79	6.70	0.05	0.01
dried skim milk	6.57	6.59	0.01	0.69
dried whole whey	6.24	6.25	0.01	0.60
overall means (46 ingredients)	6.77	6.75	0.02	0.01

[a] Statistical significance at $P \leq 0.05$, with a trend between $P \geq 0.06$ and $P \leq 0.10$.

Relationships Between in Vitro hP Values and Published in Vivo P Availabilities for Feed Ingredients. *Plant Origin Feed Ingredients.* The regression and correlation (*r*) coefficients and the probability values for the relationships between the TT method with 0.25 g samples (both the 2 and 4 h pancreatic incubation times), the DT method with 1.0 g samples (mainly the 4 h pancreatic incubation time), and the published in vivo values for swine (*29*) are presented in **Table 6**. The hP values for both the TT and the DT methods were highly correlated ($r = 0.93-0.95$, $P < 0.001$) with published in vivo aP values (*29*). Also, the 2 and 4 h hP values for the TT method were highly correlated with each other and with the 4 h hP values from the DT method ($r = 0.96-0.99$, $P < 0.001$). Therefore, these correlations based on the hP data from 41 plant origin feed ingredients confirm the conclusion in our companion TT methods paper (*20*) that our simplified in vitro TT method is comparable to our in vitro DT method for estimating aP and is a valid alternative to conducting in vivo studies to determine the availability of P in plant origin feed ingredients fed to swine. For the DT method, the hP values for the 2 and 4 h incubations were highly correlated ($r = 1.00$, $P < 0.001$). This indicates that the 2 and 4 h hP responses for the DT method had similar slopes even though the 2 h values have less validity (below in vivo values) as compared with the 4 h values.

Table 6. Correlations between the hP Values from Two in Vitro Methods and Published in Vivo P Availabilities for Feed Ingredients Fed to Swine[a]

X value	*Y* value	regression coefficient	correlation coefficient (*r*)	*P* value
plant origin feed ingredients				
TT, 2 h	NRC, 1998	$Y = 4.14 + 0.96X$	0.94	<0.001
TT, 4 h	NRC, 1998	$Y = 4.91 + 0.95X$	0.93	<0.001
DT, 4 h	NRC, 1998	$Y = 2.33 + 1.32X$	0.95	<0.001
TT, 4 h	DT, 4 h	$Y = 0.51 + 0.76X$	0.97	<0.001
TT, 4 h	TT, 2 h	$Y = 1.41 + 0.99X$	0.99	<0.001
TT, 2 h	DT, 4 h	$Y = -0.92 + 0.76X$	0.96	<0.001
DT, 4 h	DT, 2 h	$Y = -1.03 + 0.85X$	1.00	<0.001
animal origin feed ingredients				
DT, 4 h	NRC, 1998	$Y = 91.35 + 0.04X$	0.46	0.54
TT, 2 h	NRC, 1998	$Y = 91.87 + 0.01X$	0.07	0.93
TT, 4 h	NRC, 1998	$Y = 90.83 + 0.03X$	0.13	0.87
TT, 2 h	DT, 4 h	$Y = 163.67 - 1.62X$	0.76	0.14
TT, 4 h	DT, 4 h	$Y = 165.17 - 1.63X$	0.71	0.18

[a] Forty-one plant origin and five animal origin feed ingredients with sample sizes of 0.25 g for the TT and 1.0 g for the DT methods and pancreatin incubation times of 2 or 4 h.

Animal Origin Feed Ingredients. The hP values obtained by the in vitro TT and DT methods for the five animal origin ingredients were not significantly correlated ($P \geq 0.44$, **Table 6**) with published in vivo values (*29*), and hP values from the TT method were not correlated with hP values from the DT method ($P \geq 0.18$). Therefore, these correlations confirm our hP results that found the in vitro TT and DT methods were not valid for estimating P availability in animal origin feed ingredients for swine. Liu et al. (*18*) also found that the hP values for animal origin ingredients from our original DT method were not significantly correlated with published in vivo aP values.

Correlations between the Calculated aP Values and Two in Vitro Methods. Veum et al. (*8*, *9*) and Bollinger et al. (*20*) have suggested that the analytical values for phytic acid P and tP (*22*) could be used to calculate the estimated percentage availability of P in barley grains, corn hybrids, and soybean products. In this experiment, significant correlations ($P < 0.001$) were obtained between the calculated aP values for the plant origin feed ingredients [(tP − phytic acid P/ tP) × 100], hP values from the DT method using 1.0 g samples, hP values from the TT method using 0.25 g samples, and published in vivo aP values for swine (*29*), with correlations ranging from 0.73 to 0.80 as shown in **Table 7**. This confirms our earlier suggestion that calculated aP values may provide useful estimates of the percentage of tP that is available in plant origin feed ingredients

Table 7. Correlations between the Plant Origin Feed Ingredient Values for Calculated aP, hP from Two in Vitro Methods, and Published in Vivo aP Values for Swine[a]

X value	*Y* value	regression coefficient	correlation coefficient	*P* value
calcd aP	NRC, 1998	$Y = 8.28 + 0.94X$	0.77	<0.001
calcd aP	DT, 4 h	$Y = 3.32 + 0.70X$	0.80	<0.001
calcd aP	TT, 2 h	$Y = 7.90 + 0.82X$	0.73	<0.001
calcd aP	TT, 4 h	$Y = 5.46 + 0.86X$	0.77	<0.001

[a] Calculated available (aP) was determined by subtracting phytic acid P from tP, using the values from the 41 plant origin feed ingredients reported in **Table 1**. The two in vitro methods were the TT with 0.25 g samples and the DT with 1.0 g samples.

for swine. However, the correlation coefficient obtained between the calculated aP and the published in vivo aP values for plant origin feed ingredients for swine (*29*) averaged 0.77. This correlation was not as strong as the correlations obtained between the in vitro methods and the in vivo aP values for plant origin ingredients for swine (**Table 6**) that ranged from 0.93 to 0.96. Therefore, both of our in vitro methods for estimating P availability have a higher validity and accuracy than the calculated aP value obtained by subtracting the percentage of phytic acid P from the percentage of tP.

Nutrient digestibility (nutrient availability) in animals is controlled by complex physiological mechanisms that include the neural and endocrine systems and gastrointestinal factors such as pH, enzyme production, rate of passage, and species differences (*34*−*36*). Therefore, an in vitro method will not be able to duplicate the in vivo digestion process exactly for all ingredients (*36*, *37*). For these reasons, in vitro methods provide a relative rather than an absolute estimate of nutrient availability that may be very useful for ranking purposes (*18*, *36*, *37*).

In conclusion, both the DT and the TT in vitro methods produced hP values that were valid and accurate estimates of P availability in plant origin feed ingredients fed to swine. The simplified TT method is faster and easier to use than the DT method. However, neither the DT nor the TT in vitro methods are valid for estimating P availability in animal origin feed ingredients fed to swine.

Our simple in vitro TT procedure and our modified DT in vitro method are valid and reliable alternatives to conducting in vivo studies to estimate P availability in plant origin feed ingredients. As compared with the DT method, the simple TT procedure reduces preparation time, eliminates the complicated and time-consuming manipulation of digesta transfer to DT, and shortens the pancreatic incubation (digestion) time.

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