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The impact of estimation methods on phytase phosphorus equivalency for commercial layer hens

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Abstract An experiment was performed to evaluate the calibration curve (CC) and compare the negative and positive controls (CNP) as a major method for estimating the phytase phosphorus equivalencies for layer hens. Three hundred and sixty 70-wk-old layer hens (W-36Hy-line) were used in a completely randomized design. Evaluated methods were setting two regression equations for NPP-supplemented and phytase supplemented treatments with two sub-methods by including the calibration curve (CC) or excluding the phosphorus content of the basal diet (CC-BD) in calculations, and exploring enzyme equivalency by comparing phosphorus deficient diet as a negative and supplemented diet by inorganic phosphorus sources as a positive control group (CNP). The experiment included nine treatments (a phosphorus deficient basal diet containing 0.12% available phosphorus (Av. P), 4 basal diets containing 200, 300, 400 and 500 FTU/kg phytase, and four basal diets supplemented with 0.20, 0.27, 0.35 and 0.43% Av. P). Each treatment was replicated five times with eight birds per replicate. Estimation method had a significant effect on phosphorus equivalency estimation ($P<0.0001$). Fitted regression equation models that deducted the P content of the basal diet (CC-BD) provided more rational values than those method ignore it (CC) (0.432% *vs* 0.564% for 500 FTU/kg phytase for layer hens) ($P<0.0001$). Of the three methods used, the CC method provided the highest estimated values ($P<0.0001$). Regardless of the mathematical methods, there were no significant differences for egg production performance and egg quality traits served as the response criteria. It was concluded that the phosphorus equivalent value of enzyme varied according to the estimation methods; therefore, using the matrix values of enzymes for accurate feed formulation depends on a variety of circumstances, and decision making requires comprehensive information.

Keywords: enzyme, feed formulation, calibration curve, matrix estimation method

Introduction

Phytase is the most commonly used enzyme in poultry diets aimed at increasing the availability of plant origin phosphorus content of feed ingredients of plant origins. The standard phytase activity is defined as the amount of enzyme that releases 1 µ mol of inorganic phosphate from 5 mM sodium phytate substrate per minute at pH 5.5 and 37 °C, and is expressed as FTU, FYT or OUT per kg of feed (Wealleans et al., 2016). However, because many factors affect phytase functionality in practical nutrition, it is not an appropriate predictor of *in-vivo* efficiency of phy-

tase (Bedford and Patridge, 2011). Dersjant-Li et al. (2019) reported that the optimal range of pH for various phytases can be remarkably different. Phosphorus equivalency illustrates the potential of the enzyme to adding phosphorus to the diet or phosphorus contribution of a given unit of phytase *in-vivo* (Bedford and Cowieson 2020). Numerous studies have determined the P equivalencies of various phytases in poultry feeds. Interestingly, these values have been influenced by the source of phytase (Rodriguez et al., 1999a, b; Tran et al., 2011), source of in-organic P (Li et al., 2015), P and Ca contents of the basal diet, Ca:P ratio of basal diet (Li et al., 2013), phytase inclusion rates in the diets (Abd El-Hack et al., 2018), intended strain (Leske and Coon, 1999) and finally, the manner of estimation (Dersjant-Li et al., 2019). Phytase action is not limited to the phosphorus release solely. It was reported that supplementation of phytase in poultry diet, not only improved the phosphorus availability but also the bioavailability of some other minerals, protein, amino acids and even energy (Jalal and Scheideler, 2001; Newkirk and Classen, 2001; Rutherfurd et al., 2004a, b; Liu et al., 2009; Ghosh et al., 2016; Zaghari et al., 2009; Zaghari et al., 2015). Therefore, the matrix value should estimate the releasing extent of the first limiting nutrient (i.e., P) and secondly Ca, Na, protein, AME and some other minerals in body using the recommended dose of enzyme.

Matrix values have been determined under controlled *in-vivo* experiments; however, the claimed nutrient saving values must be guaranteed by a significant degree of confidence. Besides the variations resulted from different experimental assays adopted for P equivalency estimation (i.e., directly through digestibility tests or indirectly using a biological response criterion) (Bedford and Cowieson, 2020), it seems that within a distinct manner of measurement, the method of P equivalency calculation may affect the estimated values. Several performance trials, fully described by Bedford and Cowieson (2020), have been employed to determine the nutrient equivalency of a pure phytase in hens. Therefore, the objective of the present study was to compare three different methods within calibration curves as a major method that has been adopted to calculate the P equivalency values of phytase.

Materials and methods

Three hundred and sixty 70-wk-old layer hens (W-36Hy-line) were used in an experiment to estimate the phosphorus equivalency of phytase. Hens were selected from a healthy commercial flock based on the relatively same average body weight (1450±25 g) and egg production (72±1 %) which had been fed a diet (0.4% Av. P) without phytase. Hens were allotted to nine treatments and five replicates in a completely randomized design. Treatments were: a basal diet with 0.12% available P (Av. P), four diets containing increasing levels of 0.07, 0.15, 0.21 and 0.31% NPP (equivalent to 0.20, 0.27, 0.35 and 0.43 % Av. P, respectively), and four diets containing increasing doses of phytase (0.002, 0.003, 0.004 and 0.005 g/kg feed equivalent to 200, 300, 400 and 500 FTU/kg). The phytase used was a mixture of bacterial and fungal phytase. Phytase activity was measured following ISO 2009 (reference number, ISO 30024:2009(E)). Compositions of the experimental diets are shown in Table 1. Daily feed intake was 100 g/bird in mash form. All diets were iso-energetic and iso-nitrogenous by substituting inert filler with dicalcium phosphate (DCP) and phytase.

During 6 weeks of the experiment, total egg production (all laid eggs) and total saleable egg production (total laid eggs excluding the dirty, cracked, under-sized, miss-shaped, over-sized and soft-shelled) were recorded daily. The eggs were weighed once a week. Two samples from each replicate were selected to measure the eggshell thickness, using the mean of measurements on three points on the shell (Zaghari, 2009). Egg mass was calculated as egg production rate × egg weight. Weekly feed intake (g) and egg mass (g) were used to calculate the feed conversion ratio (Zaghari, 2009).

Hens were housed in a cross-ventilation system house in which they were exposed to 16 h of incandescent light at 10 Lux and 8 h of darkness per day. There were 90 cages, and each cage contained 4 hens. Two adjacent cages containing 8 hens were considered as a replicate. The cage dimensions were 30 × 64 cm (equal to 1920 cm^2 of floor space). Each hen had 480 cm^2 of floor space. Free access to water was supplied by nipple drinkers. The experiment was conducted for a 6 weeks period from 70 to 75 week. The daily average ambient temperature was 24°C, and the relative air humidity ranged between 30 to 40% throughout the experiment.

Statistical analysis

Data were analyzed by using the GLM procedure (SAS, 2004), and mean separation was performed using the Duncan's multiple range test at ($P<0.05$). Two regression equations (calibration curves) were created for two classes of treatments (NPP-supplemented and phytase-supplemented).

Three different methods were used to calculate the phosphorus release values.

Method one (Calibration Curve (CC)): Phosphorus equivalency was calculated by putting Y=treatment mean values into regression equations created for NPP-supplemented treatments as described by Fernandez et al., (2019) and solved as follows:

Calculations were performed based on the present study data (Tables 1, 2 and 3).

Linear function: $Y= a + bX$

$Y_{egg\ production} = 37.53 + 69.57 \times Phosphorus$

$Y_{egg\ production} = 77.11$ (Treatment mean, supplemented with 500 FTU/kg phytase)

Table 1. Diet composition and nutrient analysis of the experimental diet for layer hens.

	Treatments (Av. P %)								
Ingredients	0.12	0.43	0.35	0.27	0.20	0.12	0.12	0.12	0.12
Corn grain	50.5	50.5	50.5	50.5	50.5	50.5	50.5	50.5	50.5
Soybean meal (44%)	24.4	24.4	24.4	24.4	24.4	24.4	24.4	24.4	24.4
Barely	10	10	10	10	10	10	10	10	10
Fat powder[1]	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6
DCP[2]	-	1.8	1.35	0.9	0.45	0	0	0	0
$CaCO_3$	9.45	9.45	9.45	9.45	9.45	9.45	9.45	9.45	9.45
Common Salt	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.38
Sodium Bicarbonate	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
Vit+Min premix[3,4]	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
DL-Methionine	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
Phytase	-	-	-	-	-	0.02	0.03	0.04	.05
Neutral Filler[4]	1.8	0	0.45	0.9	1.35	1.7998	1.7997	1.7996	1.7995
Total	100	100	100	100	100	100	100	100	100
Nutrients (%)									
Calculated									
ME (kcal/kg)	2700	2700	2700	2700	2700	2700	2700	2700	2700
CP	13.00	13.00	13.00	13.00	13.00	13.00	13.00	13.00	13.00
Ca	3.91	4.35	4.24	4.13	4.02	3.91	3.91	3.91	3.91
Total P	0.323	0.631	0.554	0.477	0.400	0.323	0.323	0.323	0.323
Av. P	0.121	0.429	0.352	0.275	0.198	0.121	0.121	0.121	0.121
Phytate P	0.202	0.202	0.202	0.202	0.202	0.202	0.202	0.202	0.202
Analyzed									
Ca	3.35	3.97	3.32	3.34	3.10	2.79	2.81	2.96	2.86
Total P	0.255	0.540	0.484	0.414	0.337	0.256	0.253	0.246	0.245

[1] 8100 kcal/kg ME, 11% Ca.
[2] Di-Calcium Phosphate:24% Ca, 17.1% P, 0.06% Na.
[3,4] Mineral premix provided 75 mg Mn, 75 mg Fe, 60 mg Zn, 0.868 mg I, 0.2 mg Choline-Cl per Kg of diet. Vitamin premix provided 8800 IU Vit A, 2500 IU$vitD_3$, 11 IU vit E, 2.2 mg $vitK_3$, 1.5 mg Thiamine, 4 mg Riboflavin, 8 mg Niacin, 35 mg; Pantothenic acid, 2.462 mg Pyridoxine, 0.504 mg Folacin, 0.01 mg $vitB_{12}$, 0.15 mg Biotin, 200 mg Choline-Cl, 1 mg B.H.T
[4] Washed and sterilized sand.

77.11=37.53 + 69.57 × Phosphorus
Phosphorus= (77.11 -35.53)/69.57
Phosphorus= 0.569

Method two (Calibration Curve-Basal Diet Phosphorus (CC-BD)): Phosphorus equivalency was calculated by setting the two regression equations equal according to following procedure as described by Zaghari et al. (2008):

$Y_{egg\ production}$= 37.53 + 69.57 × Phosphorus,

$Y_{egg\ production}$= 52.28 + 4395.27 × Enzyme,
37.53+69.57 Phosphorus=52.28 + 4395.27 × Enzyme
69.57 × Phosphorus=14.75 + 4395.27 × Enzyme
Phosphorus=0.21 + 63.17 × Enzyme
Phosphorus=0.21+63.17 (0.005)
Phosphorus=0.21 +0.315
Phosphorus=0.525 – 0.121(Phosphorus content of basal diet)
Phosphorus= 0.404

Method three (Negative Control-Positive Control (CNP)) as described by Zaghari et al. (2008): The third method is the product of the difference in Av. P content between the negative and positive controls, multiplied by the percentage of performance improvement of the phytase supplemented treatment compared to the positive control.

Results and discussion

Effects of different levels of Av. P and phytase on performance and egg quality are shown in Table 2. Dietary treatments had no significant effects on egg production and egg quality variables during weeks 70 to 73 ($P>0.05$). However, different levels of Av. P and phytase led to improvements in FCR at weeks 74 and 75 ($P<0.05$). At week 75, number of produced eggs, egg production rate, saleable egg percentage and FCR in treatments containing graded levels of Av. P were significantly different from the negative control (without NPP and phytase) ($P<0.05$). Layer hens fed with 500 FTU/kg added phytase recorded egg production percentage (EPP), saleable egg production percentage (SEP) and FCR equal to the birds fed the positive control (0.43% Av. P), as expressed previously by Shet et al. (2017). But such an effect was not seen in treatment fed the lowest doses of phytase (200 FTU/kg). The above results regarding the addition of phytase to the NPP deficient diet are consistent with the results obtained by Um and Paik (1999) and Shet et al. (2017) who reported that in very low phosphorus diets (approximately 0.12 % Av. P), higher dose of phytase (500 FTU/kg) could maintain laying performance without supplemental NPP, while lower doses (e.g., 250 FTU/kg) resulted in moderate improvement in performance.

On the other hand, the insignificant effects of phytase in P deficient diets during weeks 70 to 73 might be due to the releasing of Ca and P from medullary bone into blood stream (Whitehead and Fleming, 2000), which have decreased the efficacy of dephytinization. Fernandez et al. (2019) have stated at time lag the medullary bone resources compensate P and Ca requirements for egg production.

Table 3 shows the phosphorus equivalencies of phytase in layer hens at week 75 obtained from three different methods, i.e., after solving the regression equations with or without considering the P content of basal diet and by comparing phosphorus contents of the positive and negative controls. Phosphorus equivalencies in the second method were calculated by subtracting the amount of available phosphorus in the basal diet (i.e., 0.12%) from the obtained values. Eggshell thickness, FCR, total egg production, egg production percentage and total saleable egg percentage showed a greater relationship with their respective regression equations compared to egg quality variables. The variable most dependent on the P and phytase levels was FCR (R^2=0.53 and 0.67).

The amount of released P by phytase in 200 FTU/kg supplemented diet was lower than 500 FTU/kg. These findings are in accordance with Fernandez et al. (2019) and Vieira et al. (2015) who reported that phytase P releasing values went up with increasing the dosage of phytase.

Table 2. Effects of different levels of Av. P and phytase on laying hen performance and egg quality (week 75).

	Treatments									SEM	P-value
Av. P	0.12	0.43	0.35	0.27	0.20	0.12	0.12	0.12	0.12		
Phytase (FTU/kg)	-	-	-	-	-	200	300	400	500		
Variables											
Total Egg	20.29^{c}	41.20^{a}	36.80^{ab}	37.20^{ab}	32.20^{bc}	34.80^{b}	37.40^{ab}	35.20^{b}	41.20^{a}	1.80	0.0006
EPP[1]	52.14^{c}	77.65^{a}	65.71^{b}	66.42^{b}	63.49^{b}	62.14^{b}	66.78^{b}	64.74^{b}	77.11^{a}	3.385	0.0002
SEP [2]	47.50^{d}	74.13^{ab}	64.64^{bc}	63.57^{bc}	60.07^{c}	57.85^{c}	64.28^{bc}	62.24^{c}	75.61^{a}	3.581	0.0001
FCR	3.32^{a}	2.17^{cd}	2.59^{bc}	2.56^{bc}	2.80^{b}	2.63^{b}	2.54^{bc}	2.56^{bc}	2.13^{d}	0.134	<0.0001
Egg weight (g)	58.27	59.63	59.94	59.72	57.14	61.17	59.03	60.88	61.39	1.008	0.132
Yolk (%)[3]	28.27	29.73	28.14	28.52	28.09	27.76	28.47	28.80	28.32	0.0696	0.732
Egg shell thickness (mm)	0.344^{c}	0.366^{ab}	0.362^{abc}	0.362^{abc}	0.353^{bc}	0.372^{a}	0.352^{bc}	0.358^{abc}	0.366^{ab}	0.0057	0.045

^{a,b}Within rows, means with common superscript(s) do not differ (P>0.05).
[1]Egg production percentage
[2]Saleable egg production percentage
[3](Yolk weight/egg weight) ×100.

Using eggshell thickness as the response variable resulted in larger P equivalency value compared with other response variables in both CC and CC-BD methods, but not in CNP method. Estimated P equivalencies obtained from CC, CC-BD and CNP methods were slightly (about 10%) higher than the values reported by Simons and Versteegh (1992, 1993) and Waldroup (1999), which could be attributed to the differences in the method of determination and experimental assays (digestibility *vs* performance trials) (Dersjant-Li et al., 2019), adopting different response criteria (Adedokun et al., 2004), diet ingredients (Francesch et al., 2005), phytase type (Igbasan et al., 2000; Selle and Ravindran, 2000; Ribeiro et al., 2016), phosphorus source (Li et al., 2015), bird age (Bedford and Cowieson, 2020) and protein and energy effect of phytase (Ravindran et al., 1999; 2000; Nahm, 2002; Liu et al., 2009). The latter item needs more attention when interpreting the P equivalencies of phytase, because phytase may influence the performance independently of the phytate-bound P release (Wu et al., 2004); therefore, it probably results in over-estimation of P equivalency of a given phytase.

In the case of current study, it seems that supplementation of a P deficient barley-based layer hen diet with phytase, resulted in higher mean P absorption as stated by Francesch et al. (2005) compared with the studies that used a maize based diet. Moreover, there are evidences for the presence of a complementary effect between intrinsic phytase of barley and supplemental phytase (Zyla, 1993; Näsi et al., 1999).
Table 4 represents the effect of calculation method on phosphorus equivalencies of phytase at the level of 500 FTU/kg phytase. The CNP provided the lowest P equivalencies at all phytase levels (P<0.0001), indicative of underestimation of values obtained by the CNP method in layer hens. On the other hand, the values obtained by the CC method (without subtracting P content of basal diet) might be conflicting and may overestimates the P equivalency of phytase, because theoretically, it exceeded phytate phosphorus content of the basal diet (i.e., 0.202%). It may be concluded that CC method estimates total P release value in phytase-supplemented treatments, while part of the NPP supplied by the basal diet are assumed to have been released by phytase. Moreover, in both the CC and CNP methods, -

Table 3. Regression equations and estimated phosphorus equivalency values of phytase in layer hens at week 75.

	Egg shell thickness	FCR	Saleable egg percentage	Egg production percentage		
Equation	Y=0.29+0.13P	Y = 3.99 - 3.27P	Y=32.085+75.50P	Y=37.53+69.57P		
R^2	0.40	0.53	0.52	0.45		
P	0.0007	<0.0001	<0.0001	0.0002		
Equation	Y=0.33+7.45E	Y= 3.23 - 211.70E	Y=47.38+5041.21E	Y=52.28+4395.27E		
R^2	0.36	0.67	0.58	0.62		
P	0.0015	<0.0001	<0.0001	<0.0001		
Phytase (FTU/kg)		**P equivalence (Method CC)[1]**				
200	0.500	0.382	0.341	0.353		
300	0.500	0.452	0.426	0.420		
400	0.500	0.393	0.437	0.434		
500	0.568	0.553	0.568	0.569		
Phytase (FTU/kg)		**P equivalence (Method CC-BD)[2]**				
200	0.298	0.238	0.170	0.215		
300	0.356	0.303	0.240	0.187		
400	0.413	0.367	0.306	0.341		
500	0.470	0.422	0.372	0.404		
Phytase (FTU/kg)		**P equivalence (Method CNP)[3]**				
500[4]	0.293	0.313	0.314	0.308	**P-Value**	**SEM**
Average P equivalence[5]	0.433	0.359	0.352	0.480	0.3802	0.0354

[1]Calibration Curve: Calculated by solving obtained regression equations for P by Y=treatment means.
[2]Calibration Curve-Basal Diet phosphorus: Calculated by setting the regression equations for P equal with those created for phytase, followed by subtracting phosphorus content of basal diet.
[3] Negative Control-Positive Control: Difference in Av. P content between negative and positive controls multiplied by the percentage of performance improvement of the phytase supplemented treatment compared to the positive control.
[4]Only 500 FTU/kg phytase resulted in identical performance to the positive control (0.43% Av. P) for all response criteria.
[5]Average for all response criteria.

statistical influences of other doses of phytase were not considered where P equivalencies of a given dose were calculated. Therefore, it is not surprising that calculated values are not supported by the phytate content of the basal diet. Bedford and Cowieson (2020) stated that calculation of P equivalencies of phytase through CNP method, may not be as accurate as using the multiple calibration curves, because it strictly depends on the differences in the P content of the negative control and positive control and real P requirements. The mean P release value of 500 FTU/kg phytase was 0.230% in layers. Available phosphorus content of the layer hen basal diet was approximately 3.5 times lower than recommended P requirements at this age (0.121% *vs* 0.40 to 0.42%). Therefore, the slopes for egg production equation derived from NPP-supplemented treatments in the current study, were slightly higher (69.75 *vs* 67.6) than the slopes derived for data reported by Fernandez et al. (2019). Consequently, the slopes for egg production equation created for phytase-supplemented treatments will increase exponentially and seems that CC-BD method results in larger values, when equations are set equal to obtain P equivalencies of phytase. Therefore, the obtained values may not be representative of commercial status performed by the end user.

Zaghari (2009) showed that formulating diets, using the claimed nutrient equivalencies of a commercial enzyme, resulted in different responses in broiler chickens compared to layer hens. Overall, the results of the current study showed that there are some interfering factors, such as inclusion of the basal diet Av. P in the equation or disregarding it and the method of calculation, which result in significant differences between computed P equivalencies of a specific phytase. Recommendation of a single P equivalency for all strains and diet types is ambiguous for the end user to include the matrix value of enzyme claimed by the supplier in the diet formulation.

Conclusions

1- In layer hens, the minimum value of phosphorus equivalency for 500 FTU/kg phytase was recorded for the CNP method (0.308) whereas the CC method predicted the highest equivalency value (0.569).
2- Estimation of the method that considers and deducts the amount of phosphorus content in the basal diet (CC-BD) seemed more reliable than the CC method for practical feed formulation.
3- There was significant difference between the estimation methods and even between two subclasses of a major method of calculation (i.e.,

Table 4. Comparison of different methods for estimating the phosphorus equivalencies (contribution in the diet).

Method of calculation	Layer hens 500 FTU/kg
[1] CC	0.564[a]
[2] CC-BD	0.432[b]
[3]CNP	0.304[c]
SEM	0.01
P-value	<0.0001

[a,b]Within rows, means with common superscript(s) do not differ (P>0.05).
[1]Calibration Curve: Calculated by solving obtained regression equations for P by Y=treatment means.
[2]Calibration Curve-Basal Diet phosphorus: Calculated by setting the regression equations for P (treatments 1 to 3) equal with those created for phytase followed by subtracting the phosphorus content of the basal diet
[3]Positive Control-Negative Control: Calculated by comparing the phosphorus contents of positive and negative controls.

calibration curves of performance response) of P equivalencies of phytase.

4- Different traits had no significant influence on P equivalencies of phytase.

Conflict of interest

There is no conflict of interest to declare.

Ethics statement

All procedures including animal welfare, husbandry and experimental procedures were evaluated and approved by the Institutional Animal Care and Ethics Committee of the Iranian Council of Animal Care (Care ICoA 1995).

Data availability statement

The data that support the findings of this study are available from the corresponding author, upon reasonable request, subject to restrictions and conditions.

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