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Effect of Citric Acid, Phytase and Calcium Levels on the Calcium and Phosphorus Content in Egg: Yolk-Albumen and Shell, Yolk Color and Egg Quality in Diets of Laying Hens

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Abstract

To evaluate the effect of different levels of citric acid (CA) combined with phytase and calcium levels in the diets of laying hens on the calcium and phosphorus (P) contents of eggs: yolk-albumen and shell, yolk color and egg quality. A study was conducted with 24-week-old laying hens fed with a diet based on sorghum and soybean meal with 2.7 Mcal of EM/kg, 15% crude protein, 3.25% calcium and 0.25% available P. The levels of CA, phytase and calcium were 0%, 0.6% and 1.2%; 0 and 300 units of phytase (PHYU)/kg; and 3.0% and 3.25%, respectively. Phytase was added as an ingredient into to the diets, contributing 0.1% P and 0.3% calcium. A completely randomized factorial experiment was performed with a 3 × 2 × 2 design and 4 replications. The CA increased ($P < 0.05$) the P in the shell, and the combination of 1.2% CA, 300 PHYU phytase and 3.25% calcium increased ($P < 0.05$) the calcium in the eggshell. There was a CA × phytase × calcium interaction ($P < 0.05$) affecting egg yolk pigmentation; there were no differences in egg quality. It was concluded that adding CA to the diets of laying hens increases eggshell calcium content, and improves the phytase response. 1.2% CA × Phytase × 3.25% calcium significantly increases shell calcium by 0.56 g. Under the conditions of the present investigation, it would imply greater resistance of the eggs to be broken in the handling from the farm to the sales centers and consumer. Phytase interacts negatively with 0.6% CA at low calcium levels to degrade the yellow pigmentation of the yolk.

Keywords

Citric Acid, Phytase, Calcium, Laying Hens, Eggshell

1. Introduction

The phytic acid (myoinositol hexaphosphate) present in vegetable seeds reduces the availability of phosphorus (P) and, at a certain pH interferes with the dispensability of minerals [1], which are excreted into the environment. This, in turn, constitutes a global ecological problem.

Studies show that phytase improves P availability [2] [3] [4] [5] [6], but a determining factor in phytic acid hydrolysis is pH. The ability of phytic acid to chelate at alkaline pH is strong, and it forms insoluble salts with different minerals [1].

Studies show that citric acid (CA) reduces the chelation potential of phytic acid by binding to certain minerals, which results in lower excretion and greater availability of P, calcium and nitrogen [7]-[13].

However, high levels of calcium and a neutral pH allow calcium to bind to phytic acid and form calcium phytate salt, an insoluble compound, which makes calcium unavailable to laying hens and as well as inaccessible to phytase [14]. Although many factors influence the percentage of broken eggs, the nutrition is an important factor. The egg shell, composed of 94% of calcium carbonate, has an economic impact on the poultry industry; on average the percentage of broken eggs oscillates in 8% of the production, which represents great economic losses.

In a study of the effects of CA combined with phytase excretion and availability of calcium and P in the diets of molting laying hens, it is concluded that 2% CA increases the content of calcium in the shell and P in the yolk-albumen of the egg, and reduces N excretion; and 2% CA combined with 600 phytase units (PHYU) reduces P excretion and increases egg weight [9].

The above led to the proposed study with the following objectives: 1) evaluate the effect of CA on the calcium and P content of the egg including the yolk-albumen and shell, pigmentation of the yolk and the quality of the egg; 2) evaluate the effect of CA on the phytase response in diets of laying hens from the ages of 24 to 39 weeks; 3) evaluate the effects of the diets on egg calcium and P contents in the yolk-albumen, shell, pigmentation of the yolk and egg quality in laying hens aged 24 to 39 weeks.

2. Materials and Methods

The research was carried out at INIFAP's "Valle de México" experimental station in-Chapingo, Mexico. One hundred and forty-four Hy-line W98 24-week-old hens were housed in individual metal cages with automatic feed and water troughs. The hens were evaluated for 15 weeks and provided with 12 hours of natural light in addition to a sufficient duration of artificial light to attain 17 hours of total light during the peak laying period.

Diets were based on sorghum + soybean meal, with a 15% protein, 0.25% available phosphorus and 3.25% calcium and a total of 2.7 Mcal EM/kg (Table 1).

Diet components were analyzed and N was determined through Kjeldahl's method, P by spectrophotometry[1] and calcium by atomic absorption[2] [15].

Sixty grams/t of phytase was added to the diet, and phytase was considered as a diet's ingredient based on the dose to be used, 300 PHYU (phytase unit) and on its potential product, Natuphos 5000[3]. One phytase unit (PHYU) is defined as the amount of enzyme activity that liberates 1 mmol of inorganic P/minute from a 0.5 mM Na-phytate solution

Table 1. Composition of the basal diet fed to laying hens from 24 to 39 week of age*.

Ingredient	%
Sorghum	73.5700
Soy bean	14.5600
Calcium carbonate	9.1300
Orthophosphate	1.8000
Salt	0.3500
DL-Methionine	0.1500
Vitamin Premix†	0.1000
Mineral Premix‡	0.1000
Oil	0.1000
L-Lysine	0.1400
Pigment	0.0015
Calculated nutrient	
Crude protein	15.00
Metabolizable Energy (Mcal/Kg)	2.7
Lysine	0.690
Methionine + Cysteine	0.580
Threonine	0.470
Calcium	3.250
Nonphytate Phosphorus (AP)	0.250
Total Phosphorus (TP)	0.500

*The dietary treatments were: 0.0% CA, 0.0 PHYU, 3.25% Ca; 0.0% CA, 0.0 PHYU, 3.00% Ca; 0.0% AC, 300 PHYU, 3.00% Ca; 0.0% CA, 300.0 PHYU, 3.25% Ca; 0.6% CA, 0.0 PHYU, 3.00% Ca; 0.6% CA, 0.0 PHYU, 3.25% Ca; 0.6% CA, 300 PHYU, 3.00% Ca; 0.6% CA, 300 PHYU, 3.25% Ca; 1.2% CA, 0.0 PHYU, 3.00% Ca; 1.2% CA, 0.0 PHYU, 3.25% Ca; 1.2% CA, 300 PHYU, 300% Ca; and 1.2% CA, 300 PHYU, 3.25% Ca. Citric acid and phytase were added at the expense of sorghum. Phytase was added as a dietary ingredient to relevant treatments based on the dose of 300 PHYU and on its potential product, Natuphos 5000 (BASF, México, D.F). For diets containing phytase, the P and calcium levels were subtracted from the orthophosphate concentrations. Phytase leads to the release of approximately 0.1% P and 0.3% calcium (Parr, 1996). †Vitamin premix per kg of feed: A (retinol), 12,000 IU; D (cholecalciferol), 2400 IU; E (DL-alpha-tocopherol), 20 IU; K (menadione), 1.2 mg; thiamine, 1.6 mg; riboflavin, 8.0 mg; niacin, 32 mg; pyridoxine 3 mg; pantothenic acid, 11.2 mg; cyanocobalamin, 16 µg; folic acid, 1.6 mg; choline, 250 mg. ‡Mineral premix per kg of feed: Mn, 60 mg; Zn, 50 mg; Fe, 30 mg, Cu, 5 mg; I, 1.0 mg; Se, 0.1 mg.

[1]Genesys 10SUV-VIS, Madison, Wisconsin, USA.

[2]Varian Spectra Inc. AA 240 FS. Palo Alto, CA, USA.

[3]BASF, D.F, Mexico.

at pH 5.5 and 37.5˚C. P and Ca values freed through phytase's action were approximately 0.1% and 0.3% [16].

Food grade anhydrous CA was added to the experimental diets. The diet's pH was determined on a 20 g sample by means of a potentiometer at the start, midway point and final stage of the experiment.

2.1. Variables Measured

Egg quality in Haugh units (HU) was determined every 2 weeks. Albumen height was measured with a micrometer and estimated with the following equation [17]:

$$HU = 100 \log H$$

where $H = h + (7.685 - 1.7\ W^{0.37})$;

h = observed albumen height (mm), and W = egg weight.

The specific gravity was measured with a hydrometer every 2 weeks. The values in the solutions ranged from 1.068 to 1.100 with an increase of 0.004.

The color and brightness of the egg was measured twice with a reflectance colorimeter: once halfway through the experiment and once at the end.

P content in the yolk-albumen, eggshell, and diets was determined colorimetrically; calcium content was determined by atomic absorption; and N content was determined by the Kjeldahl method [15].

At 60 and 110 days, which were the first and second sampling days, respectively, 4 hens per treatment were selected to assess the calcium and P contents of the yolk-albumen and shell, and these samples were collected each day for 3 days. Subsequently, the shell and the yolk-albumen were separated and dried at 55˚C and the samples were incinerated for the corresponding analyses.

The ashes obtained from the samples were solubilized with 50% and 10% HC land evaporated to a volume not less than 10 ml, and the solution was transferred to 50-ml volumetric flasks for subsequent dilution. Molybdic acid and p-methylaminophenol sulfate, were used to determine the P content, and dilutions were made depending on the part of the egg to be analyzed. The samples were then read in a UV spectrophotometer at 660 nm. To determine calcium, lanthanum oxide was used, and the dilutions were made according to the part of the egg to be analyzed. Samples were then read by atomic absorption spectrophotometry at a wavelength of 425 nm.

2.2. Statistical Analyses

Treatments were randomly assigned to the experimental units according to a completely randomized factorial design with a 3 × 2 × 2 arrangement: 3 levels of CA (0%, 0.6% and 1.2%), 2 levels of phytase (0 and 300 PHYU) and 2 levels of calcium (3.0% and 3.25%) with 4 replicates per treatment and 3 hens per replicate. The means were compared using Tukey's test [18], and the data were analyzed using the program SAS [19].

3. Results and Discussion

There were no differences ($P > 0.05$) in productive performance: feed consumption,

feed conversion, egg weight and egg production (data not shown).

3.1. Phosphorus and Calcium Content in Eggs: Yolk-Albumen and Shell

There were no significant differences ($P > 0.05$) in the P and calcium contents of the yolk-albumen (Table 2). However, [9] in their study of molting laying hens, found an effect of CA and phytase on P and calcium, respectively, in yolk-albumen of egg; factors such as age and physiological state of the birds affect the response, in molting birds, calcium metabolism and its deposition in the egg increases [20]. Other important factors include the genetic line, diets, among others.

For P content in the shell, 1.2% CA × 300 PHYU × 3.25% calcium, 1.2% CA × 0 PHYU × 3.0% calcium and 0.6% CA × 300 PHYU × 3.0% calcium, increased the amount of P in the shell ($P < 0.05$) to 0.0067, 0.0067 and 0.0065 g respectively, in contrast with 0.0% CA × 300 PHYU × 3.0% calcium and 0.6% CA × 0 PHYU × 3.0% calcium with 0.0041 and 0.0040 g respectively (Table 3).

CA linearly increased the calcium content in the shell ($P < 0.05$) from 1.72 to 1.97, and 2.0 g for 0%, 0.6% and 1.2% respectively (Table 3). 1.2% CA × 300 PHYU × 3.25% calcium diets increased the eggshell calcium content ($P < 0.05$) to 2.25 g, in contrast with 0% CA × 0 PHYU × 3.0% and 3.25% calcium and the 0% CA × 300 PHYU × 3% calcium with 1.68, 1.69 and 1.72 g respectively (Table 3).

The pH of the diets with addition of CA decreased, from 5.4 in the control group to

Table 2. Phosphorus and calcium contents in the egg yolk-albumen (g/hen/day) in 24 to 39 week-old hens supplemented with citric acid, phytase and calcium*.

Variable	Sample			
	First		Second	
	P	Ca	P	Ca
CA × PHYU × Ca				
0.0 0.0 3.00	0.107	0.036	0.104	0.032
0.0 0.0 3.25	0.111	0.037	0.106	0.032
0.0 300 3.00	0.113	0.038	0.101	0.031
0.0 300 3.25	0.103	0.035	0.099	0.029
0.6 0.0 3.00	0.110	0.034	0.108	0.029
0.6 0.0 3.25	0.095	0.028	0.099	0.031
0.6 30 3.00	0.093	0.033	0.106	0.033
0.6 300 3.25	0.115	0.041	0.105	0.034
1.2 0.0 3.00	0.107	0.037	0.104	0.030
1.2 0.0 3.25	0.091	0.032	0.108	0.034
1.2 300 3.00	0.099	0.035	0.097	0.028
1.2 300 3.25	0.101	0.032	0.096	0.032
SE	0.02	0.003	0.004	0.003

*No interaction. CA = citric acid; Ca = calcium; P = phosphorus. SE = standard error.

Table 3. Phosphorus and calcium contents in the eggshell (g/hen/day) of 24 to 39 week-old laying hens supplemented with citric acid, phytase and calcium.

Variable	Sample			
	First		Second	
	P	Ca	P	Ca
Citric acid (%)				
0.0	0.0052	1.73[b]	0.0049[b]	1.72[b]
0.6	0.0048	1.92[ab]	0.0051[b]	1.97[a]
1.2	0.0050	2.03[a]	0.0061[a]	2.00[a]
Phytase (PHYU)				
0	0.0051	1.83	0.0051[b]	1.91
300	0.0049	1.94	0.0057[a]	1.90
Calcium (%)				
3.0	0.0051	1.94	0.0055	1.92
3.25	0.0049	1.84	0.0052	1.89
CA × PHYU × Ca				
0.0 0.0 3.00	0.0053	1.57	0.0054[abc]	1.68[b]
0.0 0.0 3.25	0.0050	1.63	0.0054[abc]	1.69[b]
0.0 300 3.00	0.0050	1.80	0.0041[bc]	1.72[b]
0.0 300 3.25	0.0054	1.82	0.0049[abc]	1.77[ab]
0.6 0.0 3.00	0.0050	1.79	0.0040[c]	2.05[ab]
0.6 0.0 3.25	0.0052	1.83	0.0044[abc]	1.91[ab]
0.6 300 3.00	0.0051	1.92	0.0065[a]	2.05[ab]
0.6 300 3.25	0.0041	2.12	0.0057[abc]	1.89[ab]
1.2 0.0 3.00	0.0056	1.96	0.0067[a]	2.19[ab]
1.2 0.0 3.25	0.0045	2.08	0.0044[abc]	1.81[ab]
1.2 3003.00	0.0047	1.91	0.0064[ab]	1.99[ab]
1.2 300 3.25	0.0051	2.17	0.0067[a]	2.25[a]
SE	0.0005	0.12	0.0005	0.11

[a,b,c]Averages with distinct letters within columns are significantly different ($p \leq 0.05$). CA = citric acid; Ca = calcium; P = phosphorus. SE = standard error.

4.7 and 4.3 with 0.6% and 1.2% CA, respectively, which most likely acidified the gastrointestinal tract as indicated by [21] [22]. For phytase [23] conclude that the addition of phytase increases the pH of the gastrointestinal tract, gizzard, duodenum, jejunum and ileum, which would affect the solubility of calcium, as calcium solubility depends on the chemical form of the calcium salts and the pH in the intestinal region [24]. Citric acid improved the phytase response, making calcium more available, with greater calcium deposition in the shell.

The addition of CA could promote calcium solubility by creating an acidic environment in the gastrointestinal tract that facilitates the absorption of calcium ions, thus

decreasing calcium excretion increasing its availability. This is supported by [13] in their study of laying hens as well as by the higher calcium content in the shell observed in the present study. Calcium is completely soluble under an acidic pH and does not bind to phytic acid [25].

On the other hand [26] increased calcium levels from 2.5% to 3.5% and 4.5% in the diets of laying eggs observed a significant improvement in shell quality; therefore, birds supplemented with higher levels of calcium retained more of this mineral for shell composition.

In terms of phytase, in a study with laying hens in the tropics, [27] indicate that phytase does not affect shell parameters, [3] [28] find that eggshell quality tends to decrease in hens fed phytase. In the current study, 0% CA, 300 PHYU and 3% calcium decreased eggshell calcium, possibly due to increased P availability, which interferes with calcium and thereby reduces the quality of the shell [29]. High blood serum P levels are associated with low-quality shells, which can be attributed to the inhibited precipitation of calcium ions in the serum due to the presence of P ions, and at high levels, the phosphate ion competes with the carbonate ion for calcium by inhibiting the formation and development of calcite crystals [30].

3.2. Egg Quality and Yolk Pigmentation

There were no differences in the specific gravity of the eggs ($P > 0.05$) (**Table 4**). For phytase a similar result was found by [31]. [28] report that the specific gravity of the eggs in birds fed phosphate-deficient diets supplemented with phytase is lower than that in a control group. In contrast, [32] indicate that the specific gravity of eggs was reduced in chickens fed P-deficient diets, and the combination of 0.1% available P with addition of phytase improved the specific gravity.

1.2% CA × 300PHYU × 3.25% calcium and 1.2% CA × 0PHYU × 3.0% calcium diet yielded the highest specific gravity values, without differences ($P > 0.05$). [33] conclude

Table 4. Yolk pigmentation and egg quality in 24- to 39-week-old laying hens supplemented with citric acid, phytase and calcium.

Treatments		Pigmentation				Egg quality			
		Brightness (L*)		Yellow (b*)		Haugh Units		Specific Gravity	
CA (%)	(PHYU/Kg)	Calcium (%)							
		3.00	3.25	3.00	3.25	3.00	3.25	3.00	3.25
0	0	61.7	62.3	40.8^{ab}	39.7^{ab}	100.5	100.5	1.084	1.085
	300	61.7	61.5	41.4^{a}	40.4^{ab}	97.4	99.1	1.085	1.085
0.6	0	62.0	61.7	41.8^{a}	39.8^{ab}	101.8	101.2	1.086	1.086
	300	61.2	61.0	36.6^{b}	40.8^{ab}	102.8	100.7	1.087	1.086
1.2	0	62.0	61.9	41.5^{a}	40.8^{ab}	100.1	104.6	1.088	1.087
	300	61.8	60.9	40.8^{ab}	40.7^{ab}	99.5	100.5	1.086	1.088
SE		0.51		0.95		1.77		0.002	

^{a,b}Averages with distinct letters between rows and columns are significantly different ($p < 0.05$). SE = standard error.

that organic acids and prebiotic, that lower pH in the diet and intestinal content, may have a positive effect on the quality of the shell. In contrast, [34] report that organic acids do not improve shell quality.

Haugh units were not influenced by levels of CA, phytase and/or calcium ($P > 0.05$) (**Table 4**), however [35] indicates that acidifying substances increase Haugh units and primarily act on the pH and the proportions of albumen calcium and magnesium and reports that few are the substances food-related factors.

Similar results for phytase were found by [28], who detected no difference in albumen quality. In contrast, [36] in a study of 22- to 35-week-old hens fed low levels of available P (0.20%) supplemented with phytase, found significant differences in the height of the albumen, which decreased with high levels of available P (0.40%) and phytase.

For the yellow pigmentation of the yolk, 1.2% CA × 0 PHYU × 3% calcium, 0.6% CA × 0 PHYU × 3% calcium and 0% CA × 300 PHYU × 3% calcium improved ($P < 0.05$) yellow pigmentation, with 41.5, 41.8 and 41.4 respectively, in contrast with 0.6% CA × 300 PHYU × 3% calcium with 36.6 (**Table 4**). Phytase negatively interacts with CA at low calcium levels and interferes with the yellow pigmentation of the yolk, associated with the possibly interacting effects that both compounds have on the availability of certain nutrients.

In animals that do not make good use of fat, the digestibility of carotenoid esters is low, so the efficiency of fat absorption in birds influences the rate of carotenoid deposition [37].

Research in this area involving CA is scarce, However [7] found that lipid metabolism is not modified by the addition of organic acids.

About, [38] report that extracting pigment from marigold flowers, *Tagetes erecta*, pre-treatment with a CA solution produces higher pigment yield and greater carotenoid retention. Moreover in some carotenoid-producing fungi, the addition of organic acids raises the content of β-carotene [39], the activity of the CA cycle and a high respiratory rate is associated with an increase in the production of compounds that improve the production of carotenoids [40].

On the other hand, there was a better response of yolk pigmentation to low levels of calcium, which is consistent with another researcher [41], who indicates that increasing the levels of calcium in the diet negatively affects the absorption of carotenoids.

4. Conclusions

The results of this research indicate that adding CA to the diets of laying hens during the first stage of production increases eggshell calcium content, and improves the phytase response. 1.2% CA × Phytase × 3.25% calcium significantly increases shell calcium by 0.56 g. Under the conditions of the present investigation, it would imply greater resistance of the eggs to be broken in the handling from the farm to the sales centers and consumer.

Phytase interacts negatively with 0.6% CA at low calcium levels to degrade the yellow pigmentation of the yolk.

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