



## Assessment of *In vitro* Solubility of Stone Grit- a Major Calcium Source in Poultry Feed

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### ABSTRACT

*In-vitro* solubility estimation experiments were conducted on 156 stone grit samples to analyze the major factors influencing solubility and their strength of correlation by using five different strengths of hydrochloric acid (HCl at 0.1N, 0.2N, 0.3 N, 0.5 N, and 1N), three different volumes of HCl (200mL, 100mL and 50mL), two different reaction duration (10 minutes and 30 minutes), two different particle sizes (2mm and 4mm) and two different sample weights (1g and 2g) based on the weight loss method. Solubility data analyzed in multiple linear regression model revealed that the variables such as stone grit size (X1), strength of HCl (X2), volume of HCl (X3), and reaction duration (X4) were statistically significant ( $P < 0.01$ ) and the sample weight (X5) was statistically not significant. The strength of HCl, volume of HCl, and duration were positively correlated, whereas the stone grit size was negatively correlated with solubility. The regression equation ( $Y = -21.993 - 3.888 X_1 + 99.607 X_2 + 0.123 X_3 + 1.83 X_4 + 2.295 X_5 + \mu$ ) was derived to predict the solubility values for different particle sizes of stone grit easily with known sample weight, HCl volume and strength, and reaction duration. In order to obtain the accurate solubility values of stone grit, the estimation procedure was modified in the existing weight loss method by taking 2 grams of sample to react in 100mL of 0.2N HCl for 10 minutes duration. This modified procedure was ideal and suggested for analyzing the solubility for various particle sizes of stone grits available in the market.

**KEYWORDS:** Calcium, *In vitro* solubility, Particle size, Poultry feed, Stone grit.

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### INTRODUCTION

Stone grit is primarily used in the poultry industry for its calcium content (38%). It is a viable alternative to familiar calcium sources such as shell grit and calcite because of its low cost and easy availability. Stone grit is obtained from sedimentary rocks composed of different crystal forms of calcium carbonate. It is resistant to heat, pressure, wear, and impact due to its hardness, porosity, and specific heat capacity, whereas it is soluble in hydrochloric acid (HCl) secreted in the bird's stomach when ingested. The *in vivo* solubility depends upon various factors like particle size, pH strength of HCl, duration of retention time in the gizzard, and the inclusion level of calcium in the feed.

The solubility of calcium sources *in vitro* is highly correlated with solubility *in vivo* (reverse relationship) and calcium bioavailability (Rao and

Roland, 1989). Since the available calcium is responsible for the egg shell quality, skeletal maintenance, and overall health performance in poultry, there is a strong justification for selecting the ideal calcium source to perfectly match the calcium requirements based on *in vitro* calcium solubility. Kim et al. (2018) observed that feeding birds with pulverized limestone resulted in an increase in pH of the gizzard due to the more rapid solubilization of calcium carbonate when limestone particles are finely ground. Finer particles have a larger surface area, which accelerates the dissolution process in the acidic environment of the gizzard. They also concluded that the apparent ileal digestibility of calcium was dependent on the particle size of limestone. So, assessing the solubility of stone grit of various particle sizes before its use in poultry feed is a proactive step to ensure the calcium availability

and utilization by the birds. Kim et al (2019) concluded that in addition to particle size, distribution and origin of limestone physical properties of the rock, geological origin, and its chemical composition influenced *in vitro* solubility and thereby the *in vivo* calcium digestibility. Previous studies (Scott et al., 1991; Cheng and Coon, 1990; Zhang and Coon, 1997 and Kim et al., 2019) described the different modified methods for estimating the *in vitro* solubility of limestone. As the solubility mainly depends on the origin and chemical composition of the rocks (Kim et al., 2019), the finding of ideal method for estimating the solubility of stone grits available in different sizes in the market will benefit the poultry nutritionists as well as farmers for feed formulation. Hence, the present study is undertaken in stone grit to determine the significance and correlation of various factors influencing *in vitro* solubility and to find out the prediction equation to estimate the *in vitro* solubility instantly and to suggest the modified procedure for estimation of *in vitro* solubility for stone grit with the ideal combination of the major influencing factors.

## MATERIALS AND METHODS

The stone grit samples collected from various traders around Namakkal, Tamil Nadu were analyzed for chemical composition, particularly calcium, manganese, and acid-insoluble ash by adopting AOAC (2023) laboratory procedures. The size was sorted out into 2 millimetre (mm) and 4mm sizes by using the specific standard sieves and the uniformity of size was ensured throughout the experiment period.

The solubility experiments on stone grit were conducted by using 5 different normality (N) strengths of HCl (0.1N, 0.2N, 0.3 N, 0.5 N, and 1N), 3 different volumes of HCl (200ml, 100ml and 50ml), 2 different reaction timings (10 minutes and 30 minutes), 2 different particle sizes of stone grit (2mm and 4mm) and 2 different sample weights (1g and 2g). Freshly prepared solutions of different HCl concentrations by diluting the concentrated HCl with deionized distilled water were used in this experiment.

Stone grit sample of about 1g /2g was weighed in an electronic balance with an accuracy of 0.001g was taken into a 400mL Erlenmeyer flask. The flask was placed into a 42°C shaking water bath for 10 minutes and 200mL/100mL/50mL of pre-warmed (42°C) 0.1N/ 0.2N/ 0.3 N/ 0.5 N/ 1N HCl solution was added into the flask that was shaken vigorously to maximize mixing without losing solution for 10 minutes/30 minutes. Then the digestion was stopped by adding 100 mL of chilled deionized distilled water into the flask and immediately pouring all contents through a pre-weighed and Whatman No.41 filter paper. Additional ice-cold distilled water was added to flush any remaining pieces of limestone in the Erlenmeyer flask. The filter was placed in a pre-weighed, glass petridish and then dried at 100°C for a minimum of 8 h. A total of 156 samples were estimated for their solubility by the weight loss method as described by Zhang and Coon (1997) and calculated in the formula given below.

Solubility of stone grit (%) =  $1 - \frac{\text{dried remaining stone grit}}{\text{dry initial stone grit}} \times 100$

## Statistical Analysis

### Multiple linear regression model

To assess the factors influencing the solubility of stone grit, a multiple linear regression model was fitted. The functional form was as below:

$$Y = a + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \mu$$

Where,

Dependant / Independent Variables

Y Solubility of stone grit

X1 Stone grit size in mm

X2 Strength of HCl in N

X3 The volume of HCl in mL

X4 Reaction Duration in minutes

X5 The weight of the sample in g

A Intercept

$\beta_i$  Regression coefficients to be estimated

$\mu$  Error term

### Univariate analysis of variance (ANOVA)

The chemical composition of different particle sizes of stone grit samples and the mean solubility percent values across different particle sizes, sample weights, HCl strengths and volumes, and reaction time were analyzed by univariate ANOVA for its significance across different dependent variables.

### RESULTS AND DISCUSSION

The chemical composition of various stone grit samples of different sizes is given in Table 1. The present study revealed that the calcium and magnesium content did not differ significantly ( $P>0.05$ ) with the particle sizes and the average mean values were 38.69% and 0.91% respectively, whereas the acid insoluble ash content differed significantly ( $P<0.05$ ) among the various sizes of stone grit. Kim et al. (2019) estimated the 3 limestones originating from various locations had

differences in calcium concentrations (37.22, 35.03, and 37.40%), whereas the magnesium content was highest in LIME-3 (1.19%). Inoti et al. (2020) collected limestone samples from two different sources of Kenya (Ukunda in the Kenya South coast (UKC) and Athi River (AR) near Nairobi and estimated the acid-insoluble ash as 3.39% and 23.17% respectively and Ca content in UKC limestone was 420 g/kg while in AR it was 340 g/kg. The high acid insoluble ash in AR limestone caused lower *in vitro* solubility  $29.43 \pm 0.23\%$  as compared to UKC limestone ( $63.04 \pm 0.34\%$ ). The magnesium content below 1% in all sizes of stone grit in this study indicates that the stone grit can be used as a feed supplement without any adverse effects. The observation of an increase in acid-insoluble ash with smaller particle sizes may be due to the presence of sand and silica segregated from the surface of grit chips as fine powder during the milling process and may affect the *in vitro* solubility

Table.1 Chemical composition of various stone grit samples

S.No	Size of stone grit	Acid- insoluble ash%	Calcium%	Magnesium%
1	2mm	0.51 <sup>b</sup> ±0.087	38.7± 0.07	0.92±.010
2	4mm	0.29 <sup>a</sup> ±0.04	38.7±0.04	0.89±0.01
3	6mm	0.28 <sup>a</sup> ± 0.05	38.7±0.03	0.92±0.017
	OVERALL MEAN	0.36± 0.04	38.7±0.03	0.913±.008
	F VALUE	3.9*	0.01 <sup>NS</sup>	1.52 <sup>NS</sup>

Values in each row having a common superscript do not differ significantly. \*  $P<0.05$ , NS – Not significant

The results of the multiple linear regression model fitted to find out the factors influencing the solubility of stone grit are presented in Table 2. The model showed a good fit with the adjusted  $R^2$  of 0.729, indicating that 72.9% of the variation in the dependent variable was explained by the independent variables used in fitting the model. The ANOVA also showed that the model was statistically significant with an 'F' value of 84.291 ( $P<0.01$ ). Among the five variables chosen for model definition, the variables, viz., stone grit size, strength of HCl, volume of HCl, and duration were found to have statistically significant ( $P<0.01$ ) correlation with the solubility. Among these variables, strength of HCl, volume of HCl, and duration of reaction were found to be

positively correlated with solubility. The variable, stone size was found to be negatively correlated with solubility. The variable, the weight of the sample was found to be statistically not significant ( $P>0.05$ ).

On perusal of the standardized coefficient, it was found that the strength of HCl was the most significant factor influencing the solubility of stone grit followed by the duration of reaction, the volume of HCl, and stone size in descending order. The regression equation derived to predict the solubility of stone grit is of the following form.

$$Y = -21.993 - 3.888 X_1 + 99.607 X_2 + 0.123 X_3 + 1.83 X_4 + 2.295 X_5 + \mu$$

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Table 2. Multiple linear regression model for interpreting the factors influencing *in-vitro* solubility of stone grit

Variables	Unstandardized Coefficients		Standardized Coefficients	t-value	P value	Significance
	B	Std. Error	Beta			
Constant	-21.993	9.838		-2.236	0.027	*
Stone size (mm)	-3.888	1.431	-0.124	-2.717	0.007	**
Strength of HCL (N)	99.607	6.296	0.798	15.821	0.000	**
Volume of HCL (ml)	0.123	0.028	0.233	4.433	0.000	**
Duration in minutes	1.830	0.171	0.600	10.687	0.000	**
Weight of the sample (g)	2.295	3.501	0.037	0.656	0.513	NS
N				156		
R <sup>2</sup>				0.738		
Adjusted R <sup>2</sup>				0.729		
F – Value				84.291**		

\*- P<0.05, \*\* - P<0.01, NS- Not significant

**Factors influencing the *in vitro* solubility of stone grit**

**1. Particle size (X<sub>1</sub>)**

The mean values of solubility of stone grit of different sizes in two different HCl strengths are tabulated in Table 3. The mean solubility values of 2mm and 4mm stone grit in this study (Table 3) indicated a significant difference (P<0.05) between particle size and solubility values. Since the particle size of stone grit determines the retention time in the gizzard, the larger the particle size, the lesser the *in vitro* solubility and the more retention time in the gizzard. The smaller the particle size, the greater the *in vitro* solubility but the lesser retention time in the gizzard. The negative correlation (R<sup>2</sup>= -0.124) of particle size with *in-vitro* solubility obtained in this study (Table 2) coinciding with the findings of Cheng and Coon (1990), Zhang and Coon (1997), and Ajakaiye et al. (1996).

Cheng and Roland (1990) reported that there was a significant difference in calcium retention in layers when fed with two calcium sources of the same

particle size with different *in vitro* solubility and confirmed that the retention of calcium is more related to *in vitro* solubility than the particle size. Sinclair-Black et al. (2019) also recently observed that a coarse limestone with having 1.5 mm Gross Mean Diameter (GMD), and 30% solubility at 30 min increased blood ionizable calcium concentration in laying hens overnight when more calcium is needed for eggshell formation, in comparison to a fine limestone (0.2 mm GMD 86% solubility at 30 min). On the other hand, Kim et al. (2019) found lesser solubility in the limestone sample having a GMD of 0.326mm than the limestone sample of a different source having a GMD of 0.633mm in 5 minutes reaction time with 0.2N HCl. This might be due to the difference in the geological origin, physical properties, and intrinsic characteristics of the rocks from which limestone is made. Gilani et al. (2022) found that there was a weak negative correlation between mean particle size and *in vitro* solubility for fine limestone after 5, 15, or 30 min of incubation with HCl buffered to pH=3 (R<sup>2</sup>=0.52, 0.3, and 0.09 respectively). However, for the coarse limestone,

there was no correlation between particle size and solubility at any of the incubation time points (30, 90, or 150 min) Similarly, Ajakaiye et al. (1996) revealed that particle size did not affect the solubility of the most soluble sources, namely calcium carbonate and marble dust. But there was an increase in solubility of the bivalve, periwinkle eggshell, and oyster shells, when particle size was reduced, whereas the solubility of snail meal was not improved even after finer grinding.

Reducing the particle size will result in increased solubility, but the retention time in the gizzard is poor, moreover, the calcium ions will form complexes with phytate or other minerals and become unavailable, and the calcium absorption capacity of birds is unable to match with the rapid release of calcium ions into the intestines and get excreted finally. The slow and consistent release of calcium is needed in case of layers for optimum eggshell formation, the prolonged exposure time to large particles of calcium supplements to HCl acid in the gizzard, due to their slower transit time, resulted in an increased dissociation of  $\text{CaCO}_3$  into bio-available  $\text{Ca}^{2+}$  that could be utilized by the bird for eggshell calcification as well as replenishment of medullary bone reserves. The large particles accumulating in the

gizzard during the day would dissolve slowly throughout the night, thereby maintaining a higher blood calcium level than would occur in hens which received high calcium levels only during the daytime hours.

Recommendations regarding the ideal limestone particle size for commercial laying hens were under continued investigation and ranged generally between 1.40 and 5.60 mm, depending on the production status and age of the laying hens. The minimum particle size of 1mm would supply adequate calcium ions in later stages of production. (De Witt et al., 2009). In broilers, particle size between 137 $\mu\text{m}$  and 388  $\mu\text{m}$  improved the FCR and weight gain in the first 28 days and particle size less than 28  $\mu\text{m}$  with more than 70% *in-vitro* solubility caused the phosphorous deficiency by limiting the phytate phosphorous hydrolysis. Budgayci et al. (2022) used limestone grit having a size of 2.041 mm as ad-libitum in the feeding of 5-week-old Japanese quail layers in their experiment and found no significant difference in egg production. Egg mass, egg quality, and egg yolk cholesterol. The ideal particle size of stone grit can be selected based on the age, stage of production, and genotype of the birds in addition to the *in vitro* solubility values.

Table 3. Mean ( $\pm$ S.E.) of solubility (%) of stone grit at different particle sizes

S.No	Particle size	Mean solubility (%)	Number of samples	F value
1	2mm	55.02 <sup>a</sup> $\pm$ 0.31	96	5.96*
2	4mm	51.02 <sup>b</sup> $\pm$ 0.51	60	

Values in each row having a common superscript do not differ significantly, \*  $P < 0.05$

## 2. Strength of HCl ( $X_2$ )

The mean solubility values of 2mm and 4mm stone grit at different strengths of HCl are given in Table 4. In the present study, the solubility was increased linearly with a significant increase ( $P < 0.01$ ) in the normality strength of HCl from 0.1N to 0.5N as

indicated in Table 4. The strength of the acid determines the speed of solubility. The amount of  $\text{H}^+$  ions will be increased with increasing the normality of HCl and more  $\text{H}^+$  ions are required for the chemical reaction of calcium carbonate with HCl for faster solubility.

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Table 4. Mean ( $\pm$ S.E.) of solubility (%) of stone grit at different strengths of HCl

S.No	Particle size	Strength of HCl	Mean solubility (%)	Number of samples	F value
1	2mm	0.1N	24.0 <sup>a</sup> $\pm$ 0.62	18	472.56**
2	2mm	0.2N	41.9 <sup>b</sup> $\pm$ 0.74	30	
3	2mm	0.3N	53.3 <sup>c</sup> $\pm$ 1.48	18	
4	2mm	0.5N	87.7 <sup>d</sup> $\pm$ 0.22	30	
		Total		96	
5	4mm	0.1N	14.4 <sup>a</sup> $\pm$ 0.21	18	1467**
6	4mm	0.2N	28.3 <sup>b</sup> $\pm$ 0.17	6	
7	4mm	0.3N	56.5 <sup>c</sup> $\pm$ 0.14	6	
8	4mm	0.5N	82.4 <sup>c</sup> $\pm$ 0.57	18	
9	4mm	1N	67.4 <sup>d</sup> $\pm$ 1.61	12	
		Total		60	

Values in each row having a common superscript do not differ significantly. \*\* - $P < 0.01$

The positive correlation between the strength of HCl and solubility obtained in this study ( $R^2 = 0.79$  as per Table 2) is consistent with the findings of Zhang and Coon (1997) who estimated the solubility of limestone at 0.1, 0.2, and 0.3N HCl. Kim et al. (2018) found that the pH value of gizzard ranges from 1.94 to 2.36 based on the calcium inclusion level in the diet, whereas the pH value of 0.2 N HCl solution is about 0.78 and provides sufficient  $H^+$  ions to neutralize the  $Ca^{2+}$  ions. The more physiological pH buffering at 3 to maintain a stable pH will simulate the gizzard pH and improve the correlation between *in vitro* and *in vivo* calcium solubility. In another study, Kim et al. (2019) performed solubility experiments in pulverized limestone with a more acidic condition (0.2N HCl) and a pH similar to the gizzard condition (pH=3 buffered HCl) and noticed a similar solubility range in both the solutions at 1 and 30 minutes duration and significantly higher ( $P < 0.05$ ) solubility in pH= 3 buffered HCl solution at 5, 15 minutes. On perusal of the standardized coefficient (Table 1), it could be inferred that the strength of HCl was the most significant factor ( $P < 0.01$ ) influencing the solubility of stone grit. The solubility range values for different strengths of HCl are given in Table 5. The ideal strength of HCl in this study was 0.2N because it provided a wider solubility range (24.9-87.07%) for stone grit samples (Table 5) and greater solubility separation between different stone grit samples when compared to other strengths of HCl.

Table 5. Range of solubility % at different strengths of HCl

Strength of HCl	Range of solubility %	Range type
0.1N	7.81-39.5	Moderate
0.2N	24.9 - 87.07	Wide
0.3N	43.9-91.3	Moderate
0.5N	68.4-96.3	Moderate
1N	48.7-89.3	Moderate

### 3. Volume of HCl in mL ( $X_3$ )

The mean solubility values of stone grit experimented with different volumes of HCl are given in Table 6. The volume of HCl was found to significantly ( $P < 0.01$ ) influence the solubility of stone grit of both 2 mm and 4 mm sizes (Table 6). The volume of HCl contributes to the quantity of  $H^+$  ions needed for the neutralization of the  $Ca^{2+}$  ions during the solubility reaction. Tion et al. (2005) modified the method of Roland and Rabon (1985) by taking one gram of sample with 100mL 12N HCl for 30 minutes. They repeated the same solubility experiments with a 0.5g sample with 50 ml of 12N HCl and a 0.25g sample with 25 mL of 12N HCl and found similar values of solubility. This indicates that the volume of HCl could be reduced if the weight of the sample was reduced proportionately.

The positive correlation ( $R^2=0.23$ ) of the volume of HCl with solubility (Table 2) is consistent with the findings of Zhang and Coon (1997) who did solubility experiments in oyster shell and limestone with different particle sizes with 100 mL and 200 mL of 0.2N HCl for 10 minutes reaction time and inferred that the solubility values range concerning with the volume is wider when the particle size is increased. Since the speed of solubility of stone grit

depends upon the surface area available for HCl reaction, the volume of HCl did not significantly influence the solubility of the fine form of calcium source when compared with the coarse form. In this study, the solubility values of 2mm and 4mm stone grit in 100mL volume were more or less the same, which inferred that 100 mL was the ideal volume that provided sufficient  $H^+$  ions for optimum solubility.

Table 6. Mean ( $\pm$ S.E.) of solubility (%) of stone grit at different volumes of HCl

S.No	Particle size	Volume of HCl	Mean solubility (%)	Number of samples	F value
1	2mm	200mL	55.8 <sup>b</sup> $\pm$ 1.16	24	160.94**
2	2mm	100mL	63.3 <sup>a</sup> $\pm$ 1.05	48	
3	2mm	50mL	26.3 <sup>c</sup> $\pm$ 1.47	24	
		Total		96	
5	4mm	200ml	85.7 <sup>a</sup> $\pm$ 1.16	24	227.24**
6	4mm	100ml	64.6 <sup>b</sup> $\pm$ 1.13	18	
7	4mm	50ml	31.5 <sup>c</sup> $\pm$ 1.13	18	
		Total		60	

Values in each row having a common superscript do not differ significantly. \*\* - $P < 0.01$

#### 4. Reaction Duration ( $X_4$ )

The mean solubility values of 2mm and 4mm stone grits experimented with two different incubation times are given in Table 7. The results inferred that the time duration of the reaction significantly ( $P < 0.01$ ) influenced the solubility of both 2mm and 4mm stone grit. The duration of the reaction of HCl with stone grit was positively correlated ( $R^2=0.6$ ) with solubility values (Table 2). This result was coherent with the study of Gilani et al. (2022) who conducted a global survey on limestone and measured the solubility of limestone of different origins as per the method suggested by Kim et al. (2019) with three incubation time points at 5, 15 and 30 min for the fine limestone and at 30, 90 and 150 min for the coarse limestone. The mean in vitro solubility observed in coarse limestone by Gilani et al. (2022) was less than 70%

after 30 min, less than 95% after 60 min, and more than 95% after 150 minutes, whereas in fine limestone, it was 68.4%, 80% and 90% at 5, 15, 30 min incubation. Kim et al. (2019) calculated the solubility in pulverized limestone as 68% and 96% at 1 and 30 minutes of reaction with 0.2N HCl and 51% and 97% at 1 and 30 minutes of reaction with buffered HCl pH=3. This indicates that *in-vitro* solubility values can be increased by increasing the incubation duration which is similar to the *in vivo* condition that more retention time in the gizzard will lead to more solubility as described by Rao et al. (1992). The time duration of 10 minutes was considered ideal for segregating the more soluble from less soluble stone grits since the finer grits will be soluble completely before the 30 minutes reaction duration.

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Table 7. Mean ( $\pm$ S.E.) of solubility (%) of stone grit at different reaction durations

S.No	Particle size	Duration of reaction	Mean solubility (%)	Number of samples	F value
1	2mm	10min	34.7 <sup>b</sup> $\pm$ 0.46	42	61.61**
2	2mm	30min	70.8 <sup>a</sup> $\pm$ 0.50	54	
Total				96	
5	4mm	10min	45.0 <sup>b</sup> $\pm$ 0.62	42	323.55**
6	4mm	30min	65.05 <sup>a</sup> $\pm$ 1.97	18	
Total				60	

Values in each row having a common superscript do not differ significantly. \*\* -P<0.01

### 5. Weight of the sample ( $X_s$ )

The mean solubility of 2mm and 4mm sized stone grit at two different sample weights (1g and 2g) are given in Table 8. The above results revealed that the solubility of both 2mm and 4mm stone grit are significantly ( $P<0.01$ ) influenced by the weight of the sample. As the sample weight increases, the solubility values decrease, but the difference in the solubility values between two sample weights is greater in 2mm and lesser in 4mm stone grit. This might be due to the lesser surface area available for HCl reaction in large-sized stone grit.

Since the solubility is measured based on the weight loss of stone grit before and after the reaction, the initial weight of the sample taken will alter the solubility values. Cheng and Coon (1990) experimented with 2g of limestone allowed to react with 100mL of 0.1M HCl for 15 min in a 42°C water

bath in weight loss method (WLM) and 2g of limestone allowed to react with 98 mL of 0.75 M acid for 10 min in pH change method (PHM). Scott (1991) modified the WLM method by decreasing the sample quantity from 2g to 0.5g to alleviate the problem of H<sup>+</sup> ions deficiency observed in the WLM method, But the reduction of the sample weight will lead to an inaccurate measurement in case of large-sized particle samples in SWLM. Zhang and Coon (1997) suggested a newly developed method by taking 2g of sample reacting with 200mL of 0.2N HCl for 15 minutes to get a wider solubility range (20.96-78.15%) when compared with WLM, PHM, and SWLM. This study also revealed that the ideal weight of the sample could be taken as 2 grams to ensure more accuracy in the representation of large-sized particles and also to get the optimum range of solubility values.

Table 8. Mean ( $\pm$ S.E.) of solubility (%) of stone grit at two different sample weights

S.No	Particle size	Sample weight	Mean solubility (%)	Number of samples	F value
1	2mm	1g	64.3 <sup>a</sup> $\pm$ 1.50	42	144.96**
2	2mm	2g	32.2 <sup>b</sup> $\pm$ 1.42	54	
Total				96	
5	4mm	1g	76.6 <sup>a</sup> $\pm$ 0.99	24	668.89**
6	4mm	2g	45.2 <sup>b</sup> $\pm$ 0.69	36	
Total				60	

Values in each row having a common superscript do not differ significantly. \*\* -P<0.01

## CONCLUSION

Based on the strength of correlation of different factors determining the *in vitro* solubility of stone grit samples, the regression equation was derived and it can serve as a practical tool for predicting the solubility of stone grit for different particle sizes instantly without performing the time consuming laboratory experiments and to explore further research in applications of poultry nutrition. In order to obtain the accurate solubility values of stone grit, the estimation procedure was modified in the existing weight loss method by taking 2 grams of sample to react in 100mL of 0.2N HCl for 10 minutes duration. This improved method is considered as ideal and suitable for stone grit because, it provides wide range of solubility values for different particle sizes. This study revealed the valuable insights in optimizing the inclusion of suitable particle size of stone grit in the poultry diet based on *in vitro* solubility values. The ideal particle size of stone grit can be selected based on the age, stage of production, and genotype of the birds in addition to the *in vitro* solubility values.

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## REFERENCES

Ajakaiye, A., Atteh, J.O and Leeson, S. 1997. Effects of calcium source, particle size and time on *in-vitro* calcium solubility of some indigenous Nigerian mineral ingredients for poultry diets. *Animal Feed Science Technology*. 65: 293-298.

- AOAC. 2023. Official Methods of Analysis, 22nd Edn. Association of Official Analytical Chemists. Washington, D.C, USA.
- Cheng, T. K., and Coon, C.N. 1990. Comparison of various *in vitro* methods for the determination of limestone solubility. *Poultry Science*. 69:2204-2208.
- De Witt F.H., Kuleile, N.P., Van Der Merwe, H.J., and Fair, M.D. 2009. Effect of limestone particle size on egg production and eggshell quality of hens during late production. *South African Journal of Animal Science*. 39: 37–40.
- Gilani, S., Mereu, A., Li, W., Plumstead, P.W., Angel, R., Wilks., G and Dersjant-Li, Y. 2022. Global survey of limestone used in poultry diets: calcium content, particle size and solubility. *Journal of Applied Animal Nutrition*. 10 (1): 19-30.
- Inoti, D.K., Mbugua, P.N., Gachui, C.K., and Maina, J.G. 2020. Physical and Chemical Characteristics of Limestone for use in Layer Feeds in Kenya. *Indian Journal of Animal Nutrition*. 37 (3): 242-246.
- Kim, S.W., Li, W., Angel, R., and Proszkowiec-Weglarz, M. 2018. Effects of limestone particle size and dietary Ca concentration on apparent P and Ca digestibility in the presence or absence of phytase. *Poultry Science*. 97: 4306-4314.
- Kim, S.W., Li, W., Angel, R and Plumstead, P.W. 2019. Modification of a limestone solubility method and potential to correlate with *in vivo* limestone calcium digestibility. *Poultry Science*. 98: 6837-6848.
- Rao, K.S, and Roland, D.A. 1989. Influence of dietary calcium level and particle size of calcium source on *in vivo* calcium solubilization by commercial Leghorns. *Poultry Science*. 68: 1499-1505.
- Rao, K.S., Roland, D.A., Adams, J.L and Durboraw, W.M. 1992. Improved Limestone Retention in the Gizzard of Commercial Leghorn Hens. *Journal of Applied Poultry Research*. 1: 6-10.

- Scott, M. 1991. How can calcium be supplied to high-producing hens? *Feedstuffs*. 63 (39): 16-18.
- Sinclair-Black, M., Angel, R., Li, W. and Plumstead, P. 2019. The effect of limestone particle size, phytase inclusion, and time post oviposition on ionized blood calcium levels in commercial laying hens. *Proceedings of the 22nd European Symposium on Poultry Nutrition*. June 10-13, 2019.
- Zhang, B., and Coon, C.N. 1997. Improved in vitro methods for determining limestone and oyster shell solubility. *Journal of Applied Poultry Research*. 6: 94–99.