Comparison of sample preparation methods and chemical assays for determination of titanium, phosphorus and calcium in broiler diets and digesta

Samat, N.[1]* and H. Miller[2']
[1]Livestock Science Research Centre, MARDI, Persiaran MARDI-UPM, 43400 Serdang, Selangor, Malaysia.
[2]School of Biology, University of Leeds, Leeds LS2 9JT, United Kingdom
*E-mail: nsamat@mardi.gov.my

Abstract

In understanding the effectiveness of phytase in improving nutrient digestibility, digesta samples from each segment of the gastrointestinal tract (GIT) need to be analysed for several different nutrients which require a large amount of dry matter. This study aimed to develop a typical sample preparation that could minimize the amount of digesta samples required and enable sequential chemical analyses. The specific objectives were to compare the content of titanium (Ti), phosphorus (P) and calcium (Ca) in diet and digesta samples after being separately prepared using different acid digestion and analysed by different chemical methods. In this study, gizzard and terminal ileal digesta were collected from 20 d old Ross 308 male broilers fed with wheat/corn diets either with or without supplemental phytase at 1500 FTU/kg. Diets and digesta samples were digested in hydrochloric acid (HCl), and sulphuric acid (H_2SO_4) and the contents of Ti, P and Ca were determined by colourimetry and ICP-OES methods. The present study showed that H_2SO_4 could be used to replace HCl in sample digestion before mineral analysis. Furthermore, digestion of diet and digesta using H_2SO_4 enabled sequential analysis of Ti, P and other minerals.

Keywords: phosphorus, calcium, titanium, ileal nutrient digestibity

Introduction

Efficacy of supplemental phytase on performance and nutrient digestibility in broilers fed wheat-based diet is well documented (Cabahug *et al.*, 1999; Svihus *et al*., 2013; Dessimoni *et al*., 2019). Most studies on nutrient digestibility concerning phytate degradation have focused on the ileal digesta (Ravindran *et al*., 2000, Rutherfurd *et al*., 2002, Leytem *et al*., 2008, Walk and Olukosi, 2019). In further understanding, the effectiveness of supplemental phytase in elevating the adverse influence of wheat phytate on nutrient digestibility, digesta samples from each segment of the GIT were collected and analysed for several different nutrients (Rutherfurd *et al*., 2002, Walk *et al*., 2012, Zeller *et al*., 2015). The analyses may include proximate analysis, amino acids, polysaccharides, minerals, dietary markers, phytate and inositol-6-phosphate esters. Most of the analyses require separate sample preparation and a large amount of sample which is the main constraint concerning the amount of digesta obtained from each segment of GIT. Having a common sample preparation for digesta samples that later could be used in several analyses minimise the sample required.

Morgan *et al*. (2014) have successfully shown that an inductively coupled plasma optical emission spectrophotometer (ICP-OES) assay can replace the UV-spectroscopy assay for rapid analysis of TiO_2 in broiler feed

and ileal digesta samples and incorporate the measurement of TiO_2 into the analysis of other minerals. The ICP-OES assay is more sensitive at quantitative analysis with improved detection limits, less time-consuming and enables simultaneous measurement of several elements. However, this rapid and efficient technique required high technology and specialized instrument and UV-spectroscopy assays probably be the most doable methods for determining Ti (Short *et al.*,1996), P and Ca (AOAC, 2000) in a basic chemistry laboratory. Therefore, in order to have a common sample preparation for diet and digesta samples prior to Ti, P and Ca assays, this study was conducted to determine the concentration of Ti, P and Ca of diet and digesta samples from two different acid digestions (HCl versus H_2SO_4) using colourimetric methods. The use of H_2SO_4in sample digestion for P and Ca assays was also evaluated using ICP-OES assay as the reference method to replace HCl in sample digestion and enable simultaneous Ti measurement. It was hypothesised that P and Ca's content following either HCl or H_2SO_4 digestion would not differ from each other. Similarly, the content of P, Ca and Ti in H_2SO_4as determined by colourimetry and ICP-OES methods would be similar.

Material and methods

All experimental procedures were complied with The Animals (Scientific Procedures) ACT 1986, under Animal Ethical Review Committee of University of Leeds.

Experimental diets and husbandry

Ross 308 male broilers fed on wheat/corn diets either with or without supplemental phytase at 1500 FTU/kg were used in this study. The diet was formulated to meet the specification for Ross 308 Broiler (Aviagen, 2007) except with lower content of available P (0.35%) and Ca (0.86%) and contained 0.5% titanium dioxide (TiO_2). The crumble diets were given *ad libitum* to chickens from day old to 23 d, randomly allocated in 16 pens with 5 chicks in a pen for each diet. Initial temperature (32 ± 2°C) of the chicken shed was gradually reduced to 21°C on d 23. Continuous lighting with the light intensity of 40 lux was applied at the start of the trial, followed with 18 h light and 6 h dark (18L:6D) lighting program on d 4 and light intensity reduction to 10 lux after d 7. Water was available all the time. On d 20, 3 birds were randomly selected, individually weighed, killed by intravenous injections of pentobarbital sodium followed with cervical dislocation. The digesta samples were collected from the gizzard and terminal ileum. Terminal ileum was defined as the segment between a distal two-third of ileum away from Meckel's diverticulum to about 2 cm from ileocaecal junction. The samples were stored at -20°C prior to further processing and analysis.

Experimental design and chemical analysis

The overall flow of work is shown in Figure 1. Part 1 of this study, 2 different acid digestions were done prior to the determination of Ti, P, and Ca in all diets and ileal digesta samples. The sample preparation methods were; (1) HCl digestion according to AOAC Official Method 965.17 for determination of P in animal feed and pet food and (2) H_2SO_4 digestion as in method by Short *et al.* (1996) for determination of TiO_2 in chicken digesta, both with slight modification. Briefly, for HCl digestion, 0.1 g of sample was ashed at 600°C overnight and cooled before being digested with 10 ml of 5M HCl at boiling point for 30 min. For H_2SO_4 digestion, 0.1 g sample was ashed at 600°C overnight and cooled before being digested with 10 ml of 7.4M H_2SO_4 at boiling point for 1 h. After

cooling, the digested sample solutions were poured through Whatman No.541 filter paper into 100 ml volumetric flask, diluted to 100 ml and analysed for Ti, P and Ca following ICP-OES method of AOAC Official Method 985.01 (AOAC, 2000).

For Part 2, in order to compare 2 colourimetric methods for determining Ti content in H_2SO_4 digestion samples, digested samples from Part 1 were analysed by AOAC Official Method 973.36 Titanium in cheese, whereas a separate set of samples were prepared and analysed following as the method of Short *et al*. (1996). For Part 3, digested samples from Part 1 were analysed for P according to the method of AOAC Official Method 965.17 and Ca according to the method of AOAC Official Method 927.02 and Attin *et al*. (2005) with slight modifications.

Molybdovanadate reagent wasprepared to dissolve 25 g ammonium molybdate and 1.25 g ammonium vanadate separately using ultrapure water. Molybdate solution was gradually added into vanadate solution with stirring on a hot plate, and the mixture was then diluted to 500 ml. Prior to the analysis, 20 ml molybdovanadate reagent and 20 ml 5 M HCl were added to 120 ml ultrapure water and mixed well. Forty µl of digested samples were pipetted into 96-wells flat-bottom microplates before adding 160 µl of diluted molybdovanadate solution. A pale-yellow colour was allowed to develop for 10 min before being measured by UV spectrophotometer at the wavelength of 405 nm.

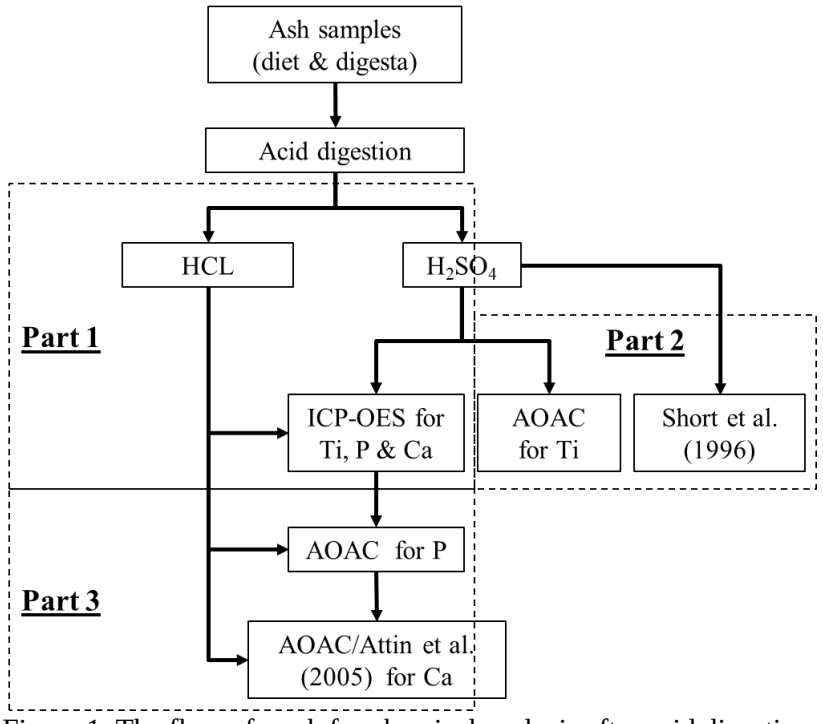

Figure 1. The flow of work for chemical analysis after acid digestion.

For calcium contents analysis, Arsenazo III reagent was prepared by separately dissolving 19.4 mg of Arsenazo III and 3.4 g Imidazole with ultrapure water. Arsenazo solution was added to Imizadole buffer solution with stirring, and the pH of the

mixture was adjusted to 6.5 with 3.0 M NaOH or 3.0 HCl dropwise before being diluted to 500 ml. Arsenazo III buffered reagent should contain 0.05 mM Arsenazo III and 0.1 M Imidazole buffer. The content of Ca was determined by mixing each sample with Arsenazo III reagent at the ratio of 1 to 50. Four µl of digested samples were pipetted into 96-wells flat-bottom microplates before adding 200 µl of Arsenazo III buffered reagent. A bluish-purple colour was allowed to develop for 10 min before being measured by UV spectrophotometer at the wavelength of 650 nm.

All the data were analysed using Minitab 17 Statistical Software (Minitab Inc, 2014). Diet and digesta content of Ti, P and Ca were first subjected to normality test and the non-normal data were transformed by the best estimate λ value prior to the analysis of variance (ANOVA) using general linear model (GLM). Significant differences between means were identified at $P \leq 0.05$ by Multiple Comparison of Means Tukey's Method. The Pearson's correlation coefficients of Ti and P contents from chemical assays following two digestion methods were also calculated before a fitted regression line was plotted to illustrate the relationship between 2 sets of data resulting from the chemical assays.

Results and Discussion

Effect of different acid digestion on Ti, P and Ca contents

There were no significant differences observed between P content of the diets measured by ICP-OES assay following HCl or H_2SO_4 digestion (Table 1). Different acid digestion significantly affected ($P<0.05$) ileal P content and Ca and Ti content in both diet and ileal digesta. Contents of P and Ca were lower in HCl compared to H_2SO_4 and the differences in P and Ca content between the two acid digestions were larger in digesta compared to those in diets. This is probably due to different boiling times (Morgan *et al*., 2014) and the high concentration of TiO_2 in digesta samples. Based on our observation, the greyish-white opaque solution was produced when acid was added to ash containing TiO_2 and H_2SO_4 solution became clear after 60 min of boiling, indicating TiO_2 was completely dissolved. For digesta sample with a higher concentration of TiO_2, boiling time was about 90 min before the acid solution became clear, while HCl solution remained cloudy after boiling for 60 min. Very low Ti was detected in HCl digested samples which were unexpected and unexplainable since Ti is insoluble in HCl (FAO JECFA Monographs 13, 2012).

Table 1. Total phosphorus (P), calcium (Ca) and titanium (Ti) in diets and ileal digesta of broilers as prepared by different acid digestion methods.

Acid digestion	Diet			Ileal digesta		
	P (%)	Ca (%)	Ti (%)	P (%)	Ca (%)	Ti (%)
HCl	0.63±0.03	0.83±0.04^{b}	0.03±0.04^{b}	0.76±0.05^{b}	1.43±0.12^{b}	1.10±0.14^{b}
H_2SO_4	0.68±0.01	0.94±0.04^{a}	2.98±0.15^{a}	0.98±0.02^{a}	1.90±0.07^{a}	10.78±0.38^{a}
SEM	0.02	0.03	0.35	0.04	0.09	1.27
P-value	0.158	0.023	<0.001	<0.001	<0.001	<0.001

^{a,b}Means ± SE with a different letter within a column were significantly different, $P< 0.05$

Effect of different colourimetric assays on Ti contents

In the present study, Ti's content in H_2SO_4 digested diet samples by ICP-OES method was lower than the expected value, i.e., 0.5%, therefore the samples were analysed further using colourimetric methods. The content of Ti measured according to the method of Short *et al*. (1996) and AOAC official Method 973.36 Ti in cheese are presented in Table 2. The content of Ti in diets and ileal digesta were significantly affected by different Ti assays. Contents of Ti in diets measured by AOAC method were higher than those measured by Shorts *et al.* (1996), but both measured values were close to the amount of Ti added to the diets. Ileal Ti measured by AOAC method was higher than those measured by Short *et al*. (1996) method. It was also observed that the difference in Ti contents between the two methods was larger in ileal digesta compared in diets (Table 2).

Table 2. Titanium (Ti) in diets, gizzard and ileal digesta of broilers as determined by Short et al. (1996) and AOAC Method 973.36 (Ti in cheese)

Ti assays	Diet (%)	Gizzard digesta (%)	Ileal digesta (%)
Short *et al*. (1996)	0.46±0.01[b]	0.44±0.02	1.61±0.02[b]
AOAC 973.36	0.50±0.02[a]	0.49±0.03	1.80±0.04[a]
SEM	0.02	0.02	0.03
P-value	<0.001	<0.001	<0.001

[a,b]Means ± SE with a different letter within a column were significantly different, $P < 0.05$

Effect of different assays on P and Ca contents

There was no significant effect of different assays on P content in H_2SO_4 digested samples (Table 3). On the other hand, the concentration of P in samples digested with HCl was significantly lower ($P < 0.05$) when measured by the molybdovanadate method compared to the ICP-OES method. H_2SO_4 digestion resulted in more consistent results in both assays of P than HCl digestion, particularly for ileal digesta. These results suggested that either molybdovanate method or ICP-OES method used in this study was equally efficient in determining P content in diets and digesta of broilers hydrolysed by H_2SO_4.

Table 3. Phosphorus (P) in diets, gizzard and ileal digesta of broilers as determined by methods of molybdovanadate[1] and ICP-OES[2].

Sample type	P assays	P (%)	
		H_2SO_4 hydrolysis	HCl hydrolysis
Diet	Molybdovanadate	0.58±0.13^{ab}	0.54±0.03^{b}
Diet	ICP-OES	0.65±0.03^{ab}	0.62±0.04^{ab}
Gizzard digesta	Molybdovanadate	0.45±0.03^{b}	0.28±0.05^{c}
Gizzard digesta	ICP-OES	0.34±0.02^{c}	0.27±0.02^{c}
			To be continued…
Ileal digesta	Molybdovanadate	0.80±0.06^{a}	0.56±0.03^{b}
Ileal digesta	ICP-OES	0.83±0.06^{a}	0.78±0.06^{a}
Main effects			
P assays			
Molybdovanadate		0.58±0.05	0.44±0.03^{b}
ICP-OES		0.55±0.06	0.51±0.05^{a}
Sample type			
Diet		0.61±0.05^{a}	0.58±0.03^{a}
Gizzard digesta		0.39±0.02^{b}	0.28±0.03^{b}
Ileal digesta		0.82±0.04^{a}	0.66±0.05^{a}
P-value			
P assays		0.447	0.032
Sample type		<0.001	<0.001
P assays x sample type		0.015	0.086

^{a,b,c} Means ± SE with a different letter within a column were significantly different, $P< 0.05$;
[1]AOAC Official Method 965.17 Phosphorus in Animal Feed and Pet Food,
[2]AOAC Official Method 985.01 Metals and other elements in plants and pet foods (ICP-OES).

The advantages and disadvantages of the ICP-OES method over the colourimetric method were reported by Morgan *et al*. (2014). In the present study, colourimetric methods were shown to be as efficient as ICP-OES in analysing Ti and P in H_2SO_4 digested samples, although the ICP-OES method is unquestionably more sensitive and capable of analysing multi-elements simultaneously. Besides P, colourimetric methods could be used for determining other minerals, for example, Arsenazo III method (AOAC Official Method 927.02, Attin *et al*., 2005) could be used in analysing Ca content in H_2SO_4 digested samples. In the present study, Ca content in digested samples prepared in Part 1 was determined by Arsenazo III method. The content of Ca in HCl digested samples was highly correlated with those determined by ICP-OES method (Figure 2).

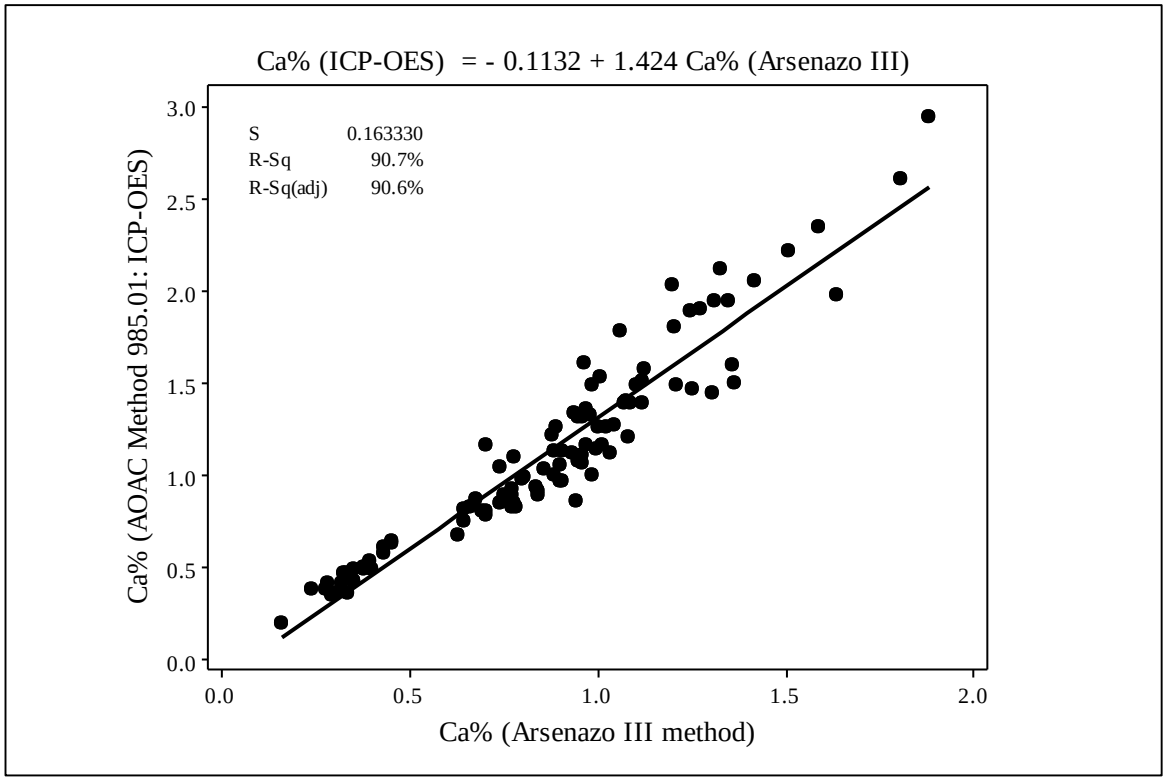

Figure 2. Relationship between Ca content of diet and digesta as determined by Arsenazo III method and AOAC Official Method 985.01 (ICP-OES). HCl digestion (Pearson correlation = 0.952; P-value <0.001).

Conversely, Arsenazo III method was not successful in analysing Ca content in H_2SO_4 digested samples although H_2SO_4 solution itself did not interfere with the analysis as various levels of Ca in H_2SO_4 solution were effectively determined by Arsenazo III for the construction of Ca standard curve (data not shown). The Ti concentration could be one of the interfering factors for Ca determination by Arsenazo III in H_2SO_4 digested sample as TiO_2 was highly dissolved in H_2SO_4 compared to in HCl (Table 2) and this warrants further investigation.

In common practice, samples are prepared separately for different analyses. For example P content in a diet containing TiO_2 is analysed using the molybdovanadate method that involves HCl digestion, but TiO_2 does not fully dissolve in HCl. Therefore, Ti content is determined separately by the method of Short *et al*. (1996). Consequently, no sample will be left for any other analysis as the total volume of the sample prepared is used in this method. On the other hand, it was reported that sequential analysis of several nutrients could minimize the unavoidable samples losses when the analyses were performed in a single container instead of using several containers (de Coca-Sinova *et al*., 2011). Based on the findings of the study, therefore, the sequential analysis is proposed for determining DM, total ash, Ti and other minerals for diet and digesta. Briefly, a 20 ml borosilicate glass vial is used to determine DM and ash content of a sample and followed by acid digestion. The vial's total content is filtered through a filter paper into a 100 ml volumetric flask and after diluted to

100 ml with ultra-pure water, the sample is ready for analysis of minerals using either colourimetric or ICP-OES methods. The filtered residue can be further ashed for AIA determination if required.

Conclusion

In conclusion, it is suggested that H_2SO_4 could be used in sample digestion prior to P and Ca analysis. Digestion of diet and digesta using H_2SO_4 enabled sequential analysis of Ti, P and other minerals.

References

Attin, T., Becker, K., Hannig, C., Buchalla, W. and Hilgers, R. 2005. Method to detect minimal amounts of calcium dissolved in acidic solutions. Caries Research. 39(5), pp.432-436.

Aviagen. 2007. Ross 308 Broiler: Nutrition Specification. [Online]. [Accessed 9 February 2012]. Available from: http://en.aviagen.com/ross-308/

Cabahug, S., Ravindran, V., Selle, P. H. and Bryden, W. L. 1999. Response of broiler chickens to microbial phytase as influenced by dietary phytic acid and non-phytate phosphorus levels. I. Effects on bird performance and toe ash content. *British Poultry Science*. 40, pp.660-666.

De Coca-Sinova, A., Mateos, G. G., González-Alvarado, J. M., Centeno, C., García, R. L., & Moreno, E. J. (2011). Comparative study of two analytical procedures for the determination of acid insoluble ash for evaluation of nutrient retention in broilers. Spanish journal of agricultural research, (3), 761-768.

Dessimoni, G. V., Sakomura, N. K., Donato, D. C. Z., Goldflus, F., Ferreira, N. T., & Dalólio, F. S. 2019. Effect of supplementation with Escherichia coli phytase for broilers on performance, nutrient digestibility, minerals in the tibia and diet cost. Semina: Ciências Agrárias, 40(2), 767-780.

Joint FAO/WHO Expert Committee on Food Additives. 2013. Compendium of food additive specifications. FAO JECFA monographs 13. [Online]. Accessed 1 July 2015. Available from: http://www.fao.org/food/food-safety-quality/ scientific-advice/jecfa/

Leytem, A. B., Widyaratne, G. P. and Thacker, P. A. 2008. Phosphorus utilization and characterization of ileal digesta and excreta from broiler chickens fed diets varying in cereal grain, phosphorus level, and phytase addition. Poultry Science. 87(12), pp.2466-2476.

Minitab 17 Statistical Software 2014. [Computer software]. State College, PA: Minitab, Inc. www.minitab.com.

Morgan, N. K., Scholey, D. V. and Burton, E. J. 2014. A comparison of two methods for determining titanium dioxide marker content in broiler digestibility studies. Animal. 8(04), pp.529-533.Ravindran *et al*., 2000,

Official Methods of Analysis of AOAC INTERNATIONAL .2000. 17th Ed., *AOAC INTERNATIONAL*, Gaithersburg, MD, USA, Official Method 985.01.

Official Methods of Analysis of AOAC INTERNATIONAL .2000. 17th Ed., *AOAC INTERNATIONAL*, Gaithersburg, MD, USA, Official Method 965.17.

Official Methods of Analysis of AOAC INTERNATIONAL .2000. 17th Ed., *AOAC INTERNATIONAL*, Gaithersburg, MD, USA, Official Method 927.02.

Official Methods of Analysis of AOAC INTERNATIONAL .2000. 17th Ed., *AOAC INTERNATIONAL*, Gaithersburg, MD, USA, Official Method 973.36.

Rutherfurd, S. M., Chung, T. K. and Moughan, P. J. 2002. The effect of microbial phytase on ileal phosphorus and amino acid digestibility in the broiler chicken. British Poultry Science. 43(4), pp.598-606.

Short, F. J., Gorton, P., Wiseman, J. and Boorman, K. N. 1996. Determination of titanium dioxide added as an inert marker in chicken digestibility studies. *Animal Feed Science and Technology*. 59(4), pp.215-221.

Svihus, B., Lund, V. B., Borjgen, B., Bedford, M. R. and Bakken, M. 2013. Effect of intermittent feeding, structural components and phytase on performance and behaviour of broiler chickens. *British Poultry Science*. 54(2), pp.222-230.

Walk, C. L., & Olukosi, O. A. (2019). Influence of graded concentrations of phytase in high-phytate diets on growth performance, apparent ileal amino acid digestibility, and phytate concentration in broilers from hatch to 28 D post-hatch. *Poultry Science*, *98*(9), 3884-3893.

Walk, C. L., Bedford, M. R. and Mcelroy, A. P. 2012. Influence of limestone and phytase on broiler performance, gastrointestinal pH, and apparent ileal nutrient digestibility. Poultry Science. 91(6), pp.1371-1378.

Zeller, E., Schollenberger, M., Kühn, I. and M. Rodehutscord. 2015. Hydrolysis of phytate and formation of inositol phosphate isomers without or with supplemented phytases in different segments of the digestive tract of broilers. *Journal of Nutritional Science.* 4, pp.e1.