Use of Zn concentration in the gastrointestinal tract as a measure of phytate susceptibility to the effect of phytase supplementation in broilers

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ABSTRACT

Zinc (Zn) is the most vulnerable cation to complexation with phytate. An experiment was conducted to evaluate the potential of measurements of Zn concentration in the gastrointestinal tract as a marker to assess the anti-nutritional impact of phytate and susceptibility of phytate to phytase in broilers. Ross 308 broilers (n = 180) were fed one of 5 experimental diets with differing phytase activity levels, analyzed at 605, 1150, 1804, 3954 and 5925 U/kg. Broiler performance and Zn concentration, pH and amount of phytate hydrolyzed in the gizzard, duodenum and ileum were analyzed at d21 post hatch. Phytate susceptibility to phytase degradation was determined *in vivo* and *in vitro* by measuring total phytate-P hydrolyzed in the tract or in conditions that mimicked the tract, respectively. Phytase activity level had a significant ($P < 0.05$) impact on Zn concentration and phytate hydrolyzed in the gizzard and ileum, but not in the duodenum. Strong relationships were observed between the amount of phytate hydrolyzed and Zn concentration in the gizzard in birds fed the diets with 1804 U/kg or higher levels of phytase. Phytate and phytase effects could therefore potentially be evaluated by measuring Zn concentration in the gizzard. Susceptible phytate levels measured *in vivo* and *in vitro* were almost identical in the diet with phytase activity of 5925 U/kg, but in the diets with lower phytase activity levels the *in vitro* assay overestimated the amount of P released. There were strong relationships between *in vivo* susceptible phytate level and pH and amount of phytate hydrolyzed in the gizzard, duodenum and ileum and Zn concentration in the gizzard and ileum. This illustrates that phytate susceptibility directly effects mineral availability in the gastrointestinal tract. Measurements of Zn concentration in the gastrointestinal tract, particularly in the gizzard, can potentially be used as a marker to assess the anti-nutritional impact of phytate and susceptibility of phytate to phytase in broilers.

Key words: broiler, zinc, phytate, phytase

INTRODUCTION

The current commonly used methods for measuring phytate concentration and phytase activity in poultry diets and digesta are colorimetric, whereby the color formation between molybdate and released inorganic orthophosphate is measured. There is however large variation and poor reproducibility using these methods. This is because free or water-soluble P results in high background and hinders degradation of phytate, the absorbance readings are often unstable as the background color of the blank is strong (and thus the color contribution by phytase hydrolysis is reduced) and the samples do not mix very well as the presence of fat and carbohydrate causes an oil layer to form (Kim, 2005). High performance liquid chromatography (HPLC) is also widely used for phytate analysis (Cowieson et al., 2006), but each analysis requires expensive and time-consuming anion-exchange purification. Alternative methods for measuring phytate concentration include using measurements of turbidity as an indicator of phytate complex hydrolysis, based on the principle that stable protein-phytate complexes form turbid solutions (Tran et al. 2011), heating samples with a standardized amount of $FeCl_3$ and measuring the amount of precipitated ferric phytate (Wheeler and Ferrel, 1971), electrophoresis (Blatny et al. 1995), paper chromatography (Agostinho et al. 2016) and nuclear magnetic resonance (Leytem et al. 2008). The flaws with these methods are that they are non-specific to phytate and require a large volume of sample. The most sensitive method for phytate analysis is the use of metal dye detection reagents (Mayr, 1988) but the extreme sensitivity of this method makes it complex and expensive. Alternative methods for measuring phytate and phytase activity in poultry diets and digesta which are sensitive, low cost and require small sample size are therefore required.

This study explores the hypothesis that it may be advantageous to measure the impact of phytate and phytase on mineral availability and the gastrointestinal environment as opposed to measuring phytate and phytase directly. Zinc (Zn) is the most vulnerable of the essential

cations to complexation with phytate (Park et al., 2013). Zn deficiency in broilers occurs only when high concentrations of phytate are present (Kornegay, 2001; Oberleas and Harland, 1996); phytate binds to the positively charged Zn resulting in the formation of insoluble Zn-protein-phytate complexes (especially in the small intestine) that cannot be absorbed in the digestive tract (Schlegel et al., 2010). This suggests that the degree of Zn deficiency, due to the vulnerability of Zn to phytate, could be used as an indicator of the chelating capacity and hence anti-nutritional impact of phytate. The presence of calcium aggravates the effect of phytate on Zn utilization (Linares et al. 2007), which may be because it increases pH and insoluble calcium-phytate-Zn complexes form which prevent Zn absorption.

Measuring the total phytate-P concentration of a diet following phytase addition may be misleading because the total amount of phytate in the diet does not necessarily represent the quantity of phosphorus available for hydrolysis. This is because insoluble phytate-mineral precipitates and soluble mineral–phytate complexes may be resistant to phytase hydrolysis (Maenz et al., 1999). Measurements of the susceptibility of phytate to phytase may give a better indication of the impact of phytate and phytase and should potentially be taken into account when formulating diets with phytase. Phytate susceptibility is determined *in vitro* by exposing samples to conditions that mimic the bird gastrointestinal tract and then measuring phytate-P released. This is then assumed to be the proportion of total phytate that is susceptible to phytase degradation. In this study, the relationship between *in vitro* and *in vivo* phytate susceptibility and Zn is investigated to examine if measurements of gastrointestinal Zn levels could be used as a tool to indicate phytate susceptibility to phytase. This was carried out by observing if there was a direct relationship between level of Zn and level of susceptible phytate; in the presence of phytase the higher the level of Zn suggests the more phytate has been degraded as more Zn has been released from phytate, and hence the more susceptible the phytate is to the effects of phytase. This would be advantageous as enables predictions and assessments of phytase

success and extent of the impact of phytate in different ingredients and combinations to be carried in a relatively cheap and simple way.

Dose response curves for the effects of phytase on Zn utilisation are not as clearly understood as they are for Ca and P and there is lack of understanding and consistency amongst studies about the extent to which phytase improves bioavailability and digestibility of Zn. Some studies have found that phytase improves Zn digestibility and retention. For example, Yi et al. (1996) found that approximately 0.9 mg of Zn was released per 100 U of phytase over the range of 150 to 600 U of phytase. Also, Thiel and Weigand (1992) found that the addition of 800 U/kg phytase to a diet containing 27mg Zn/kg significantly increased Zn retention and reduced Zn excretion in chicks. Thiel et al. (1993) reported that Zn content of chick femurs was similar between birds fed diets containing 30mg Zn/kg with 700U/kg phytase and those fed diets containing 39mg Zn/kg without supplemental phytase. Biehl et al. (1995) found that phytase and 1,25-dihydroxycholecalciferol supplementation in chicks increased growth rate and tibia Zn to the same degree. Some studies however have found that there is a negative relationship between Zn and phytase, for example Augspurger et al. (2004) reported that 800mg/kg of Zn supplementation in chick diets reduced the phosphorus-releasing efficacy of phytase, resulting in decreased tibia ash (14%) in the presence of phytase. This study illustrates a potent inhibitory effect of $Zn^{2}+$ on phytate hydrolysis by phytase. Furthermore, Roberson and Edwards (1994) did not observe consistent improvements in Zn absorption when broilers were fed 600-750U/kg phytase in diets containing 32mg Zn/kg. When dietary Zn level is greater than the requirement for growth, plasma and tibia concentration, at approximately 45mg/kg (Mohanna and Nys, 1999), it is excreted, which has negative implications both economically and environmentally, as excess Zn can cause phytotoxicity in soil. The findings from these studies suggest a lot more investigation is required into the relationship between phytate and phytase and Zn in poultry.

Physiological conditions and transit time in the digestive tract contribute towards Zn solubility and availability in the digestive tract. Phytate-mineral complexes are soluble at low pH (< 3.5) and become less soluble as pH increases along the gastrointestinal tract, forming insoluble complexes at the pH of the small intestine (pH 5-7). The shift of pH from the gizzard to the duodenum causes phytate to readily seek cations to complex and precipitate with (Kornegay, 2001). When the rate of Zn secretion into the duodenum via the pancreas exceeds the rate at which dietary Zn is consumed, Zn deficiency occurs in the presence of high quantities of phytate (Yu et al., 2010). This suggests that measurements of phytate and phytase effects in the duodenum, as opposed to other sections of the gastrointestinal tract, may give the best indication of the extent of the anti-nutritional impact of phytate on Zn bioavailability. This paper investigates whether this is true, or if it is more advantageous to determine the relationship between Zn and phytate in the gizzard or ileum.

The aim of this study was to evaluate whether measurements of Zn concentration in the gastrointestinal tract can be used as a marker to assess the anti-nutritional effect of phytate and phytate susceptibility to the effects of phytase in broilers. An additional objective was to establish where in the gastrointestinal tract measures of phytate-Zn reactions give the best indication of the effects of phytate and phytase.

MATERIALS AND METHODS

Birds and Husbandry

Ross 308, male broilers (n = 180) from a 43-week-old breeder flock were obtained from a commercial hatchery at day of hatch. Chicks were randomized by weight and placed in 0.64 m^2 floor pens in groups of 4, bedded on clean wood shavings. Pen allocation was randomized across the room. On arrival birds were individually weighed and allocated to a pen. Three birds per pen (n = 135) were sampled in the study, with one spare bird per pen. Total pen weight and mean chick body weight (**BW**) were calculated, and diet allocation was arranged to ensure

there was no significant difference in BW by pen across diets. Birds were allowed ad libitum access to the treatment diets and water for the duration of the trial. The room was thermostatically controlled to produce an initial temperature of 32°C on d1 and reduced in steps of 0·5°C per d, reaching 21°C by day 14. The lighting regimen used was 24 hours light on d1, with darkness increasing by 1 hour a day until 6 hours of darkness was reached, which was maintained throughout the remainder of the study. All birds sampled were euthanized by cervical dislocation on d21 post-hatch. This occurred after at least 6 hours of light, to ensure maximal gut fill. Total pen weight and feed intake (**FI**) was determined on d21 post-hatch and was used to calculate feed conversion ratio (**FCR**). Mortality was recorded daily, and any birds culled or dead were weighed. Institutional and national guidelines for the care and use of animals were followed and all experimental procedures involving animals were approved by the University's College of Science ethical review committee.

Dietary Treatments

Diets were formulated to produce total phytase levels of approximately 400, 700, 2000, 4000 and 6000 U/kg. This study was conducted primarily to examine a novel variety of high phytase wheat (*Triticum aestivum L.*) and has been reported elsewhere (Brinch-Pedersen et al., 2012). The control diet, with a predicted phytase activity of 400 FTU/kg, had no supplemental phytase added (Table 1). The diet with a predicted phytase activity of 700 FTU/kg was the control diet with added supplemental phytase (Quantum Blue^{TM}, AB Vista Feed Ingredients). The diets formulated to have predicted phytase levels of 2000, 4000 and 6000 U/kg were the control diet with a proportion of the wheat replaced with wheat that had higher intrinsic phytase content. A P-adequate control diet was used in this study so direct comparisons could be made between the endogenous phytase activity provided by the novel wheat compared to standard wheat. The conventional wheat had a measured phytase level of 1060 U/kg and the higher

phytase wheat had a measured phytase level of 6196 U/kg. These treatments also enabled differences between phytase effects from microbial sources compared to plant sources to be assessed. The measured phytase levels of the diets were 605 U/kg for the control diet and 1150, 1804, 3954 and 5925 U/kg for the treatment diets. The diets had total phytate levels ranging from approximately 10-12g/kg DM (Table 2), dietary Ca levels of approximately 7.80g/kg DM (as dicalcium phosphate and limestone) (Table 1) and non-phytate-P levels of approximately 2.50 g/kg DM (Table 2). There were five treatments with each treatment replicated by 9 pens of 3 birds each (27 birds/dietary treatment). Diets were mixed in house and fed as mash. They were analyzed for gross energy by bomb calorimetry (Latshaw and Moritz, 2009), dry matter and protein content (calculated as nitrogen multiplied by 6.25) by the AOAC standard methods (930.15 and 990.03, respectively). Zn, P and Ca content of the diets were analyzed by inductively coupled plasma-optical emission spectroscopy (ICP-OES) following an aqua regia digestion step (AOAC 985.01, Thomas and Ravindran, 2010). Titanium dioxide (TiO_2) was added at a rate of 0.5% to act as an inert marker for evaluation of dietary phytate hydrolyzed and the dietary content of TiO_2 was quantified by ICP-OES following aqua regia digestion, by the method of Morgan et al. (2014). Total phytic acid content was analyzed by a K-Phyt assay kit (Megazyme^{TM}, Wicklow, Ireland, UK). This assay quantitatively measured available phosphorus released from the samples. Briefly, inositol phosphates were acid extracted followed by treatment with a phytase specific for IP_6-IP_2 and alkaline phosphate added to ensure release of the final phosphate from myo-inositol phosphate (IP_1). The total phosphate released was measured using a modified colorimetric method and given as grams of phosphorus per 100 g of sample material. *In vitro* susceptible phytate content was analysed by adding warmed acetate buffer (2.5M acetic acid and 2.5M sodium acetate, pH 4.5) to the diet samples, incubating and centrifuging the solution, neutralizing the supernatant with 0.25M NaOH, and subsequently using the K-Phyt^{TM} assay to analyze phytate content. Phytase activity was

186 analyzed according to the method of Engelen et al. (2001). Diet composition and analyzed GE,
187 CP and mineral values for the diets are shown in Table 1. Analyzed phytase, phytate and
188 susceptible phytate values for each of the diets are shown in Table 2.

189 ***Response Variables***

190 Two birds per pen were individually weighed and marked with a colored pen for
191 identification purposes post-euthanasia. Gizzard, duodenum and ileum pH of these two birds
192 was determined by inserting a spear tip piercing pH electrode (Sensorex, CA) with digital pH
193 meter (Mettler-Toledo, Leicester, UK) directly into the digesta in the gut lumen as soon as they
194 had been excised. Readings were repeated three times per section of gut per bird (ensuring the
195 probe did not touch the gut wall) and average pH was calculated.

196 Gizzard, duodenum and ileum digesta contents from 3 birds per pen were then collected
197 by gentle digital pressure into one pot per section of tract per pen, and stored at -20°C prior to
198 freeze drying. Once freeze dried, the samples were ground to a fine powder with a pestle and
199 mortar. Zn and titanium dioxide content of the digesta was determined by ICP-OES following
200 aqua regia digestion as previously discussed for the diets. Total phytate content of the gizzard,
201 duodenum and ileum digesta samples were analyzed by a K-Phyt assay as previously discussed
202 and the amount of dietary phytate hydrolyzed was calculated using the equation:
203 Dietary phytate (g/kg DM)*(1-(digesta phytate (g/kg DM) *$TiO_{2\ diet}$ (g/kg DM)) / (TiO_2 digesta
204 (g/kg DM)* dietary phytate (g/kg DM))
205 These values were used to calculate the *in vivo* susceptible phytate level by calculating the
206 proportion of total phytate that had been successfully hydrolyzed in the gizzard, duodenum and
207 ileum.

208 ***Data Analysis***

209 All data were analyzed using IBM SPSS statistics version 22. After Kolmogorov–
210 Smirnov testing to confirm normality, one-way ANOVA was used to test the equality of the

means and multiple ANOVA was used to test interactions. When means were significantly different, Duncan post-hoc tests were conducted to differentiate between them. Following polynomial contrast tests to test polynomial patterns in all of the data, correlations between measured factors were analyzed by bivariate correlations using Pearson product- moment correlation coefficient. Interpretations of the strength of the relationships between the factors were based on determining a very strong relationship as r = 0.800 to 1.000, strong as r = 0.600 to 0.790, moderate as r = 0.400 to 0.590, weak as r = 0.200 to 0.390 and very weak as r = 0.000 to 0.190. Statistical significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

Table 2 shows that the susceptible phytate levels measured *in vitro* and *in vivo* were almost identical in the diets with phytase activity of 5925 FTU/kg, but at the lower dietary phytase levels the *in vitro* assay used in this study overestimated how much of the total dietary phytate was susceptible to the effects of phytase. This was particularly the case in the diet with phytase activity of only 605 FTU/kg, in which it was predicted that approximately 5% more phytate would be hydrolyzed than was actually observed *in vivo*. Similar findings of observed stronger relationships between *in vitro* and *in vivo* methods in diets with phytase levels of 5000 FTU/kg or higher compared to diets with lower phytase levels were also observed by Morgan *et al.* (2014b). This is likely to be because the *in vitro* assay was not able to completely mimic conditions in the tract. This is because the *in vitro* assay was a closed system in which P ions released by phytate hydrolysis were not removed, so minor phytase effects appear to be more influential than would be observed *in vivo*. This suggests that the *in vitro* assay was successful at detecting P release when there was near complete phosphate hydrolysis, which likely occurred at this high phytase activity level, but was not sensitive enough to measure only partial phytate hydrolysis when low levels of P were released. The 6 phosphate groups on the inositol ring of phytate are hydrolysed in sequential order, with 6-phytases, such as the ones featured

236 in this study, starting at position 6, then position 1, 2, 3, 4 and 5. Degrading IP_6 to IP_4 eliminates
237 most of the anti-nutritional effects of phytate, but does not generate the full phosphorus
238 hydrolyzing capacity. In the diets with lower phytase activity the partial phytate hydrolysis
239 would result in a higher presence of lower inositols (namely IP_4 and IP_3) and hence a lower
240 concentration of IP_6 for the phytase to act on. IP_4 and IP_3 form weaker mineral complexes and
241 bind to fewer minerals than IP_5 and IP_6. It may be possible therefore that the impact of the
242 alkaline phosphatase was more pronounced in the *in vitro* assay due to the higher presence of
243 lower inositols and the degradation of these would occur more rapidly compared to degradation
244 of IP_5 and IP_6, resulting in overestimation by the *in vitro* assay. In the *in vivo* assay however
245 the effects of the alkaline phosphatase addition were less extreme as the phytate had already
246 been exposed to both phytase and alkaline phosphatase in the bird's gastrointestinal tract.
247 Further work is needed to improve the quality of the *in vitro* assay, especially at lower levels
248 of dietary phytase. It was noted that the 3954 FTU diets recorded the lowest phytate
249 susceptibility which may be because these diets had the highest total phytate content, resulting
250 in the phytate and Zn dissociating to a greater extent in these diets (Schlegel et al. 2010).
251 Table 3 shows that as phytase activity level increased more phytate was hydrolyzed and
252 more Zn was released from phytate-Zn complexes. In this study there were strong correlations
253 between the amount of phytate hydrolyzed and Zn concentration in the gizzard in birds fed the
254 diets with 1804 FTU/kg ($r = 0.689$, $P = 0.040$), 3954 FTU/kg ($r = 0.632$, $P = 0.048$) and 5925
255 FTU/kg ($r = 0.735$, $P = 0.032$). Phytate readily seeks cations, chiefly Zn, to complex with, so
256 in the presence of phytate the rate of Zn secretion into the duodenum via the pancreas exceeds
257 the rate at which dietary Zn is consumed. This suggests that measuring Zn level could be used
258 as a tool to indicate the chelating capacity of phytate and phytate susceptibility to phytase,
259 because any Zn bound to phytate would be excreted, so the higher the phytate presence the
260 lower the amount of free Zn present in the GIT. Phytase activity had a significant effect on the

amount of phytate hydrolyzed and Zn concentration in both the gizzard and ileum but not in the duodenum. Previously it was hypothesized that the duodenum may be the most appropriate area of the tract to determine the relationship between phytate and Zn, but findings from this study suggest that the gizzard gives the optimum location in the gastrointestinal tract for assessing the relationship between phytate and Zn, despite the upper part of the small intestine being the main site of Zn absorption (Schlegel et al. 2010). This is potentially because protein-phytate-mineral complexes, which are difficult to hydrolyze, do not readily form at gizzard pH but form at higher pH, meaning phytate is most susceptible to the effects of phytase in the gizzard (Amerah et al. 2014). This is advantageous because there is significantly more digesta sample in the gizzard, resulting in better homogeneity of the sample, allowing greater replication and potentially reducing the number of birds required for sample collection. In the duodenum and ileum however there were weak and non-significant relationships between the amount of phytate hydrolyzed and Zn concentration (Table 3). Further investigation is required to assess the impact of method of euthanization on Zn and phytate levels in the different sections of the tract, as it is possible that cervical dislocation resulted in issues such as mixing of digesta between segments due to peristalsis and mucosal shedding, which may have reduced the accuracy of the measurements in the digesta. Additionally, the observed effects in birds fed the diets with 1804, 3954 and 5925 U/kg phytase but not at the lower phytase levels may be partly because the phytase in these diets is from a plant source as opposed to a microbial source, but this requires further investigation.

The lack of strong correlations between gizzard pH and phytate hydrolysis in this study illustrates that gizzard pH was sufficiently low in all diets to allow for phytate hydrolysis (Table 4). This study was not designed to distinguish performance differences between the diets and P-adequate dietary treatments were fed, hence why significant performance effects were not observed, although FCR was numerically improved in the presence of phytase (Table 5).

Strong relationships were observed between pH and phytate susceptibility *in vivo* and *in vitro* in the gizzard (r = 0.749 P = 0.045 and r = 0.966 P = 0.007 respectively) and ileum (r = 0.664 P = 0.021 and r = 0.861 P = 0.041 respectively), and between duodenum pH and phytate susceptibility measured *in vivo* (r = 0.609 P = 0.026) (Table 6). This may be because it is addition of H+ ions to the weak acid phosphate groups of phytate that convert it from being resistant to susceptible to the effects of phytase. This study was in agreement with the work of Schlegel et al. (2010) who found that lower gizzard pH directly related to higher Zn solubility and that phytase was efficient in increasing digestive Zn and improving Zn status in the bird. Zn-phytate complexes dissociate as soon as pH is below 4 (Ellis et al. 1982), so the results presented for pH (Table 4) and phytate hydrolyzed (Table 3) for the 605 FTU/kg phytase diet suggest that the low pH in the gizzard allowed for Zn-phytate complexes to dissociate, even in the presence of very low levels of phytase. At higher pH, such as in the duodenum and ileum, phytate must first be hydrolyzed by phytase before Zn can be released. In this study the level of Zn was the same in each treatment in order to determine the direct effect of phytate and phytase on intestinal Zn levels, as opposed to the impact of dietary Zn presence on any of the parameters studied, but it would be advantageous in future studies to observe the effects of different Zn concentrations and define the optimum level of Zn for a successful marker.

In the gizzard, *in vivo* phytate susceptibility correlated moderately with Zn concentration (r = 0.583 P = 0.025) and strongly with phytate hydrolyzed (r = 0.733 P = 0.014) (Table 6). This suggests that measuring Zn level in the gizzard could be used as an indicator of phytate susceptibility to phytase and hence used to predict the anti-nutritional effect of phytate and the ability of phytase to combat its effects. In this study over 80% of the dietary zinc was utilized in the presence of phytase and as phytase level increased the strength of the relationship between phytate hydrolysis and zinc increased, highlighting heightened zinc release from phytate. This suggests that Zn deficiency could potentially be almost completely eliminated by

supplementing diets with high levels of phytase (Linares et al., 2007; Adams et al, 2002). A further advantage is that the ICP-OES assay is less time consuming and more sensitive than the colorimetric assay for measuring phytate and Zn can be measured concurrently with other mineral analysis. Zn availability is dependent on solubility in the digesta which is dictated by pH and dietary Zn concentration as discussed, but also the source of the supplemented Zn and interactions between diet Zn and other components in the diet (Schlegel et al., 2010). Further investigation is therefore needed into the interaction between phytate and phytase with different Zn sources and diet combinations. These findings also illustrate that additional phytase delivered in the form of an endogenous enzyme in wheat was effective at increasing zinc availability to the bird.

It can be concluded that measurements of Zn concentration in the gastrointestinal tract, particularly in the gizzard, have the potential to be used as a marker to assess the anti-nutritional impact of phytate and phytate susceptibility to the effects of phytase in broilers. Phytate susceptibility to phytase effects has a direct effect on Zn availability in the gastrointestinal tract and hence bird performance. It was hypothesized that measurements in the duodenum would give the best indication of phytate susceptibility to phytase and the anti-nutritional impact of phytate on Zn bioavailability, but it was found that that measurements of Zn in the gizzard provided the best indicator of phytate and phytase effects. This study also illustrated that additional phytase delivered in the form of an endogenous enzyme in wheat is effective at increasing Zn availability. Further work in needed to improve the quality of the susceptible phytate assay and to assess the relationship between Zn and phytate in diets with differing ingredient combinations and hence differing Zn and phytate levels.

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Table 1. Composition of control diet

	Phytase, U/kg[1]				
Ingredient	605	1150	1804	3954	5925
Standard wheat, 10% CP	57.21	57.21	38.11	19.11	0
Higher phytase wheat	0	0	19.10	38.10	57.21
Soyabean meal, 48% CP	35	35	35	35	35
Soy oil	3.78	3.78	3.78	3.78	3.78
Limestone	1.28	1.28	1.28	1.28	1.28
Salt	0.17	0.17	0.17	0.17	0.17
Sodium bicarbonate	0.26	0.26	0.26	0.26	0.26
Monocalcium phosphate HCl	1.23	1.23	1.23	1.23	1.23
Lysine HCl	0.21	0.21	0.21	0.21	0.21
Methionine	0.32	0.32	0.32	0.32	0.32
Threonine	0.13	0.13	0.13	0.13	0.13
Econase XT	0.01	0.01	0.01	0.01	0.01
Quantum Blue Phytase	0	0.01	0	0	0
Vitamin/Mineral Premix[1]	0.40	0.40	0.40	0.40	0.40
Titanium Dioxide	0.50	0.50	0.50	0.50	0.50
Analyzed composition					
CP, %	26.73	26.92	27.28	27.45	27.69
Gross energy, kcal/kg	4687	4686	4842	4905	4886
Total Zn, mg/kg	314.93	359.90	329.04	338.01	355.23
Total P, %	0.58	0.57	0.52	0.56	0.59
Total Ca, %	0.78	0.80	0.77	0.78	0.78

[1]Supplied per kilogram of diet: 10g calcium, 4.5g phosphorus, 1.5g sodium, 4.00g potassium, 1.5g chloride, 0.6g magnesium, 60mg manganese, 50mg Zn, 80mg iron, 6mg copper, 0.5mg iodine, 0.2mg molybdenum, 0.15mg selenium, 2.25mg retinol, 37.5µg cholecalciferol, 10mg tocopherol, 3.0mg menadione, 3.0mg thiamine, 5.0mg riboflavin, 10mg pantothenic acid, 4.0mg pyroxidine, 30mg niacin, 10µg cobalamin, 1.5mg folic acid, 0.15mg biotin, 1.4g choline, 125mg amprolium, 125mg antioxidant.

Table 2. Proximate phytate and phytase composition of experimental diets and *in vitro* and *in vivo* evaluation of phytate susceptibility to phytase on a dry matter basis

	Phytase, U/kg[1]				
	605	1150	1804	3954	5925
Phytate, %	1.01	1.01	1.02	1.21	1.19
Phytate-P, %[2]	0.29	0.29	0.29	0.34	0.34
Non-phytate-P, %[3]	0.30	0.28	0.24	0.22	0.21
In vitro Susceptible Phytate, %	0.56	0.57	0.58	0.64	0.65
In vitro Susceptible Phytate (% of phytic acid)	55.25	56.14	56.96	52.89	54.71
In vivo[4] Susceptible Phytate, %	0.51	0.54	0.56	0.59	0.64
In vivo[4] Susceptible Phytate (% of phytic acid)	50.20	53.37	55.29	48.84	54.12

[1]Total phytase activity was analysed by a colorimetric enzymatic method and calculated as (net optical density at 415nm*dilution volume)/(slope of standard curve*mass*incubation time) (Engelen et al. 2001).

[2] Phytate-P was calculated as 28.2% of phytate (Harland and Oberleas, 1986).

[3] Non-phytate P was calculated as the difference between total P and phytate-P.

[4] *In vivo* means represent the average response of 27 birds/treatment.

Table 3. Effect of phytase concentration on amount of dietary phytic acid hydrolyzed and remaining after hydrolysis (g/kg dry matter) and Zn concentration in the gizzard, duodenum and ileum (mg/kg dry matter) and correlation between the amount of phytate hydrolyzed and Zn concentration at d21[1]

		Phytase, U/kg[1]						
		605	1150	1804	3954	5925	SEM	*P*-value
Gizzard	Remaining	5.24^{b}	4.98^{b}	4.89^{b}	6.17^{a}	6.08^{a}	0.24	<0.001
	Hydrolyzed	3.68^{c}	3.96^{bc}	4.19^{abc}	4.44^{ab}	4.75^{a}	0.16	0.030
	Zn	35.61^{c}	37.13^{c}	41.28^{b}	41.48^{b}	46.34^{a}	1.68	<0.001
	Correlation[2]	0.138	0.634	0.689	0.632	0.735		
	P-value	0.053	0.066	0.040	0.048	0.032		
Duodenum	Remaining	4.85	4.56	4.44	5.81	5.49	0.24	0.202
	Hydrolyzed	4.07	4.38	4.64	4.80	5.34	0.19	0.063
	Zn	58.00	61.41	60.27	60.61	62.51	0.67	0.897
	Correlation[2]	0.079	0.413	0.234	0.297	0.301		
	P-value	0.841	0.269	0.544	0.438	0.998		
Ileum	Remaining	3.85^{b}	3.55^{b}	3.44^{b}	4.70^{a}	4.38^{a}	0.22	<0.001
	Hydrolyzed	5.07^{c}	5.39^{bc}	5.64^{b}	5.91^{b}	6.44^{a}	0.21	<0.001
	Zn	88.49^{c}	94.36^{bc}	98.36^{abc}	103.09^{ab}	106.81^{a}	2.88	0.021
	Correlation[2]	0.013	0.188	0.317	0.005	0.084		
	P-value	0.973	0.387	0.529	0.990	0.276		

$^{a–c}$ Means within the same column with no common superscript differ significantly ($P \leq 0.05$). 2-way ANOVA and Duncan Post-Hoc test were used to differentiate between means.

[1]Means represent the average response of 27 birds/treatment.

[2]Strength of the relationship between the amount of phytate hydrolyzed and Zn concentration in the gizzard, duodenum and ileum.

Table 4. Effect of phytase concentration on gizzard, duodenum and ileum pH and correlation between gastrointestinal pH[2] and the amount of phytate hydrolyzed[2] in broilers from d 0 to 21

		Phytase, U/kg[1]					
		605	1150	1804	3954	5925	*P*-value
Gizzard	pH	2.83	2.59	2.57	3.05	2.89	0.099
	Correlation[3]	0.276	0.190	0.292	0.351	0.108	
	P-value	0.472	0.625	0.446	0.141	0.782	
Duodenum	pH	6.19	6.18	6.20	6.17	6.24	0.826
	Correlation[3]	0.482	0.148	0.269	0.719	0.349	
	P-value	0.189	0.703	0.484	0.029	0.357	
Ileum	pH	6.91	7.08	6.92	7.96	7.10	0.583
	Correlation[3]	0.011	0.627	0.303	0.092	0.086	
	P-value	0.978	0.041	0.428	0.815	0.825	

[1]Means represent the average gastrointestinal pH of 18 birds/treatment.

[2] Means represent the average response of 27 birds/treatment.

[3]Strength of the relationship between the amount of phytate hydrolyzed and pH in the gizzard, duodenum and ileum.

Table 5. Effect of phytase concentration on bird performance in broilers from d 0 to 21[1]

	Phytase, U/kg[1]						
	605	1150	1804	3954	5925	SEM	*P*-value
FI (g)	1238.01	1162.25	1139.00	1124.24	1073.92	24.03	0.070
BWG (g)	764.53	722.19	737.75	726.91	790.41	11.47	0.268
FCR	1.63	1.57	1.54	1.49	1.46	0.03	0.317

[1]Means represent the average response of 36 birds/treatment

Table 6. Strength of the relationship between susceptible phytate levels measured *in vitro* and *in vivo* (g/kg) and gastrointestinal Zn concentration (g), phytate hydrolyzed (g) and pH in broilers from d 0 to 21

		Zn		Phytate Hydrolyzed		pH	
	Susceptible Phytate Method	Correlation	*P*- value	Correlation	*P*- value	Correlation	*P*- value
Gizzard	*In vitro*	0.244	0.692	0.430	0.470	0.966	0.007
	In vivo	0.603	0.025	0.733	0.014	0.749	0.045
Duodenum	*In vitro*	0.087	0.890	0.335	0.582	0.188	0.762
	In vivo	0.484	0.409	0.607	0.034	0.609	0.026
Ileum	*In vitro*	0.435	0.465	0.395	0.511	0.861	0.041
	In vivo	0.750	0.197	0.681	0.253	0.664	0.021