In vitro versus *in situ* evaluation of the effect of phytase supplementation on calcium and phosphorus solubility in soybean and rapeseed meal broiler diets

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Abstract

1. *In vitro* assays provide a sensitive and economic tool to evaluate dietary effects, but have limitations. In this study, the effect of phytase supplementation on solubility, and presumed availability, of calcium (**Ca**) and phosphorus (**P**) in soybean meal (**SBM**) and rapeseed meal (**RSM**) based diets was evaluated both *in situ* and by a 2-step *in vitro* digestion assay that simulated the gastric and small intestine (**SI**) phases of digestion.

2. Comparison of the *in vitro* findings to *in situ* findings was used to evaluate the *in vitro* assay. Ross 308 broilers (n = 192) were fed one of 6 SBM or RSM diets supplemented with 0, 500 or 5000 FTU/kg phytase from 0 to 28 d. The 6 diets and raw SBM and RSM were exposed to a two-step *in vitro* assay. Ca and P solubility and pH in the gizzard and jejunal digesta and in the gastric and SI phase of *in vitro* digestion were measured.

3. Both *in vitro* and *in situ* analysis detected that Ca solubility was lowest ($P < 0.05$) when diets were supplemented with 500 FTU/kg phytase, compared to the control diets and diets supplemented with 5000 FTU/kg phytase. Phosphorus solubility increased ($P < 0.05$) with increasing phytase level. Both methods also identified that mineral solubility plateaus in the gastric phase.

4. Overall relatedness of the two methods was strong for both determination of gastric phase Ca and P solubility ($r = 0.96$ and 0.92, respectively) and SI phase Ca and P solubility ($r = 0.71$ and 0.82, respectively). However mineral solubility and pH were higher ($P < 0.05$) when measured *in vitro* than *in situ*, and the *in situ* assay identified an interaction between phase, protein source and phytase inclusion level on Ca solubility that the *in vitro* assay did not detect.

5. This 2-step *in vitro* assay successfully predicted phytase efficacy, but to determine detailed response effects in the animal *in situ* data is still required.

Introduction

Dietary ingredients, phytate concentration and gastrointestinal pH are among the factors that dictate availability of dietary calcium and phosphorus. Mineral solubility is presumed to indicate availability following the studies of Walk *et al.* (2012a), Shafey *et al.* (1991) and Tamim *et al.* (2004). Despite having one of the lowest affinities with phytate, dietary calcium has the greatest practical impact on phytate-P availability due to its high inclusion levels in broiler diets (Maenz *et al*., 1999). High dietary Ca levels instigate an increase in gastric pH which shifts the pH away from the optimum (pH 2.8) for pepsin activity (Bohak, 1969). Large ratios of Ca: P have also been associated with reduced phytase efficacy (Tamim and Angel, 2003, Tamim *et al*., 2004) and hence reduced mineral availability (Selle *et al*., 2000) and bird performance (Huff *et al*., 1998; Cabahug *et al*., 1999; Cowieson *et al*., 2006b).

Low intrinsic phytase activity in the small intestine (**SI**) of poultry, and low phytase activity in common poultry feed ingredients, does not allow sufficient utilisation of phytate bound P without the use of exogenous phytase. Endogenous phytase activity has been detected in all sections of the SI, predominantly in the duodenum of broilers, indicating optimum efficiency at pH 5.5-6.5. However, as phytate-mineral complexes are most soluble in the crop, proventriculus and gizzard, hydrolysing phytate at this point in the gastrointestinal tract increases potential for mineral utilisation (Tamim *et al*., 2004). As the pH of this gastric phase of the tract is less than 4 (Selle *et al*., 2000), commercially available microbial phytases demonstrate optimum efficiency at this pH.

Digesta pH is one of the major gastrointestinal factors influencing Ca and P solubility (Pang and Applegate, 2007). Small deviations from the normal pH range throughout the gastrointestinal tract, such as those initiated by the high acid buffering capacity of limestone,

may have significant negative implications on Ca and P absorption (Bristol, 2003). As pH increases along the gastrointestinal tract, the affinity of phytate for Ca is increased and more phytate-Ca complexes are formed. This may subsequently result in a decrease in hydrogen ions released, an increase in gastrointestinal pH, and thereby reduce the mineral: phytate molar ratio required to precipitate phytate (Maenz *et al.,* 1999).

The concentration of Ca ions in the SI is thought to determine the rate of hydrolysis of phytate-P by endogenous phytase (Maenz and Classen, 1998). For example, intestinal phytase activity was 9% greater when birds were fed diets containing 4g/kg compared to 9g/kg Ca from either $CaCO_3$ or Ca malate (Applegate *et al.,* 2003). Phytate-P hydrolysis by endogenous phytase is consequently reduced in the presence of high Ca; for example apparent absorption of P was 24.9% when broilers were fed diets containing 5g/kg Ca compared to 65.2% when Ca was not added (Tamim and Angel, 2003). These findings suggest that the potential impact of high levels of Ca (commonly used in poultry diets) on exogenous phytase efficacy requires investigation.

In vitro assays have the potential to act as sensitive and cost effective tools for the evaluation of both phytase efficacy and Ca and P availability in dietary ingredients. There are however limitations to *in vitro* methodologies; it is impossible to reconstruct exactly the variability and interactions present *in situ*, so *in vitro* assays are able to measure degradability only and not digestibility. The overall aim of this study was to compare *in vitro* and *in situ* quantification of Ca and P solubility in a range of diets. The first objective of the study was to examine the solubility of Ca and P in pure soybean meal (**SBM**) and rapeseed meal (**RSM**) using a 2-step *in vitro* assay procedure developed by Walk *et al*. (2012a). The second objective was to determine the influence of 3 levels of phytase on Ca and P solubility in SBM- and RSM-based diets both *in vitro* and *in situ*. The final objective was to evaluate the

2-step *in vitro* assay by comparing Ca and P solubility determined *in vitro* to Ca and P solubility measured *in situ* in both the gastric and SI phase.

Materials and Methods

Birds and Husbandry

Institutional and national guidelines for the care and use of animals were followed and all experimental procedures involving animals were approved by the Nottingham Trent University College of Science ethical review committee.

Ross 308, male broilers (n = 192) from a 42-week-old breeder flock were obtained from a commercial hatchery at day of hatch. Chicks were randomised by weight and placed in 0.64 m^2 floor pens in groups of six, bedded on clean wood shavings. Birds were allowed *ad libitum* access to the treatment diets and water for the duration of the trial (d 0 to 28). The room was thermostatically controlled to produce an initial temperature of 32°C and reduced to 21°C by d 21. The lighting regimen used was 24 hours light on d 1, with darkness increasing by 1 hour per d until 6 hours of darkness was reached and this was maintained throughout the remainder of the study.

Dietary Treatments

Experimental diets were arranged as a 2 x 3 factorial including 2 dietary protein sources (SBM or RSM) and 3 levels of phytase (0, 500, or 5000 FTU/kg). This resulted in a total of 6 treatment groups replicated by 8 pens of 4 chicks each (32 chicks/dietary treatment). Diets were fed in mash form and formulated to meet or exceed Ross 308 nutrient requirements (Table 1). Diets were mixed in house and analysed for P and Ca content by ICP-OES (ICP-MS model PQ Excell VG Elemental) following an aqua regia digestion step (AOAC, 985.01; Leytem *et al*. 2008). Titanium dioxide was added as an inert marker for nutrient digestibility evaluation and the dietary content quantified by the method of Short *et*

al. (1996). Total phytate content was analysed by Megazyme^{TM} K-Phyt assay and phytase activity was analysed according to modified methods of Engelen *et al*., 2001 (Enzyme Services and Consultancy, Ystrad Mynach, UK). Formulated and analysed values for each diet are shown in Table 2. The phytase used in the experiment was an *Escherichia coli* 6-phytase with an expected activity of 5000 FTU/g (Quantum Blue, AB Vista Feed Ingredients, Marlborough, UK).

***In situ* Procedure**

Immediately post euthanasia on d 28, the gizzard was removed intact and a digital pH meter (Mettler-Toledo, UK) with spear tip piercing pH electrode (Sensorex, California, USA) was inserted directly into the digesta in the lumen of the proximal gizzard (proventricular opening), ensuring the pH electrode did not touch the gizzard wall, to record pH. This measure was repeated six times in different areas of the gizzard (mean variability +/- 0.07). SI pH was recorded in the same way from the medial jejunum (the intestinal section distal to the duodenal loop and proximal to the Meckel's diverticulum; mean variability +/- 0.06). After pH measurements were obtained, gizzard or jejunal digesta was pooled per pen and frozen at -20°C prior to freeze drying (LTE Scientific, UK) for 5 days to uniform weight.

Soluble Ca and P content was determined in the gizzard and jejunal digesta and in the feed by a method based on Kleinman *et al*. (2007) and Self-Davis and Moore (2000), respectively. Briefly, 2 g of sample was weighed into a pre-weighed bottle and 200 ml ultra-pure water (ICW 3000 water purifier for ion chromatograph, Millipore) was added. The sample was placed on a shaker set at 200 epm for 60 minutes prior to being centrifuged at 3,000 rpm for 10 minutes. The supernatant was then filtered through Whatman #541 filter papers before measurement by ICP-OES, with wavelengths for Ca and P set at 317.933 nm and 213.617 nm, respectively. Six replicates were analysed for each sample. ICP standards were made by diluting 1,000 ppm standard (Fisher Scientific, UK) and ultra-pure water. If it

was not possible to carry out the ICP analysis immediately, 5 drops of concentrated HCl per 20 mL extract were added to acidify the samples. The percent Ca or P solubility was calculated according to the following equation:

(Soluble Ca or P in digesta supernatant/ Total Ca or P in the diet) * 100

***In vitro* Procedure**

A 2-step *in vitro* assay procedure was used to investigate solubility of Ca and P in the gastric and SI phases of digestion in samples of RSM and SBM and in the six complete diets fed in the previously mentioned *in situ* study. This assay was based on that of Walk *et al*. (2012a) and Bedford and Classen (1993), except soluble Ca and P were analysed by ICP-OES as opposed to colorimetric analysis. Briefly, each feed ingredient or diet was ground through a 1 mm screen and 2.5 g was weighed into pre-weighed tubes. For each phase (gastric, indicating proventriculus and gizzard or SI, indicating the duodenum and jejunum), a minimum of 9 sub-samples were analysed for each diet and feed ingredient. Samples were analysed for Ca and P content in triplicate on the ICP-OES. To mimic the gastric phase, 4.5 ml of 0.13 N HCl, with 2,000 U pepsin/ml (Sigma-Aldrich, UK) was added to the pre-weighed samples before incubating at 41°C for 20 minutes. Sample pH was then obtained in triplicate using a spear tip piercing pH electrode (Sensorex, California, USA) to ensure samples were within the target range of pH 3.5-4.5. Samples were then diluted to 20 ml with 0.1 N HCl before centrifugation at 3,400 rpm at 4°C for 1 minute and the tubes weighed. Post centrifugation, the supernatant was collected into a separate pre-weighed tube. The sample was again diluted to 20 ml with 0.1 N HCl, re-centrifuged, and the supernatant collected. The pooled supernatant was filtered through a 0.22 µm filter (Fisher Scientific, UK) and stored at -20°C until further analysis. The filtered supernatant was diluted at 1:10 with water and analysed for soluble Ca and P using ICP-OES set at 213.617 nm for P and 317.933 nm for Ca

as previously described. The percent gastric Ca or P solubility was then calculated according to the following equation:

(Soluble Ca or P in the gastric phase/ Total Ca or P in the diet) * 100

For the intestinal phase, the samples were weighed and incubated with the HCl/pepsin as for the gastric phase and incubated for 20 minutes at 41^{o}C. Immediately after incubation 1.5 ml of $NaHCO_3$ containing 2 mg of pancreatin/ml (Sigma-Aldrich, UK) was added to each sample. The samples were mixed and incubated at 41^{o}C for an additional 60 minutes. The tubes were then made up to 45 ml with 0.32 M $HClO_4$ to stop the enzymatic reaction and weighed before centrifugation at 4˚C at 2,400 RPM for 1 minute. The supernatant was immediately filtered through Whatman 541 filter paper and diluted 1:10 with HNO_3. The diluted samples then analysed for Ca and P using ICP-OES as described previously. The percent of soluble Ca or P in the SI was calculated according to the following equation:

(Soluble Ca or P in the SI/ Total Ca or P in the diet) * 100

Statistical Analysis

All data was analysed using IBM SPSS statistics version 19. Multiple ANOVA was used to determine the effect of protein source and phytase inclusion on solubility of Ca and P in the gastric and SI phase of both the *in vitro* and *in situ* samples. The statistical model included protein source, phytase inclusion level and digestion phase to investigate all 2- and 3- way interactions. Independent sample t-tests were used to directly compare the findings from the *in vitro* and *in situ* methodologies. The relatedness of the methodologies was investigated using Pearson product-moment correlation coefficient and interpretations of the strength of the relationship between the two methods was based on guidelines by Cohen

(1988); weak relationship r = 0.10 to 0.29, medium relationship r = 0.30 to 0.49 and strong relationship r = 0.50 to 1.0. Significance was accepted at $P < 0.05$.

RESULTS AND DISCUSSION

Solubility of Ca and P in SBM and RSM determined by *in vitro* methodology

Calcium and P solubility is dictated by total dietary mineral content, gastrointestinal pH, and the precipitation of free Ca and P by phytate (Maenz *et al.*, 1999; Walk *et al*., 2012b). The *in vitro* solubility of Ca and P in pure RSM or SBM were observed to be lower ($P < 0.05$) in the SI phase than in the gastric phase (Table 3). This is in agreement with the work of Walk *et al*. (2012a) who reported that Ca and P solubility reached a plateau in the gastric phase. Mineral solubility in the gastric phase therefore dictates availability of those minerals in the rest of the gastrointestinal tract. Phytate, Ca and P are relatively soluble at gastric pH and are hence unlikely to precipitate. However, at higher pH (pH 4 to 7) phytate-mineral complexes are more insoluble (Selle *et al*., 2000) and precipitation of Ca, P and phytate is likely in the SI phase, thereby reducing Ca and P solubility and absorption. The pH range to which the samples were exposed in this study were within the range found in the gastrointestinal tract of poultry (Table 3) and are similar to those found in published *in situ* studies (Pang and Applegate, 2007; Shafey, 1999) In addition, Ca solubility in SBM has been previously reported as approximately 93% soluble (Zhang and Coon, 1997) and P as approximately 52% soluble (Ciurescu, 2009), which is comparable to 89 and 45% soluble Ca and P, respectively, observed in this study (Table 3).

Rapeseed meal contained slightly more soluble Ca and P than SBM, in both the gastric and SI phases (Table 3). This may be because both gastric and SI pH was significantly lower when the RSM was subjected to the *in vitro* assay, resulting in less precipitation of Ca-

phosphate or Ca-phytate from the RSM samples than from the SBM samples. The observed lower pH in the RSM samples may be partly because RSM contains more sulphur than SBM (1.14% compared with 0.44% in SBM; Mushtaq *et al*., 2007). Sulphur has an acidogenic effect which lowers dietary cation-anion difference and stimulates Ca homeostasis (Guéguen *et al*., 2000). RSM also has approximately double the endogenous phytase activity of SBM; 16 U/kg compared to 8 U/kg (Eeckhout and De Paepe, 1994), although these values are negligible in comparison to the phytase activity demonstrated by exogenous phytases. Therefore Ca ion release may have been higher in the RSM diets, hence the observed increase in Ca solubility.

Solubility of Ca and P in SBM and RSM based diets supplemented with phytase

The *in vitro* solubility of Ca was lowest in the diets supplemented with 500 FTU/kg phytase compared to the other diets in the gastric and SI phase (Table 4). This resulted in a phase x phytase ($P < 0.05$) interaction; the reduction in Ca solubility between the diets supplemented with 500 FTU/kg phytase and the other diets was greater ($P < 0.05$) in the gastric phase than the SI phase. This effect was overcome when the diets were supplemented with 5000 FTU/kg of phytase and may be associated with the molar Ca concentration of the diets and the Ca to P ratio. Increased production of inert Ca-phytate complexes (Tamim and Angel, 2003), particularly due to the non-parallel release of Ca and P from phytate (Walk *et al*., 2012b), may have promoted free phosphate or phytate precipitation of Ca, causing the observed reduced Ca solubility when the diets with supplemented with 500 FTU/kg. Most microbial phytases in current use start phytate degradation at the 6-phosphate position of the phytate molecule and only partially continue round the inositol ring (to yield predominantly IP4 and IP3) (Cowieson *et al*., 2011). However, at 5000 FTU/kg, the enzyme is present in sufficiently high concentration to complete phosphate hydrolysis of each molecule, leaving a phytate free diet and increasing free phosphate to a ratio above that of the free Ca. Partial

hydrolysis of phytate is particularly relevant at 500 FTU/kg because at this phytase supplementation level there may have been a high presence of IP4 and IP3, and consequently lower concentration of IP6 for phytase to act on, resulting in only partial release of Ca (Luttrell, 1993). It is therefore possible that Ca released from the 500 FTU/kg promoted Ca-phosphate and Ca-phytate precipitation and reduced the magnitude of P released from 500 FTU/kg of phytase (Table 5). In the non-phytase diets, the majority of the Ca may have been complexed to phytate (Selle *et al*. 2009) and in the 5000 FTU/kg supplemented diet the majority of the dietary Ca may have been free (Manangi *et al*. 2009; Walk *et al*. 2012b).

The observed difference on *in vitro* Ca solubility between the two protein sources was greater ($P < 0.05$) in the gastric phase than the SI phase. This resulted in a phase x protein source ($P < 0.05$) interaction and may be due to the greater difference in pH ($P < 0.05$) between the gastric phase and the SI phase, and the precipitation of Ca and phytate at higher pH. Interactions ($P < 0.05$) were observed on *in situ* Ca (Table 4) and P solubility and *in vitro* P solubility (Table 5) between phytase supplementation, protein source and digestion phase. In the gizzard, Ca solubility was reduced in both SBM and RSM diets supplemented with 500 FTU/kg phytase, compared to the other diets and reduced in SBM diets compared with RSM diets. However, there was no effect of diet or protein source on *in situ* Ca solubility in the SI phase, except when RSM was supplemented with 5000 FTU/kg phytase, which was higher than SBM at 500 FTU/kg (Table 4). As mentioned previously, the Ca to available P ratio may have a substantial influence on Ca and P solubility. In the SBM diets, the Ca to P ratio was approximately 3.5:1 and reduced in the RSM diets to 2.8:1. The wider Ca to P ratio in the SBM diets may have resulted in the reduced Ca solubility in the SBM diets compared to the RSM diets (Tamim *et al*. 2004; Qian *et al*. 1997).

The *in vitro* studies indicated that, in the SBM based diets, variance in P solubility between the two phases was not influenced by phytase inclusion level. Conversely, in the

RSM based diets, as phytase level increased, the difference in P solubility between the gastric and SI phase increased ($P < 0.05$). As phytase level increased, the difference in gastric P solubility between the two protein sources decreased ($P < 0.05$) and no differences were observed in the SI phase at any phytase supplementation level (Table 5). This again highlights that mineral solubility in the gastric phase determines subsequent mineral absorption in the SI, and suggests that, within the SI itself, diet composition has no direct effect on mineral solubility.

In situ P solubility in the gizzard and jejunum increased with increasing phytase supplementation and was higher in the SBM diets than the RSM diets at all phytase inclusion levels. However, in the gizzard an increase in P solubility compared with the non-phytase supplemented SBM diet was higher only in the presence of 5000 FTU/kg phytase (Table 5). Phytase supplementation increased *in situ* P solubility in the RSM diets comparable to the non-phytase supplemented SBM diets, but only at 5000 FTU/kg. This may be partly because the solubility and susceptibility of phytate to degradation by phytase differs between SBM and RSM; the phytate present in SBM is potentially more soluble and susceptible to the effects of phytase than the phytate present in RSM (Classen *et al*., 2010; Maenz *et al*., 1999). Differences in non-starch polysaccharide content may also impact on mineral availability; gelling cell wall polysaccharides, such as arabinoxylans and β-glucans, may entrap minerals (Meng *et al*. 2005). RSM has a higher non-starch polysaccharide content than SBM (Ciurescu, 2009) suggesting that phytase was able to access the phytate-mineral complex substrates in the SBM more readily than in the RSM.

Comparison between *in vitro* and *in situ* methodologies

There were both consistencies and differences between the *in vitro* and *in situ* methods featured in this study. There were some strong relationships between the two

methods; particularly for Ca and P solubility in the gastric phase (r=0.963 and 0.917 respectively), and for Ca and P solubility in the SI phase (r=0.713 and 0.824). While tables 4 and 5 show predominantly strong and medium relationships between the two methods in the gastric phase, the SI phase shows a higher proportion of weak relationships between the methods. This suggests that the *in vitro* assay is more comparable to *in situ* measures for mineral solubility in the gastric phase, rather than the SI phase, most likely due to the lack of absorption and secretion in the SI phase *in vitro* compared to *in situ*.

Both the *in vitro* and *in situ* assays detected that, in the gastric and SI phase, Ca solubility was lowest in the diets supplemented with 500 FTU/kg and was highest in the diets supplemented with 5000 FTU/kg, and that P solubility increased with increasing phytase supplementation level. Both methods also identified that Ca solubility was higher in the RSM based diets compared to the SBM diets, and that P solubility was higher in the SBM based diets (Table 4 and 5). A phase x protein source x phytase inclusion level interaction on P solubility (Table 5) and phase effect on pH (Table 4) was also detected by both methods. The pH ranges to which the samples were exposed to in the *in vitro* method were within the range found *in situ* (Table 4). These findings indicate that this *in vitro* assay could be a proficient tool to indicate the efficacy of phytase at increasing Ca and P solubility.

However there were some contrasting findings between the *in vitro* and *in situ* analysis. The *in vitro* assay is a closed system where the hydrolysis of phytate may alter the P and Ca ion concentration as minerals are not absorbed from the supernatant (Walk *et al*., 2012a). The likelihood of Ca-phosphate precipitation is higher *in vitro* than *in situ* because high accumulated Ca levels *in vitro* promote both precipitation and increased pH, causing a reduction in mineral solubility (Selle *et al.,* 2009). This implies that dietary effects could be amplified in the *in vitro* samples, indicating that minor phytase and protein source effects may appear to have more influence on mineral solubility than would occur *in situ*. This may

explain why interactions between protein source and phytase inclusion level were observed on Ca solubility in the gastric phase (Table 4) and P solubility in the SI phase (Table 5) when measured *in situ*, but were not identified when measured *in vitro*. No relationship was observed between the two methods for the measurement of Ca solubility in the SBM control diets in the SI phase (Table 5). This could be due to the lack of phytate hydrolysis in the *in vitro* samples (where no phytase was present) so Ca was readily precipitated leading to calcium phosphate accumulation in the closed *in vitro* system.

The relationships between the two methods for the measurement of P solubility in the SI phase was stronger in the RSM based diets compared to the SBM diets. This may be attributable to the higher Ca to free P ratio (2.39:1) of the SBM based diets which caused phosphate to be readily precipitated and reduced release of free P. In the *in vitro* system, the release of these small amounts of free P is detectable, as there is accumulation, whereas *in situ* the free P would have been readily absorbed. Phytases are most active at low pH, so the hydrolysis of phytate-calcium and phytate-phosphorus complexes was likely to be less when determined *in vitro* than *in situ*, due to the higher pH in the *in vitro* samples. This would result in greater Ca-phosphate formation and precipitation in the SI, and hence lower mineral solubility *in vitro* compared to *in situ*. In future studies, pH may need to be manipulated in the *in vitro* assay, based on the properties (namely Ca, P and phytate content) of the diet being analysed, in order to accurately identify phytase efficacy. There is also variation in retention time between the two methods, which may explain why a phase x protein source x phytase supplementation level interaction was found when Ca solubility was measured *in situ*, but was not when detected *in vitro* (Table 4). In the *in situ* samples there are minor amounts of endogenous phytase in the SI phase that contribute towards mineral release from phytate, which may go some way towards explaining why protein source and phytase supplementation had an effect on SI phase pH when determined *in situ*, but not when measured *in vitro* (Table

4). This indicates the *in vitro* assay is more proficient at measuring mineral solubility in the gastric phase than the SI phase.

The 2-step *in vitro* assay in this study was successful at identifying the effect of phytase supplementation on Ca and P solubility in the gastric and SI phase, signifying it can be used a tool to indicate dietary effects on the GIT environment. This assay is however unable to measure digestibility as is a closed system so cannot completely mimic bird GIT conditions, suggesting *in situ* analysis is still required to fully distinguish phytase effects on mineral solubility. Dietary levels of Ca, P and phytate dictate precipitation of soluble Ca and P and hence availability of these minerals, therefore calcium phosphate precipitation occurs if the Ca: P ratio is not balanced and if the pH is high. The effects of phytase on dietary free Ca and P and phytate are positive *in situ,* but potentially cause increased calcium phosphate precipitation *in vitro,* which reduces the reliability of this assay.

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Table 1. Ingredient and nutrient composition of experimental diets

Ingredient, g/kg	SBM[1] diet	RSM[2] diet
Wheat	605.9	605.2
Fishmeal, 72%	30.0	30.0
Rapeseed extruded	0.00	250.0
Soybean meal, 46%	180.0	0.00
Soy hulls	70.0	0.00
Soy oil	5.59	5.44
Salt	3.6	3.6
Valine	2.1	2.2
DL methionine	4.1	2.9
Lysine HCl	5.0	6.5
Threonine	2.4	2.4
L-tryptophan	0.1	0.2
Glycine	3.2	5.1
L-arginine HCl	3.6	5.5
Isoleucine	1.9	2.8
Limestone	10.0	8.5
Dicalcium phosphate	13.0	11.4
Coccidiostat	0.2	0.2
Vitamin/trace mineral premix[3]	4.0	4.0
Titanium dioxide	5.0	5.0
Formulated composition		
Crude protein, g/kg	201.1	198.6
ME, MJ/kg	12.8	12.8
Calcium, g/kg	10.0	10.0
Total P, g/kg	6.8	7.7
Available P, g/kg	4.5	4.5
Phytate P, g/kg	5.4	6.4
Lys, g/kg	13.3	13.3
Analysed composition		
Crude protein, g/kg	238.7	231.4
ME, MJ/kg	13.9	13.5
Dry matter, g/kg	870.6	869.4
Calcium, g/kg	15.3	12.7
Total P, g/kg	6.7	7.5
Phytate P, g/kg	5.4	6.5

[1] Soybean meal is the primary vegetable protein source.

[2] Rapeseed meal is the primary vegetable protein source.

[3] Supplied per kilogram of diet: manganese, 100 mg; zinc, 80 mg; iron (ferrous sulphate), 20 mg; copper, 10 mg; iodine, 1 mg; molybdenum, 0.48 mg; selenium, 0.2 mg; retinol, 13.5 mg; cholecalciferol, 3 mg; tocopherol, 25 mg; menadione, 5.0 mg; thiamine, 3 mg; riboflavin, 10 mg; pantothenic acid, 15 mg; pyroxidine, 3.0 mg; niacin, 60 mg; cobalamin, 30 µg; folic acid, 1.5 mg; and biotin 125 mg.

Table 2. Recovery of phytase activity in experimental diets

Diet	Phytase[1] recovery (FTU/kg diet, as-fed)
Soybean meal (SBM)	138
SBM + 500 FTU/kg phytase	457
SBM + 5000 FTU/kg phytase	4830
Rapeseed meal (RSM)	<50
RSM + 500 FTU/kg phytase	877
RSM + 5000 FTU/kg phytase	5390

[1]One unit of phytase activity (FTU) is defined as the quantity of enzyme that liberates 1 mole of inorganic P per minute from rice bran phytate at pH 4.5 and 37°C. Each result is the mean of 3 replicates.

Table 3. Solubility of P and Ca (g/100g) liberated from soybean meal (SBM) or rapeseed meal (RSM) subjected to a 2-step *in vitro* assay procedure[1]

In vitro phase	Soluble Ca	Soluble P	pH
Gastric			
Soybean meal	0.89[a]	0.45[a]	3.96[c]
Rapeseed meal	0.91[a]	0.48[a]	3.88[d]
Small intestine			
Soybean meal	0.63[c]	0.29[c]	6.70[a]
Rapeseed meal	0.67[b]	0.33[b]	6.59[b]
SEM	0.02	0.03	0.05
P-value			
Protein source	0.042	0.030	0.026
Phase	<0.001	<0.001	<0.001

[1]Soybean meal and rapeseed meal were exposed to a 2-step *in vitro* assay as described by Walk *et al*. (2012) with slight modifications. Means represent the average of 10 replicates per feed ingredient.

[a-d] Means within a column with no common superscript are different ($P < 0.05$).

Table 4. Interaction of digestion phase, phytase and protein source on gut pH and solubility of Ca liberated (g/100g) from soybean meal (SBM) and rapeseed meal (RSM) based diets subjected to the gastric and small intestine phase of a 2-step *in vitro* assay procedure[1] and in the gizzard and jejunum of 28-d-old broilers[2]

	Dietary treatment		Method			pH		
Phase	Protein source	Phytase (FTU)	*In vitro*	*In situ*	r[3]	*In vitro*	*In situ*	r[3]
Gastric	SBM	0	0.68	0.42[b]	0.398	3.75	2.85	0.899
		500	0.52	0.34[c]	0.893	3.98	2.99	0.771
		5000	0.68	0.43[b]	0.862	3.68	2.88	0.301
	RSM	0	0.78	0.55[a]	0.497	3.15	2.43	0.205
		500	0.65	0.40[b]	0.467	3.40	2.45	0.045
		5000	0.81	0.56[a]	0.580	3.14	2.34	0.946
		SEM	0.022	0.033		0.045	0.037	
Small intestine	SBM	0	0.26	0.21[de]	0.002	7.34	6.00	0.755
		500	0.24	0.17[e]	0.996	7.61	6.14	0.077
		5000	0.28	0.24[de]	0.156	7.42	6.08	0.313
	RSM	0	0.30	0.22[de]	0.883	7.57	5.91	0.704
		500	0.29	0.19[de]	0.186	7.60	6.03	0.082
		5000	0.31	0.25[d]	0.136	7.47	5.99	0.474
		SEM	0.014	0.011		0.040	0.030	
Gastric	SBM		0.63[b]	0.40		3.80	2.91	
	RSM		0.75[a]	0.50		3.23	2.41	
Small intestine	SBM		0.26[d]	0.21		7.46	6.07	
	RSM		0.30[c]	0.22		7.55	5.98	
Gastric		0	0.73[a]	0.49		3.45	2.64	
		500	0.59[b]	0.37		3.69	2.72	
		5000	0.75[a]	0.50		3.41	2.61	
Small intestine		0	0.28[c]	0.22		7.46	5.96	
		500	0.27[d]	0.18		7.61	6.09	
		5000	0.30[c]	0.25		7.45	6.04	
Gastric			0.69	0.45		3.52[a]	2.66[a]	
Small Intestine			0.28	0.22		7.50[b]	6.03[b]	
P-value								
Phase x protein source			<0.001	NS		NS	NS	
Phase x phytase			<0.001	NS		NS	NS	
Protein source x phytase x phase			NS	<0.001		NS	NS	
Phase						<0.001	<0.001	

[1] Diets were exposed to a 2-step *in vitro* assay as described by Walk *et al*. (2012) with slight modifications (see Materials and Methods section). Means represent the average of a minimum of 9 replicates per feed ingredient.

[2] Represent the average response of 32 birds per diet, 8 pens of 4 birds per diet, and 192 birds in total. Means represent the average of 6 replicates per pen.
[3] Strength of the relationship between the *in vitro* and *in situ* readings for each mineral solubility measured in each diet where confidence in the result is $P < 0.05$.
[a-e] Means within the same column with no common superscript differ significantly ($P < 0.05$).

Table 5: Interaction of phase, phytase and protein source on solubility of P liberated (g/100g) from soybean meal (SBM) and rapeseed meal (RSM) based diets subjected to the gastric and small intestine phase of a 2-step *in vitro* assay procedure[1] and in the gizzard and jejunum of 28-d-old broilers[2]

	Dietary treatment		Method		
Phase	Protein Source	Phytase (FTU)	*In vitro*	*In situ*	r[3]
Gastric	SBM	0	0.63^{b}	0.48^{bc}	0.988
		500	0.67^{b}	0.49^{b}	0.965
		5000	0.71^{a}	0.54^{a}	0.563
	RSM	0	0.45^{d}	0.39^{e}	0.544
		500	0.52^{c}	0.42^{d}	0.722
		5000	0.66^{b}	0.45^{c}	0.159
		SEM	0.021	0.011	
Small Intestine	SBM	0	0.28^{e}	0.24^{hi}	0.284
		500	0.29^{e}	0.25^{g}	0.115
		5000	0.29^{e}	0.29^{f}	0.604
	RSM	0	0.26^{f}	0.22^{j}	0.363
		500	0.27^{f}	0.23^{i}	0.567
		5000	0.28^{ef}	0.25^{g}	0.998
		SEM	0.002	0.009	
P-value					
Protein Source x Phytase x Phase			0.037	<0.001	

[1] Diets were exposed to a 2-step *in vitro* assay as described by Walk *et al*. (2012) with slight modifications (see Materials and Methods section). Means represent the average of a minimum of 9 replicates per feed ingredient.

[2] Represent the average response of 32 birds per diet, 8 pens of 4 birds per diet, and 192 birds in total. Means represent the average of 6 replicates per pen.

[3] Strength of the relationship between the *in vitro* and *in situ* readings for each mineral solubility measured in each diet where confidence in the result is $P < 0.05$.

[a-b] Means within the same column with no common superscript differ significantly ($P < 0.05$).