Interactions between phytase and different dietary minerals in *in vitro* systems

Marjina Akter 1*, Hadden Graham 2 and Paul Ade Iji 1

1 School of Environmental and Rural Science, University of New England, Armidale, NSW 2351, Australia. 2 AB Vista, 3 Woodstock Court, Marlborough, Wilts SN8 4AN, UK. *e-mail: marjinajahivet@gmail.com

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**Abstract**

An *in vitro* experiment was conducted to evaluate the effect of different dietary minerals on phytate hydrolysis by microbial phytase enzyme and to measure the residual phytate content after phytate digestion. Calcium (0, 6, 8, 10 g kg⁻¹ of diet), Fe (0, 70, 80, 90 mg kg⁻¹ of diet), Zn (0, 30, 40, 50 mg kg⁻¹ of diet) or Na (0, 1.5, 2.5 and 3.5 g kg⁻¹ of diet) were incubated with a Na-phytate solution, with phytase enzyme (500 FTU kg⁻¹ of diet) at pH 2.5 or 6.5. The phytate concentration was set at 0.27% to mimic the phytate level in a typical corn-soybean meal-based poultry diet. Inclusion of Ca, Zn and Fe significantly (p < 0.05) reduced the phytate phosphorus hydrolysis at both pH values but the effect was strongest at pH 6.5. Irrespective of the pH, adding Na also inhibited (p < 0.05) phytate phosphorus hydrolysis; however, the negative effect was more pronounced at pH 2.5 than 6.5. Residual phytate content (mg) after phytate digestion was higher (p < 0.05) at pH 6.5 than at 2.5 in the presence of Ca, Zn and Fe. The reverse was the case in the presence of Na. These *in vitro* results demonstrated that increased concentrations of dietary Ca, Fe, Zn and Na could reduce phytate phosphorus hydrolysis, but such a response would need to be tested in animal feeding trials.

**Key words:** *In vitro*, phytate, phytase, minerals, pH.

**Introduction**

Phytic acid (PA, myo-inositol hexaphosphoric acid, IP6) is a natural reservoir of phosphorus in plants, especially grains 1. Phytate phosphorus (PP) represents around 50-80% of total P in the diet 2 and is poorly utilized by poultry 1. The strongly reactive PA also limits the bioavailability of other dietary minerals (Ca, Fe, Zn, Cu, etc.) by precipitating them in the digestive tract as evaluated by Pontoppidan *et al.* 3 and Selle and Ravindran 4. Formation of insoluble Ca-phytate complexes in the small intestine causes a decline in Ca and P availability, resulting in rickets or osteomalacia in poultry 5. Bioavailability of the phytate-bound minerals largely depends on enzymatic dephosphorylation by phytase. Efficient endogenous phytase activity renders the non-ruminant animals more vulnerable to the negative effect of phytate 6. Angel *et al.* 8 stated that besides this aforementioned fact, increasing feed cost and negative impact on the environment by excessive excretion of P, the use of phytase has been considered a better option than inorganic supplements to alleviate the anti-nutritive effect of phytate in poultry.

Phytase sequentially releases phosphorus and other minerals from phytate 9 and makes them available for poultry 10. A significant number of reports have documented the improved digestibility of Ca 11, 12 and phytate P 13 and enhancement of absorption and retention of Zn 14, Fe, Mg and Cu 12,15 by phytase-induced phytate destruction in non-ruminants. Although the beneficial effect of phytase in poultry nutrition has already been established, there is still some controversy regarding its effectiveness under the influence of factors such as pH of the media, mineral levels, temperature, duration of incubation, agitation and mineral:phytate ratio 5. Phytic acid is able to chelate nutritionally important di- and trivalent cations such as Ca, Mn, Cu, Fe, Zn and Mg in the digestive tract and form soluble or insoluble mineral-phytate complexes as concluded by Angel *et al.* 8. The solubility of phytate minerals is a prerequisite for enzymatic hydrolysis, as phytase cannot easily hydrolyze the precipitated substrate 2. On the other hand, pH appears to be the most important factor regulating the solubility of these phytate-mineral complexes as determined by Maenz *et al.* 16 and Maenz 17. Selle *et al.* 18 stated that a relatively high pH (4-7) more readily facilitates the formation of insoluble mineral-phytate complexes than low pH (< 3.50). Dietary minerals are another influential factor in phytate hydrolysis as observed by Sandberg *et al.* 19, whereas Ca plays a crucial role in regulation of phytase efficacy in the poultry gut as reported by Angel *et al.* 8.

Excessive amounts of dietary Ca (9-10 g kg⁻¹) reduced phytate hydrolysis by phytase due to the formation of insoluble Ca-phytate complex at high pH (6.5) than at low pH (2.5) 20. Similarly, degradation of ileal phytate by mucosal phytase decreased with the addition of 9 g kg⁻¹ Ca in diet 21. In chickens, formation of insoluble Zn-phytate complex due to high concentration of Zn (200 ppm) at high pH 22 reduced efficacy of phytase 21. The pH of duodenum and upper jejunum facilitated Zn-phytate complex formation 24 and consequently limited their hydrolysis by phytase 25. Similarly, Tang *et al.* 26 showed that phytate-resistant phytate-mineral precipitants have been observed with Fe³⁺, Fe²⁺, Cu²⁺ and Zn²⁺.

From the above findings, it can be hypothesized that the formation of pH-dependent soluble or insoluble mineral-phytate complexes could hinder phytase-mediated phytate hydrolysis and reduce the phytate P and other mineral digestibility. Although
the negative effect of Ca on phytase efficacy has already been assessed in both in vitro and in vivo system 27,28, there is a lack of information on other key minerals such as Na, Fe and Zn, especially in poultry. Therefore, the present study was undertaken to investigate how dietary minerals affect the efficacy of phytase at different pH and incubation periods under defined in vitro conditions.

Materials and Methods

The general procedure of Kim and Lei 29 was followed in the assessment of in vitro hydrolysis of phytate, with some modifications, as suitable for different minerals. The tested mineral concentrations reflected the level of minerals used in typical poultry diets. The two pH conditions were chosen to simulate the gastric and upper intestinal pH levels.

Effect of Ca on phytate hydrolysis by phytase: Typically, corn-soybean meal-based broiler starter diets contain 0.27% phytate phosphorus (PP) 30. On this basis, the substrate solution was prepared by adding 4.84 mM sodium phytate to 200 mM of glycine buffer (pH 2.5) or 40 mM mes-bis-tris buffer (prepared by mixing 40 mM of 2-(N-morpholino)ethanesulfonic acid and 50 mM of bis-(2-hydroxyethyl)amino-tris (hydroxymethyl) methane (pH 6.5). CaCO₃ was added to the substrate solution to obtain three concentrations of Ca 150, 200 and 250 mM (equivalent to 6, 8, 10 g kg⁻¹, respectively). In order to obtain a solution containing 500 FTU of phytase g⁻¹ (Quantum Blue, AB Vista), 0.1 ml of phytase enzyme was added to 99.9 ml of glycine buffer or mes-bis-tris buffer and stirred well to achieve the desired phytase activity (5 U/ml).

Fifty µl of phytase solution was added to the test tube containing 4 ml of substrate solution containing the Ca. The resulting mixture was vortexed and then incubated for 30, 60 or 90 min at 41°C. After incubation, 1 ml of ice-cold 50% (w/v) trichloroacetic acid (TCA) was added to terminate the reaction. The mixture was centrifuged at 2000 x g (Beckman Allegra 6R, New Hampshire, USA) for 10 min. After centrifuging, 1 ml of supernatant was transferred into fresh test tubes for measurement of residual phytate. Two ml of fresh colour reagent (prepared by adding three volumes of 1 M sulphuric acid to 1 volume of 10% ascorbic acid and then 1 volume of 2.5% ammonium molybdate was added and mixed thoroughly) was added to the remaining solution of the test tube and thoroughly mixed. The mixture was incubated at 41°C for 30 min and cooled to room temperature. The absorbance was read on Hitachi 150-200 UV spectrophotometer (Hitachi Science System Ltd., Ibaraki, Japan) at 660 nm and liberated inorganic P (Pi) was measured and quantified through comparison with an inorganic P standard curve (P8810, Sigma Aldrich Co., LLC). All incubations were done in quintuplicate and absorbance was read in duplicate from each replicate.

Similarly, the effect of Fe, Zn and Na on phytase activity was tested separately by following the same procedure mentioned above except their concentrations. Three concentrations of Fe solutions 0.84, 0.96 and 1.08 mM (equivalent to 70, 80, 90 mg kg⁻¹ diet, respectively), Zn solutions 0.48, 0.64 and 0.81 mM (equivalent to obtain 30, 40 and 50 mg equivalent of Zn kg⁻¹ diet, respectively) and Na solutions 65, 108 and 152 mM (equivalent to 1.5, 2.5 and 3.5 g kg⁻¹ diet, respectively) were prepared from FeSO₄·12H₂O, ZnO and NaCl and added to substrate solution. One of the levels of these minerals were selected based on NRC 30 requirement and two other levels were selected as immediately below and above the dietary specification.

As a further measure of the digestibility of phytate, the level of residual phytate from the substrate solution was measured according to the method described by Haug and Lantzsch 31. Briefly, 2 ml of ammonium iron sulphate solution (made by dissolving 0.2 g of ammonium iron (III) sulphate, 12H₂O (Sigma Aldrich Co., LLC, F3629) in 100 ml of 2 N HCl and made up to 1000 ml with Milli-Q water) was added to the test tube containing 1 ml of substrate solution that was taken after centrifuging. The tubes were sealed with stoppers and tightly closed with clips. Tubes were then placed in boiling water for 30 min, taking care to keep the stoppers on within the first 5 min. After cooling at room temperature, the contents were mixed and centrifuged at 2000 x g for 30 min. One ml of supernatant was transferred to another test tube and 1.5 ml of 2,2-bipyridine solution (prepared by dissolving 10 g of 2,2-bipyridine (Sigma Aldrich Co., LLC, D216305) and 10 ml thiglycolic acid (Sigma Aldrich Co., LLC, T3758) in Milli-Q water and made up to 1000 ml) were added. This solution was mixed and absorbance was read at 519 nm against a blank. The apparent degradation of phytate was then calculated as:

\[
\text{Initial concentration of phytate - Residual phytate} / \text{Initial concentration of phytate} 
\]

Statistical analysis: The effect of each of the ions (Ca, Fe, Zn and Na) was assessed separately in a 3x2 factorial (3 levels of each ion and 2 levels of pH) design with 5 replicates per treatment. The data were analyzed for each time (30, 60 and 90 min) separately by the GLM procedure of Minitab 16. 32 Differences between means were determined using the Fisher’s least significant difference test. Pairwise comparison was conducted using Tukey’s HSD test, to compare means within each pH/mineral level. Differences between mean values were considered significant at p ≤ 0.05.

Results

Effect of Ca on phytate hydrolysis by phytase: The effects of added Ca at two pH levels (2.5, 6.5) on phytate hydrolysis by phytase are summarized in Table 1. Addition of Ca significantly (p < 0.001) reduced phytate hydrolysis over all incubation periods, especially at an inclusion level of 1% Ca per kg of diet. Phytate hydrolysis was lower (p < 0.001) at pH 6.5 than at pH 2.5, regardless of incubation period. The interaction between Ca and pH after 30 (p < 0.019) and 90 (p < 0.001) min of incubation was significant.

Effect of Zn on phytate hydrolysis by phytase: Phytate-P release was reduced (p < 0.001) by increasing levels of Zn in the media, with all periods of incubation (Table 2). Irrespective of incubation times, low pH (2.5) enhanced (p < 0.001) enzymatic degradation of phytate, whereas the reverse was the case with pH 6.5. There was a significant (p < 0.05) interaction between Zn and pH over the 90-min incubation period.

Effect of Fe level on phytate hydrolysis by phytase: Hydrolysis of phytate was significantly (p < 0.001) impaired as the concentration of Fe increased after 30 and 60 min of incubation (Table 3). Phytate degradation was lower (p < 0.001) at pH 6.5 than at pH 2.5 over all incubation periods. The interaction between Fe level and pH was also significant (p < 0.001).
There was a significant interaction between level of Na and pH after 60 min (p < 0.05) and 90 min (p < 0.001) of incubation.

Effect of Na level on phytate hydrolysis by phytase: Table 4 summarizes the effect of Na and pH on hydrolysis of phytate. After 90 min of incubation, both low pH and high Na levels significantly reduced (p < 0.001) the phytate hydrolysis. In contrast, after 60 of incubation, high pH (6.5) lowered (p < 0.001) the release of phytate-P by 5.6% compared to low pH (2.5). There was a significant interaction between level of Na and pH after 60 min (p < 0.05) and 90 min (p < 0.001) of incubation.

Effect of Ca on the degradation of phytate: The degradation of phytate, measured as loss in phytate content, was unaffected by the Ca level over all incubations periods, except after 60 min incubation (Table 5). Regardless of incubation time, the loss of phytate was higher (p < 0.001) at pH 2.5 than at 6.5. There was significant interaction between Ca and pH with the 30 (p < 0.001) incubation period.

### Table 1. Effect of Ca level on in vitro phytate phosphorus hydrolysis (µM of P released/min) by phytase at pH 2.5 and 6.5 after 30, 60 or 90 min of incubation.

<table>
<thead>
<tr>
<th>Items</th>
<th>Duration of incubation (min)</th>
<th>pH</th>
<th>Ca (%)</th>
<th>SEM</th>
<th>Main effects</th>
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<tr>
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<td>4.2b</td>
<td>a, b, c Means within a column without a common superscript are significantly different at the levels shown.</td>
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<tr>
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<td>0.19</td>
<td>0.09</td>
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</table>

**Source of variation** Probability > F

- Ca: 0.001
- pH: 0.001
- Ca x pH: 0.001

### Table 2. Effect of Zn level on in vitro phytate phosphorus hydrolysis (µM of P released/min) by phytase at pH 2.5 and 6.5 after 30, 60 or 90 min of incubation.

<table>
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<th>Zn (mg)</th>
<th>SEM</th>
<th>Main effects</th>
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<td>a, b, c Means within a column without a common superscript are significantly different at the levels shown.</td>
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<td>SEM</td>
<td>0.32</td>
<td>0.19</td>
<td>0.09</td>
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<td></td>
</tr>
</tbody>
</table>

**Source of variation** Probability > F

- Zn: 0.001 0.001 0.001
- pH: 0.001 0.001 0.001
- Zn x pH: 0.298 0.345 0.011

### Table 3. Effect of Fe level on in vitro phytate phosphorus hydrolysis (µM of P released/min) by phytase at pH 2.5 and 6.5 after 30, 60 or 90 min of incubation.

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<th>Items</th>
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<th>Fe (mg)</th>
<th>SEM</th>
<th>Main effects</th>
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<td>6.2</td>
<td>4.2b</td>
<td>a, b, c Means within a column without a common superscript are significantly different at the levels shown.</td>
</tr>
<tr>
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</table>

**Source of variation** Probability > F

- Fe: 0.001 0.001 0.547
- pH: 0.001 0.001 0.001
- Fe x pH: 0.001 0.001 0.001

### Table 4. Effect of Na level on in vitro phytate phosphorus hydrolysis (µM of P released/minute) by phytase at pH 2.5 and 6.5 after 30, 60 or 90 min of incubation.

<table>
<thead>
<tr>
<th>Items</th>
<th>Duration of incubation (min)</th>
<th>pH</th>
<th>Na (%)</th>
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</table>

**Source of variation** Probability > F

- Na: 0.320 0.001 0.001
- pH: 0.668 0.001 0.001
- Na x pH: 0.152 0.019 0.001

**Note**: Means within a column without a common superscript are significantly different at the level shown.
Effect of Zn on degradation of phytate: Table 6 summarizes the rate of phytate loss over the three different times of incubation. The different concentrations of Zn in the reaction solution had no effect (p > 0.05) on phytate loss over all incubation periods, except after 60 min (p < 0.05) incubation. The digestibility of phytate was more rapid (p < 0.001) at pH 2.5 than at 6.5, but when considered over only 90 min of incubation. The interaction between Zn and pH was significant (p < 0.05) with the 30 and 90 min of incubation.

Effect of Na on degradation of phytate: Table 7 indicates that a high Na (0.35%) level in the reaction media significantly increased phytate degradation over all incubation periods. High pH (6.5) increased (p < 0.001) phytate degradation after 60 (p < 0.001) and 90 (p < 0.05) min of incubation. There was a significant interaction (p < 0.001) between Na and pH after 60 and 90 min period of incubation.

Effect of Fe on the degradation of phytate: High concentrations of Fe, particularly 90 mg kg⁻¹ of diet, reduced (p < 0.05) the degradation coefficient of phytate over all incubation time (Table 8). Regardless of incubation time, phytate degradation was lower (p < 0.001) at pH 6.5 than at pH 2.5. There was a significant (p < 0.05) interaction effect observed between Fe concentration and pH only after 90 min of incubation.
Discussion

The anti-nutritional effect of PA in animal and human nutrition is well documented. Nolan et al. showed that phytic acid contains twelve dissociate hydrogen ions, of which six are strongly acidic (pK value 1.1-2.1), three weakly acidic (pK value 5.7-7.6) and the remainder have pK values of 10-12. Therefore, over a wide range of pH, ionization of hydrogen ions enables PA to bind with different cations (e.g. Zn, Ca, Mg, Fe, Cu and Mn) and proteins. This results in the formation of poorly soluble or stable mineral-phytate and protein-phytate complexes. 

The present study revealed that phytase-mediated phytate hydrolysis and subsequent release of inorganic P was highly pH-dependent. Importantly, phytate dephosphorylation by phytase under high pH conditions was considerably impaired, which is in agreement with Maenz et al. . The increased concentration of minerals (particularly Ca, Zn and Fe) also had significant negative effects on phytate hydrolysis by phytase, particularly at high pH. Excessive Ca concentration (1%) significantly reduced phytate hydrolysis, which is in agreement with Tamim and Angel . A subsequent increase in pH and Ca:PA ratio due to the high Ca level inhibits phytate hydrolysis by precipitating Ca as well as Ca-phytate complex as explained by Tamim et al. and Wise . In the current study, the Ca:PA molar ratios were equivalent to 31.2:1, 41.7:1 and 52.1:1 and the highest inhibition (15%) of phytate hydrolysis was found at 52.1:1 Ca:PA molar ratio at pH 6.5. This result is not consistent with the findings of Tamim and Angel, but partly agrees with Maenz et al. , who reported that about 36.1 and 7000:1 Ca:PA molar ratio was required for 50% inhibition of PP hydrolysis at pH 6.5 and 4, respectively. The results of these studies indicate that a relatively high Ca:PA molar ratio is required for a 50% inhibition of PP hydrolysis at pH below 5. The possible explanation may be that at low Ca:PA molar ratio, preferably mono-, di- or tri-Ca-phytate complexes are formed, which are soluble even over a wide range of pH (2-9) due to presence of potentially ionizable H ions in their structure.

There was a significant reduction in phytate hydrolysis in the media containing increased concentration of Zn, especially at high pH, which is in agreement with Gifford and Clydesdale . Greiner et al. showed that formation of Zn-phytate complex at neutral pH is one of the causes of reduced phytase activity. According to Oberleas and Harland , the Zn:PA molar ratio could influence the availability of Zn in diets. Maenz et al. reported that in the current study phytate hydrolysis was reduced by 12% at 0.17:1 Zn:PA molar ratio (equivalent to 50 mg Zn kg diet); a similar study reported 50% inhibition of phytate hydrolysis at 0.35:1 Zn:PA molar ratio at pH 6.5. This dissimilarity between the studies could be partly explained by the difference in their Zn:PA molar ratios. The observed negative role of excessive Fe on phytate hydrolysis at high pH in the current study is in agreement with Pang and Applegate . Moreover, Maenz et al. reported that Fe had a greater inhibitory effect on phytate hydrolysis at high pH, whereas Fe showed similar effect at low pH, which is consistent with the present study. Phytate chelation with trivalent cations (Fe) is more resistant to hydrolysis than with divalent cations (Fe, Ca), because the intermolecular bond between Fe and phytate is so strong and is not weakened through a reduction in media pH as researched by Maenz et al. and Dao . Under such circumstances, phytase activity would be reduced. 

The pattern of response to pH on phytate in the presence of Na was irregular. No clear reasons can be found for this, but it has been suggested by Jog et al. that NaCl may alter the structural conformation of fungal or alkaline phytases over a wide range of pH (1.9-6.0) by changing the electrostatic repulsion of media, which subsequently facilitates phytate hydrolysis. The hydrolysis of phytate in the current study was unaffected at high concentration of NaCl (108 mM) at low pH (2.5), which is consistent with the findings of Ullah et al. Although the same authors found severe inhibition of E. coli phytase activity at pH above 3, the current study observed only a 10% reduction of hydrolysis at pH 6. This discrepancy may be due to the variation in the concentration of NaCl and type and dose of enzyme used between the studies.

Residual phytate was measured to estimate the actual loss (degradation) following hydrolysis with phytase, as further confirmation of the release of Pi. The lower degradation of phytate at pH 6.5 with all the tested minerals (Ca, Zn and Fe) further explains the inability of phytase to hydrolyze phytate at higher pH. According to Simons et al. , complete hydrolysis of phytate in canola meal was not achieved at pH 5.8. As most of the microbial phytase remains active within pH 4.5 to 6.0, changing the pH to below 3.0 or above 7.5 can dramatically reduce the stability of phytase and subsequently reduce phytate digestibility as investigated by Greiner et al. and Liu et al. .

However, the measurement of phytate degradation could be over or underestimated, because phytate exists in a very fine colloidal state, which requires high centrifugal forces to pellet out of the solution as explained by Maenz et al. and Champagne and Fisher. In the present study, about 70-80% of phytate was degraded at the pH and incubation conditions tested, which partly agrees with Newkirk and Classen, who reported that rapid hydrolysis (70-80%) of phytate occurs within 30 min of incubation after which the process process took long time to hydrolyze. This may be due to the accumulation of lower inositol phosphate (IP, IP, IP, IP) in the reaction mixture, which is known to reduce the solubility of both Ca and Zn-phytate as explained by Xu et al. . The method that was used in the current study for phytate measurement is unable to separate the different inositol phosphate esters but instead only measures the leftover phytate as ferric phytate. Therefore, it is unclear whether the accumulated lower inositol phosphate in the reaction solution had any effect on the phytase activity. More importantly, in the present study, all incubations were done discretely to the end point rather than continuously with the same media. Therefore, it is not certain whether the process could impair phytate hydrolysis due to buildup of lower inositol phosphate in the reaction mixture. In support of this, Konietzny and Greiner have observed that a phytase with broad substrate specificity could prevent the accumulation of intermediate inositol phosphate esters by effective degradation of phytate to myo-inositol monophosphate.

Conclusions

Minerals that were tested in this study showed inhibitory effects towards phytate degradation, especially at pH 6.5. A reduction in degradate of phytate at high pH (6.5) supports the hypothesis of this study that high pH in addition to high mineral concentration synergistically reduces the solubility of phytate, rendering it inaccessible to phytase degradation. Besides, the minerals tested in this study showed minimal or no inhibitory effect on phytate
hydrolysis at the levels recommended in poultry diets. Although the \textit{in vitro} results may not fully reflect the \textit{in vivo} system, the findings of this study clearly indicate that mineral levels should be carefully considered for optimum benefit from supplemental phytases. These relationships would need to be tested in animal feeding trials.

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References
15Nolan, K. B., Duffin, P. A. and McWeeny, D. J. 1987. Effects of phytate on mineral bioavailability. \textit{In vitro} studies on Mg\textsuperscript{2+}, Ca\textsuperscript{2+}, Fe\textsuperscript{3+}, Cu\textsuperscript{2+} and Zn\textsuperscript{2+} (also Cd\textsuperscript{2+}) solubilities in the presence of phytate. J. Sci. Food Agric. 40:72-85.


