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The impact of processing on phytic acid, *in vitro* soluble zinc and Phy/Zn molar ratio of faba bean (*Vicia faba* L.)

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Abstract

Faba bean is a major source of micronutrients in many rural areas. Unfortunately the bioavailability of zinc from faba bean is low. Influence of soaking, germination and fermentation with expectation of increasing its bioavailability was investigated. Fermentation treatments were most effective in decreasing phytic acid (48-84%), followed by soaking at 10°C after preheating (36-51%). Steeping of faba beans for 24 h at 25°C had the least effect on phytic acid removal (9-24%). With increased germination time at 30°C, phytic acid progressively decreased from 9 to 69%. Most wet processing procedures, except soaking after wet preheating, caused losses of dry matter and zinc (8-22%). *In vitro* zinc solubility, as a percentage of total zinc in soaked faba bean after dry preheating, was significantly higher than in raw faba bean ($P < 0.05$). Probably complex association between dietary fiber and zinc is the reason for the poor bioavailability of zinc in faba bean.

Introduction

The faba bean is one of the oldest crops that ranks sixth in production among the different legumes grown in the world (Concepcion *et al*., 1998). Faba beans are a good source of energy, proteins, vitamins, minerals and dietary fiber. They are relatively inexpensive compared to meat foods and as they have a high carbohydrate content (50–65%). Among that 50-65% carbohydrates only about 40% are starch and sugars, and other parts are dietary fibers. Therefore, faba beans are beneficial for human nutrition perspectives because they are good source of energy, protein and dietary fiber. In China, plant foods provide at least 50% of the dietary energy and nutrients, and faba bean is one of the most important legumes (Ma *et al*., 2005). Faba beans are a good source of dietary minerals, such as phosphorus, calcium, sulphur, zinc and iron. However, the utilization of the minerals are limited by the presence of phytic acid. Phytic acid (myoinositol 1, 2, 3, 4, 5, 6 hexakidishydrogen phosphate) is common in faba beans and is the principal storage form of phosphorus in many dry beans. The typical phytic acid content in faba beans is 8.58 mg g^{-1}. A nutritional concern about the presence of phytic acid in dry beans arises from the fact that phytic acid decreases the bioavailability of essential minerals and may possibly interfere in the utilization of proteins due to phytate-protein and phytate-mineral-protein complexes.

The bioavailability of minerals from foods is defined as the proportion of the minerals that can be absorbed and utilised within the body. Solubility of minerals, pH of intestinal lumen, dietary factors and retention time at the digestion and absorption site influence the bioavailability of minerals (Larsson *et al*., 1997). Solubility of zinc could be predicted by molar ratios of phytic acid to zinc, HCl (hydrochloric acid)-extractability and *in vitro* solubility of zinc. The inhibitory effect of phytate on zinc absorption can also be predicted *in vitro* by the molar ratio of phytate to zinc (Phy/Zn). Davies and Olpin (1979) showed that molar ratios above 10-15 progressively inhibited zinc absorption and were associated with suboptimal zinc status in rats fed with egg-albumenbased diets with added phytate (0-7.43 g kg^{-1}) or zinc (18-144 mg kg^{-1}).

Zinc is the fourth important micronutrient after vitamin A, iron and iodine, and is now receiving increasing global attention. Zinc deficiency, although not completely assessed, is believed to be as widespread as that of iron and is a cause for concern, especially in the developing countries. Although many factors are responsible for iron deficiency, the most

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likely cause of this nutritional problem in developing countries is the poor bioavailability of dietary iron (Gibson, 2000; Sandberg, 2002). Animal foods are rich sources of zinc in our diet, but this micronutrient is derived mainly through food grains by a majority of the population in developing countries. Staple foods in developing countries include cereals and legumes, which are the main sources of zinc for most of the population, but even if net zinc intake appears adequate, compromised zinc status is common (Gibson *et al*., 1998).

Phytate is especially known as a chelating agent that reduces the bioavailability of divalent cations. It was reported that wet processing (including soaking, germination and fermentation) leads to a reduction in phytic acid and increase the solubility of zinc in foods, which could thus improve bioavailability of minerals in cereals and legumes (Honke *et al*., 1998; Lestienne *et al.*, 2005b).

Although a complete removal of phytic acid has not been reported, wet processing technologies can help to reduce phytic acid so that solubility of minerals in foods could be increased. To our knowledge, there is little information in the literature on wet processing of faba bean. The objectives of the present study were 1) to investigate the effects of wet processing (soaking, germination and fermentation) on the dry matter, zinc and phytic acid content in faba bean and 2) to study the effects of phytic acid content and Phy/Zn molar ratio in raw and wet processed faba beans on the solubility of zinc.

Materials and Methods

Materials

Faba bean (Qidou 2, cultivated in Jiangsu province and harvested in 2007) was purchased at a local market in Nanjing (Nanjing Jiangsu).

Soaking

Faba bean was either heated as is, in a drying oven for 30 minutes at 100°C (dry-preheated), or mixed with demineralized water (1:1, w/v) and autoclaved for 10 min at 115°C (wet-preheated). After this step, the faba bean was soaked at 10°C with demineralised water (1:5, w/v). The mixture was either left as is, natural (pH 5.9), or adjusted pH to 3.5 with 5N HCl. Faba bean was soaked for either 1 (wet preheated samples) or 7 (dry heated samples) days. After soaking, the faba bean was freeze-dried and stored in sealed plastic bags at 4°C for further analysis. Table 1 summarizes the different soaking conditions used. All soaking treatments were done in triplicates.

Table 1. Preheating and soaking conditions

Treatment Code	Preheating		Soaking medium		Soaking time(d)	
	Dry heated[A]	Wet heated[B]	Natural demineralized water(pH 5.9)	Acidic solution[C]	1	7
SDN	√		√			√
SDA	√			√		√
SWN		√	√		√	
SWA		√		√	√	

SDN: Dry heated+Natural demineralized water (pH 5.9) + Soaked 7 days.
SDA: Dry heated +Acidic solution + Soaked 7 days.
SWN: Wet heated+ Natural demineralized water (pH 5.9) + Soaked 1 day.
SWA: Wet heated + Acidic solution + Soaked 1 day.
[A]:Faba bean was heated in drying oven at 100°C for 30 min before soaking.
[B]: Mixture of faba bean and demineralized water (1:1, w/v) was heated in autoclave at 115°C for 10 min.
[C]: pH of mixture was adjusted to 3.5 with 5N HCl before soaking.

Germination

Steeping and sprouting

Steeping and sprouting were carried out following the procedure developed by Capanzana and Buckle (1997). Approximately 50 g of faba bean were used. At the steeping phase, faba bean was soaked in 150 ml of demineralised water in plastic boxes at 25°C. Small samples were taken out from the incubator at intervals of 4, 8, 12 and 24 h. After steeping for 24 h, faba bean was separated by decanting and placed in plastic boxes and covered with punctured lids for sprouting for 120 h at 30°C. Samples were taken at 24, 48, 72, 96 and 120 h. Treatments of steeping and sprouting were carried out in duplicates. After steeping and sprouting, samples were freeze-dried and stored in sealed plastic bags at 4°C for further analysis.

Fermentation

Mixtures of faba bean and demineralized water (ratio 1:5, w/v) were fermented naturally for 24 h at 30°C. Starting on the second day and after each consecutive day, a fresh mixture was inoculated with 10% of the water of the previously fermented mixture, in order to obtain an accelerated fermentation by enrichment of acidifying microbiota (Nche *et al*., 1994). All treatments were carried out in triplicates. After fermentation, samples were freeze-dried and stored in sealed plastic bags at 4°C for further analysis.

Zinc analysis

Total zinc content

Total zinc contents were determined by atomic absorption spectrophotometry (Varian SpectrAA 200, Victoria, Australia) after dry mineralization for 2 h at 530°C. Depending on the different treatments, 2-4 g of ash were weighed in a silicon evaporating dish. Next, the ashes were wet-acid digested with nitric acid on a hot plate and solubilized with 25 ml of 0.5N HCl.

In vitro *soluble zinc content*

In vitro soluble zinc was defined as the relative amount of zinc that becomes soluble after enzymatic treatment. Faba bean samples were sequentially digested with enzymes, including amylase, pepsin, pancreatin and bile, under certain conditions following the enzymatic degradation procedure described by Kiers (2000). Mixtures were centrifuged at 5000 *g* for 15 min at 4°C. The resulting supernatant was filtered (0.30 μm membrane, FP 030/3, Kaijie, Hangzhou, Zhejiang) and frozen until further analysis. Zinc levels were analysed by atomic absorption spectrophotometry. Each sample was enzymatically extracted in duplicates.

Phytic acid

Phytic acid contents were determined by the method of Haug and Lantzsch (1983). The sample extract (with 0.2N HCl) was heated with an acidic iron (III) solution of known iron content (0.2 g ammonium iron (III) sulphate-12 H_2O was dissolved in 100 ml 2N HCl and volume made up to 1000 ml with distilled water). The phytic acid was precipitated with an acidic iron-III-solution of known iron content. Phytic acid content in the supernatant was measured as the decrease in absorbance of iron content using 2,2-bipyridine (Dissolve 10 g 2,2'-bipyridine and 10 ml thioglycollic acid in distilled water and make up to 1000 ml) at 419 nm.

Molar ratio

Molar ratios of Phy/Zn were calculated by first converting the levels of phytic acid, zinc in mg g^{-1} to millimoles g^{-1} using molecular mass unit of 660.8 for phytic acid and atomic mass unit of 65.3 for zinc respectively. Corresponding molar ratios were then obtained using these values.

Statistical analysis

Data were analysed with SPSS 13.0 for windows. The mean and standard deviation of means were calculated. The data were analysed by one-way analysis of variance (ANOVA). Duncan's multiple range test was used to separate means. Significance was accepted at probability $P < 0.05$.

Results

Effect of soaking, fermentation and germination on dry matter, phytic acid and zinc

Dry matter

Table 2 shows the different results obtained. Wet treatments (soaking, fermentation and germination) decreased dry matter content of faba bean by 43.8-79.4 g kg^{-1} ($P < 0.05$). Dry matter reduction in accelerated fermented faba bean was 46.3-54.9 g kg^{-1} ($P < 0.05$). Germination cause a higher dry matter loss in faba bean than the other treatments. Especially faba beans germinated for 120 h reduced dry matter by 79.4 g kg^{-1} ($P < 0.05$). Dry matter reduction during soaking ranged from 50.3-69.0 g kg^{-1} ($P < 0.05$).

Phytic acid

The concentrations of phytic acid in raw and treated faba beans are presented in Table 2. Level of phytic acid in untreated faba bean was 8.36 mg g^{-1}, while it ranged from 1.34 to 7.70 mg g^{-1} in treated samples. Soaking decreased the phytic acid content to 4.10-5.35 mg g^{-1} ($P < 0.05$) with wet preheated faba bean having lower levels than dry preheated. Faba bean fermented with enrichment starter (AF3) had the lowest phytic acid content (1.34 mg g^{-1}), but natural fermentation (NF) also reduced the initial phytic acid level by 4.02 mg g^{-1} ($P < 0.05$). Contents of phytic acid slightly decreased from 6.86 to 6.12 mg g^{-1} ($P > 0.05$) in the steeping phase during germination, and decreased further from 7.70 to 2.66 mg g^{-1} ($P < 0.05$) in sprouting phase during germination over a period of 24 h to 120 h.

Zinc

Zinc contents of raw and processed faba beans are presented in Table 2. Zinc contents of the most processed faba beans were ranged from 25.3 to 28.4 mg kg^{-1}, while the raw faba bean contained 32.6 mg kg^{-1}. However, faba bean soaked after wet heating contained significantly less zinc (13.2 mg kg^{-1}, $P < 0.05$). Further data analysis showed (Table 2) that sprouting and accelerated fermentation had significant effects on decrease of phytic acid ($P < 0.05$).

Molar ratio of phytic acid to zinc (Phy/Zn) and in vitro solubility (IVS) of zinc

The molar ration of Phy/Zn in untreated raw faba bean was 25.3, but it ranged after various processing from 4.8 to 33.4. The lowest ratio was obtained by fermentation (AF3), and the highest in soaked faba bean after wet preheating. The amount of IVS-zinc of treated faba bean is assumed to provide an indication of the amount of zinc available for absorption *in vivo*. The IVS is expected to be more relevant than the phytate to zinc ratio, which is only based on contents. Untreated faba bean had 10.3 mg kg^{-1} of IVS-zinc and, after the various treatments, IVS-zinc levels ranged from 4.9-13.1 mg kg^{-1} (Table 3).

Discussion

In this study, we found that soaking, germination and fermentation could significantly decrease phytic

Table 2. Effects of soaking, germination and fermentation on dry matter, phytic acid and zinc content in raw and processed faba beans.[A,B]

Treatments	Methods	Dry matter(g kg^{-1})[B,C]	Phytic acid(mg g^{-1})[B,C]	Zinc(mg kg^{-1})[B,C]
Untreated	None	584.5±3.6	8.36±0.2	32.6±2.7
Soaking	SDN	534.2±4.1^a	5.35±0.4^a	27.9±1.4^a
	SDA	530.1±2.8^a	5.10±0.4^a	27.6±0.6^a
	SWN	520.8±3.4^a	4.60±0.5^a	13.6±0.8^b
	SWA	515.5±1.9^a	4.10±0.5^a	13.2±0.4^b
	Sig(df=3)	0.295	0.118	0.000
Natural and Accelerated Fermentation	NF	536.6±2.6^a	4.34±0.3^a	28.4±0.7^a
	AF1	538.3±2.4^a	3.42±0.4^b	28.1±0.5^a
	AF2	535.4±2.1^a	2.60±0.2^c	28.3±0.3^a
	AF3	529.6±2.2^a	1.34±0.2^d	27.8±1.2^a
	Sig(df=3)	0.823	0.000	0.089
Germination: (1) Steeping	ST4	540.7±2.7^a	6.86±0.5^a	27.6±1.2^a
	ST8	535.9±3.1^a	6.56±0.3^a	27.1±1.8^a
	ST12	532.4±2.7^a	6.35±0.4^a	26.8±1.0^a
	ST24	528.4±2.9^a	6.12±0.4^a	26.8±1.1^a
	Sig(df=3)	0.703	0.068	0.062
Germination: (2) Sprouting	G24	530.0±1.8^a	7.70±0.6^a	25.3±0.9^a
	G48	521.3±2.7^a	6.77±0.5^a	26.8±0.7^a
	G72	517.3±1.3^a	4.60±0.4^b	26.4±0.6^a
	G96	510.3±1.6^a	3.43±0.3^c	27.2±0.4^a
	G120	505.1±2.0^b	2.66±0.2^c	27.3±1.1^a
	Sig(df=4)	0.182	0.000	0.076

Abbreviations: SDN, SDA, SWN, SWA: see Table 1; NF: natural fermentation without starters addition at 30 °C for 24 h; AF1/AF2/AF3: accelerated fermentation with one/two/three cycles of starter enrichment for 24 h and fermented at 30°C for 24 h; ST4/ST8/ST12/ST24: faba bean was steeped for 4, 8, 12 and 24 h, respectively; G24/G48/G72/G96/G120: faba bean was germinated for 24, 48, 72, 96 and 120 h, respectively.

[A]: All data are expressed on dry matter basis.

[B]: Contents of dry matter, zinc and phytic acid in all materials: mean ± standard deviation (n = 3).

[C]: Values within a column without common superscripts are significantly different ($P < 0.05$).

Table 3. *In-vitro* solubility of zinc and molar ratios of phytic acid to zinc in untreated and treated faba beans

Treatments	Methods [A]	*In vitro* soluble zinc (mg kg^{-1})[B,C]	Solubility (%)[C,D]	[Phy]/[Zn][C,E]
Untreated	None	10.3±0.2	31.6	25.3
Soaking	SDN	13.1±0.6^a	46.8^b	18.9^b
	SDA	11.9±0.4^a	43.1^b	18.3^b
	SWN	4.9±0.7^c	36.2^c	33.4^a
	SWA	9.1±0.2^b	68.9^a	30.7^a
	Sig(df=3)	0.000	0.000	0.000
Natural and Accelerated Fermentation	NF	7.8±0.3^b	27.5^c	15.1^a
	AF1	11.4±0.4^a	40.6^b	12.0^b
	AF2	12.5±0.2^a	44.3^a	9.1^c
	AF3	8.1±0.5^b	29.2^c	4.8^d
	Sig(df=3)	0.002	0.000	0.000
Germination: (1) Steeping	ST4	8.9±0.5^b	32.1^b	24.6^a
	ST8	8.5±0.2^b	31.5^c	23.9^a
	ST12	9.5±0.3^b	35.6^b	23.4^a
	ST24	11.6±0.5^a	43.2^a	22.6^a
	Sig(df=3)	0.001	0.000	0.235
Germination: (2) Sprouting	G24	10.8±0.4^b	42.8^b	30.1^a
	G48	9.0±0.3^b	33.6^d	25.0^b
	G72	9.8±0.4^b	37.2^c	17.2^c
	G96	8.0±0.5^c	29.3^c	12.5^d
	G120	13.0±0.4^a	47.6^a	9.6^c
	Sig(df=4)	0.000	0.000	0.000

[A]: Abbreviations of treatments are the same as in Table1 and Table 2.

[B]:Contents of zinc in all materials: mean ± standard deviation (n = 3).

[C]: Values within a column without common superscripts are significantly different ($P < 0.05$).

[D]:Expressed on dry matter basis.

[E]: Calculated on molar ratio of phytic acid to zinc in untreated and treated faba beans.

acid in faba bean. Our results agreed well with reports about similar treatments on cereals, such as fermentation of white rice flour (Reddy and Salunkhe, 1980), and steeping and sprouting of oats or corn (Larsson and Sandberg, 1995; Fageer *et al.*, 2004). We found that soaking, germination and fermentation have different efficacies in reducing the content of phytic acid. Accelerated fermentation, especially fermentation with starters, enriched three times is the most effective approach for reducing phytic acid in faba bean. In sorghum and brown rice, soaking and natural fermentation had similar impacts on phytic acid (Mahgoub and Elhag, 1998; Liang *et al.*,2008) which is in line with our results with NF.

Numerous authors have reported that processes, such as soaking and germination, activate the endogenous phytases which are able to hydrolyse IP6 to free myo-inositol and inorganic phosphate via lower inositol phosphate esters (IP5–IP1) (Kozlowska *et al.*, 1996; Honke *et al.*, 1998). Although decreases in phytic acid content in cereals and legumes caused by fermentation and germination are mainly due to the action of phytase, some authors suggested that diffusion is a cofactor which affected the hydrolysis of phytic acid in the soaking process (Henderson and Ankrah, 1985; Mahgoub *et al.*, 1998). However, other studies have proposed that the activity of endogenous phytase was the main factor leading to reduction of phytic acid during soaking (Lestienne *et al.*, 2005b). Soaking of millet, soya bean, maize, sorghum, and mung bean at 30°C for 24 h decreased the contents of phytic acid by 4-51% (Lestienne *et al.*, 2005a; Lestienne *et al.*, 2005b), and soaking of sorghum flour (80% extraction) at room temperature for 24 h reduced phytic acid levels by 16-21% (Mahgoub *et al.*, 1998). Soaking of pounded maize for 1 h at room temperature already led to a reduction of phytic acid by 51% (Henderson *et al.*, 1985). In a study by Carlson and Poulsen (2003) in which heated and non-heated barley and wheat were soaked, reduction of phytic acid was clearly higher in the non-heated treatment. They ascribed the difference in phytate degradation to the inactivation of endogenous phytase. Our results demonstrated that the reduction in phytic acid content during germination was a time-dependent process, which agrees with previous studies reporting that the activity and/or production of phytase increased during steeping (Henderson *et al.*, 1985; Larsson *et al.*, 1997). Abdalla *et al.* (1998) suggested that enzymatic hydrolysis of phytic acid by both endogenous phytase and microbial phytase (lactic acid bacteria can produce significant levels of

phytase) may account for most of the loss of phytic acid during fermentation (Abdalla *et al.*, 1998; Towo *et al*., 2006). Our observation that faba bean contained a significantly lower phytic acid after fermentation is similar to results obtained with fermented whole wheat flour (Lestienne *et al*., 2005c). Collectively, these findings suggest that both endogenous phytase and microbial phytase may have contributed to reduction in phytic acid in fermented faba beans. We hypothesized that during accelerated fermentation, endogenous phytase was activated and accumulated and, at the same time, with growth of micro organisms, microbial phytase also accumulated. From Tables 4 and 2 we observed that a longer fermentation in a lower pH medium decreased phytic acid content in faba beans. From Table 2 and Table 4 we also observed that natural fermentation (NF) was much less effective for phytate removal ($P < 0.05$) and had higher pH than fermentation with cycle 3 starter (AF3). The reasons for different phytic acid degradation in fermentation might be the conditions of hydrolysis chosen (pH, reaction mediums). Optimal pH values for plant phytase and microbial phytase were about 5 ± 0.5 and 3 ± 0.5, respectively. So we suggested that relatively lower pH values were beneficial for the hydrolysis of phytic acid.

Table 4. Initial and final pH values after natural, and accelerated fermentation of faba beans

Treatment[A]	Initial Ph[B]	Final Ph[B]
NF	7.5(7.7, 7.3)	5.4(5.2, 5.6)
AF1	6.4(6.5, 6.3)	5.3(5.1, 5.5)
AF2	6.0(6.2, 5.8)	5.2(5.1, 5.3)
AF3	5.7(5.6, 5.8)	4.8(4.5, 5.1)

[A]: Abbreviations are the same as in Table 2.
[B]: Average (values of replicates).

The retention and solubility of individual zinc was also affected by processing conditions. For zinc, only soaking after wet processing resulted in significantly lower retention compared with other treatments. This can be explained by the effect of wet preheating. Whereas dry preheating only results in minor physical damage, such as fissures (or cracks) in the kernels, wet preheating induced swelling of the kernel. The swollen kernel would allow better zinc and soluble fractions diffusion and we expect this to be the main reason for the higher loss of zinc and dry matter in soaked faba bean after wet preheating compared with dry preheated faba beans.

For accelerated fermentation, the Phy/Zn molar ratios were all below 15 which consequently may have an acceptable level of zinc bioavailability. We expected that, with lower molar ratios of Phy/Zn, the *in vitro* solubility of zinc would increase, but there were no relationship between Phy/Zn and *in vitro* soluble zinc content. This discrepancy was also observed by authors who studied cereals, such as sorghum, millet and millet bran fractions (Lestienne *et al.*, 2005b).

Faba beans are rich in dietary fibers (Concepcion *et al*., 1998). Dietary fibers are known to bind with nutritionally significant minerals. Dietary fibers such as cellulose, hemicellulose, pectins, other polysaccharides, and lignin may form insoluble complexes with mineral elements and thus reduce bioavailability of minerals (Rendleman and Grobe, 1982; Persson *et al*., 1987). Earlier studies reported that the availability of calcium, iron, and zinc from cereal foods was very poor (Camire and Clydesdale, 1982; Rendleman, 1982; Rendleman *et al*., 1982; Maha *et al*., 1997) and that the affinity of dietary fibers for different mineral elements varied (Maha Lakshmi *et al.*, 1997). Ekholm *et al.* (2003) found the solubility of zinc from high dietary fiber oat samples was very poor. The higher dietary fiber content of the oat bran sample bound the zinc tighter than the oat flake sample with a lower dietary fiber content. According to the results of our study, decreases in phytic acid content of faba beans in wet processing treatments did not increase the solubility of zinc. It seems that componets of dietary fiber other than phytic acid are more important in binding zinc and cause poor solubility of zinc in faba bean. Probably complex association between dietary fiber and zinc might be the reason for the poor bioavailability of zinc in faba beans.

From this study, we conclude that neither soaking, germination, nor fermentation significantly improve the apparent bioavailability of zinc in faba bean. It may be of interest to combine wet processing methods, including phytase treatment, with other approaches, such as the application of uptake enhancers, to improve the availability of zinc in faba bean.

Conclusions

Soaking, germination and fermentation decreased phytic acid content in faba bean. The most effective approach, which was accelerated fermentation 3 could reduce 7.02 mg g^{-1} of total phytic acid and could decrease the molar ratio of phytic acid to iron below 5.

The sharp decrease of Phy/Zn ratio in fermented faba beans would suggest that fermentation could be a successful way to increase zinc bioavailability. However, results from in vitro solubility measurement of zinc showed little improvement in fermented faba beans over untreated raw faba bean. This could result from the presence of components such as dietary fiber leading to the formation of insoluble zinc complexes.

It remains to be investigated to what extent this would affect *in vitro* zinc-solubility and bioavailability.

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