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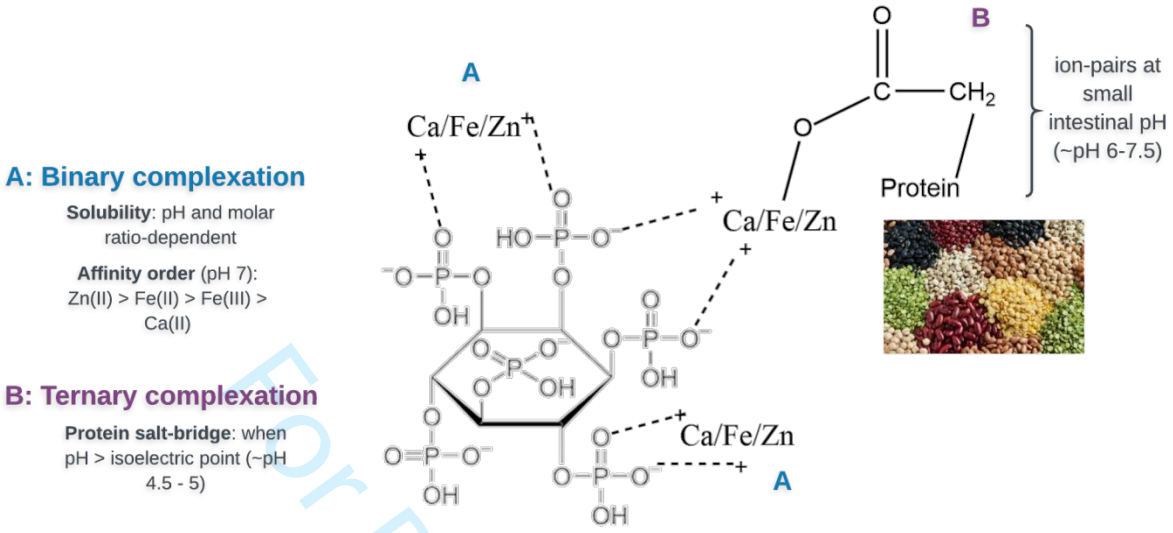
Graphical Abstract



Revisiting Phytate-Element Interactions: Implications for Iron, Zinc and Calcium bioavailability, with emphasis on legumes

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Myo-Inositol hexakisphosphate or phytic acid concentration is a prominent factor known to impede divalent element bioavailability in vegetal foods including legumes. Both *in vivo* and *in vitro* studies have suggested that phytic acid and other plant-based constituents may synergistically form insoluble complexes affecting bioavailability of essential elements. This review provides an overview of existing investigations on the role of phytic acid in the binding, solubility and bioavailability of iron, zinc and calcium with a focus on legumes. Given the presence of various interference factors within legume matrices, current findings suggest that the commonly adapted approach of using phytic acid-element molar ratios as a bioavailability predictor may only be valid in limited circumstances. In particular, differences between protein properties and molar concentrations of other interacting ions are likely responsible for the observed poor correlations. The role of phytate degradation in element bioavailability has been previously examined, and in this review we re-emphasize its importance as a tool to enhance mineral bioavailability of mineral fortified legume crops. Food processing strategies to achieve phytate reduction were identified as promising tools to increase mineral bioavailability and included germination and fermentation, particularly when other bioavailability promoters (e.g. NaCl) are simultaneously added.

Keywords: phytic acid; mineral bioavailability; legumes

1. Introduction

Essential element deficiencies constitute a large sector of micronutrient deficiencies that affect 2 billion people globally (Bailey, West, & Black, 2015). It has been reported that approximately a fifth of the population are at risk of zinc deficiency, whilst 30% are anaemic, largely as a result of iron deficiency (United Nations & FAO, 2019). Global calcium intake has also reported to be low in many countries (Balk et al., 2017),

paralleled with the 200 million people estimated to suffer from osteoporosis (Sözen, Özışık, & Başaran, 2017). Legumes are a key contributor to nutrient intake amongst cereals in populations consuming monotonous plant-based diets and is thus a food target to alleviate essential element deficiencies. However, a large body of evidence suggests that the bioavailability of Iron (Fe), Zinc (Zn) and Calcium (Ca) are relatively low from legumes, even when they are present at elevated concentrations (DellaValle & Glahn, 2014; DellaValle, Glahn, Shaff, & O'Brien, 2015; Feitosa et al., 2018; Petry, Egli, Zeder, Walczyk, & Hurrell, 2010; Vaz-Tostes, Verediano, de Mejia, & Brunoro Costa, 2016; Weinborn et al., 2015).

Myo-Inositol hexakisphosphate (IP_6), also known as phytic acid (PA), is a hexaphosphoric ester of cyclohexane. It naturally occurs in legumes at high levels and is often ascribed as a key factor in poor element bioavailability through reducing solubility at the lumen level (Ahmed, Randhawa, & Sajid, 2014; Sandberg, 1991). Phytic acid is known to form insoluble complexes with divalent cations such as iron, zinc and calcium that cannot be dissociated for absorption within the small intestine and particularly the duodenum, the site where most elemental absorption occurs (Kiela & Ghishan, 2016). The significance of phytic acid in impeding element absorption led to the development of a database by FAO in 2018 (Dahdouh et al., 2019), supporting that legumes are one of the highest sources of phytate (de Almeida Siqueira, Mendes, & Arruda, 2007; Petry et al., 2016). Although the level of seed phytate can be reduced through genetic approaches, it cannot be fully eliminated (Raboy, 2020). Low phytate mutant phenotypes are also often linked to undesirable effects on the plant, leading to reduced germination and stress tolerance, as well as poor seed filling (Panzeri et al., 2011). Furthermore, an increase in seed phytic acid has also been shown in crops grown under projected climate change conditions (e.g. elevated CO_2) (Dietterich et al.,

2015), which is concomitant with a reduction in the level of Fe, Zn and Ca (Loladze, 2014). Together, these factors infer that phytate will continue to be a debilitating component of human diets in the imminent future.

Previous literature reviews on phytic acid have only briefly reviewed interactions in the context of food matrices (Cheryan & Rackis, 1980; Kumar, Sinha, Makkar, & Becker, 2010; Lönnerdal, 2002; Weaver & Kannan, 2002), and many have contributed to the knowledge that its reduction likely enhances element bioavailability from vegetal matrices (Abdulwaliyu et al., 2019; Gibson, Bailey, Gibbs, & Ferguson, 2010; Oatway, Vasanthan, & Helm, 2001). However, an understanding of the composition of insoluble complexes and how it is linked to low bioavailability remains limited. Although phytic acid is considered a major inhibitory factor against iron absorption, some human studies support a role of interactions between phytic acid and other plant factors in reducing element bioavailability, which are particularly evident with legumes. For example, Hurrell et al. (1992) examined iron absorption in a human intervention trial. They found that iron absorption increased four- to fivefold when phytic acid was reduced to < 0.01 mg/g of isolate, but even residual phytate were strong inhibitor. The study indicated that even after removal of virtually all the phytic acid, iron absorption from soy protein was still only half that of the egg white control. Consequently, it was concluded that other factors might contribute to the poor bioavailability of iron from soy protein. Another human study by Petry et al. (2010) demonstrated that in beans, dephytinization without simultaneously decreasing polyphenols did not lead to an increase in Fe absorption. Similarly, dephytinization was only found to partly mitigate some inhibitory effects of polysaccharide fractions of Faba bean in reducing Zn absorption in rodent models (Rubio, Grant, Dewey, Bremner, & Pusztai, 1994). Several other analyses of *in vitro* Fe-binding in pea and faba have also

found poor correlation between element solubility and phytic acid concentration (Bangar et al., 2017; Luo, Gu, Han, & Chen, 2009). Findings of these investigations indicate that the inhibitory properties of phytic acid may be synergistic with other components of the legume matrix, which can exacerbate its complexation properties towards essential element reducing bioavailability. These factors may include certain polyphenols, tannins, oxalates, fibres as well as microstructure and state of aggregation (Rousseau, Kyomugasho, Celus, Hendrickx, & Grauwet, 2019). Additionally, food processing techniques, such as thermal treatments may mediate or reduce the degree of interactions between phytate and these factors (Feitosa et al., 2018).

The current review aims to provide an updated narrative on studies examining the effects of phytic acid on Fe, Zn and Ca bioavailability, its chemical properties as it relates to legumes, and the existing knowledge of phytate binding behaviour in the presence of these cations. Due to the absence of reviews in this area, legume proteins and their role in the impacts of phytate on Fe, Zn and Ca bioavailability are also comprehensively discussed. We postulate that the role of phytate in these elements' bioavailability is highly complex, and that a standard predictive approach based on the phytate to element molar ratio can only be of limited value in predicting element bioavailability.

2. Phytic acid in legume seeds

Inositol hexaphosphate (IP_6) is invariably present in legume seeds as source of inositol and phosphorous to be utilised during germination (Lott, Ockenden, Raboy, & Batten, 2000). In the human body, IP_6 can be internalised by cells thus can affect intercellular processes; whiles its metabolites inositol is a precursor for intracellular messaging molecules, and phosphorous a macronutrient constituent of key energy molecules such as ATP (Schachtman, Reid, & Ayling, 1998). Whilst there is little IP_6 in the immature

legume seed (Moore et al., 2018), a substantial quantity is synthesized and accumulated during maturation, constituting 60-90% of the total seed phosphorous at dormancy (Kumar et al., 2010). Subsequently, positive correlations between IP_6 and total phosphorous have been observed in legumes. The IP_6 content of legumes vary significantly between species, genotypes, and growth location (Oomah et al., 2011), and is shown in **Table 1**.

IP_6 is found complexed to divalent elements such as magnesium, calcium, iron and zinc *in planta* in various proportions (Gabaza, Muchuweti, Vandamme, & Raes, 2017; Skoglund, Carlsson, & Sandberg, 2009). The complex is named phytin, and its mineral composition vary with plant origin (Sandberg & Scheers, 2016). There are some reports of phytin being extractable from legume seeds by soaking (Hummel et al., 2020; Luo & Xie, 2013), whilst others have also demonstrated little or no effect of such extraction (Lathia, Hoch, & Kievernagel, 1987; Shi, Arntfield, & Nickerson, 2018). Unlike cereal grains where phytin is accumulated mostly in the aleurone fraction, phytin is eventually distributed throughout the dicotyledonous legume seed (Gupta, Gangoliya, & Singh, 2015). Decortication is thus ineffective in eliminating phytin (DellaValle & Glahn, 2014), unless the seed coat comprises a large fraction of overall seed, e.g. in lupin (Gad, Mohamed, El-Zalaki, & Mohasseb, 1982)). Phytin is found amongst different compartments of plant cells, with localisation within in globoid crystals of protein storage vacuoles, as well as in nonvacuolar compartments such as the cytosol and nucleus (Mitsuda & Mitsunaga, 1974). In soybean, phytin was found to be present in two fractions; a poorly soluble portion embedded in globoids and involved with mostly minerals and the 7S protein, and a soluble fraction composed of phytin-protein salts (Prattley & Stanley, 1982). As a result, the phytin levels of legume seeds have

been found to be positively correlated with total seed protein content, although the degree of correlation varies amongst species (Chitra, Vimala, Singh, & Geervani, 1995).

3. General properties of phytic acid

IP_6 degrades rapidly into orthophosphoric acid and inositol in its free form (Reddy, Pierson, Sathe, & Salunkhe, 1989). The IP_6-element complex is however insoluble and stable, resisting degradation by heat up to 120 ^oC (Hull, Gray, & Montgomery, 1999), and can thus escape decomposition through most food thermal processing. IP_6 has an empirical formula of $C_6H_{18}O_{24}P_6$ and the structure shows 12 dissociable protons (**Figure 1a**). Six of these protons have a pK_a of 1.5, while 2 to 3 protons have pK_a of 5.7-7.6 and up to 4 protons with pK_a >10 (Humer, Schwarz, & Schedle, 2015). This approximates to a net charge of -3 at pH 1.5, and -8 at pH 7.5 (Tamim & Angel, 2003), giving rise to a pronounced binding capacity with counterions or metal complexation at the near-neutral pH of the duodenum. Although IP_6 itself is water-soluble and can be absorbed, metabolised and excreted in humans (Grases, Costa-Bauza, & Prieto, 2005; Wcislo & Szarlej-Wcislo, 2014), the formation of insoluble complexes can impede absorption of both both IP_6 and bound elements in the small intestines, reducing their bioavailability.

The presence of six phosphate groups endow IP_6 with a highly anionic nature, and most IP_6 interactions with biomolecules are primarily electrostatic (Crea, De Stefano, Milea, & Sammartano, 2008; Martell & Hancock, 2013; Urbano et al., 2000). IP_6 can form ionic complexes with divalent elements such as Fe(II), Zn(II) and Ca(II), trivalent Fe(III), as well as with ligand atoms in proteins (including enzymes) and carbohydrates (Silva & Bracarense, 2016). Both direct and multidentate phosphate-element coordination have been observed in IP_6-cation complexes through IR and Raman spectroscopy, as well as computational studies (Torres et al., 2005). NMR

spectroscopic and molecular modelling have found that the inositol and phosphate group on IP_6 tend to adopt a sterically stable conformation in solution, with predominantly five C-O bonds in the equatorial position and one in the axial position on the inositol across a dynamic pH range (pH 0.5-10.5) (Bauman, Chateauneuf, Boyd, Brown, & Murthy, 1999; Paton, Noailly, & Mossoyan, 1999; Volkmann et al., 2002). Inositol ring inversion into a sterically hindered 5-axial, 1-equatorial conformation is also observed, but only occurs when > 5 groups of the inositol are phosphorylated, usually under high pH (> pH 9) and ionic strength (Bauman et al., 1999; Reinmuth, Pramanik, Douglas, Day, & Bowman-James, 2019). This form is stable where IP_6 can form chelation cages with counter ions, in the presence of *syn*-1,3,5-triaxial trisphosphate (**Figure 1b**) (Volkmann et al., 2002). Crystallographic studies have demonstrated the inversion phenomenon in both Zn complex and Na^+ salts of IP_6 (Blank, Pletcher, & Sax, 1971; Cai et al., 2017). These structural properties support strong binding of elements under a range of conditions, particularly under high ionic strength.

While IP_6 is the predominant form found in plants, various less phosphorylated myo-inositol species are also present due to its dephosphorylation. Enzymatic hydrolysis by phytase is the most prominent dephosphorylation mechanism, which can also take place during some major steps in food processing (soaking, germination, malting or fermentation) (Mahgoub & Elhag, 1998) or human digestion. Phytases are high molecular weight enzymes (~40 to 500 kDa) that catalyze the hydrolysis of phytic acid into myo-inositol phosphate intermediates (Kaur, Kunze, & Satyanarayana, 2007). They can be present intrinsically within germinating seeds, and/or released by microbial activity. Whilst most microbial phytases typically dephosphorylate from the 3- position of the inositol ring (EC.3.1.3.8), plant phytases tend to initiate hydrolysis at the 6-

position (EC.3.1.3.26) (Brejnholt, Dionisio, Glitsoe, Skov, & Brinch-Pedersen, 2011; Konietzny & Greiner, 2002). Although inositol monophosphate is the final end product of the enzymatic hydrolysis (Bohn, Meyer, & Rasmussen, 2008), different isomeric phosphorylated products can be generated from partial hydrolysis, leading to disparate behaviour on IP_6's ability to aggregate and form insoluble complexes with elements affecting their absorption kinetics throughout the human gastrointestinal tract (Sandström & Sandberg, 1992). Considering the importance of the small intestine as a major uptake site, phytase activity in food prior to ingestion, or during gastric (acid phosphatases) and small intestinal (alkaline phosphatases) digestion are thus important determinants in elemental bioavailability (Greiner & Konietzny, 2006). However, the human gut microbiome also expresses high levels of phytase activity (Markiewicz, Honke, Haros, Świątecka, & Wróblewska, 2013), which can aid in the release of phytate bound elements in the colon.

4. Studies on Fe, Zn and Ca bioavailability

The effects of IP_6 on element bioavailability are studied through either *in vitro* or *in vivo* methods. *In vitro* assessments generally characterise bioaccessibility (i.e. the amount released from the food matrix during digestion (Hemalatha, Platel, & Srinivasan, 2007)), through predicting the type of prospective interactions between one or more components with IP_6 that can take place throughout the gastrointestinal tract. Caco-2 cell models are sometimes employed following simulated digestion to examine cell uptake of the element (Walters, Esfandi, & Tsopmo, 2018). Various animal models such as rodent, broiler and swine have been used to study IP_6 effects on element bioavailability *in vivo*, although these animals may possess higher levels of endogenous phytase activity compared to the human small intestine (Iqbal, Lewis, & Cooper, 1994). Whilst rodent models are the most common, they are postulated to absorb some

elements more effectively [(e.g. Fe, reported by Tako, Blair, and Glahn (2011)]. The swine is reported to be the most suitable experimental model, as they degrade and absorb IP_6 primarily in the stomach, upper small intestine and colon, and are thus physiologically similar to humans (Silva & Bracarense, 2016).

Studies involving human intervention trials possess greater complexity, where findings often differ between populations, types of study design, and the bioavailability marker. The most common marker in human studies is fractional element absorption following single-meals, which can be measured by using radioactive-labelled isotopes and whole-body radioactivity scintillation counting. In the case of Fe, absorption into erythrocytes can be used as a biomarker, whilst biomarker for Zn is by liver uptake. Nonetheless, fractional absorption of an element is strongly influenced by host-related factors (Petry et al., 2010). For example, infants are known to be less affected by dietary IP_6 due to a range of mechanisms, such as higher gastric pH during digestion (Miller, Hambidge, & Krebs, 2015). Results from adult studies will thus be referred to throughout this review.

The inhibitory effect of phytate on element absorption has been generally confirmed by radioactive and stable isotope *in vivo* studies, although it is apparent that these effects diversify with the element and food matrix involved. IP_6 is most known for its deleterious effects on Zn absorption in human adults. Single-meal studies have demonstrated an inhibitory effect of endogenous or added IP_6 in meals on Zn absorption from complex food matrices or semi-purified foods (Sandström, Almgren, Kivistö, & Cederblad, 1987; Sandström & Sandberg, 1992; Turnlund, King, Keyes, Gong, & Michel, 1984), whilst others have demonstrated an improvement in absorption through IP_6 degradation in food (Egli, Davidsson, Zeder, Walczyk, & Hurrell, 2004; Thacher et al., 2009). Zn absorption may be less affected by IP species with lower degree of

phosphorylation, as corroborated by another study that demonstrated no effect on short-term Zn absorption when IP_5 was added to food (Sandberg et al., 1999). Long-term intervention studies (3-4 months) have reported reduced Zn absorption in both young and elderly women due to an approximate two-fold increase in dietary phytate intake (Hunt, Johnson, & Fariba Roughead, 2009; Kim et al., 2007). Similarly, reduced absorption or uptake have also been observed in rodent studies involving both added and endogenous IP_6 in food matrices (Davies & Olpin, 1979; Lönnerdal, Sandberg, Sandström, & Kunz, 1989; Rubio et al., 1994).

Adverse impacts of IP_6 on inorganic Fe absorption are also apparent in human studies, but with some mixed findings. Several single meal studies have demonstrated reduced Fe bioavailability in the presence of IP_6 (Hallberg, Brune, & Rossander, 1989; Sandberg et al., 1999; Siegenberg et al., 1991), and its elimination from food matrices has generally led to better Fe absorption (Hallberg, Rossander, & Skånberg, 1987; Hurrell et al., 1992; Hurrell, Reddy, Juillerat, & Cook, 2003; Sandberg & Svanberg, 1991). However, some studies have found inconsistent effects on Fe absorption (i.e. no improvements) when IP_6 was removed (Hurrell, Reddy, Burri, & Cook, 2002; Petry et al., 2010). Similar to observations with Zn, inositol species with lower levels of phosphorylation, such as IP_3 were found to have no effect on Fe absorption when added to food (Sandberg et al., 1999).

The effects of IP_6 on calcium has been more nebulous. Unlike Fe and Zn that are primarily transcellularly absorbed in the small intestine, Ca is unique in its reliance on paracellular uptake in both the small intestine and the colon, so its absorption kinetics likely vary to a greater extent with physiological factors such as transit time and colonic availability (Kiela & Ghishan, 2016). To date, there has been only one study using IP_6 in isolation, which exhibited mixed effects on Ca bioavailability in humans

(Reinhold, Lahimgarzadeh, Nasr, & Hedayati, 1973). Other studies with reported negative effects on Ca absorption have either administered phytate with or within various food matrices (Bronner, Harris, Maletskos, & Benda, 1954; Heaney, Weaver, & Fitzsimmons, 1991; Kim et al., 2009; Reinhold, Faradji, Abadi, & Ismail-Beigi, 1976; Weaver, Heaney, Martin, & Fitzsimmons, 1991). Animal studies have also exhibited mixed results, with some authors reporting reduced Ca uptake in rodent (Lönnerdal et al., 1989; Rimbach, Pallauf, Brandt, & Most, 1995) and broiler (Tamim & Angel, 2003) models. However, other rodent models have also found no significant differences (Lopez, Leenhardt, Coudray, & Remesy, 2002; Shockravi, Mohammad Shirazi, Abadi, Seyedain Ardebili, & Kimiagar, 2012).

In all elements, knowledge regarding the effects of reducing IP_6 on elements availability are largely confounded by the dephytinization method used. Numerous investigations have used microbial phytase to reduce or eliminate IP_6 (Troesch, Jing, Laillou, and Fowler (2013), and the phytase are known to exert effects on the food matrix beyond dephosphorylation of phytic acid. A notable 'extra-phosphoric' effect is a concomitant increase in protein solubility apparently from loosening phytate-protein interactions in the food matrix (Rosa-Sibakov, Re, Karsma, Laitila, & Nordlund, 2018), which may enhance element bioavailability as a soluble protein ligand. Other IP_6 reduction methods, such as through genetic modification of plants have been linked to changes in the distribution of minerals (Cominelli et al., 2020; Sakai et al., 2015) and phytochemical composition (Tako, Beebe, Reed, Hart, & Glahn, 2014) within the seed, which can also affect element bioavailability. These factors imply that some of the bioavailability enhancement effects implicated in IP_6 reduction can be attributed to changes to the vegetal matrix due to IP_6 depletion, highlighting the need to understand some of these interactions.

5. Molar ratios as an index to predict bioavailability

A standard predictor of element bioavailability in a plant-based food or diet is to determine its molar ratio with IP_6. The concept was initially used for Zinc, where the high phytate-to-zinc ratio (PA:Zn) was recognized in 1976 as a causal factor linked to impeded growth in rats feeding on legumes, cereals and semi-purified diets (Lee, Williams, Cartwright, Prasad, & Oberleas, 1976; Prasad, 2013). The PA:Zn, as well as a PA:Ca:Zn ratio was then suggested to be applied to humans following a review of apparent Zinc absorption and balance in animal and human studies (Morris & Ellis, 1989). Later, the inhibitory effects of IP_6 on both Fe and Zn absorption were found to be dose-dependent (Fredlund, Isaksson, Rossander-Hulthén, Almgren, & Sandberg, 2006; Hallberg et al., 1989), which led to extrapolation not only with Fe, but also with Ca-to phytate molar ratios.

The formula to calculate IP_6 to mineral molar ratio is as follows (Abdulwaliyu et al., 2019):

$$PA{:}Element\ Molar\ ratio = \frac{PA/MwPA}{Element/MwElement}$$

Where PA is the amount of IP_6 found in the food sample, MwPA is the molecular weight of IP_6 (0.6 kDa), Element is the amount in the food sample, and MwElement is the molecular weight of the element of interest (65 for Zn, 56 for Fe and 40 for Ca).

Out of the three elements, only the PA:Zn ratio has been validated using various regression models of existing isotopic human studies (Hambidge, Miller, Westcott, & Krebs, 2008; Miller, Krebs, & Hambidge, 2013). Reasonable absorption reportedly occurs between the range 5:1 to 15:1 (Dary & Hurrell, 2006; Gibson, 2006; Lonnerdal, 1998), although 18:1 has been proposed to be optimal by the International Zinc Nutrition Consultative Group (IZiNCG) (Brown et al., 2004). In Fe, the PA:Fe ratio

determined by human studies has been reported to be < 1:1 or ideally 1:2 or 2.5 to significantly enhance absorption (Abdulwaliyu et al., 2019; Hurrell & Egli, 2010). Some isotopic studies have shown for this critical threshold to be a good index of fractional absorption in non-haem Fe within vegetal matrices (Abizari et al., 2012; Petry, Egli, Campion, Nielsen, & Hurrell, 2013), whilst others have demonstrated poor predictability of bioavailability both *in vivo* (de Almeida Siqueira et al., 2007) and *in vitro* (Bangar et al., 2017). The critical PA:Ca ratio has been suggested to range from 4.17:1 to 5.88:1 (Abdulwaliyu et al., 2019; Ma et al., 2007; Sandberg & Svanberg, 1991), although its utility has been of controversy (Erba, Manini, Meroni, & Casiraghi, 2017), most likely due to the ambiguous effects of IP_6 on calcium bioavailability.

Considering the presence of other components that IP_6 may bind to, prior to or during digestion, the mixed findings amongst studies on these elements are unsurprising.

6. Phytic interactions with elements

Here we evaluate the existing knowledge on the mechanisms of phytate-cations interactions, and some structure-activity relationships between the binding of IP6 with elements are discussed. Much of the current discussions on phytate and element bioavailability are founded on a partially dissociated structure proposed by Weingartner and Erdman (1978), which suggests that various cations can fully interact with the six partially deprotonated phosphate groups at neutral pH. This can be achieved through element chelation intramolecularly *via* electrons donation from two oxygen atoms from the same or different phosphate groups (**Figure 1c**), and/or intermolecularly via element bridging between two P–O groups on different IP6 molecules. However, speciation studies have identified several factors that govern the nature and composition of complexes formed between phytic acid and cations. These include the molar mass ratio,

degree of phosphorylation, pH and ionic strength (Crea et al., 2008; Crea, De Stefano, Milea, Porcino, & Sammartano, 2007). As these factors likely vary amongst food matrices, the types of interactions responsible for insoluble complexes that lead to reduced element bioavailability *in vivo* may not be facile as once believed.

The properties of IP_6-element complexes have been comprehensively characterised *in vitro* using a range of physical techniques. For example, isothermal titration calorimetry (ITC) is used to determine the thermodynamic and chemical parameters of binding affinity and stoichiometry, respectively, as well as enthalpy changes during the binding process. Potentiometric and thermal titration studies also provide insights into stoichiometry and for binding sites on the IP_6 molecule, whilst differential scanning calorimetry (DSC) can be used to assess structural stability by measuring the overall formation constant at the equilibrium temperature.

To assist the interpretation of *in vitro* studies in this section, we made specific references to the solubility behaviour of IP_6 at different pH, along with structural requirements and binding affinity. Many binding studies have utilised a medium with physiological ionic strength at or near 0.1 M, which corroborates that found in human gastric and intestinal fluids (Mudie, Amidon, & Amidon, 2010). The following discussion will be built predominantly on investigations employing these mediums wherever possible. It is noted that whilst the approximate pH of human gastric (pH 1.3 – 2.5) and intestinal fluids (pH 6.5) at fasted state are well established (Kong & Singh, 2008; Riethorst, Brouwers, Motmans, & Augustijns, 2018), these ranges are dynamic in accordance with food consumption (Mudie et al., 2010).

6.1. Binary phytate-element complexes

6.1.1. Solubility behaviour and structural requirements

The molar ratio between IP_6 and cations conspicuously differentiates the nature of complexation. Under equimolar or molar excess of IP_6 or IP_5, potentiometric studies under physiological ionic strength have generally exhibited a phenomenon of exclusively forming high-affinity, soluble hydroxo-mononuclear species (1:1) with Fe(III), Zn(II) and Ca(II) (Torres et al., 2005; Veiga et al., 2015; Veiga, Torres, Godage, et al., 2009). These complexes contain only the elements coordinated with water and the phosphate group(s) from IP_6 as ligands, following a general stoichiometry of $Phy[M(H_2O)_n]^{z+}$, where Phy stands for IP_6, M is the element, and z is the charge on the phytin species (Crea, de Robertis, de Stefano, & Sammartano, 2006; Graf, Empson, & Eaton, 1987). However, the emphasis on thermodynamic properties in many investigations inadvertently provides a level of characterisation based on metastable states, when the dehydration rate constants of the complexed cations (particularly small ions) can be small, due to a fragile but well-defined second hydration shell to the cations (Israelachvili, 2015). This can lead to slow formation of insoluble equilibrating species, which is consistent with thermodynamic findings that the entropy in each protonation step of IP_6-cation binding greatly exceeds that of the corresponding enthalpy (De Stefano, Milea, & Sammartano, 2004), and the entropy tends to increase with time. A more consistent observation is observed in the case of cations in molar excess to IP_6, where insoluble polymetallic IP_6 species of unknown composition are formed, except for Mg(II) (Torres et al., 2005). Due to a large amount of coordinated water to the element, the structure of the polynuclear species are difficult to characterise (Bohn et al., 2008; Graf et al., 1987). However, the IP_6-element stoichiometries have also been found to vary depending on the initial reactants and pH (Bebot-Brigaud,

Dange, Fauconnier, & Gérard, 1999).

IP_6 can oxidise coordinated Fe(II) to Fe(III), where its higher positive charge produces greater interaction with IP_6 (Torres et al., 2005). According to Bretti, Cigala, Lando, Milea, and Sammartano (2012), when IP_6 is in molar excess of Fe, mononuclear ferric phytate species primarily dissociates into iron hydroxide and free iron ions at gastric pH (below pH 3.5). Above this pH, it reassociates into soluble forms of ferric phytate, with solubility declining above pH 6 (small intestinal pH). Correspondingly, monoferric phytate possesses an extent of bioavailability similar to non-haem Fe in humans (Cook et al., 1973; Dold et al., 2020; Simpson, Morris, & Cook, 1981). This signifies that soluble IP_6-Fe(III) can either be absorbed intact, or the iron dissociates for trans-cellular uptake. On the other hand, a molar excess of Fe(III) to phytate exhibits high solubility of 90% under pH 1.5, with Fe solubility decreasing as the pH reaches neutrality (Cheryan & Rackis, 1980). The binding of Fe(III) with IP_6 is known to lessen with decreasing degree of phosphorylation on the inositol ring. A minimum of three phosphate groups are required to bind Fe(III), which several authors have proposed to be the 1,2,3-equatorial-axial-equatorial triphosphate motif (Mansell et al., 2010; Saiardi, 2017; Veiga, Torres, Mansell, et al., 2009; Yu, Cowieson, Gilbert, Plumstead, & Dalsgaard, 2012). Šala, Makuc, Kolar, Plavec, and Pihlar (2011) have shown through ^{31}P NMR studies that in the interaction of IP_6 with Fe(III), P1,2 and 3 are involved in the initial coordination process, followed by P4,5 and 6. They found aggregation to be initiated at high Fe(III):IP_6 ratio in which intermolecular bonds between P-O groups from different IP molecules are formed. Concomitantly, weak or little complexation appears to occur < IP_4, as tri- and tetraferric phytate appear to increase the solubilised fraction of Fe(III) after simulated *in vitro* digestion, regardless of molar ratio (Sandberg, Carlsson, & Svanberg, 1989). The weak binding likely contributes to the findings that

isolated myo-inositol phosphates with < 3 phosphate groups do not inhibit Fe(III) absorption *in vivo* (Sandberg et al., 1999).

Both Zn(II) and Ca(II) have been found to form soluble species with IP_6 under equimolar or excess of IP_6 across pH 2-11 (Crea, De Stefano, Milea, & Sammartano, 2009; Crea, De Stefano, Milea, Porcino, & Sammartano, 2007). However, there have been reports of Zn-phytate precipitating between pH 5 and 7 in molar excess of IP_6, generally at or below the ratio of 10:1 of PA:Zn (Allred, Kratzer, & Porter, 1964; Champagne, Fisher, & Hinojosa, 1990; Wise, 1983). This can be explained by differences in the coordination kinetics. Zn(II) coordination has been suggested to favour the less dominant 5-axial, 1-equatorial conformation at pH 7, which may hinder the rate of complex formation due to low concentrations of IP_6 in this conformation (Champagne & Fisher, 1990; Wise, 1995). Zn(II) has also been implicated in the formation of a salt bridge between 2 IP_6 molecules when IP_6 is in molar excess (Erdman & Poneros-Schneier, 1989; Vasca et al., 2002). Both the conformation and molar excess of IP_6 may have contributed to precipitation. Pierce Jr (1985) found that at 1:5 PA:Zn ratio and pH 5-6, precipitation is kinetically slow but are observed after 1-3 hours. High ionic strength, which promotes the 5-axial, 1-equatorial conformation, is thus promotive in the formation of IP_6 polynuclear species (Crea et al., 2009). This is noteworthy considering the higher levels of ionic strength in the chyme, as enhanced by both electrolytes from the food itself and endogenous secretions. Zn(II) coordination appears to be a bidentate chelate in reference to the phosphate ligand (Rodrigues-Filho et al., 2005), favouring the P2 or P5 group (**Figure 1b**) (Bebot-Brigaud et al., 1999; Champagne & Fisher, 1990). Similar to Fe(III), binding of Zn(II) drastically decreases at lower degrees of inositol phosphorylation, like the IP_3 and IP_4 species (Persson, Türk,

Nyman, & Sandberg, 1998). This corresponds with human studies that IP species below IP_5 do not negatively affect Zn bioavailability (Sandström & Sandberg, 1992).

IP_6 complexation with Ca(II) has been found to be endothermic and reversible between pH 2-12, forming soluble IP_6-Ca(II) species regardless of molar ratios below pH < 5 (Siener, Heynck, & Hesse, 2001). At pH 2.5, a tri-phosphate salt is formed within the same IP_6 molecule when Ca is in excess (Byrd & Matrone, 1965). However, this does not occur in the absence of a NaCl salt in buffer (Martin & Evans, 1986), indicating a role of Na^+ in potentiating Ca(II) complexation to IP_6 at low pH. Under conditions of IP_6 excess, it does not complex Ca(II) at pH 2.8 or below but complexation is gradual as pH increases (Champagne, 1987). At equimolar or cation excess, Ca(II) is chelated by IP_6 even at pH 2, either as 1:1 or 2:1 Ca:PA stoichiometry (Graf, 1983). The IP_6-Ca mononuclear complexes formed at pH 7 are soluble, whilst precipitation of polynuclear species occurs at molar ratios of Ca:PA above 2:1 (Graf, 1983). Above pH 6, the greatest calcium precipitation occurred at molar ratios between 4 and 6.5, reaching a saturation at approximately 5 mol of Ca(II) per mol of IP_6 at high Ca:PA ratios (Bullock, Duffin, Nolan, & Smith, 1995; Grynspan & Cheryan, 1983; Martin & Evans, 1986). The initial binding sites for Ca(II) appear to be the P4,5 or 6, where maximal binding under Ca(II) excess favours the inverse 5-equatorial/1-axial conformation (Champagne et al., 1990). However, the stoichiometry may be modulated in legume food matrices with other food components such as protein or other elements also binding to IP_6. This phenomenon has indeed been described by Dendougui and Schwedt (2004) who found 3 mol of Ca(II) binding to each mol of IP_6 in chickpea and soybean matrices.

These complexation behaviours form an interesting case in legumes amongst plant matrices. Quantities of Ca are variable but can be in excess to IP_6 (**Table 1**),

supporting the formation of the insoluble species under neutral pH, corresponding to low bioavailability in legumes. On the other hand, IP_6 occurs in molar excess to Fe and Zn in legume matrices, yet generally displays low solubility after *in vitro* digestion. In non-food based systems, highly soluble Fe(III) complexes have indeed been observed *in vitro* after simulated gastrointestinal digestion at PA:Fe ratio from equimolar up to 40:1 (Engle-Stone, Yeung, Welch, & Glahn, 2005; Glahn, Wortley, South, & Miller, 2002), and found to be bioavailable in rodents (Graf & Eaton, 1984). However, disparate results have been observed in the translation of solubility towards cell up take and ferritin formation in Caco-2 cell models. Whilst some authors have reported no significant changes in Fe cell uptake (Andrews, Briones, Jaramillo, Pizarro, & Arredondo, 2014; García-Casal, Leets, & Layrisse, 2000), marked reductions in uptake and ferritin formation were also observed in a number studies (Engle-Stone et al., 2005; Han, Failla, Hill, Morris, & Smith Jr, 1994; He, Feng, Li, & Yang, 2008; Jin, Frohman, Thannhauser, Welch, & Glahn, 2008), which were shown to be the result of Fe(III) not being accessible to ferric reductase (*Dcytb*) for reduction into the ferrous form for uptake. The low solubility of Fe(III) in digested food matrices is thus likely due to the formation of polynuclear complexes in the presence of other ligands in the digested legume matrix, which leaves a small quantity of Fe(III) remaining in solution for uptake or paracellular absorption. It is established that the formation of these polynuclear complexes are slow and largely kinetic intermediates from the soluble mononuclear aquo species (Krężel & Maret, 2016). Similarly, given sufficient quantities of Zn(II) to bind the various phosphorylated form of IP_6 , the formation of a stable precipitate over the time course of orofecal transit of 90-180 min (Sellin, 2016) under neutral pH is highly likely, even in excess of IP_6. Here, the lower phosphorylated inositol species with their reduced phosphate binding sites may be less likely to polymerise, regardless

of the cation involved. Paradoxically, these IP species may keep the cations in solution *via* weak binding or as counter ions, as observed with Fe(III) both *in vitro* (Gupta et al., 2020; Sandberg et al., 1989) and in humans (Dold et al., 2020).

6.1.2. Binding affinity

Due to the likely presence of different cations in a dietary context, competitive binding of IP_6 to the various cations can contribute to cation insolubility, hence affecting absorption essential for the cations' bioavailability. The order of preferential affinity by dietary cations in forming complexes with IP_6 can be determined *in vitro* by potentiometric measurements or alkaline titration curves of the free IP_6 acid, which shows change in the pH or electrical potential in the presence of the cation. The findings from multiple investigations were generally consistent, following the Irving-Williams series of stability based on aquo-exchange of transition elements with the IP_6 ligand (Williams, 2002). The conditions covered in these investigations included a range pH (2-11) and from equimolar to IP_6 in excess, which showed the order of Cu(II)>Zn(II)>Mn(II)>Fe(III)>Ca(II) (Bebot-Brigaud et al., 1999; Persson et al., 1998; Tamim & Angel, 2003; Vohra, Gray, & Kratzer, 1965). Maddaiah, Kurnick, and Reid (1964) reported an identical sequence with these cations, except for Zn(II), which was found to have a higher affinity than Cu(II). For IP_6, the difference between Cu and Zn was reportedly small in the range pH 3-7, and their stabilities could be related to the number of cations bound to IP_6 (Champagne & Hinojosa, 1987). Under mononuclear conditions where one cation is associated with one IP_6, some authors have reported the same sequence with Mg(II) positioned before Fe(III) (Torres et al., 2005), whilst the polynuclear Ca(II) complex where more than one cations is associated with IP_6 was found to be more stable than Mg(II) (Odani, Takamido, & Yamauchi, 1997). This finding was replicated by Maenz, Engele-Schaan, Newkirk, and Classen (1999), who

measured the cation inhibitory potency of phytase hydrolysis and identified them to be in the order of Zn(II)>Fe(II)>Mn(II)>Fe(III)>Ca(II)>Mg(II) at pH 7. The same series was also reported by Lyon (1984), who extracted the mineral elements from high phytate cereal products at pH 2, and measured the extent of precipitates formed under pH 7. Interestingly, unlike previous studies that reported strong IP_6 binding with Cu(II), Lyon (1984) showed that food-extracted Cu(II) was poorly precipitated by phytate. Their findings suggest that Cu(II) extracted from the food matrix may be present with other compounds that interfere with the precipitation process, and may be the reason why IP_6 does not inhibit Cu(II) absorption to *in vivo* (Egli et al., 2004; Lönnerdal, 2002).

The order of element binding affinity supports the observation from human studies that Zn bioavailability is indeed the most negatively affected by phytate binding which produces insoluble complexes. The binding stability constants of Fe(III), Fe(II), Zn(II) and Ca(II) to IP_6 as reported by Torres et al. (2005) are reported in **Table 2,** which demonstrates that species formed by 1:1 complexes of Fe(III) tend to have the greatest relative stability. However, as Torres et al. (2005) have only reported values of the mononuclear species in Fe(III), it is possible that the stability of these species is modulated at different molar ratios. It can also be seen that Ca(II) complexes are more easily dissociable than that of other cations, which could contribute to greater release throughout the gastrointestinal tract, particularly in the colon where uptake still ensues.

7. Ternary phytate-element complexes

The predominantly ionic, yet high affinity nature of mononuclear cation-IP_6 species that were discussed earlier (section 6.1.2) can give rise to the formation of insoluble ternary complexes with other biomolecules. Although IP_6 reportedly interact with polysaccharides (Rubio et al., 1994) and fibre (Hallberg et al., 1987), interactions

between different elements and protein are the most thoroughly investigated *in vitro*.

7.1. The phytate-protein axis

One mode of ternary complexation is through the ability of cations to mediate co-complexation between proteins and IP_6 acting as a coordinated linker between the two. The presence of proteins in a food matrix is generally considered beneficial to element bioavailability, due to the ability of digestion released soluble amino acids and peptides to desorb insoluble cations from precipitates into soluble bound form (Roohani, Hurrell, Kelishadi, & Schulin, 2013). However, proteins with restricted digestibility do not provide soluble ligands for cations but instead play a negative potentiating role in terms of mineral absorption by co-complexation with IP_6 mediated by the cations. These complex formation involving anionic amino acids of protein neutralises its surface charge that decreases protein solubility and hydration in solution, and indeed thermodynamically stable complexes resistant to digestion can be formed (Reddy et al., 1989).

Several complementary theories currently ascribed for protein interactions with IP_6 prior to complexation with cations (Selle et al., 2012). The first proposes that negatively charged IP_6 directly reduces protein solubility at neutral pH by neutralising positively charged protein groups *via* binding to these groups. Under acidic pH, negatively charged groups may not be ionised while all amino groups are protonated. The positively charged basic residues (free α-amino group, ε-amino group of lysine, guanidino group of arginine, imidazole group of histidine) could then interact with the negatively charged phosphate groups. These interactions are predominantly charge-charge interactions, and weak due to the absence of concomitant hydrogen bonding (Darby, Platts, Daniel, Cowieson, & Falconer, 2017). As the pH rises during intestinal digestion, the protein-IP_6 pair is dissociated as the charge on the protein decreases

above the isoelectric point. At neutral pH, electrostatic association between proteins and IP_6 again takes place (Bye, Cowieson, Cowieson, Selle, & Falconer, 2013). Here, the presence of a bridging divalent cation (e.g. Fe, Zn or Ca) can form a stable complexation linkage between IP_6 and ionised carboxyl groups of aspartic and glutamic acid on protein. The resultant IP_6-element-protein complexes are resistant to hydrolysed by proteolytic enzymes (Kumar et al., 2010), and thus rendering the element not absorbable affecting bioavailability.

Additional theories have been reported to explain the indirect mechanisms of promoting interactions between protein and IP_6. Based on the Hofmeister series, the presence of selected ions is classified as having chaotropic (destabilising) or kosmotropic (stabilising) properties based on the thermodynamics of the milieu in disrupting water structure (Marcus, 2009). Kosmotropes are capable of breaking the structure of water held by water-water hydrogen bonds, as the ions display stronger interactions with water molecules in a charge-dipole interaction than with water with itself in hydrogen bonding. The phosphate groups on IP_6 are considered as a kosmotropes that stabilize native protein structure by interacting with protonated amine groups in charge-charge interactions, or other weaker interactions such as charge-dipole from protein OH groups. The binding of IP_6 to protein makes it resistant to protease hydrolysis. However, the degree of this effect on proteolysis would also depend on the properties of other ions within the milieu that also interacts with the protein groups [(thoroughly reviewed by Selle et al. (2012)]. Additionally, studies *in vitro* have found that IP_6 and lower phosphorylated species can also hinder proteolysis of various proteins by pepsin and trypsin (Deshpande & Damodaran, 1989; Vaintraub & Bulmaga, 1991), and reduce the conversion rate of the pepsin precursor pepsinogen itself into the active pepsin (Liu & Cowieson, 2011). These effects are known to be reduced with

decreasing number of phosphate groups on the inositol (Knuckles, Kuzmicky, Gumbmann, & Betschart, 1989).

7.1.1. Phytic acid interactions with legume proteins and elements

Phytate-induced modulations in protein properties are known to be source dependent (Carnovale, Lugaro, & Lombardi-Boccia, 1988). Although IP_6 does not interact with all proteins, it appears to interact with legume proteins *in vitro* to greater extents than milk proteins such as casein (Yu et al., 2012), and the IP_6 content of legume seeds have been strongly negatively correlated with *in vitro* protein digestibility (Chitra, Singh, & Rao, 1996; Chitra et al., 1995). Proteins from soybean, dry bean, faba bean, lentil and chickpea have been found to exhibit paralleled solubility behaviour to IP_6, exhibiting an inversely skewed distribution with high solubility below pH 2, the lowest solubility at pH 3.5-4 around the isoelectric point of most legume proteins, and becoming soluble again at pH 7 correlating to the change in the net charge on the proteins through these pH changes (**Figure 2**). Both dialysis and ultrafiltration experiments have demonstrated difficulties in separating the IP_6-protein pairs at neutral pH (Carnovale et al., 1988; Champagne et al., 1990).

The predominant fraction of legume proteins comprises of globulins that constitute up to 65-85% of total protein, and characterized by two dominant sedimentation coefficients 7S and 11S. The quaternary and tertiary structure of both globulins are modulated by pH and ionic strength (González-Pérez & Arellano, 2009), although they both contain a high proportion of peptides in the β-sheet conformation in their native tertiary structures (Carbonaro, Maselli, & Nucara, 2015). Such structural properties can greatly hinder the exposure of hydrophilic groups, and thus the digestibility of legume proteins even in the absence of IP_6 (Gilani, Xiao, & Cockell, 2012). Gastric processing in acid conditions play a significant role in the solubilisation

of proteins. However, if proteolysis of legume proteins is further impeded by binding to IP_6, the poorly digested high molecular weight products may actively promote ternary complex formation with cations upon entering the small intestinal lumen. Current evidence suggests that certain moieties, and/or amino acid composition of legume proteins could be more prone to interactions with IP_6 and synergistically inhibit element bioavailability by metal complexation. For example, a series of human isotope studies by Cook, Juillerat, Hurrell, Dassenko, and Lynch (1994) and Hurrell et al. (1992) showed that dephytinization of glycinin (11S globulin) significantly improved non-haem Fe absorption from soy proteins in humans, whilst the conglycinin (7S globulin) component was still partially inhibitory following dephytinization. This demonstrates that IP_6 exerts synergistic effects with legume protein fractions in entrapping Fe particularly with the glycosylated 7S legume proteins, which have been identified to have element-binding properties (de Souza Ferreira et al., 2018).

Similarly, investigations *in vitro* have demonstrated a tendency of IP_6 to interact with specific protein groups in legume proteins. Studies on black gram (*Phaseolus mungo*) albumin by Reddy and Salunkhe (1981) observed binary protein-IP_6 interactions at pH 2.8 after simulated gastric digestion, and the ternary complex as mediated by divalent cations at pH 8.4. The IP_6-element-protein complexation is also observed by Lombardi-Boccia, Schlemmer, Cappelloni, and Lullo (1998) and Lombardi-Boccia, Carbonaro, Lullo, and Carnovale (1994), who subjected white bean (*Phaseolus vulgaris*) protein fractions containing IP_6 to *in vitro* gastric and intestinal digestion. They found low Fe and Zn dialysability in digestates of the albumin fraction, which was enhanced after the IP_6-element-protein complexes were disrupted through dephytinization. However in a different study, the authors have also observed the globulin fraction of six different legume species enhances Fe bioavailability (Lombardi-

Boccia, Ruggeri, Aguzzi, & Cappelloni, 2003). These findings are likely a result of the extraction method used, where the fractions have not been purified following fractionation. The water-extractable albumin fraction thus likely contains a fraction of the globulins due to cross-contamination (Rubio et al., 2014), and the proteins responsible for complexation with elements and IP_6 could be found in either fraction.

It is apparent through these studies that interactions between IP_6, proteins and cations could be enhanced by limited, but incomplete proteolysis. Without unfolding of the native structure, these interactions are minimised due to hidden binding sites in the folded protein. Protein hydrolysis would expose additional sites and increase the stoichiometry for binding with IP_6 and complexation with cations and IP_6 (Darby et al., 2017).

7.2. Binding with multiple elements and protein

As exemplified with Na^+ in Section 6, the presence of secondary cations can either increase or reduce binding on IP_6 depending on the molar ratio. While IP_6 is a reasonably acidic molecule but with six held phosphate protons with low pKa, cation displacement can increase its acidity by promoting dissociation of these strongly held protons enhancing its chelation power (Marolt & Pihlar, 2015). However, competition between divalent cations for binding to IP_6 can also lead to increased availability of the target element not bound to IP_6 due to occupied IP_6 binding sites. Such dynamics are observed in interactions of Zn and Ca in the presence of IP_6, where the molar ratio again plays a decisive role in IP_6-cation interactions. Byrd and Matrone (1965) showed that when IP_6 is in molar excess of Zn(II) (IP6 5 mM, Zn 0.25 – 0.5 mM), the addition of Ca(II) at high concentrations (30 mM) enhanced Zn incorporation onto IP_6 above pH 6. However, when the Zn:Ca ratio was adjusted to 1:2, the amount of Zn precipitated with IP_6 was greatly reduced. This corresponds with a series of *in vitro* and *in vivo* rodent

studies by Graf and Eaton (1984), who showed that IP_6 must be in excess of both cations in order for the Ca-Zn-IP_6 complex to be soluble at pH 7.4 in 0.1 M ionic medium. Hunt and Beiseigel (2009) also demonstrated through a human isotope study that whilst IP_6 can affect Zn bioavailability, high quantities of Ca does not impair Zn absorption.

Interestingly, a similar trend is observed in the presence of soy protein. Gifford-Steffen and Clydesdale (1993) have found that when IP_6 was in equivalent or in molar excess to Zn(II) (IP_6 at 5 mM), the addition of Ca(II) at high concentrations (30 mM) increased Zn (5.0-2.5 mM) incorporation onto IP_6 at both pH 2.0 and 5.5. They also found that the fraction of soluble IP_6 was much higher at lower levels of Ca addition at pH 5.5. This suggests that whilst low amounts may form soluble complexes, large quantities of Ca(II) may have promoted increased complexation through salt bridging involving multiple IP_6 to the metals. In a study by Grynspan and Cheryan (1989), IP_6 in molar excess was found to form insoluble complexes with Ca(II) above pH 7, whilst the protein in the mix remained soluble. This observation may be exclusive to the 1 M NaCl medium utilised in that study, which could have contributed to protein but not Ca(II) solubilisation in the presence of IP_6. As discussed in the previous section, the magnitude of interactions may be dependent on properties of the protein/s involved. Different binding behaviour could be expected had the soy protein undergone hydrolysis in these studies.

8. Conclusion and implications for enhancing element bioavailability

The findings in this review support that protein properties, as well as concentrations of the cations present could be responsible for the insolubility of the resultant protein-metal-IP_6 complexes formed throughout gastrointestinal digestion affecting the elements' bioavailability. The review highlighted the synergistic role of protein and IP_6

in inhibiting element bioavailability, a phenomenon well-established in animal feed science but less discussed in the context of element bioavailability in human nutrition.

Due to the propensity of IP_6 to form insoluble complexes with divalent cationic at high element concentrations, strategies that involve concomitant reduction of IP_6 and/or an enrichment of cation concentrations *via* agronomic or fortification approaches is warranted. In fact, enhanced IP_6-cation interactions could be a possible contributor to the reason several human (Junqueira-Franco et al., 2018; Vaz-Tostes et al., 2016) and animal (Tako et al., 2014) studies have demonstrated no significant difference in the absorption of Fe and Zn between conventional and biofortified beans. In support of this hypothesis, Glahn et al. (2017), who measured Fe uptake by Caco-2 cells observed that ferritin formation was consistently lower in beans containing low to middle levels of IP_6, compared to those with high (normal) levels. An understanding of the IP_6-cation relationship is thus ultimately necessary in understanding element bioavailability in legumes.

The element:IP_6 molar ratio could be a poor predictor of Fe, Zn and Ca bioavailability, given that cationic binding by IP_6 appear to be modulated by other food ligands. In particular, differences between protein properties, and molar concentrations of other interacting cations are likely responsible for the disparate effects observed on Zn, Fe and Ca bioavailability. For example, in a mathematical model of Zn(II) absorption based on human isotopic studies, both dietary Ca and protein were found to be modest enhancers (Miller et al., 2013), whilst a previous analyses showed that neither adds significant prognostic power to the algorithm predicting Zn absorption (Brown et al., 2004). There is yet to be an analysis of studies investigating the effects of IP_6 on Fe and Ca absorption, although differences amongst food matrices could also

be explanatory of the disparate effects of IP_6 on element bioavailability, as reviewed above.

Protein digestibility within the legume matrix can be leveraged to enhance element bioavailability, not only directly as a soluble ligand for elements (Lv, Bao, Yang, Ren, & Guo, 2008; Torres-Fuentes, Alaiz, & Vioque, 2012), but also through reducing the formation of insoluble complexes with IP_6 (Rosa-Sibakov et al., 2018). Enhancements to protein solubility can be achieved agronomically through breeding cultivars that possess a frangible intracellular matrix, which has been shown to improve Fe uptake by Caco-2 cell models (Glahn, Tako, Cichy, & Wiesinger, 2016; Wiesinger, Cichy, Tako, & Glahn, 2018). Similarly, food processing approaches such as germination and fermentation can contribute to protein solubility, as well as simultaneously elicit the phytases that can dephosphorylate to the < 3 phosphate groups required for the complexation of cations on each IP_6 molecule (Ruel & Levin, 2002).

The effects of ionic strength on element absorption appear to be a double-edged sword. The presence of moderate level of salts such as NaCl can lead to reduction of the extent of deprotonation on IP_6 (Crea et al., 2008), and lead to enhanced protein solubility by increasing protein surface charge by the formation of salt-ion pairs with protein charge groups. Chloride can also compete with cations in solution for association with IP_6 reducing complexation of cations (De Stefano, Milea, Pettignano, & Sammartano, 2003). However, the salt solubility behaviour can differ amongst different legume proteins. Salt ionic strength can potentiate the formation of insoluble IP_6 complexes with cations, even when IP_6 is in molar excess. Whilst the effects of modulating ionic strength is not well studied in relation to element bioavailability, nascent investigations by Hemalatha et al. (2007) demonstrated that by the addition of NaCl at 5%, the positive effects of soy protein in Zn bioavailability was enhanced, and

mitigated its negative effects on Fe bioavailability (likely due to IP_6 and protein). This shows that the common salt, may reduce divalent cation binding and/or enhance protein solubility, as a bioavailability promotor in legume foods. Further investigations are required to examine its potential against other known promotors of element bioavailability, such as organic acids (Sandberg, 2002).

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Table 1. Phytic acid, Iron (Fe), Zinc (Zn), Calcium (Ca) content of various legumes

Legume	Phytic acid (mg g $^{-1}$)	Fe (mg g $^{-1}$)	Zn (mg g $^{-1}$)	Ca (mg g $^{-1}$)	Source
Field pea (*Pisum sativum)*	4.9 – 7.1	0.046 – 0.054	0.039 – 0.063	0.622 – 1.219	Amarakoon et al. (2012)
	2.2 – 11.3	0.035 – 0.09	0.017 – 0.064	0.5 – 1.1	Wang (2004)
	6.7 – 13.5	0.022 – 0.058	0.021 – 0.057	0.46 – 1.57	Humer and Zebeli (2015), Harmankaya et al. (2010)
Soybean (*Glycine max*)	1.77 – 4.86	0.079 - 0.116	0.057 - 0.092	2.55 - 4.05	Frank et al. (2009), Thavarajah et al. (2009)
	2.6 – 3.7	0.026 – 0.027	0.016 – 0.017	1.38 – 1.41	Humer and Zebeli (2015), Luo et al. (2014)
	7.98 – 8.02	0.021 – 0.029	0.015-0.016	2.94 - 3.17	Ma et al. (2007)
Lentil (*Lens culinaris*)	0.7 – 1.2	0.058 – 0.108	0.015 – 0.047	0.387 – 0.49	Ramírez-Ojeda, Moreno-Rojas, and Cámara-Martos (2018)
	0.3 – 1.20	0.043 – 3.415	0.026 – 0.059	0.1 – 0.97	Wang (2004)
	2.29	0.045 - 0.12	0.032 – 0.056	1.28	Thavarajah et al. (2009); Urbano et al. (1999)
Faba bean (*Vicia faba*)	1.62 – 3.59	0.024 – 0.027	0.015	1.31 – 1.33	Luo et al. (2014); Selle, Walker, and Bryden (2003)
	6.4	0.066 - 0.068	0.063 - 0.064	1.26 – 1.27	Abd El-Hady and Habiba (2003); Kassegn, Atsbha, and Weldeabezgi (2018); Aranda et al. (2004)
	8.36 – 8.57	0.035 – 0.047	0.033 – 0.039	1.26 – 1.27	Luo and Xie (2013); Vidal-Valverde et al. (1998)
Lupins (*Lupinus spp*.)	0.5 – 3.5	0.018 - 0.039	0.014 - 0.051	2.01 - 3.71	Humer and Zebeli (2015); Karnpanit et al. (2017)
	13.6 – 16.5	0.038 – 0.049	0.039 – 0.058	0.93 – 1.34	Zhang et al. (2018); Embaby (2010)

Legume	Phytic acid (mg g^{-1})	Fe (mg g^{-1})	Zn (mg g^{-1})	Ca (mg g^{-1})	Source
Beans (*Phaseolus vulgaris*)	11.03	0.071–0.078	0.035 - 0.038	1.46 – 1.62	Abd El-Hady and Habiba (2003); Sebastiá et al. (2001)
	1.05 – 21	0.042 – 0.049	0.021 – 0.037	0.881 – 1.162	Hummel et al. (2020); Ramírez-Ojeda, Moreno-Rojas, and Cámara-Martos (2018)
Chickpea (*Cicer arietinum*)	8.21	0.034 – 0.05	0.026 – 0.049	0.575 – 1.08	Ramírez-Ojeda, Moreno-Rojas, and Cámara-Martos (2018); Abd El-Hady and Habiba (2003)
	0.39 – 1.25	0.043 - 0.05	0.031 - 0.036	0.96 – 1.09	Wang (2004); Sebastiá et al. (2001)
Pigeon pea (*Cajanus cajan)*	3.14	0.09 – 0.99	0.059 – 0.063	1.97 – 1.98	Duhan, Khetarpaul, and Bishnoi (2002); Sangronis and Machado (2007)
Molar Range per gram legume	5.91×10^{-7} – 3.18×10^{-5}	3.22×10^{-7} – 6.12×10^{-5}	2.14×10^{-7} – 9.79×10^{-7}	2.49×10^{-6} – 1.01×10^{-4}	

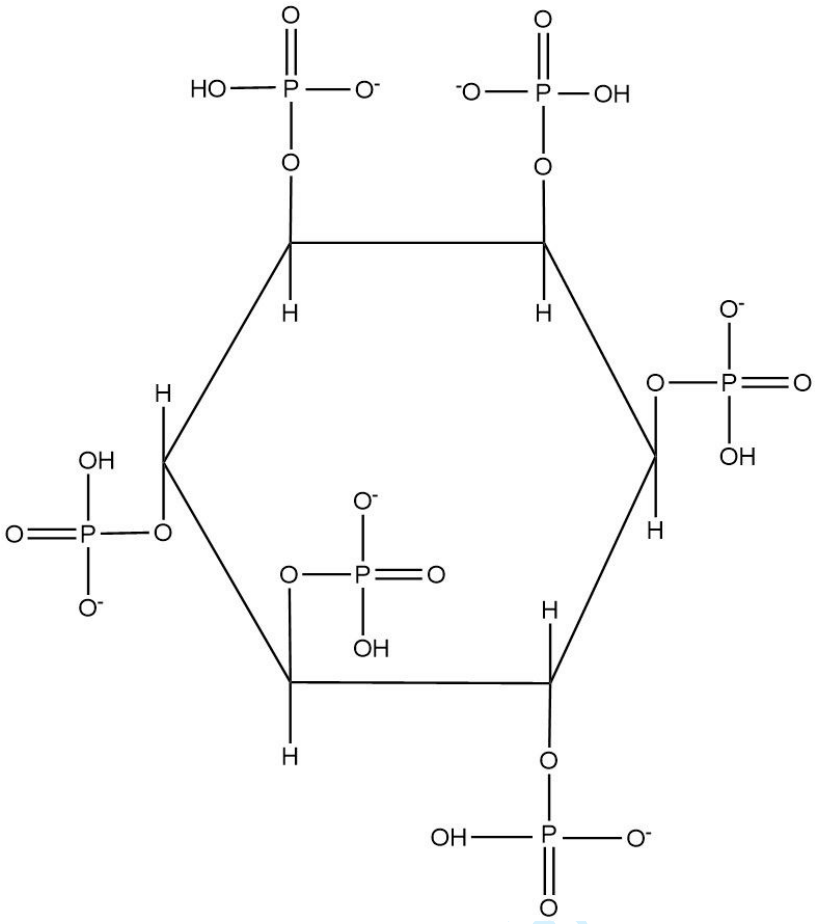

Figure 1a. Structure of phytic acid showing 6 ionized hydroxyl groups, as originally proposed by Anderson (1914)

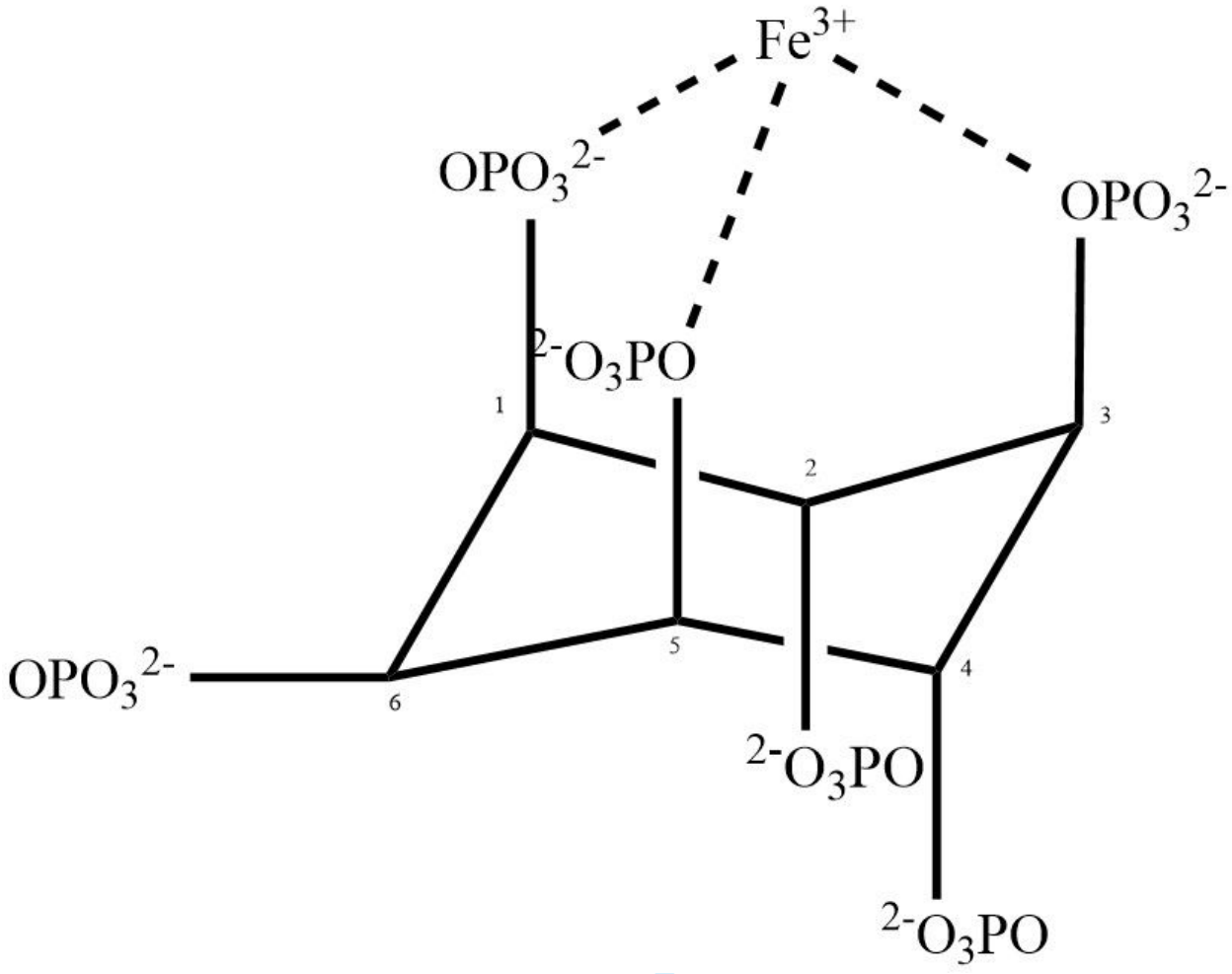

Figure 1b. A 'chelation cage' of ferric iron coordinated by phosphate from the *syn*-1,3,5-triaxial trisphosphate position of the 5-axial, 1-equatorial conformation of IP_6, which in the absence of the metal ion is otherwise sterically unstable.

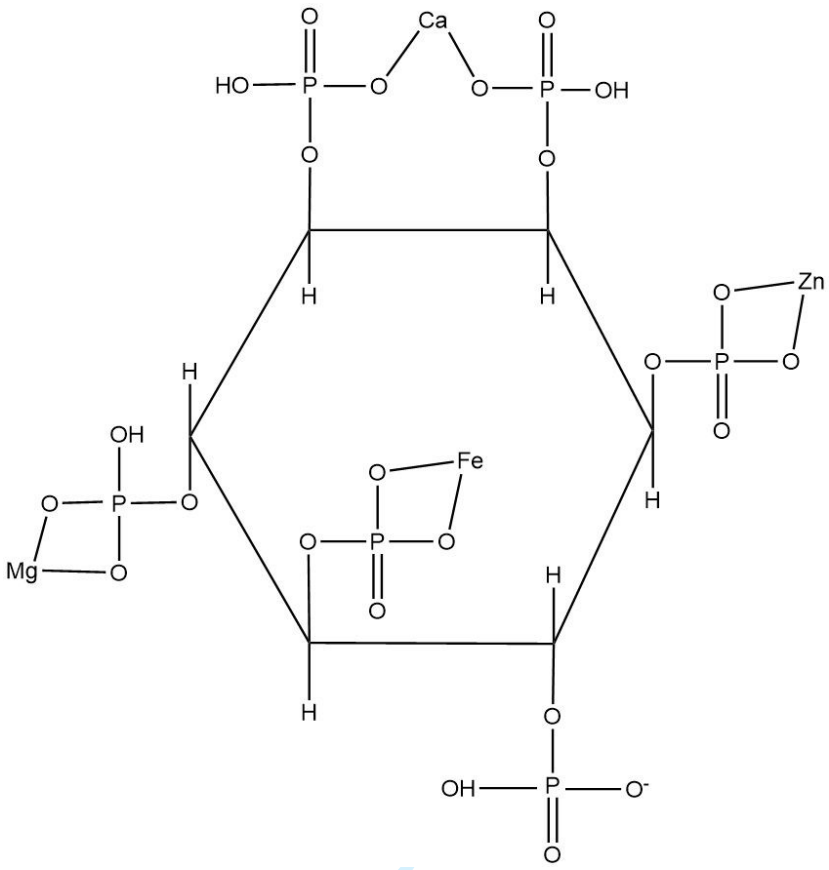

Figure 1c. The element-binding model suggested by Weingartner and Erdman (1978)

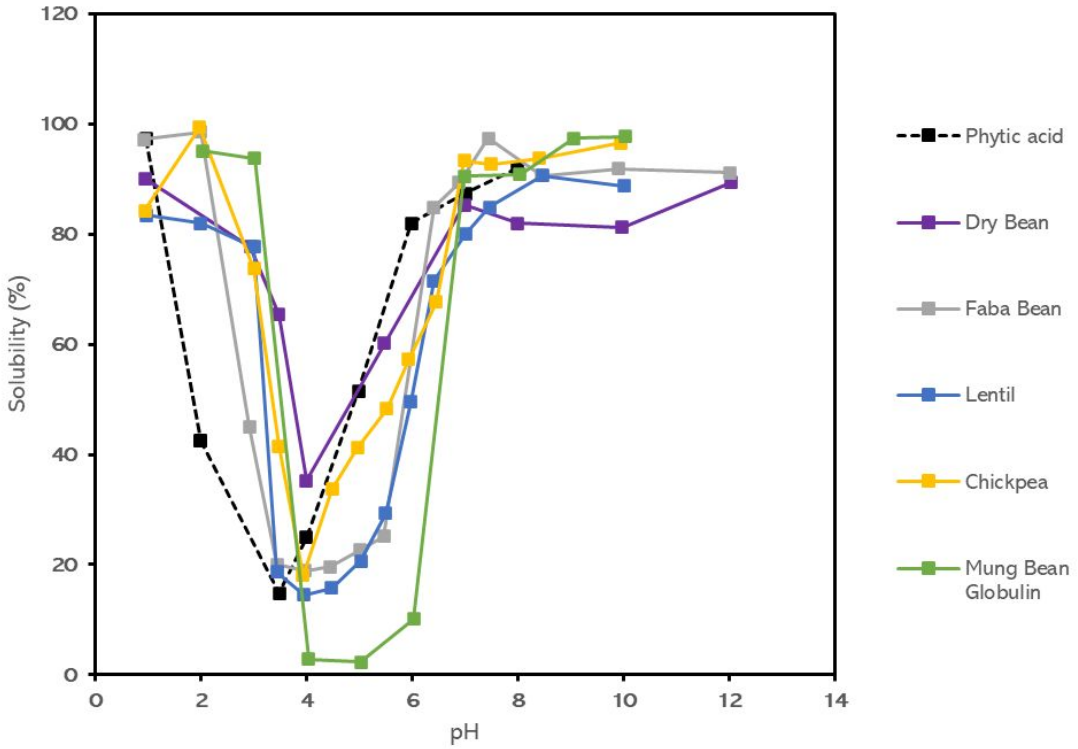

Figure 2. Solubility curve of phytic acid and various legume proteins in water, redrawn with data from Carnovale, Lugaro, and Lombardi-Boccia (1988); Carbonaro et al. (1997); Tang and Sun (2010).

Many thanks for all the valuable comments and suggestions from the editor and reviewers. We have addressed and implemented all these comments/suggestions as summarised in the table below and highlighted changes in yellow throughout the manuscript.

Response to reviewers' comments for manuscript BFSN-2020-5787-R1

Comments from Reviewer: 1	Responses
Present review is focusing on the effect of Phytic acid on minerals bioavailability (Iron, Zinc and Calcium) with special focus on legumes. However, some points needs to be rectify/elaboration for better understanding of the content in the review. 1. In the manuscript, several times mentioned the terminology "Bioaccessibility" and "Bioavailability" which is confusing to understand the impact of Phytic acid. Please use the term "Bioavailability" uniformly throughout the manuscript as it is the key of nutritional efficiency of any food and cover both bioaccessibility and bioactivity.	We thank reviewer 1 for their constructive feedback. We understand that 'bioaccessibility' and 'bioavailability' refers to different terms and agree that bioavailability is ultimately the key to nutritional efficacy. We have replaced most of the 'bioacessibility' terms throughout the text with 'bioavailability', except for one exception (Page 9) where we have defined the term 'bioaccessibility', consistent with the fact that most *in vitro* studies only reflect bioaccessibility.
2. L 36-41 (page 2): Clarify the sentence- "Food processing strategies to achieve phytate reduction were identified as promising tools to increase mineral bioaccessibility and included germination and fermentation, particularly when combined with promoter addition."	We have reworded the sentence to 'Food processing strategies to achieve phytate reduction were identified as promising tools to increase mineral bioavailability and included germination and fermentation, particularly when other bioavailability promoters (e.g. NaCl) are simultaneously added.'
3. L 45-59 (page 4), L 1-17 (page 5): It has been mentioned and well understood that Phytic acid only is not responsible for lower minerals bioavailability. Mention and discuss other components (apart from protein) and their effect on minerals bioavailability.	We agree that phytic acid is not the only factor responsible for lower mineral bioavailability and have added the following sentences: 'These factors may include certain polyphenols, tannins, oxalates, fibres as well as microstructure and state of aggregation. Additionally,

	food processing techniques, such as thermal treatments may mediate or reduce the degree of interactions between phytate and these factors." in L5 – 9 on page 5.
4. L 8-29 (page 11), L 3-8 (page 12): Clarify and properly discuss the mixed finding of Phytic acid on Fe, Zn and Ca bioavailability..	We have clarified several sentences that may have been confusing, including: L 25, and L1-2 on pages 10 and 11 "Zn absorption may be less affected by IP species with lower degree of phosphorylation, as corroborated by another study that demonstrated no effect on short-term Zn absorption when IP5 was added to food" L 15-19 on page 11: "However, some studies have found inconsistent effects on Fe absorption (i.e. no improvements) when IP6 was removed (Hurrell, Reddy, Burri, & Cook, 2002; Petry et al., 2010). Similar to the observations with Zn, inositol species with lower levels of phosphorylation, such as IP3 were found to have no effect on Fe absorption when added to food (Sandberg et al., 1999)."
5. Graphical abstract is required for better understanding of Phytate-element interaction affecting minerals bioavailability.	A graphical abstract has been prepared and attached.
Comments from Reviewer 2	
The structure of IP6 and its effect on bioavailability of different minerals including iron and zinc have been well narrated. Not found much correction. Some of the corrections include:	Thank you for the kind comments and constructive feedback. We have replaced the 2006 reference with a more recent reference (Bailey, West & Black, 2015.

1. Page 2-Line 55: the reference is too old (2006). try to add current diffeciency status	
2. Page 3-Line 8: Here also replace the old (2007) reference to current status.	The 2007 reference was replaced with Sozen, Ozisik & Basaran, 2017.