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Full title: Opportunities for Plant-derived Enhancers for Iron, Zinc and Calcium Bioavailability: a review

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ABSTRACT: Understanding of the mechanism of interactions between dietary elements, their salts and complexing/binding ligands is vital to manage both deficiency and toxicity associated with essential element bioavailability. Numerous mineral ligands are found in both animal and plant foods and are known to exert bioactivity *via* element chelation resulting in modulation of antioxidant capacity or micobiome metabolism amongst other physiological outcomes. However, little is explored in the context of dietary mineral ligands and element bioavailability enhancement, particularly with respect to ligands from plant-derived food sources. This review highlights a novel perspective to consider various plant macro/micro-nutrients as prospective bioavailability enhancing ligands of three essential elements (Fe, Zn and Ca). We also delineate the molecular mechanisms of the ligand-binding interactions underlying mineral bioaccessibility at the luminal level. We conclude that despite current understandings of some of the structure-activity relationships associated with strong mineral-ligand binding, the physiological links between ligands as element carriers and uptake at targeted sites throughout the gastrointestinal tract still requires more research. The binding behaviour of potential ligands in the human diet should be further elucidated and validated using pharmaco-kinetic approaches and gastrointestinal models.

1 Introduction

Over twenty inorganic elements are essential to human health as dietary nutrients, facilitating an array of biological functions within the human body (White & Broadley, 2005). These essential elements function as structural elements of biomolecules and tissues, and some also with physiological functions. Examples include Ca, P and Mg in bone health; Fe, Zn and Cu as metalloprotein and enzyme cofactors; Ca and P as intracellular signalling messengers; Na, K, Ca, Se in redox and acid-base balance and K, P and Na in osmoregulation (Replogle, Fleet, & Salt, 2011). Despite their widespread presence in biological systems, essential element deficiency remains a highly prevalent global concern that affects up to a third of the world's population (Jariwalla, Niedwiecki, & Rath, 2010). The physiological and metabolic impairments associated with element deficiency affects socioeconomic development and productivity at the individual, societal and national level (Troesch et al., 2015).

The bioavailability of an element is defined as the total fraction in a given food or diet that is available to the body for physiological function after ingestion (Guo et al., 2014; La Frano, de Moura, Boy, Lönnerdal, & Burri, 2014). Bioavailability is central to the development of cogent element fortification and supplementation strategies, as the absorption of elements are associated to dosage, chemical form, delivery matrix, and pharmacokinetics. These factors are central to element bioavailability from both dietary and supplemental sources, particularly with divalent cations (Rafferty, Walters, & Heaney, 2007). Similarly, elemental

content is a poor indicator of bioavailability in food matrices (Nosratpour & Jafari, 2019), as other bioavailability factors in foods are involved, such as absorption enhancers and inhibitors. Oral administration through dietary or supplement routes is considered the most acceptable approach to reverse mineral deficiency, but is limited by bioavailability barriers. Additionally, approaches to address deficiency through supplementary salts can produce adverse sensory effects and dose-dependent bioavailability issues (C. Wang, Li, & Ao, 2012), and some may cause toxicity from overdose (Zand, Christides, & Loughrill, 2015).

In the past decade, food-derived components as bio-based delivery systems for enhancing element bioavailability have been gaining increasing consumer attraction as desirable substitutes to element supplements (Guo et al., 2014). Some isolated food polymer fractions comprise physicochemical properties, such as binding, bulking and fermentation ability, solubility and gel formation, which can facilitate elemental release and absorption (Baye, Guyot, & Mouquet-Rivier, 2017; Belitz, Grosch, & Schieberle, 2009). Fractions that may enhance elemental bioavailability have been characterized in a diverse number of novel food sources, such as calcium-binding peptides from milk (Cross, Huq, & Reynolds, 2007), soybean (Y. Lv, Bao, Liu, Ren, & Guo, 2013) and shrimp (Huang, Ren, & Jiang, 2011). However, bioactive supplements from plant-derived sources have not been extensively explored (Eckert, Bamdad, & Chen, 2014; Eckert et al., 2016). Plant metabolites such as peptides and polyphenols are known for their element chelation properties, although

studies generally demonstrate source-dependent and inconsistent effects on element bioavailability.

The essential dietary elements Fe, Zn and Ca are well documented in a global deficiency (Shenkin, 2008; Suzuki, Landowski, & Hediger, 2008) and are often associated with bioavailability issues rather than a lack of quantity, especially from plant sources (Drago, 2017; Vavrusova & Skibsted, 2014). The bioavailability of elements from animal foods are fairly adequate, however the majority of populations worldwide relies on plant-based diets, particularly in developing countries with lower income (Solomons, 2000). Fe, Zn and Ca intake are critical for optimal wellbeing of all individuals, but particularly towards adequate development in vulnerable populations including pregnant women and children, to prevent intergenerational cycles of micronutrient malnutrition (Keeley, Little, & Zuehlke, 2019). Furthermore, the prevalence Fe, Zn and Ca deficiency as a public health predicament may be exacerbated under the context of global climate change, where field studies under projected conditions have demonstrated reduced soil uptake of these elements into commonly edible crop tissues (Dietterich et al., 2015; Loladze, 2014).

Nutritional bioavailability enhancement using recipient food-grade complexes as delivery agents has been a subject of research in recent years (F. Liu, Ma, Gao, & McClements, 2017; McClements et al., 2015). This current review builds upon existing knowledge aiming to further examine the role of plant-derived fractions in the bioavailability of Fe, Zn and Ca.

First, we discuss the fundamental antagonistic causes leading to reduced bioavailability of Fe, Zn and Ca. We then focused on the chemistry of plant biomolecules that may be naturally present or derived and that may help promote the absorption of elements from foods. We propose that the identified plant-derived element binding ligands would greatly benefit from the use of biorelevant simulations throughout all stages of the digestive tract, which would allow characterization of their abilities as element bioavailability enhancers. Finally, a general outlook and some practical limitations toward their application in food systems are described.

2 Overview of element bioavailability

2.1 The biological roles of Iron, Zinc and Calcium

Iron (Fe) functions as a bound factor of many proteins, and as an active site element for over 200 metalloenzymes implicated in the primary biological processes of mammalian cells (Sheftel, Mason, & Ponka, 2012). As a component of haemoglobin, Fe plays a vital role in the binding, transport and activation of O_2 and CO_2 for cellular respiration within the human body (Tracey A. Rouault, 2003). In healthy adults, 1–2 mg of Fe are absorbed per day with a circulating pool of ~3–5 g (Sebastiani & Pantopoulos, 2011). Up to 90% of daily Fe requirements are reported to be met endogenously, primarily through the recycling of haemoglobin from senescent red blood cells. However, the absence of adequate stores engenders Fe deficiency and its associated anaemia (Clark, 2008). Fe-deficiency anaemia is classified amongst the most prevalent Fe related disorders worldwide, imperilling approximately one billion people in both developing and developed nations (Balarajan, Ramakrishnan, Özaltin, Shankar, & Subramanian, 2011; Knai, Sharan, & Baltussen, 2004). The condition can impair individuals' cognitive and physical development and productivity, which leads to morbidity and mortality in susceptible populations including women, children, and consumers of plant-based diets (Y. Li, Jiang, & Huang, 2017; Zimmermann, Chaouki, & Hurrell, 2005).

In juxtaposition with deficiency, adverse conditions involving non-hereditary Fe overload are also reported from supplemental salts and parenteral administration (Hira et al., 2018;

Yilmaz & Li, 2018a). Although Fe absorption is strictly regulated by host feedback of body stores and requirements for erythropoiesis (Nicolas et al., 2002), the lack of a physiological route for Fe excretion can result in gradual build-up of a non-chemically bound and reactive pool of Fe. Due to the high redox potential of free Fe, electron transfer processes that occur catalyse a chain of Haber-Weiss/Fenton-like reactions. The associated radical intermediates formed are known to be cytotoxic and highly oxidative, and instigate various dysfunctions associated with oxidative stress within the body (Adjimani & Asare, 2015; Fraga, 2005). An effective yet safe therapeutic window has thus been central to the development of strategies targeting Fe deficiencies, with food-derived Fe-sources recognised as nontoxic alternatives that may reduce their ability to act as pro-oxidants in generating free radicals (Eckert et al., 2014; Prentice et al., 2016).

Zinc (Zn) is required for a myriad of structural, catalytic and regulatory roles (Bel-Serrat et al., 2014), and it has an ability to bind to ~10% of proteins within the human body (Andreini & Bertini, 2012). While Fe is confined intracellularly with specific physiological roles, Zn is ubiquitously present as a co-factor and intracellular signalling ion in the functioning of more than 300 enzymes and 1000 transcription factors (Cherasse & Urade, 2017; Roohani, Hurrell, Kelishadi, & Schulin, 2013). In addition, recent evidence from meta-analyses is suggesting the role of adequate Zn status in the prevention of chronic diseases, such as diabetes mellitus (Fernández-Cao et al., 2019). Although global risks of inadequate Zn intake have been decreasing over the past decade, it is still projected to affect 16-17.5% of the world's

population (Kumssa et al., 2015; Wessells & Brown, 2012). To date, reliable biomarkers to assess Zn status remain undefined (Wieringa, Dijkhuizen, Fiorentino, Laillou, & Berger, 2015). Whilst most cases of Zn deficiency are mild with little clinical signs, it is often coupled with acute respiratory conditions, pregnancy complications, diarrhoea, and restricted growth in children (P. White & Broadley, 2011; Wilson, Grieger, Bianco-Miotto, & Roberts, 2016). The prevalence of inadequate intake amongst infants and children with pneumonia, diarrhoea or malaria has been estimated to contribute to 800,000 excess deaths (Caulfield, de Onis, Blössner, & Black, 2004). However, high Zn intakes may interfere with copper absorption (Fraga, 2005). Homeostatic adjustments of dietary Zn absorption and endogenous excretions ensue according to body demands, with multiple excretion pathways (Krebs, 2000). Although a substantial amount of endogenous Zn is reabsorbed within the gastrointestinal tract, low intake or prolonged marginal intake can augment Zn elimination (Roohani et al., 2013). Half of all Zn losses occur in the lumen, and is further exacerbated in those suffering deficiency-related diarrhoea (K. H. Brown, Peerson, & Allen, 1998).

Calcium (Ca) is the most abundant inorganic element in the human body, 99% of which are in the form of calcium phosphate in bones (Guo et al., 2014). Apart from its role in the development and maintenance of bone structure and health, Ca also participates in muscle contraction, blood clotting, enzyme regulation and intracellular metabolism (Lagarda, Cilla, & Barbera, 2016). As a result of insufficient dietary intake and bioavailability, an estimated

3.5 billion people are at risk of Ca deficiency (Kumssa et al., 2015). Sufficient dietary Ca prevents the occurrence of rickets in infants and children (Sun et al., 2016), and is particularly critical between adolescence and 30 years old as one reaches peak bone mass (L Hallberg, Rossander-Hulten, Brune, & Gleerup, 1992). This reduces the likelihood of osteoporosis during later adulthood, as skeletal demineralization gradually occurs (Drago, 2017).

Similar to Fe and Zn, Ca absorption and resorption are regulated by several host factors including the amounts ingested. Hormonal regulation through calcitriol [$1,25(OH)_2D_3$] in conjunction with parathyroid and thyroid hormones play a critical role in Ca homeostasis (Diaz de Barboza, Guizzardi, & Tolosa de Talamoni, 2015). Ca losses from both endogenous (salivary, biliary and pancreatic fluids) and dietary pools occur in the digestive lumen and *via* faecal excretion (Drago, 2017). However, cases of hypercalcemia and hypercalciuria have been common from excessive intake of supplements (Ross, Taylor, Yaktine, & Del Valle, 2011). The dysregulation associated with both deficient and excess quantities of Ca are linked to the pathogenesis of inflammatory, degenerative and metabolic diseases (Peterlik & Cross, 2009), as well as an increased risk of some cancers (Barry et al., 2017).

Both Fe and Ca are physiologically classified as type one nutrients, with inadequacies initially leading to reductions in body stores prior to functional components; while Zn is a type two nutrient where declines in plasma levels are observed following observations in clinical

symptoms (Kondaiah, Yaduvanshi, Sharp, & Pullakhandam, 2019). This reveals that clinical Zn deficiency can be particularly difficult to detect, due to the absence of an established biomarker (K. Brown et al., 2004). Nonetheless, considering the dire consequences in physiological imbalances of Fe, Zn and Ca, understanding the factors that control the bioavailability of these elements are crucial for addressing their deficiency and toxicity issues. The following sections will briefly introduce the chemistry of Fe, Zn and Ca as found within food matrices, and how their speciation determines bioavailability through the digestive tract.

2.2 The chemistry of Iron, Zinc and Calcium from food sources

2.2.1 Iron

Iron is primarily found in two readily reversible oxidation states in foods: Ferric (Fe^{3+}) and ferrous (Fe^{2+}). As a transition metal, Fe shows oxo- and thiophilicity with its ability to form coordination complexes with O-, S- and N- atoms on donor ligands (Kepp, 2017). According to Cremonesi, Acebron, Raja, and Simpson (2002), Fe in solution exists as the di- or trivalent ion in an aqua complex under anoxic conditions. However, the ferrous-aquo complex is rapidly deprotonated at intestinal pH in the presence of oxygen, forming precipitates of ferric-oxo-hydroxides. Fe is highly prone to complexing with other compounds of plant origin that can inhibit its uptake, whilst Fe of animal origin is usually complemented with factors that circumvent Fe precipitation and associated bioavailability issues. These absorption promotors from animal sources are collectively termed ”meat factors”, and

knowledge regarding their enhancing mechanisms are briefly explored in the current text in Section 3. Haem/heme, the form of organic Fe that accounts for 40% of the total Fe found in animal foods (Saunders, Craig, Baines, & Posen, 2013), is a principal component. Haem is a complex of ferrous Fe coordinated to a protoporphyrin ring and is highly bioavailable (Y. Li et al., 2017), and constitutes more than 40% of total absorbed Fe in meat-eating populations (Egli & Hurrell, 2010). Some plants also have haem group proteins, such as the leghemoglobin found in some plant roots (Kannan, Sithara, & Chandru, 2015). However, these root sources are not considered as a nutritional iron as the roots are not conventionally consumed.

Other naturally occurring bound forms of Fe include phytin in plants, and ferritin in both plant and animals. Fe-bound to phytin (myo-inositol hexakisphosphoric acid, IP), is considered to have little bioavailability in its native form (Sokrab, Mohamed Ahmed, & Babiker, 2014). This is due to its number of highly negatively charged phosphate groups on phytin, which bind with high affinity to cations at neutral physiologic conditions of the small intestine (~ pH 7) (Rosalind S Gibson, Raboy, & King, 2018). Ferritin is a ubiquitous family of Fe-storage polypeptides found in virtually all living organisms. The globular protein complex encapsulates a ferric Fe core, and has shown potential in some human studies to be a readily bioavailable form of Fe (Elizabeth C. Theil et al., 2012; Elizabeth C Theil, Davila-Hicks, & Lönnerdal, 2004). However, differences in subunit compositions amongst food sources of ferritin may confer diverse properties in relation to its digestibility, and thus bioavailability

(C. Lv, Zhao, & Lönnerdal, 2015). In a study by Jin, Frohman, Thannhauser, Welch, and Glahn (2008), both reconstituted plant- and animal- type ferritin from horse spleen apoferrin have been found to release their Fe core during *in vitro* digestion, and the Fe was subsequently prone to interactions with absorption enhancers such as ascorbic acid, and inhibitors such as phytate.

2.2.2 Zinc

Zinc is a trace element and is widespread in foods due to its pervading role in biological systems. It has remarkably high binding affinity for food proteins and nucleic acids from various plant and animal food sources (Drago, 2017). In plants, a proportion of Zn is also present in insoluble phytin bodies together with Fe (Raes, Knockaert, Struijs, & Van Camp, 2014). Non-protein bound Zn is poorly understood in respect to ligand exchange, apart for its affinity for various inorganic and organic ions due to the lack of stereochemical preferences (Krężel & Maret, 2016). The best dietary sources of Zn are derived from meat and seafood flesh, both of which provide necessary quantities with high bioavailability (Fraga, 2005; Hambidge & Krebs, 2007). Adequate intakes are provided easily with meat inclusion in one's diet, yet much of the world's population subsists on plant foods as sole sources of Zn (R.S. Gibson, Bailey, Gibbs, & Ferguson, 2010). Concentrations found in plant products are considered insufficient even in sources containing high concentrations, such as grains and legumes. These foods are known to contain Zn-binding low molecular weight compounds, which can affect its bioavailability. For example, high molar ratios of phytic

acid (0.66 kDa) to Zn are well known to inhibit Zn absorption from plant-based diets (Wessells & Brown, 2012). On the other hand, carrier proteins such as casein phosphopeptides and milk lactoferrin can act as ligands to solubilize zinc (Ainscough, Brodie, & Plowman, 1980; Delshadian et al., 2018).

2.2.3 Calcium

The chemical properties of Ca from different food sources play a critical role in its bioavailability. The primary dietary Ca contributors are dairy products, which intrinsically possess high concentrations. Calcium absorption enhancers found in milk, such as lactose, organic acids, casein and whey proteins (Buchowski, 2015), make dairy-based matrixes an efficient promoter of Ca bioavailability. Plant-derived Ca is present in vegetative or reproductive tissues (e.g. the leaves and seeds) and constitute its second highest dietary contributor (Dayod, Tyerman, Leigh, & Gilliham, 2010). However, dietary Ca bioavailability from plants is limited by its ability to readily form stable interactions with biomolecules in plants, which can act as absorption inhibitors. In plants, a large fraction of Ca can be found cross-linked in cell wall structures, or bound to various organic/inorganic anions as insoluble carbonate or oxalate salts (Philip. J. White & Broadley, 2003). In these complex structures Ca provides structural integrity to plant cellular membranes (Hepler, 2005), however, the amount of Ca that undergoes solubilisation from this composite matrix during digestion in the human digestive tract is limited. Additionally, solubilised free Ca^{2+} tends to precipitate in its carbonate form under the alkaline pH and aerobic conditions of the small intestine

(Goss, Prushko, & Bogner, 2010). Ca bioavailability is thus challenging, despite its abundant distribution and high concentrations in plant-based foods.

2.3 Bioavailability during transit through the gastrointestinal tract

2.3.1 Element absorption during gastrointestinal (GI) digestion

For an element to be bioavailable, it must first be bioaccessible for absorption. The accessible element must cross the apical mucosal membrane lining the GI tract *via* a transcellular or paracellular route, and into the portal circulation (Harvey, 2001; Krebs, 2000). From the basis of their physicochemical properties, elements can diverge significantly in their ability to be transported across the columnar epithelial cells lining the small (enterocytes) and large (colonocytes) intestines. Some general key intrinsic properties that affect their absorption and hence bioavailability include solubility in gastrointestinal fluids, chemical speciation and physical characteristics of the food matrix (Apostoli, 2006).

A number of dynamic digestive processes occur sequentially prior to impending absorption, beginning from oral processing. The oral route of element delivery takes place through the ingestion of food. Within the oral cavity, mastication contributes physically for size reduction of the food, and is assisted by salivary wetting and enzymatic amylolysis of starch (where present) by salivary α-amylase. The secretion of salivary proteins such as mucins may be important for element bioaccessibility from food matrices, as they not only

contribute to eventual elemental dissolution but may also interact with molecules that act as element enhancers or inhibitors (Çelebioğlu, Lee, & Chronakis, 2019; Delimont, Rosenkranz, Haub, & Lindshield, 2017). The bolus then travels down the oesophagus passing the oesophageal sphincter into the stomach. Here, an amalgamation of proteolytic enzymes, acidic secretions and physical churning takes place in a highly acidic environment of pH 1.7 – 2.8 (Dressman et al., 1990; Feldman & Barnett, 1991), which favours element dissolution and solubility (Miller, 2007). Its relevance to elemental bioavailability is, however, limited as little element uptake occurs during this stage in the stomach with exception of Cu^{2+} (Lowndes & Harris, 2005).

The bulk of element absorption occurs in the proximal section of the small intestine, the duodenum (Eckert et al., 2014), under neutral pH and aerobic conditions. The passage of the bolus from the stomach lowers the fasting pH in the duodenum from pH 7.0 to 5.5, which transiently favours element solubility. However, the pH gradually rises to back to ~ 7.0 at the terminal ileum section due to bicarbonate secretion (Gharibzahedi & Jafari, 2017). These conditions can lower the amount of bioaccessible element through different ways. For example, a reduction in the concentrations of free ions arises in some elements, due to the formation of poorly soluble mineral oxides and hydroxides under oxygenated conditions. The neutral pH also facilitates the accumulation of element-organic complex of various dietary or endogenously secreted compounds that act as mineral ligands.

Depending on their physicochemical properties, these ligands may reduce or enhance element solubility (Carbonaro, 2011).

2.3.2 Trans- and para-cellular element transport

Elements undergo an array of unique or shared transport pathways across the apical membrane of the enterocytes that form the small intestinal lining (Kiela & Ghishan, 2016). Transcellular and paracellular pathways are the two primary transport mechanisms, both of which are subject to regulation by nutritional and hormonal factors (Diaz de Barboza et al., 2015). The transcellular route is the predominant pathway where the intake is low, requiring active transport by specific membrane transporters on enterocytes surface and is thus saturable across a concentration gradient. However, different ions present in the lumen can be an imperative aspect in the active transport process. Some element transporters can be non-specific, where elements with similar coordination geometry and ionic radii can be competitive with each other with respect to transporter binding. This is exhibited by the interactions between Fe, Zn and Ca with the communal divalent metal transporter DMT-1, that mediates the uptake of Fe^{2+} (Tracey A Rouault, 2013) and Zn^{2+} (Espinoza et al., 2012). The affinity of DMT-1 towards various divalent cations (including Ca^{2+}) can demonstrate inhibition in the cellular uptake of these elements at certain concentrations (Shawki & Mackenzie, 2010; B. A. V. Thompson, Sharp, Elliott, & Fairweather-Tait, 2010; Yamaji et al., 2001). However, soluble mineral chelates can undergo transcellular absorption with separate transport pathways from those of the free ions. For

example, an element-bound peptide can be absorbed intact by selective peptide transporters carrying the element with it (Chakrabarti, Guha, & Majumder, 2018). This route can provide circumvention of any competition between ions for metal-specific carrier transport.

The passive non-saturable paracellular route of Fe, Zn and Ca uptake typically occurs under high intake, where an influx of intraluminal saturation can overcome the lumen-positive transepithelial potential difference (Field, Harris, & Pollock, 2010). Passive uptake may also be mediated with the presence of certain osmotic agents, such as sugars (Buchowski, 2015; Christides & Sharp, 2013) and other soluble element binding ligands where elements can co-diffuse with water into the interstitial space (Goff, 2018). Membrane permeability between cell junctions can be modulated by the presence and expression of Claudin proteins at certain segments, which form channels that can facilitate selective ion uptake (Workinger, Doyle, & Bortz, 2018). Claudin expression by epithelial tissues is host-dependent (Tsukita, Tanaka, & Tamura, 2019), but is also mediated by the presence of gut bacteria metabolites such as short-chain fatty acids (SCFAs) derived from dietary fibre (Zheng et al., 2017).

2.3.3 Apical Iron, Zinc and Calcium influx

The route of Fe uptake is highly contingent on its chemical form and elemental speciation. Haem bound ferrous iron is readily absorbed as a haem-Fe^{2+} soluble complex conceivably

involving a haem carrier protein (HCP-1). Ferric-containing ferritin is also able to enter the enterocyte intact, *via* an unspecified, saturable form of receptor mediated endocytosis (Kalgaonkar & Lönnerdal, 2009; San Martin et al., 2008). Free ferric Fe is only able to undergo transcellular absorption after its reduction to ferrous Fe, either by dietary components such as organic acids and phenolics, or through an intestinal ferric reductase (duodenal cytochrome b) at the brush border of duodenal enterocytes (Frazer, Wilkins, Vulpe, & Anderson, 2005). Reduction of the ferric ion by dietary reducing agents is conditional; for example, reduction by ascorbic acid only takes place below pH 6 (Hsieh & Hsieh, 1997). Transport into the cell takes place primarily through the action of the DMT-1 for intracellular utilization or storage (Fuqua, Vulpe, & Anderson, 2012). Due to the harmful nature of free Fe as an oxidative catalyst, absorption of Fe ions *via* passive diffusion is limited (Geisser, 2007). On the other hand, precipitated forms of ferric Fe salts are unavailable for uptake and are passed to the colon where dybiosis is experienced as a result of the iron-mediated growth of pathogenic bacteria.

Zn^{2+} ions are absorbed through binding to endogenously secreted ligands such as mucins (Food and Agriculture Organization & World Health Organization, 2004). The carrier-mediated route is considered to be the primary pathway, with paracellular uptake ensuing at higher intake (Krebs, 2000). Apical transport is thought to be mediated by the protein Zip4 (SLC39A4), which is upregulated in response to high luminal zinc concentrations (Dufner-Beattie et al., 2003; Weaver, Dufner-Beattie, Kambe, & Andrews, 2007). Zn uptake

is not generally considered to be different from organic versus inorganic sources within a food matrix, although more efficient uptake is observed from animal-based sources (King & Keen, 1999). Zinc-amino acid conjugates are known to be taken up by amino acid carriers and is mostly uninhibited by complexing agents (Sauer et al., 2017). While Fe homeostasis is structured by controlling absorption, Zn is regulated through the excretion of endogenous Zn (Kondaiah et al., 2019), as well as luminal and cytoplasmic zinc concentrations (Maares & Haase, 2020).

Small intestinal absorption of soluble Ca^{2+} is an intertwined process between transcellular and paracellular pathways. The paracellular route prevails with normal to high intake, which occurs from the duodenum to the ileum (Kiela & Ghishan, 2016). An estimated 65% of total Ca absorption takes place in the ileum, due to the relative length of the segment. Claudin proteins (Cldn-2, Cldn-12, and Cldn-15) are known to facilitate absorption through enhancing Ca^{2+} permeability in the jejunum and ileum (Fujita et al., 2008). The transcellular pathway of apical transport is upregulated at lower Ca intake, predominating in the duodenum and jejunum (Christakos, 2012). Active Ca transport is also dependent on calcitrol, which plays a role in the biosynthesis of the Calbindin-D9k that binds to Ca^{2+} ions and mediate cellular transport from the apical to the basolateral side (Bronner & Pansu, 1999). A number of other duodenal transporters have been identified to work synergistically towards apical Ca intake, including TRPV5, TRPV6, and Cav1.3 (Wongdee & Charoenphandhu, 2015). Transcellular transport is dependent on 1,25-dihydroxyvitamin D3

[1,25(OH)2D3], which upregulates the expression of these calcium transporter genes (Balesaria, Sangha, & Walters, 2009).

2.3.4 Element uptake in the colon

Insoluble elements in their matrix-bound, organically complexed or precipitated forms, which escape absorption in the small intestine are passed into the colon along with other undigested food and indigestible matter. The colonocytes transport mostly water, electrolytes and prebiotic microbial fermentation products such as SCFAs (Fairweather-Tait & Johnson, 1999), and is generally considered to be a limited site for element uptake. The more constricted colonic epithelial cell junctions impose greater difficulties in expediting paracellular uptake, although non-direct means of element uptake by the colonocytes may be facilitated through anion exchange and separate transporters (Kiela & Ghishan, 2016). The factors controlling colonic elemental absorption are not well understood in humans, with limited studies demonstrating evidence of element uptake in nutritionally significant amounts, except for Ca, Mg and possibly Fe (Alexander, Swanson, Fahey, & Garleb, 2019). Large intestinal absorption of Ca^{2+} has been suggested to account for ~10% of total calcium uptake (Bronner & Pansu, 1999), occurring *via* both active transcellular and passive diffusion routes in the rectum and distal colon (L. U. Thompson, Wolever, & Trinidad, 1996). Ca-SCFA complexes have been proposed as a route to paracellular absorption through anion exchange mechanism (Hara, 2002). Colonic Fe availability is restricted (Parmanand et al., 2019; Swinkels, Kortman, Tjalsma, & Raffatellu, 2014), likely as a protective mechanism

against free Fe-induced oxidative damage and inflammation (Yilmaz & Li, 2018b), and their utilization by pathogenic bacteria (Grinter, Walker, & Milner, 2013). Evidence has been reported in the literature regarding Fe and Zn caecal absorption in rodents (Bouglé et al., 2002; Seal & Mathers, 1989). However, there isn't enough information regarding the extent to which this occurs in humans (Carvalho et al., 2017; Gopalsamy et al., 2015).

There is accumulating evidence from both human and rodent studies suggesting the stimulation of element absorption by prebiotic fermentation products, particularly SCFAs such as acetate, butyrate and propionate (Katharina E Scholz-Ahrens, Schaafsma, van den Heuvel, & Schrezenmeir, 2001; Whisner & Castillo, 2018). SCFAs may mediate localised reductions in luminal pH (Topping & Clifton, 2001), supplementing the reduced (anaerobic) environment of the colon (Hedrich, Schlömann, & Johnson, 2011), and augment extracellular reductases' activity (Swinkels et al., 2014; Takeuchi et al., 2005) in enhancing ion solubility. In rodents, SCFAs have been linked to increases in Fe and Ca transporter expression (Carvalho et al., 2017; Fukushima, Aizaki, & Sakuma, 2008), as well as gas-induced distention of the mucosal surface area for absorption (Katharina E. Scholz-Ahrens et al., 2016).

3 Ligands in relation to bioavailability

Cations such as Fe^{2+}, Zn^{2+} and Ca^{2+} may form reversible ion-pairs or chelates with numerous endogenous molecules from the body or derived from foods. The food ligands are ordinarily characterized as compounds with a minimum of two element binding sites from electron donor atoms that coordinate to the central elemental ion. Under favourable conditions, proton displacement by the element ion can occur in the presence of acidic groups (e.g. -OH, -COOH, -SH, -NOH) and Lewis bases (e.g. -C=O, $-NH_2$, and other electron pair donors) of various biomolecules found in food (Kontoghiorghe, Kolnagou, & Kontoghiorghes, 2015; Lindsay, 2017). Regardless of the chelating ligand, the strength of binding plays an important role in mediating elemental bioavailability. Loosely coordinated complexes may dissociate during digestion, allowing for precipitation or ligand exchange prior to prospective uptake. Strong complexation may lead to elimination in the case of an insoluble element-ligand complex, or reduced intracellular efflux following uptake with a soluble element-ligand complex. In addition to being soluble, an effective chelate needs to be able to bypass co-precipitation with other insoluble complexes.

Although the literature has largely emphasized the strong mineral-ligand binding in bioavailability enhancement, weak mineral-ligand pairs that dissociate under gastric digestion may still facilitate element bioavailability through re-complexation *in situ*. This is largely determined by the availability of free ligands, dissolution/reformation kinetics and gut transit time (Harju, 1989). The presence of free gastric-dissociated soluble ligands, particularly in excess molar ratios, may encourage immediate mineral-ligand complexation

and directly undergo uptake. Where complexation occurs during early small intestinal digestion (i.e. duodenum and/or jejunum), uptake is propagated at peak efficiency and likely faster than insoluble ligands can bind to the element ion (Vavrusova, Raitio, Orlien, & Skibsted, 2013). The lag phase before precipitation, in which complex recombination can occur has been demonstrated by kinetic studies on Ca salts (Skibsted, 2016). Ca salts that rapidly reform soluble complexes in the duodenum, such as those with hydroxycarboxylic acids, often demonstrate higher fractional absorption and retention *in vivo* (Costello, Franklin, Reynolds, & Chellaiah, 2012; Hackl et al., 2016). Similarly, a proposed mechanism of the 'meat factor' as a Fe bioavailability enhancer is the fast formation of soluble ferrous complexes with both gastric-hydrolysed and cysteine-containing peptide fractions during early protein digestion (Hurrell, Reddy, Juillerat, & Cook, 2006; Kapsokefalou & Miller, 1995; Mulvihill, Kirwan, Morrissey, & Flynn, 1998; Tabatabai, Swain, & Reddy, 2002). Similarly, studies by Huh, Hotchkiss, Brouillette, and Glahn (2004); Laparra, Tako, Glahn, and Miller (2008) reported that specific carbohydrates originating from glycosaminoglycans of muscle tissues can enhance Fe bioavailability. Transit time, a factor preponderating these interactions, is also commonly considered in the absorption of Fe (Leary et al., 2017), Zn and Ca (Fleet & Schoch, 2010), despite limited literature towards its role in bioavailability enhancement *via* foods. For Fe and Zn where efficient uptake is limited to within the small intestine, lack of ligand dissolution during its transit time may be unfavourable for elemental bioavailability. Such low bioavailability would be observed for soluble ligand complexes that provide progressively latent release, or for strongly bound insoluble ligand complexes that

evade small intestinal dissolution. On the contrary, weak chelation sufficient to facilitate the dissolution of ions in solution can enhance element bioavailability (Lönnerdal, 2000).

The prevailing factors under physiological conditions that affect element-ligand complexes have been summarized in **Figure 1**. Purified biomolecular ligands have the potential to negate the bioaccessibility complexities of food matrices, while circumventing the toxicity of supplementary salts. Although there is broad literature consensus of many food compounds augmenting or inhibiting element bioavailability within the context of food matrices, the processes underlying the absorption, distribution, metabolism and excretion (ADME) of isolated food fractions remain obscure. Element-ligand interactions are largely considered as Lewis acid-base interactions (Lundgren & Stradiotto, 2016). For a given ligand, the hard and soft character are thus important dictators in the properties of complex formation. Ca^{2+} and Fe^{3+} are hard acids with affinity for ligands of the same property, while Fe^{2+} and Zn^{2+} are borderline acids. However, Fe^{3+} as a transition element with higher charge density possesses different coordination properties to Ca^{2+}. Subsequently, whilst all three element present similarities in binding target preferences, the stability, thermodynamic and kinetic affinity of bound complexes can diverge significantly. Some prospective element-ligand interactions between food components for Fe, Zn and Ca are summarized in **Table 1**.

The following sections delineate the features of some macro- and micromolecules that have been identified to interact with elements, and likely leverage their bioavailability.

Additionally, the direct and subsidiary means in which these compounds may modulate elemental absorption are highlighted.

3.1 Protein/Peptide derivatives

Proteins, peptides and amino acids (AAs) possess multiple ligand groups for element interactions, are amphoteric by their nature and able to react both as a base and as an acid. Considering that approximately 30% of biological proteins are metal- or metalloid-bound (Hauser-Davis & Parente, 2018), peptide conjugation is a broadly employed method for enhancing the cellular and nuclear entry of various biomolecules including elements (Dinca, Chien, & Chin, 2016; Puckett & Barton, 2010). The ability of protein fractions to confer elemental bioavailability stems from their element solubilizing or reducing properties at the small intestinal pH values, and/or as barriers shielding element ions from binding to other insoluble ligands. Element binding proteins and their derivatives from various food sources diverge in structure and composition.

3.1.1 Bioactive protein hydrolysates

Food-derived bioactive peptides are short amino acid sequences of 2 to 20 residues (Zarei et al., 2016), which can occur naturally or upon release from its native protein *via* heat or enzymatic processing. The element-binding property of bioactive peptides may accompany a spectrum of physiological activities observed with their ingestion (Bünning & Riordan,

1985; Carrasco-Castilla et al., 2012); including antioxidant, antimicrobial, immunomodulatory and antihypertensive activities (Chakrabarti et al., 2018; Xia, Bamdad, Gänzle, & Chen, 2012). Certain AA motifs may also exert element bioavailability enhancement within the gastrointestinal tract (GIT). Depending on the AA composition and sequence, element chelation can be achieved *via* specific or non-specific binding sites of residues with electron and ionic-rich side chains (Cai, Klamczynska, & Baik, 2001; Walters, Esfandi, & Tsopmo, 2018), or encased sterically through conformational states (Kozłowski, Bal, Dyba, & Kowalik-Jankowska, 1999). The presence of the terminal carboxyl and amine groups in all AAs and peptides are electron pair donors for coordinate bonding. The amine ligand in particular only possess one lone paired electron, which forms complexes weaker than the oxygen ligand that has two (Kawaguchi, 1988). Nonetheless, these are thus considered poor element coordination ligands relative to some functional side chains of individual AAs such as aromatic rings, which can convey more stable coulombic and non-coulombic interactions with the coordinated element (Apostoli, 2006). A peptide's native AA sequence and orientation may adopt certain conformations that may also contribute to strong element binding by directional effect towards coordinating orbitals of the central element ion (Guo et al., 2014; Walters et al., 2018).

As supplements, synthetic mineral-amino acid chelates have been found to achieve higher bioavailability than inorganic salts. For example, high bioavailability of synthetic Fe-AA chelates was reported by Jiménez-Alvarado, Beristain, Medina-Torres, Román-Guerrero, and

Vernon-Carter (2009), and also those of Zn-AA (Hennigar & McClung, 2018) and Ca-AA chelates (Ashmead, 1991). However, the high energy required for free AA transport can lead to issues with competitive uptake, saturation and osmosis (Clemente, 2000). Additionally, some AA-based chelators (e.g. ferrous bisglycinate) have been found to be poor at inhibiting complex formation with other ligands (Mellican, Li, Mehansho, & Nielsen, 2003). These issues have prompted great interest in using protein hydrolysates as element carriers instead. Compared to chemical methods, enzymatically hydrolysed proteins have the advantage of higher structural integrity and controlled hydrolytic sites (Clemente, 2000; Walker & Sweeney, 2002). The end product can generate a mix of free AAs and peptides that utilise respective pathways within the GIT, which leads to more efficient transport with less saturation (Rerat, Nunes, Mendy, & Roger, 1988). Both peptides (di-, tri- and oligo-) and small polypeptides may cross the intestinal wall intact utilising transporters or non-carrier dependent mechanisms (van der Wielen, Moughan, & Mensink, 2017), with some transporters possessing superior efficiency to those of AA transporters (Y. Li et al., 2017). Subsequently, given the requisite that the element-bound peptide fractions are themselves bioavailable, they can effectively deliver elements across the epithelial wall. Compared with native food proteins hydrolysates may also overcome protein allergenicity concerns and high resistance to proteolysis during transit in the digestive tract.

While co-ingestion of some soluble peptides has been demonstrated in human studies to enhance acute intestinal absorption of Fe (Taylor, Martínez-Torres, Romano, & Layrisse,

1986), Zn (Lönnerdal, 2000), and Ca (Hunt, Johnson, & Fariba Roughead, 2009; Kerstetter, O'Brien, Caseria, Wall, & Insogna, 2005), the efficacy of peptide-element chelates as supplements has only been examined *in vitro* and in animal models, with the exception of milk caseinophosphopeptides (CPPs) (Ait-Oukhatar et al., 2002; Miquel & Farré, 2007) and lactoferrin (Dix & Wright, 2018). To date, progress towards food-derived peptides to enhance element bioavailability has been focused on the identification of Fe-, Zn- and Ca-chelating peptides and motifs from numerous food sources, most which are of animal origin (Walters et al., 2018; C. Wang et al., 2012). Whilst only some authors have examined the digestion of element-bound peptides *in vitro* or by rodent models, most studies have been pivotal in unravelling specific peptide properties or AA sequence associated with enhanced chelation activity. For comprehensive lists of food-derived, element binding hydrolysates, readers are referred to reviews for Zn (Udechukwu, Collins, & Udenigwe, 2016), and Fe/Ca (Sun et al., 2016; Walters et al., 2018). Some of the element binding properties identified in these food-based studies have been summarized in **Table 2**.

A compilation of studies on soluble cereal and legume protein hydrolysates in relation to their element binding and reducing properties are presented in **Table 3**. These studies demonstrated a trend of chelation capacity being generally enhanced by sub-fractionation, and a moderate degree of hydrolysis (~ 7-39%). These processes are often associated with an increase in the availability of electron-rich AA residues, which effectively facilitate binding interactions and complex stability. Moreover, synergistic effects between charged

and hydrophobic/aromatic groups have also been observed by Eckert et al. (2014) and Pownall, Udenigwe, and Aluko (2010). It has been suggested that the presence of charged side chains initially attract element ions through electrostatic interactions and coordinate bonding, where its positioning within the peptide can be further stabilized by hydrophobic interactions and pi-stacking of aromatic rings (Eckert et al., 2014). Although a linear correlation between binding activity and the proportion of AAs with charged residues have been documented (Bao, Lv, Yang, Ren, & Guo, 2008; Y. Lv et al., 2013), both compositional and conformational differences are significant determinants of complexation due to possible steric effects. This explains the finding that the highest Fe-chelating activity in un-fractionated hydrolysates, despite identifying that fractionation enhanced concentrations of hydrophobic AAs (Pownall et al., 2010).

In congruence with element-chelating peptides from other food sources, a varying range of MW have been found to be effective for element chelation. Although optimal molecular weight (MW) values in relation to binding activity can be concluded from **Table 3** (e.g. 0.5-1.0 kDa for Fe binding, 8.0-14.4 for Ca binding), studies by Eckert et al. (2014) have found distinct solubility differences amongst ultrafiltered peptide size fractions complexed with Fe, Zn and Ca, which were highly dependent on the enzyme chosen for protein hydrolysis. This means that for each element, the ideal MW range offering the prime balance between binding capacity and stability is likely unique and it also depends strongly on amino acid compostion and position.

Most studies, however, examined element chelation as a function of the *in vitro* antioxidant capacity exerted by the purified hydrolysate, which only provide information on the structural properties that promote element binding under the physicochemical parameters examined. The GIT, *in situ* presents significantly different environments as endogenous secretions containing salt and electrolytes affects element binding and binding strength (Foltz, van der Pijl, & Duchateau, 2009). As an example, the presence of chloride anions in gastric and intestinal fluids (Mudie, Amidon, & Amidon, 2010) have been found to increase the binding affinity of Ca in CPPs (Recio, Guerra, Torrado, & Skibsted, 2019). It would thus be useful to re-evaluate the identified binding properties using dynamic *in vitro* simulations or animal models, prior to harnessing these characteristics in the design and optimization of soluble element-complexing agents for bioavailability.

3.1.2 Phytoferritin

Ferritins are a family of Fe-storage proteins found in the storage organelles of various organisms. All ferritins possess 24 subunits arranged in a hollow shell, with a cavity of 80 Å that allows the formation of an inorganic mineral complex (Harrison & Arosio, 1996). By nature, the ferritin complex binds up to 4500 ferric atoms in its interior as $Fe(OH)_3$ salt, although chemically prepared ferritin containing Zn and Ca in the core have also been synthesized (Mei Li, Viravaidya, & Mann, 2007; Meiliang Li, Zhang, Yang, Zhao, & Xu, 2014) and Zn (Zhen et al., 2013). Both *in vitro* and *in vivo* studies have demonstrated that the

intact ferritin polypeptide, when bioaccessible, undergoes endocytosis by intestinal cells (San Martin et al., 2008; Elizabeth C. Theil et al., 2012; Elizabeth C Theil et al., 2004). However, elemental bioavailability may be compromised if the element is released due to structural instability of the protein.

The elemental carrying capacity and structural stability of ferritins vary amongst sources. Animal ferritin is highly stable and has a slow gastrointestinal release of Fe (Mertz & Theil, 1983), thus effectively shielding the Fe against chelating ligands such as phytic acid and tannins in the GIT (Kalgaonkar & Lönnerdal, 2008). With plant-based phytoferritin, there have been mixed reports on its stability against gastric proteases, storage and heat-induced denaturation (Hoppler, Schönbächler, Meile, Hurrell, & Walczyk, 2008; X. Liu & Theil, 2005). Phytoferritin architecturally resembles animal ferritin, although animal ferritin consists of heavy (H-) and light (L) MW subunits, the latter of which is absent in phytoferritin (Zhao, 2010; Zielińska-Dawidziak, 2015). Phytoferritin is known to have one to three H- subunits (Zhou, 2017), which shares approximately 40% sequence identity with animal ferritin (Zhao, 2010), but is subject to pronounced inter-species variation in its ratio and AA sequence (Roschzttardtz et al., 2013). These H- subunits are precursors to a specific domain extension peptide (EP) on the protein exterior that stabilizes its oligomeric conformation (C. Li et al., 2009; R. Yang, Zhou, Sun, Gao, & Xu, 2015). Conformational changes in the EP domain would easily catalyse structural dissociation, which can occur during food processing and gastrointestinal digestion (Hoppler et al., 2008), or during ambient storage due to the EP's

serine protease-like activity (Haixia Yang et al., 2010). The digestive stability of the phytoferritin complex thus depends on the ratios between the different subunits (Liao, Yun, & Zhao, 2014).

Studies conducted on purified legume seed phytoferritins and their mineral bioavailability or stability are summarised in **Table 4.** Previously, multiple studies have been conducted on whole food matrices under the assumption that most of the seed iron existed as phytoferritin. However, contemporary quantification methods revealed that ferritin comprise approximately 18-69% of seed Fe, subject to high taxonomic dependence (Cvitanich et al., 2010; Hoppler et al., 2014; Hoppler, Zeder, & Walczyk, 2009). The forms of the remaining Fe vary depending on the source, and its cellular localisation and form can be modulated by physiological activity or food processing (e.g. soaking (Cvitanich et al., 2010)). Mineral bioavailability data from whole food studies have thus been excluded from the table, as well as former studies that have utilized radioactive labelling. For example, the earlier extrinsic radioactive techniques were not included in table 4, since they were recognized to result in incomplete isotopic exchange between the isotopic label and Fe within the food (Consaul & Lee, 1983; Raymond P. Glahn, Cheng, & Giri, 2015; Jin, Cheng, Rutzke, Welch, & Glahn, 2008; Messina & Messina, 2010; R. Yang et al., 2015; Zhao, 2010).

Human *in vivo* studies mostly demonstrated that the absorption of purified soybean ferritin Fe was comparable to $FeSO_4$, an iron salt known for its efficient bioavailability (Zariwala,

Somavarapu, Farnaud, & Renshaw, 2013). Phytoferritin in these studies were presumably intact, as Elizabeth C. Theil et al. (2012) showed through a simultaneous rodent study that phytoferritin endocytosis undergoes an independent, non-competing process from that of non-heme Fe. However, multiple *in vitro* studies have also shown that purified phytoferritin undergoes gastric dissociation and the released Fe interacts with various ligands (Bejjani, Pullakhandam, Punjal, & Nair, 2007; Hoppler et al., 2008). Plausible scenarios such as better digestive efficiency of *in vitro* environments and re-folding of dissociated oligomeric phytoferritin within the small intestine have been proposed (Zielińska-Dawidziak, 2015). Alternatively, since the $Fe(OH)_3$ core of phytoferritin is insoluble at intestinal pH and thus unlikely to be absorbed, efficient uptake as ferrous Fe would still ensue if oligomeric phytoferritin-catalysed reduction occurs after ferric Fe release from the mineral core. The presence of phytoferritin hydrolysis products may also limit the complexation of Fe with other insoluble ligands.

Investigations in recent years have found the stability of phytoferritin to be influenced by its interactions with other compounds, particularly with polyphenols. Polyphenol derivatives with consecutive polyhydroxy groups, from monomers such as gallic acid and (−)-epigallocatechin-3-gallate (EGCG), to oligomeric proanthocyanidins have been reported to enhance the gastrointestinal stability of phytoferritin *in vitro* (Deng et al., 2011; A. Wang, Zhou, Qi, & Zhao, 2014; Q. Wang, Zhou, Ning, & Zhao, 2016). Polyphenols appear to mediate a protein-association effect on phytoferritin that is dose-dependent and

irreversible. However, this is structure-dependent and contingent on the degree of protein-polyphenol interaction. Where there is greater affinity for polyphenol binding by minerals (i.e. under certain molar ratios and exposure of mineral *via* ferritin degradation), rapid mineral liberation can be initiated from the ferritin core, as found by some authors studying tannic acid and cinnamic acid derivatives (Meiliang Li, Jia, Yang, Deng, & Zhao, 2012; Sha, Chen, Zhang, & Zhao, 2018). Mineral liberation may have contributed to findings *in vivo* using an anaemic rat model, where proanthocyanidins have been found to inhibit Fe uptake from soy phytoferritin (Yun, Zhang, Li, Chen, & Zhao, 2011), revisiting the bioavailability discrepancies between *in vitro* and *in vivo* studies.

Knowledge governing various phytoferritins' structure-binding activity relationships can offer novel ways to enhance its stability as a mineral bioavailability enhancer. For example, several studies have demonstrated that in phytoferritins with two H- subunits (H-1 and H-2), the homopolymer of H-2 is less susceptible to degradation (Dong et al., 2008; Fu et al., 2010). Other features contributing to degradation and stability (e.g. H-subunit ratio (C. Lv et al., 2015), specific AA positioning within EP sequence (Meiliang Li, Yun, Yang, & Zhao, 2013)) have also been identified. These key determinants may be targeted in the synthesis and reassembly of mineral-containing phytoferritin. Additionally, there is vast potential in the exploitation of the inter- and intra-species variation in phytoferritin structure, in which novel characterisation from various unexplored plant sources may be valuable. Currently, most studies have only explored phytoferritin auto-degradation under storage. While

processing approaches such as thermal treatment has demonstrated potential in enhancing storage stability (Tang, Yu, Chen, & Zhao, 2019), it is imperative to examine the stability of different phytoferritins under gastrointestinal conditions to understand their ADME properties as mineral carriers.

3.2 Plant phytochemicals

Plant materials are rich in primary and secondary metabolites that constitute the plant phytochemicals, and which possess functional groups that can interact with elements through coordination and other non-covalent interactions (Ejima, Richardson, & Caruso, 2017). Whilst plant phytochemicals are generally considered to be detrimental to mineral bioavailability (Rousseau, Kyomugasho, Celus, Hendrickx, & Grauwet, 2019), there have been some mixed findings between different phytochemicals, particularly where effects from vegetal matrices are eliminated. The following sections will discuss the several phytochemicals (polyphenols, beta-carotene and nicotianamine) in relation to their element chelation properties, and the existing evidence on their potential to enhance mineral bioavailability as individual fractions.

3.2.1 Polyphenols

Polyphenols are a heterogeneous group of phytochemicals widely distributed in plants that include phenolic acids and flavonoids (Tsao, 2010). Polyphenols are generally reputed to be

inhibitors of mineral bioavailability due to their propensity to form insoluble element complexes or co-precipitates under neutral to alkaline conditions during digestion (Feitosa et al., 2018; Petry, 2014). Short-term isotopic *in vivo* studies demonstrate the inhibitory properties of polyphenol co-ingestion on non-haem Fe absorption (Gillooly et al., 1983; Kaltwasser et al., 1998; Petry, Egli, Zeder, Walczyk, & Hurrell, 2010; Samman et al., 2001). However, the mechanisms for the reduction in Fe absorption is not clear. For example, some polyphenols are known to inhibit the activity of digestive enzymes (Bennick, 2002; Manach, Williamson, Morand, Scalbert, & Rémésy, 2005), and thus it is possible that Fe remained complexed within the non-digested starch and protein molecules, rather than being complexed by the polyphenols. Moreover, insoluble complex formation involves ligand displacement from the coordinated element by polyphenols following gastric dissociation (S. Yang et al., 2014). Such ligand displacement by polyphenols may be conditional upon the presence and relative stoichiometry of certain other competing intestinal ligands generated from the partially digested food and/or microstructure (Bornhorst & Singh, 2014), and endogenous secretions released upon food ingestion. Indeed, these factors support evidence of food co-ingestion possibly hampering the oral bioavailability of certain polyphenols (Naumovski, Blades, & Roach, 2015; Takehiko, Hiromu, & Kazuhiro, 2012), and in some cases, the mineral itself, e.g. Zn (Bel-Serrat et al., 2014), and Fe (Moretti et al., 2006). Conflicting results on Zn and Fe bioavailability are found from rodent and long-term *in vivo* studies (Delimont, Haub, & Lindshield, 2017; Delimont, Rosenkranz, et al., 2017) as well as Caco-2 cells *in vitro* (Kim, Ham, Bradke, Ma, & Han, 2011; Kim, Pai, & Han, 2011; Ma, Kim, Lindsay, & Han, 2011). Similarly in Ca, polymeric polyphenols such as tannins have

been previously reported to affect Ca absorption in rodents (Chang, Bailey, & Collins, 1994; Mitjavila, Lacombe, Carrera, & Derache, 1977), although the phenolic compound genistein has been found to have anabolic effects on rodent femoral tissues *in vitro* (Yamaguchi & Jie, 2001). Human studies investigating the effects of polyphenols on Ca have been recently reviewed, with no detrimental effects on bone metabolism (Austermann, Baecker, Stehle, & Heer, 2019).

In recent years, some studies have recognized the disparity amongst heterogeneous classes of polyphenols in forming mineral complexes of varying solubility, as well as their prospective reducing properties on ferric Fe. Several authors have examined the subsequent implications on mineral bioavailability, using *in vitro* Caco-2 cell models. For example, Vlachodimitropoulou, Naftalin, and Sharp (2010) showed that the flavonoid quercetin can enhance extracellular Fe(III) reduction by ferric reductases and enhancing the import of Fe(II). Works by Hart and others (2017; 2015) and Wiesinger, Cichy, Hooper, Hart, and Glahn (2020) have characterized significant differences amongst different purified black and common bean polyphenols, where certain compounds (e.g. catechin and epicatechin) demonstrated promotive behavior in Fe cell uptake and ferritin formation regardless of Fe/polyphenol molar ratio. Some polyphenols are conditionally promotive at certain molar ratios (e.g. luteolin-7-glucoside, cyanidin-3-glucoside), while others are inhibitory to Fe uptake (e.g. malvidin), highlighting the importance of hydroxylation patterns of the ring structure in element binding. Hart, Tako, Wiesinger, and Glahn (2020) further found a

general association between Fe uptake and the ratio of promotor to inhibitory polyphenols in yellow bean seed coats, where inhibitory condensed tannins was able to proportionately counteract the promotive effect of kaempferol compounds. These binding patterns may also be dependent on the composition of the food matrix, as Laparra, Glahn, and Miller (2009) have observed an inhibition of Fe uptake by Caco-2 cells from tea extract (a source of catechin), but an increase with the catechin standard solution. Investigations by Lesjak and others (2019; 2014) have validated the increase in Fe cell uptake by isolated quercetin and its analogues, using the Caco-2 model concomitantly with rodents *in vivo*. Though the authors have also identified decreased basolateral cell efflux and expression of Fe-transporters, this effect was identified as structurally related to the analogue used. It is possible that the inhibition of Fe efflux is due to strong binding of quercetin to Fe under intracellular conditions, although this does not deduce the possibility of Fe undergoing cell efflux past the 3 h time period examined in the study.

There have been investigations of a similar type in the literature on Zn (Kim, Pai, et al., 2011). Fractionated polyphenols from various beverages have been shown to have neutral or enhancement effects on uptake by Caco-2 cells, with tannic acid and quercitin stimulating the uptake of zinc and no effect from others (Sreenivasulu, Raghu, & Nair, 2010). The study also found that polyphenols promoted the expression of metallothionein (MT), a regulatory Zn transporter associated with homeostasis. On the other hand, Kim et al. (2011) found a reduction in Zn apical uptake and transepithelial transport by purified grape seed extract,

but not from green tea extract and EGCG. Dissimilar to the previous study, the authors found no significant changes in MT expression.

The findings above are generally supported by binding studies under non-physiological contexts, which demonstrate that the affinity of specific binding sites towards Fe and Zn ions are dependent on structural features, element ion properties and pH (Andjelković et al., 2006; Dimitrić Marković, Marković, Brdarić, Pavelkić, & Jadranin, 2011; Sroka & Cisowski, 2003; Wei & Guo, 2014; S. Yang et al., 2014). On the other hand, the chelation properties of Ca have been less explored due to weaker affinity of the phenoxide group towards alkaline earth metals (Hider, Liu, & Khodr, 2001), although binding with phenolic acids has been reported (Granieri et al., 2017). Structures such as the catechol moiety and the chroman C-ring -OH and -keto groups in flavonoids can provide bidentate coordination sites for elements (Andjelković et al., 2006). Subsequently, the structural differences amongst polyphenols (e.g. degree of hydroxylation, polymerization, glycosylation) can be important determinants of mineral binding capacity, stability constants and variable affinity for different ions. The stability of some mineral-polyphenol complexes has also been reported to differ under physiological pH (Kontoghiorghe et al., 2015).

Given the diverse chemical properties of polyphenols, further studies are required to elucidate if certain polyphenols can produce Fe and Zn bioavailability enhancement in the absence of food matrices, using biorelevant simulations. This is particularly important given

that polyphenol bioavailability can differ between states of matter (i.e. solid and liquid forms) (Aguirre & Borneo, 2019; Conte et al., 2016), and the structural changes (e.g. de-conjugation of the glycosylated moiety) that occurs during passage thorough disparate segments of the GIT (Tsao, 2010). Polyphenols have also been identified to cross-link intestinal mucins, which modulates barrier properties and thus nutrient absorption (Georgiades, Pudney, Rogers, Thornton, & Waigh, 2014). This may have bioavailability implications, as mucin-binding plays an imperative role in facilitating Fe, Zn and Ca uptake.

3.2.2 Carotenoids

Carotenoids from plants, such as β-carotene are dietary retinoids that undergo enzymatic transformation into vitamin A (Adjimani & Asare, 2015; O'Byrne & Blaner, 2013). Carotenoids possess extended conjugated double bonds, and/or aromatic rings or pyrones that allow π-electron delocalization, contributing as binding sites for Fe, Zn and/or Ca, or as catalysts for Fe reduction (Llansola-Portoles, Pascal, & Robert, 2017). Their aromatic rings may play an essential role in Fe solubilisation, where the carbonyl and hydroxyl groups confer additional polarity that contribute to increased affinity for lipid-water interfaces, despite the generally lipophilic nature of carotenoids (Horiuchi et al., 2015).

Various *in vitro* studies have reported some evidence of direct metal-carotenoid or vitamin A interactions at physiological pH 6-7, with Fe, Zn and Ca. A list of some carotenoids in

relation to binding and/or bioavailability of divalent cations are provided in supplementary table 2. García-Casal et al. (2000) found a significant enhancement in Fe uptake from Caco-2 cells with the presence of β-carotene, which effectively prevented the formation of insoluble complexes with phytate or polyphenols. A similar enhancement in Fe cell uptake with β-Carotene was found in an inflamed version of the model by Katz et al. (2015), where effectual intracellular release was additionally observed. Other authors have found an increase in *in vitro* Fe and Zn bioaccessibility from dialysis following simulated gastrointestinal digestion (Gautam, Platel, & Srinivasan, 2010), or Fe in the Caco-2 model (Gargari, Razavieh, Mahboob, Niknafs, & Kooshavar, 2006) and dialysis (Singh, Bains, & Kaur, 2016). On the other hand, a series of investigations by Corte-Real et al. (2017; 2015) concluded that Ca precipitated carotenoids under simulated gastrointestinal digestion. The same authors found also that Zn binding to carotenoids was negligible, unless very high concentrations were present.

Similarly, human *in vivo* studies, have generally presented favorable effects of carotenoids on Fe/Zn bioavailability (García-Casal et al., 1998; García-Casal, 2006; Jimenez et al., 2010), however, other studies have found no influence from carotenoids (Chen et al., 2014; Walczyk, Davidsson, Rossander-Hulthen, Hallberg, & Hurrell, 2003). As previously highlighted, food matrices may be one of the confounding influences contributing to mixed effects on mineral bioavailability *in vivo*. Additionally, receptor-mediated effects have been found from rodents, where enhancements in Fe bioavailability and retention were identified

to be related to increased expression of genes involved in absorption (DMT-1, Dyct B, Ferroportin 1, ferritin) rather than direct metal-carotenoid interactions (Citelli, Bittencourt, Da Silva, Pierucci, & Pedrosa, 2012). These amalgamated effects are complications in identifying whether carotenoids can be enhancers of bioavailability in Fe and Zn, and the degree in which possible promotive effects are related to its chelation properties. Additional investigations, particularly complementary to *in vivo* and *in vitro* studies are required prior to identifying carotenoids as possible Fe and Zn promoting agents. Similarly, the behavior of carotenoid-Ca complexation also requires further substantiation.

In vitro binding studies under simulated gastrointestinal conditions may be valuable in confirming structure-activity properties of carotenoids with more relevance to digestion conditions. For instance, the partial delocalization of the pyrone oxygen in xanthophylls may be of significance as a element binding site, as it is consistently in its non-fused form in carotenoids. This is in congruence with the *in vitro* experiments by Garcia et al (1998; 2006), who discovered increased Fe solubility with cyclic xanthophylls (β-carotene, lutein and zeaxanthin) compared to non-cyclic lycopene. In the same study, the authors corroborated this relationship using Fe isotope labelling in humans *in vivo*, albeit in the presence of food matrices. Another observation that is possibly structure-related is the ability of carotenoids to consistently surpass those of Vitamin A in its bioavailability-enhancing effects, in both *in vivo* (García-Casal et al., 1998; García-Casal, 2006; Jimenez et al., 2010) and *in vitro* (García-Casal et al., 2000; Katz et al., 2015) studies. While various carotenoids and Vitamin A bear

high resemblance in chemical structure, the longer hydrocarbon chain lengths in carotenoids that provide increased contiguous element coordination sites may explain this phenomenon.

3.2.3. Nicotianamine

Some soluble, low MW element-binding ligands involved in plant mineral metabolism and transport have received some attention in bioavailability enhancement. An example is nicotianamine (NA), a non-specific element chelator known to accumulate in high concentrations in legume seeds including chickpea (Tan et al., 2018) and soybean (Nozoye et al., 2014). NA is known for its high affinity for divalent element cations, particularly ferrous iron (Fe^{2+}) whilst its biosynthetic precursor, 2'-deoxymugineic acid (DMA), can chelate Fe^{3+} (Reichman & Parker, 2002; Tsednee, Huang, Chen, & Yeh, 2016). *In vitro* investigations utilizing simulated gastrointestinal digestion and the Caco-2 cell model have found both NA and DMA to be effective enhancers of Fe from biofortified wheat (Beasley, Bonneau, et al., 2019; Eagling, Wawer, Shewry, Zhao, & Fairweather-Tait, 2014). In particular, NA has been found to enhance Fe uptake to a greater extent compared to ascorbic acid, a known enhancer of Fe absorption enhancer (Beasley, Hart, Tako, Glahn, & Johnson, 2019). Several animal studies, including rodent (Lee et al., 2012) and broiler (Beasley et al., 2020) models have also found grains biofortified with NA to be highly bioavailable and effective at enhancing Fe status. Further studies are required to validate its

potency as an enhancer of Fe bioavailability, which may possibly translate to other essential elements.

4. Outlook and potential limitations in food applications

Most of the plant-derived element binding molecules construed above are in their preliminary stages of characterisation as bioavailability enhancers. Some of the biomolecules have been investigated as stand-alone nutraceuticals in food applications, such as for bioactive peptides and polyphenols (Gonçalves, Martins, Duarte, Vicente, & Pinheiro, 2018). However, it remains undetermined to what extent the physicochemical or pharmacokinetic properties are modulated from being element-bound, and whether they would possess advantageous physico-chemical properties (e.g. better solubility) over currently employed supplementary mineral salts as directly consumed, or through excipient foods. Given current understanding of the absorption patterns at various sites for each element in the human GIT, the element-bound compounds may benefit from being loaded through delivery systems that provide additional stability and loading capacity, protection from other competing insoluble ligands and controlled release. An example of this would be nano-encapsulation, a form of which has been demonstrated to enhance the bioavailability of ferrous glycinate in a rodent model, effectively circumventing phytic acid as a competing ligand (Hualin Yang, Yi, Li, & Ding, 2017). These strategies could be explored in the next stage of development suceeding confirmation of biological efficacy.

In this section, we highlight some prospective limitations in the application of these biomolecules towards addressing essential element malnutrition in the context of different global populations. These practical constraints must be carefully considered when applying these biomolecules as stand-alone or adjunct therapies to current strategies employed to increase essential element intake in humans.

4.1. Administration form and dosage

As with any essential element supplement or fortificant, a primary barrier towards biological efficacy inside the human body is influenced by the form of administration. Most micronutrient supplements are typically vehiculated in solid, liquid or powdered forms, and/or in food matrices (Oh, Keats, & Bhutta, 2020). This depicts that even if the soluble element chelators demonstrate effective utilization of Fe, Zn or Ca *in vivo*, the true biological effects (i.e. bioequivalence) may differ when administered alone as a supplementary compound or within different foods, due to disparities in bioaccessibility and bioconversion processes (Yetley, 2007). Even if the target compound exhibit excellent bioavailability as supplements, incorporation into foods can be difficult due to changes in organoleptic properties, bioavailability and stability (Prentice et al., 2016). This is of particular concern with regards to at-risk populations in underdeveloped countries, where current micronutrient interventions typically involve biofortification of staple crops and home fortification. Element biofortification entails enhancements in the concentrations of both essential elements and the corresponding solubilizing biomolecule, typically in cereals

and legumes, through genetic engineering, traditional plant breeding, and/or agronomic approaches (Philip J. White & Broadley, 2005). Meanwhile, home fortification presents an approach where micronutrients (usually as a powder) are added to solid or semi-solid foods (De-Regil, Suchdev, Vist, Walleser, & Peña-Rosas, 2013; Dewey, Yang, & Boy, 2009). Through both approaches, the prevalence of monodiverse diets in low-income populations suggest that the element carrier may be highly prone to interactions with vegetal and fibrous food matrices, unless diet diversification or other absorption enhancers such as ascorbic acid are concomitantly applied. For example, although some flavonoids (e.g. epicatechin gallate) can form membrane-permeable complexes with iron that undergo transcellular uptake (Kim, Ham, et al., 2011), the presence of multiple *ortho*-substituted hydroxyl groups allows Fe to be co-complexed with poorly digested proteins and/or fibres in the plant matrix, forming aggregates that may be poorly soluble as discussed in section 3.2.1. Human studies have suggested the relative importance of variety selection in crop biofortification processes (Petry, Egli, Campion, Nielsen, & Hurrell, 2013; Petry et al., 2016), which takes into account of the various components of the plant matrix that also contribute to element bioavailability.

Moreover, food processing approaches may further modulate these interactions. For example, the maize germ fraction and the cotyledon cell wall of legumes have been identified as an physical inhibitor of Fe bioavailability (R. Glahn, Tako, & Gore, 2019; R.P. Glahn, Tako, Cichy, & Wiesinger, 2016). These studies indicate that processing techniques,

which have the potential to disrupt the cell wall structure and fibrous fractions, may be an additional factor compounding the ability of soluble ligands to act as absorption promotors (Wiesinger et al., 2020). A successful example that resolves both factors can be found in the Manteca yellow bean (*Phaseolus vulgaris*), a phenotype with fast-cooking properties and promotive polyphenols (kaempferol flavonoids) that has been identified to have higher bioavailability through *in vitro* Caco-2 cell models and broiler studies (Wiesinger, Cichy, Tako, & Glahn, 2018; Wiesinger et al., 2019). Food processing approaches such as enzymatic hydrolysis and thermal, shear or pressure processing may also enhance the release of soluble ligands that may act as enhancers or negators from existing plant food matrices. For example, enzymatic hydrolysates can release peptides, whilst heating methods can facilitate the release of bound polyphenols and carotenoids within the cellular matrix (Gunathilake, Ranaweera, & Rupasinghe, 2018). Indeed, in circumstances where these biomolecules are vehiculated within the plant food matrix, food processing techniques may achieve a simultaneous reduction in some endogenously occurring anti-nutritional compounds occurring within the food matrix. A prominent factor impeding elemental bioavailability from plant-based foods is phytic acid, and its elimination may be attained by soaking, thermal processing, and enzymatic hydrolysis (Gupta, Gangoliya, & Singh, 2015). Subsequently, both *in vitro* and *in vivo* studies have demonstrated enhanced Fe and Zn bioavailability with phytic acid reduction (Liang, Han, Nout, & Hamer, 2008; Petry et al., 2014; Petry et al., 2010; Vashishth, Ram, & Beniwal, 2017), which may be related to both reduction of inhibitors and release of enhancer molecules.

In relation to applications through home fortification, it is unknown as to what degree these biomolecules participate in some established interactions related to dosage and co-ingested compounds observed through existing supplemental approaches of Fe, Zn and Ca. The vulnerable populations targeted for interventions aimed at enhancing essential element intake often tend to experience severe multi-element micronutrient deficiencies, where multi-micronutrient supplementation strategies are suggested (Oh et al., 2020). This can modulate the potential of biomolecules to act as enhancers, exerting either positive or negative effects. In maternal women for example, the World Health Organization recommends both Fe and vitamin A (Tunçalp et al., 2017), which when co-administered, may interact to enhance Fe bioavailability as highlighted in Section 3.2.2. On the other hand, co-administration of multiple elements can lead to undesirable interactions during intestinal absorption. For instance, reviews of randomized controlled trials, animal and *in vitro* studies have identified reduced effectiveness of joint Fe and Zn supplementation, although the relationship can also be mutualistic depending on other factors such as therapeutic dose, element ratios, timing, and the form of vehiculation (Fischer-Walker, Kordas, Stoltzfus, & Black, 2005; Kondaiah et al., 2019). Similarly, Ca in the form of both food component and supplementary salts have been found to interefere with both haem and non-haem Fe absorption (L Hallberg et al., 1992; Leif Hallberg, Rossander-Hulthèn, Brune, & Gleerup, 1993) and status (Khan et al., 2014; van de Vijver et al., 1999) in humans, while no effects have also been reported (Abrams, Griffin, Davila, & Liang, 2001; Minihane & Fairweather-Tait, 1998). While the exact mechanisms may differ, the factors that affect competition between Zn and Fe may also alter the direction and magnitude of interactions between Ca

and Fe. This highlights a conditional nature in the efficacy of element bioavailability enhancers when co-administered with multiple micronutrients. Under such conditions the potential interactions at different doses, timing, and compatibility in common food matrices should be thoroughly examined through both *in vitro* and *in vivo* studies.

4.2. Legislative barriers

Biomolecules derived from plant foods offer great potential as a safe and biodegradable delivery system for stabilizing and enhancing essential elements' bioavailability. However, a prospective hinderence towards their application in more developed countries is the regulatory requirements associated with commercialization. Depending on their classification, there could be ambiguous boundaries as to how these biomolecules tailor into legislative frameworks at a country-level, and subsequently the parties responsible for managing their production (if permitted), distribution and quality control. These products may be classified as nutraceuticals, which are nutritional components that bring physiological or therapeutic benefits outside fundamental nutritional needs, and/or offer protection from chronic disease (Nasri, Baradaran, Shirzad, & Rafieian-Kopaei, 2014). In this case, the regulatory system may be under therapeutic goods amongst products such as complementary medicine and drugs, such as the Therapeutic Goods Administration (TGA) in Australia. In order to be lawfully supplied to consumers, the biomolecule would require registeration on an extensively regulated Register of Therapeutic Goods, and be produced in compliance with Good Manufacturing Practice (GMP) under the *Therapeutic Goods Act 1989*

(McEwan, 2007). On the other hand, if the biomolecule is to be provided and ingested through food matrices, such as through formulation of functional food products or biofortification through genetic modification, regulation of food standard and safety would be required. In Australia, this would be by Food Standards Australian & New Zealand (FSANZ). FSANZ operates within the Food Standards Code under multiple levels of governance, which comprises an independent set of legislations (*Legislation Act 2003*) related to matters prior, during and following food production (Ghosh, 2014). Separate agencies and departments at local and state government scales are often responsible for enforcement.

As such, these legislative frameworks can diverge greatly by nation. In the USA for example, the requirements applied to the novel substances that are added to either food or dietary supplements are monitored under the same system. Both applications are required to be subjected to review and approval by the Food & Drug Administration (FDA) as Generally Recognized as Safe (GRAS) prior to use (Food & Drug Administration, 2014). The FDA appointed a scientific committee that conducts independent risk assessments for novel ingredients or substances, which are available to the public through a GRAS Substances (SCOGS) Database. Similarly in the European Union, food-derived supplements or fortificants are considered as foodstuffs, and are regulated under the same system by the European Food Safety Authority (EFSA). Under the Directive 2002/46/EC, the EFSA permits the addition of Fe, Ca and Zn for nutritional purposes (Annex I), and provides an additional

list of substances that are authorised as their carriers in Annex II (European Parliament, 2011). In China, novel nutrient supplements and fortifiers, including mineral supplements are classified as health (functional) foods by the Chinese Food and Drug Administration (CFDA). The National Food Safety Standard of Health Food (GB 16740-2014) stipulates that the active ingredient and its expicient must comply with food safety standards and relevant provisions (Hu, 2017). However, all general, novel food compounds and new additives must first be assessed for safety and approved by the National Health and Family Planning Commission (NHFPC), prior to use as supplement or fortification compounds.

5 Conclusion

The identification, modulation and development of food-derived biomolecules as mineral bioavailability enhancers remain an unmapped and challenging field of study. This review has delineated the factors influencing luminal interactions between food ligands and three minerals (Fe, Zn and Ca). Additionally, it discussed some recent progress of understanding of mineral binding with nutritional fractions found in plant-based foods, many of which are found in legume seeds. In order to detect food-derived biomolecules that contribute to element bioavailability, it is important to understand the pharmacokinetic processes underlying currently effective soluble ligands for each element (e.g. citrate for Ca). Food matrix interactions and food processing strategies that may create and modulate the ability of certain ligands to act as bioaccessibility promoters, such as polyphenols or peptides, should also be thoroughly investigated. Whilst binding and release properties are important

in the efficacy of element chelators, understanding these properties under biorelevant conditions of digestions from oral to intestinal segments will assist in the identification, development and validation of both novel and currently existing mineral bioavaililty enhancers. Soluble ligands utilized for element transport within plants, such as nicotianamine, have great potential to be explored in biofortification, food fortification or supplement strategies. However, their limitations regarding potential matrix interactions and legislative constraints must be overcome in order for these biomolecules to act as effective delivery agents for Fe, Zn, Ca, and possibly other essential elements. The use of plant-derived biomolecules as essential element bioavailability enhancers offer a novel avenue towards a generation of supplementary compounds, with potentially lower toxicity and greater ease of administration.

Author Contributions (*required*)

Y.Y.Z conducted the literature research, drafted the first version of the manuscript and editing of drafts. R.S, K.N and S.A contributed to the reviewing and editing various drafts of the paper. All authors contributed to conceptualization of the review.

Conflicts of Interest

The authors declare no conflicts of interest

Data Availability

N/A

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Element	Proteins	Carbohydrates	Lipids and other metabolites	Reported enhancers *in vivo*	Reported inhibitors *in vivo*
Fe^{2+}/Fe^{3+}	Binds to N-terminus and sulphur residues of AAs/peptides, haem, phytoferritin, transferrin, nicotinamine, mucins	Binds to lignin and hemicellulose, lactose, ferric ion co-complexed with phytate, tannins (catecholic and gallolic complexation), monosaccharides	Binds to oxides/hydroxides, organic acids, phytic acid, , polyols, carbonates, phosphates, acidic phospholipids, free fatty acids	Ascorbic acid, muscle tissue, glycosaminoglycans, vitamin A and carotenoids, non-digestible carbohydrates	Phytic acid, polyphenols, Calcium, proteins
Zn^{2+}	Binds to acidic side chains, phosphorylated residues, deprotonated nitrogen and sulphur residues of AAs/peptides, ferritin, serum albumin, nicotinamine, mucins	Binds to charged polysaccharides (COOH groups), co-complexed with phytate	Binds to oxides/hydroxides, organic acids, phytic acid, phosphates, tannins (labile complexation)	Vitamin B6, animal proteins, cholesterol, dairy	Phytic acid, Calcium, Iron, Tin, proteins (bovine serum albumin, dephytinized soy), Maillard products
Ca^{2+}	Binds to acidic side chains, phosphoserine moieties of AAs/peptides, phytate-protein complexes, phytoferritin	Binds to acidic groups of pectins (and other charged polysaccharides), OH and COOH groups of lignin	Binds to oxides/hydroxides, organic acids, phosphates, phospholipids, free fatty acids, phytic acid	Lactose, prebiotic oligosaccharides, phosvitin peptides	Dietary fiber, phytate, oxalate

Properties		Mineral	Additional notes	Source
Functional groups	Thiol group (Cysteine, glutathione)	Fe/Zn	Chelating or reducing Ferric Fe Electron rich	(Agostinho, de Souza Oliveira, Anunciação, & Santos, 2016; Martínez-Torres, Romano, Layrisse, & Taylor, 1986; Pace & Weerapana, 2014; Sun et al., 2017; Tabatabai et al., 2002; Walters et al., 2018)
	Imizadole group (Histidine)	Fe/Zn	Electron rich	(Sun et al., 2017; Tabatabai et al., 2002; P. Zhang & Allen, 1995)
	Carboxyl group	Ca	Bound Ca ↑ with hydrolysate COOH content	(Bao et al., 2008)
*AA residues	Asparagine and Glutamine residues	Ca/Fe/Zn	Chelation *via* carboxylate or NH groups in the side chains	(Cortivo, Castellani, Martelli, Michelotto, & Abatangelo, 1982; Daengprok et al., 2003; Gerbino, Mobili, Tymczyszyn, Fausto, & Gómez-Zavaglia, 2011; Miquel, Alegría, Barberá, & Farré, 2005; Sun et al., 2017)
	Serine and phosphoserine residues	Ca		(Sun et al., 2016)
	Phosphorylated residues	Ca/Fe/Zn	Chelation sites for + charged ions	(Bouhallab & Bouglé, 2004; Miquel et al., 2005; Zhong et al., 2016)
	Arginine	Fe	Chelation *via* ↑ - charge	(Sun et al., 2017)
	Methionine	Fe/Zn	Chelation *via* hydrophobic residues or carrier-mediated processes	(House, Van Campen, & Welch, 1996, 1997)
Other properties	Molecular weight	Fe	MW > 10 kDa ↑binding	(Seth & Mahoney, 2000)

Properties	Mineral	Additional notes	Source
	Fe	MW < 10 kDa ↑binding	(Storcksdieck, Bonsmann, & Hurrell, 2007)
	Fe	MW < 5 kDa ↑binding	(Torres-Fuentes, Alaiz, & Vioque, 2012)
	Fe	MW < 1 kDa ↑binding	(Agostinho et al., 2016; Torres-Fuentes et al., 2012; Xia et al., 2012)
	Ca	MW 5-10 kDa ↑binding	(Eckert et al., 2014)
	Ca	MW >10 kDa ↑binding	(Y Lv, Bao, Yang, Ren, & Guo, 2008)
Charge and hydrophobicity		↑ binding	(H. Liu, Bao, Lv, Xu, & Guo, 2012; Sun et al., 2017)

Source	Protein fraction /s assayed	Element/s assayed	Peptide fractionation method	Characteristics contributing to high chelating activity	Enzymatic hydrolysis conditions	Source
Chickpea (Cicer arietinum)	Purified peptide hydrolysate	Fe and Cu	Purification: Immobilized metal affinity chromatography (IMAC) Fractionation: Gel filtration (GF) chromatography	- Purified subfractions show ↑chelation activity vs. hydrolysates - ↑ Fe chelating activity: High His content (20-30%), small molecular size (< 500 Da)	Sequential pepsin and pancreatin (37 °C, pH 2.5 for 180 min and 7.5 for 180 min, respectively)	(Megías et al., 2007; Torres-Fuentes et al., 2012)

Source	Protein fraction /s assayed	Element/s assayed	Peptide fractionation method	Characteristics contributing to high chelating activity		Enzymatic hydrolysis conditions	Source
Common bean (Phaseolus vulgaris)	Crude protein isolate-hydrolysate and phaseolin	Fe and Cu	Fractionation: Size exclusion chromatography (SEC)	-	↑ Fe chelation activity: 0.7–1.0 kDa fractions (protein isolates), and < 0.43 kDa (phaseolin)	Sequential pepsin and pancreatin (37 °C, pH 2.5 for 90 min and 7.5 for 120 min, respectively)	(Carrasco-Castilla et al., 2012)
Cowpea (Vigna unguiculata)	Desalted protein concentrate-hydrolysate	Fe and Cu	Fractionation: *GF* chromatography	-	↑ chelating capacity from hydrolysates obtained with Alcalase, Flavourzyme and sequential Alcalase-Flavourzyme vs. Pepsin-Pancreatin	Respective systems: Alcalase (pH 8) and Flavourzyme (pH 7), at 50°C, 90 min Sequential systems: Alcalase-Flavourzyme (50°C, 45 min) and Pepsin-Pancreatin (37°C, pepsin – 45 min, pH 2, pancreat	(Segura-Campos, Ruiz-Ruiz, Chel-Guerrero, & Betancur-Ancona, 2013)

Source	Protein fraction /s assayed	Element/s assayed	Peptide fractionation method	Characteristics contributing to high chelating activity	Enzymatic hydrolysis conditions	Source
					in – 45 min, pH 7.5, total: 90 min)	
Adzuki (Vigna angularis)	Protein fractions (albumins, globulins, prolamins and glutelins), desalted via dialysis (MW cut-off 5 kDa, < 7 kDa)	Fe and Cu		- Highest peptide levels/soluble peptides were obtained from albumin - Concentration of soluble peptides↓in the order of albumin > glutelin > crude > globulin > prolamin - Globulin fraction showed highest Fe chelation activity	Sequential system: oral digestion (15 min total, 37°C), gastric (2 h, 37°C), intestinal (1 h, 37°C)	(Durak, Baraniak, Jakubczyk, & Świeca, 2013)

Source	Protein fraction /s assayed	Element/s assayed	Peptide fractionation method	Characteristics contributing to high chelating activity	Enzymatic hydrolysis conditions	Source
Pea (Pisum Sativum)	Protein isolate-hydrolysate, ultrafiltered (MW < 3 kDa)	Fe	Separation: Reversed Phase (RPh-HPLC)	- Hydrolysis ↑ concentration of BCAAS and Phen - Fractionation ↑ concentration of hydrophobic AAs (BCAAS, Trp, Phe) - ↑Reducing power in fraction with non-polar, aliphatic AAs (Pro, Val, Ile, Leu, Phe) - Fe-chelating activity highest in un-fractionated hydrolysate (95%) - Metal chelating activities showed strong positive correlations with total aromatic AAs and total % of hydrophobic AAs	Thermolysin (3 h, 55°C, pH 8.0)	(Pownall et al., 2010)
African Yam Bean (Sphenostylis stenocarpa)	Protein isolate, protein isolate-hydrolysate and ultrafiltered fractions (< 1 kDa – 10 kDa)	Fe		- Peptides < 1 kDa ↑ ferric reducing power, attributed to total hydrophobic/aromatic AAs - No significant difference between Fe-chelating activity of fractions	Alcalase (pH 8, 4 h at 50°C)	(Ajibola, Fashakin, Fagbemi, & Aluko, 2011)

Source	Protein fraction /s assayed	Element/s assayed	Peptide fractionation method	Characteristics contributing to high chelating activity	Enzymatic hydrolysis conditions	Source
Broad Bean (Vicia Faba)	Protein isolate-hydrolysate	Fe		- Alcalase produced the best effect for Fe-chelation (88.22%) - Degree of hydrolysis (DH) was correlated with chelation activity	Respective systems: Papain (pH 9, 60°C), neutral protease (pH 7, 50°C) and alcalase (pH 8, 50°C) for 4 h	(X. Li, Ding, Han, & Chen, 2015)
Winged bean (Psophocarpus tetragonolobus)	Protein isolate-hydrolysate	Fe	Separation: RPh- HPLC	- Fe-chelating activity was correlated with DH - Fraction containing acidic and basic residues ↑ Fe chelating activity	Papain (pH 6.5, 70°C) for 14 h	(Yea et al., 2014)
Mung bean (Vigna radiata)	Protein isolate-hydrolysate	Ca, Fe		- DH not directly linked to Ca and Fe-binding ability - ↓ Ca and Fe-binding as DH increased above certain values (DH = ∼39%)	Respective systems: Alcalase, flavourzyme (both at pH 8, 50°C), trypsin, pancreatin (both at pH 7, 37°C), pepsin (pH 2, 37 °C), mixture of	(Budseekoad et al., 2018)

Source	Protein fraction /s assayed	Element/s assayed	Peptide fractionation method	Characteristics contributing to high chelating activity	Enzymatic hydrolysis conditions	Source
					pancreatin and alcalase (50:50), mixture of pancreatin and flavourzyme (50:50), (both at pH 8, 50 °C). All were 8 h	
Soybean (Glycine max)	Protein isolate-hydrolysate (3 – 10 kDa)	Ca	Purification: IMAC	- Bound Ca was related with Glu, Gln, Lys and Pro content	Sequential: Protease M (pH 3, 50°C for 60 min), glutaminase (pH 7, 50°C for 180 min)	(Ying Lv et al., 2013)
Soybean (Glycine max)	Isoflavone-rich isolate, protein isolate-hydrolysate	Ca		- Max. binding capacity between 10-30 min of hydrolysis - % yield of Ca-peptide complex maximum at 120-180 min - Protease M hydrolysate has a high level of Ca affinity	Respective systems: Protease M (pH 4.5 and 50°C), alcalase (pH 8 and 60°C) for 0 - 240	(M.-P. Wang, Lu, Yang, Wang, & Yang, 2017)

Source	Protein fraction /s assayed	Element/s assayed	Peptide fractionation method	Characteristics contributing to high chelating activity	Enzymatic hydrolysis conditions	Source
					min	
Soybean (Glycine max)	Protein isolate-hydrolysate	Ca	Fractionation: SEC	- Ca content in hydrolysates not significantly affected by phytate - Protease M produced maximum levels of Ca binding - Peptides with ↑ Ca binding capacity had average MW of either 14.4 kDa or 8 to 9 kDa - Ca binding capacity linearly correlated with -COOH content	Respective systems: Protease M (pH 3, 50°C), pepsin (pH 2, 37°C), neutrase and flavourzyme (pH 7, 50°C), all for 60 min	(Bao et al., 2008)
Soybean (Glycine max)	Protein isolate-hydrolysate	Fe	Separation (phytate removal): High-Performance Anion - Exchange (HPAE)	- Dephytinisation ↓ degrees of hydrolysis - Moderate DH = ↑ chelation activity (6.79%)	Respective systems: Trypsin, pepsin (37°C, pH 8 and 2, respectively), protemax, flavorzyme, neutrase and	(M.-N. Zhang, Huang, & Jiang, 2014)

Source	Protein fraction /s assayed	Element/s assayed	Peptide fractionation method	Characteristics contributing to high chelating activity	Enzymatic hydrolysis conditions	Source
					alcalase (50°C, pH 7.5, 7.1, 7.1 and 8, respectively), all for 120 min	
Wheat (Triticum aestivum) germ	Germ protein isolate-hydrolysate	Zn	Separation (desalting): IMAC	- Max. chelating activity found between 150-200 min hydrolysis - MW 1221 Da was best chelate of Zn - Simulated gastrointestinal digestion of peptide–zinc complex markedly higher than inorganic Zn salt (Caco-2 cell uptake)	Respective systems: Alcalase (pH 8.3, 55°C), flavourzyme (pH 7, 60°C), papain (pH 7, 55°C), up to 400 min Sequential systems: pepsin-pancreatin gastric (2 h, 37°C), intestinal (2 h,	(Zhu, Wang, & Guo, 2015)

Source	Protein fraction /s assayed	Element/s assayed	Peptide fractionation method	Characteristics contributing to high chelating activity	Enzymatic hydrolysis conditions	Source
					37°C),	
Wheat (Triticum aestivum)	Germ protein isolate-hydrolysate	Ca	Separation: ultrafiltration, HPAE, fractionation: GF chromatography, RPh-HPLC	- Binding capacity with DH ↑ markedly until 240 min, then ↓ rapidly - Asp and Thr possess major binding sites	Alcalase (pH 8, 50°C, 50-300 min)	(F.-R. Liu, Wang, Wang, & Chen, 2013)
Barley (Hordeum vulgare)	Hordein hydrolysate	Fe, Zn, Ca	Separation: IMAC	- A mixture of varying low MW peptides, and a combination of charged/hydrophobic residues are synergistic in ↑ binding	Respective systems: Alcalase, (pH 8, 50°C), flavourzyme (pH 7, 50°C), pepsin (pH 2, 37°C), trypsin (pH 7, 37°C), up to 2 h	(Eckert et al., 2014)

Source	Protein fraction /s assayed	Element/s assayed	Peptide fractionation method	Characteristics contributing to high chelating activity	Enzymatic hydrolysis conditions	Source
Barley (Hordeum vulgare)	Glutelin hydrolysate	Fe	Separation: RPh-HPLC	- DH and hydrophobicity not directly linked to Fe chelation - Highest Fe chelation found in fraction < 1 kDa (50 – 67% hydrophobic AA residues) - Fraction with M_w > 10 kDa showed good reducing power comparable to ascorbic acid	Respective systems: Alcalase (pH 8, 50 °C), flavourzyme (pH 7, 50°C), up to 4 h	(Eckert et al., 2016)
Rice (Oryza sativa)	Protein isolate-hydrolysate	Fe		- Pancreatin digestion ↑ Fe chelating activity to a greater extent than pepsin digestion - Hydrolysates from pepsin–pancreatin digestion exhibited a time-dependent ↑ in reducing power	Sequential system: gastric (2 h, 37°C), intestinal (4 h, 37°C),	(Y. Liu, Wang, Li, Liang, & Yang, 2016)

Source	Protein fraction /s assayed	Element/s assayed	Peptide fractionation method	Characteristics contributing to high chelating activity	Enzymatic hydrolysis conditions	Source
Rice bran (Oryza sativa)	Defatted rice bran isolate-hydrolysate	Fe		- Optimal chelation of peptides from combined enzymatic systems for moderate time (90 min) - Hydrolysates ↑ solubility/retention/transport/uptake compared to unhydrolysed protein (Caco-2) - Extended hydrolysis inhibited these parameters	Respective systems: Alcalase (pH 8, 50°C), flavourzyme (pH 7, 50°C), up to 4 h Combined systems: Alcalase (pH 8, 50°C) + flavourzyme (pH 7, 50°C), up to 4 h Sequential system: Alcalase (pH 8, 50°C) 1 h, flavourzyme (pH 7, 50°C) 3 h All systems	Foong, Imam, and Ismail (2015)

Source	Protein fraction/s assayed	Element/s assayed	Peptide fractionation method	Characteristics contributing to high chelating activity	Enzymatic hydrolysis conditions	Source
					subjected to Sequential digestion: gastric (1 h, 37°C), intestinal (3 h, 37°C)	

Source	Form	Method	Element/s assayed		Findings	In li to
Lupin (Lupinus augustifolius)	Purified	*In vivo*	Fe	-	↑ in serum parameters of healthy/anaemic rats	N
Soybean (Glycine max)	Purified	*In vivo*	Fe	-	Phytoferritin and $FeSO_4$ are equally bioavailable in healthy/anaemic humans and rats	N

Source	Form	Method	Element/s assayed	Findings	In li to
	Purified and reassembled	*In vitro* Caco-2 cell uptake	Fe	- ferritin degraded to varying extent under gastric digestion - > 50% of ferritin was intact after pepsin treatment for 45 min - Digestive stability depends on the ratio of rH-1 and rH-2 subunits - Tannic acid ↓ Fe bioavailability	P ta a
	Recombinant H-2	*In vitro* digestion, *in vitro* release/chelating activity	Fe	- Polyphenols (Gallic acid, methyl gallate and propyl gallate) with three OH groups can bind to rH-2 and ↑ digestion stability - Cinnamic acid derivatives ↑ Fe release in a structure-dependent manner - ↑ polyphenol chelating activity = faster rate of the Fe release	
Recombinant bean (Phaseolus vulgaris)	Purified	*In vitro*	Fe	- Ferritin-bound iron no longer detectable after boiling for 50 min - EDTA ↑ Ferritin-bound Fe after cooking	E
Pea (Pisum sativum)	Purified	*In vitro Caco-2 cell uptake*	Fe	- Fe core is released into digestive medium - Pea ferritin uptake inferior or indifferent to $FeSO_4$ - Ascorbic acid ↑, while phytic acid ↓ ferritin uptake	P a
	Purified	*In vitro* autodegradation	Fe	- Thermal treatment at 60 to 80 °C can ↑ storage stability to 10 days minimum - Thermal treatment > 90 °C changes ferritin structure	
Adzuki (Vigna angularis)	Purified	*In vitro* autodegradation	Fe	- Adzuki phytoferritin exhibits higher stability than soybean rH-1 - The position Ser 68 is important in the control of protein stability	N

Source	Form	Method	Element/s assayed		Findings	In li to
Broad bean (Vicia faba)	Purified	*In vitro* autodegradation	Fe	-	Stability of broad bean phytoferritin > soybean and pea ferritins	N
Chickpea (Cicer arietinum)	Purified	*In vitro* autodegradation	Fe	-	CSF not degraded at 4 °C during 39 days (Stability > SSF, PSF, and BBSF under the same conditions)	N
Lentil (Lens culinaris), chickpea (Cicer arietinum), mung bean (Vigna radiata), pea (Pisum sativum)	Purified	*In vitro* digestion	Fe	-	Most Fe released occurred during 120 min gastric digestion - Red lentil had the best stability	N
Soybean apoferritin	Reassembled	*In vitro* Caco-2 cell uptake	Ca	-	Nanocage encapsulated Ca undergoes separate uptake pathway (TfR1 receptor) compared to free Ca ions (DMT-1 related)	N