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REVIEW

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Peptide-metal complexes: obtention and role in increasing bioavailability and decreasing the pro-oxidant effect of minerals

Maria Elisa Caetano-Silva[a], Flavia Maria Netto[b], Maria Teresa Bertoldo-Pacheco[a], Amparo Alegría[c], and Antonio Cilla[c]

[a]Center of Food Science and Quality, Institute of Food Technology (ITAL), Campinas, SP, Brazil; [b]Faculty of Food Engineering, University of Campinas (UNICAMP), Campinas, SP, Brazil; [c]Nutrition and Food Science Area, Faculty of Pharmacy, University of Valencia, Valencia, Spain

ABSTRACT

Bioactive peptides derived from food protein sources have been widely studied in the last years, and scientific researchers have been proving their role in human health, beyond their nutritional value. Several bioactivities have been attributed to these peptides, such as immunomodulatory, antimicrobial, antioxidant, antihypertensive, and opioid. Among them, metal-binding capacity has gained prominence. Mineral chelating peptides have shown potential to be applied in food products so as to decrease mineral deficiencies since peptide-metal complexes could enhance their bioavailability. Furthermore, many studies have been investigating their potential to decrease the Fe pro-oxidant effect by forming a stable structure with the metal and avoiding its interaction with other food constituents. These complexes can be formed during gastrointestinal digestion or can be synthesized prior to intake, with the aim to protect the mineral through the gastrointestinal tract. This review addresses: (i) the amino acid residues for metal-binding peptides and their main protein sources, (ii) peptide-metal complexation prior to or during gastrointestinal digestion, (iii) the function of metal (especially Fe, Ca, and Zn)-binding peptides on the metal bioavailability and (iv) their reactivity and possible pro-oxidant and side effects.

KEYWORDS

Metal-binding peptides; bioavailability; mineral deficiency; bioactive peptides; hydrolysates; metal-peptide complexes

Introduction

Some minerals, such as Fe, Zn, and Ca, are essential for life, and their deficiencies can lead to several health problems. Iron is necessary for most, if not all, pathways for energy and substrate metabolism. It is an intrinsic component of myoglobin, hemoglobin, cytochromes, and several enzymes, and plays a major role in oxygen transport, electron transfer, and oxido-reductase activities. The severe Fe deficiency can trigger alterations in the structure and functions of epithelial tissue, apart from mood changes, muscle weakness, and impaired immunity (EFSA 2015a).

Iron deficiency is a major global public health problem and, although its prevalence is higher in developing countries, it is also a serious health problem in developed countries, especially for risk groups, such as the elderly, patients after bariatric surgery, children, pregnant women, and women in fertile age (Pavord et al. 2012; Shankar, Boylan, and Sriram 2010). Many factors contribute to Fe deficiency, of which low dietary Fe bioavailability is the main one.

Zinc is the second most abundant metal in the body, and it is involved in several vital physiological functions, being ubiquitous within every cell in the body (EFSA 2014). There are more than 200 metalloenzymes which require Zn for activity, mainly for carbohydrate, protein, lipid, and nucleic acid metabolism. Apart from the essential role in gene expression and the regulation of cellular growth and differentiation, it plays an important role in the immune system and male reproductive function (Alegría-Torán, Barberá-Sáez, and Cilla-Tatay 2015). Considering that Zn is essential for many core biochemical processes, it is difficult to identify specific symptoms of Zn deficiency. Growth retardation, delayed skeletal maturation, dermatitis, and vulnerability of the immune system are related to chronic deficiency of Zn, although by no means exclusively associated with this metal (EFSA 2014). The lack of Zn is prevalent, particularly, in the elderly and children, even more in a population with a plant-based diet, rich in phytate, an important inhibitor of this mineral absorption (Wang, Zhou, et al. 2011).

Calcium is an integral component of the skeleton, and it is known to be a second messenger in signal transduction. It is involved in many physiological functions such as neurotransmission, muscle contraction, and blood coagulation. The absorption of dietary Ca is influenced by several factors, such as Ca in ionized form, which is prone to form Ca precipitation in the neutral intestine environment, compromising its absorption and bioavailability. Thus, even with a high intake of this mineral, it is possible to have a Ca deficiency scenario, especially in children and the elderly, for whom Ca deficiency can easily lead to metabolic bone disease (Sun

CONTACT Maria Elisa Caetano-Silva elisacaetano4@gmail.com Center of Food Science and Quality, Institute of Food Technology (ITAL), Av. Brasil, 2880, 13070-178 Campinas, SP, Brazil; Antonio Cilla antonio.cilla@uv.es Nutrition and Food Science Area, Faculty of Pharmacy, University of Valencia, Avda. Vicente Andrés Estellés s/n, Burjassot, Valencia, Spain.

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et al. 2016). Low intake or absorption can lead to Ca resorption from the skeleton to maintain blood concentrations, which causes a reduction in bone mass, and consequently, osteopenia and osteoporosis (EFSA 2015b).

In this sense, there is a need to reduce the prevalence of these deficiencies. Food fortification is one of the strategies to control them (WHO 2015) and is identified as one of the most cost-effective and sustainable approaches (Alegría-Torán, Barberá-Sáez, and Cilla-Tatay 2015). For food fortification, the choice of the mineral compound is generally based on its solubility, no precipitation either in the food or in the organism, and stability in different pHs. As a general rule, the more soluble, the more bioavailable the compound is.

Hence, conditions which can enhance Fe solubilization and keep the metal in the ferrous form in the digestive environment can favor Fe uptake by the intestinal cells, increasing its bioavailability (Hurrell et al. 2004). Zinc salts used for fortification can be unstable and cause gastrointestinal tract (GIT) irritation, making them unsuitable for long-term intake (Udechukwu, Downey, and Udenigwe 2018). The salt chosen for supplementation can affect Zn bioavailability, which is also influenced by dietary Ca, P, oxalate, Cu, Mg, and excess Fe (Cummings and Kovacic 2009).

During gastrointestinal digestion, different minerals can interact with several components and form complexes, which can increase or decrease mineral absorption. Some compounds, such as phytic acid, can form insoluble complexes with the mineral, hindering its absorption (Lazarte et al. 2015). Nevertheless, scientific literature offers an alternative strategy for mitigating these problems: mineral complexation with some organic compounds. These complexes are more stable and less prone to interactions with the chemical environment. Among the possibilities, peptides are considered as one of the best ligands because they have carboxyl and amino groups, in addition to the diverse side chains of amino acids, that can participate in the linkage of divalent cations (Ashmead 2001).

The literature shows several studies addressing mineral complexation by peptides during simulated gastrointestinal digestion (SGID) (Argyri et al. 2007; Argyri et al. 2009; Etcheverry, Miller, and Glahn 2004; Ou et al. 2010). On the other hand, peptide-metal complexes, when synthesized before ingestion, can protect the mineral throughout the GIT and increase its bioavailability. In this case, there are several parameters which can be controlled in order to favor the best binding condition and the obtention of stable peptide-metal complexes. In the last years, many authors have studied different types of ligands, metal sources, and the conditions of synthesis of complexes with this approach (Caetano-Silva et al. 2015; de la Hoz et al. 2014; Huang, Ren, and Jiang 2014; Sugiarto, Ye, and Singh 2009; Torres-Fuentes, Alaiz, and Vioque 2011; Zhang, Huang, and Jiang 2014).

The nutritional function and bioactivity of protein and peptides have been extensively studied in the last years (Albenzio et al. 2017), wherein the approach concerning peptides with the ability to bind minerals have been receiving major importance due to their action increasing the mineral bioavailability when ingested (Walters, Esfandi, and Tsopmo 2018). These peptides, inactive within the primary structure of the protein, can be released in vivo or in vitro by enzymatic proteolysis (Hebert, Saavedra, and Ferranti 2010). There is an increasing interest in prospecting new peptides from different sources to bind minerals, considering the relevance of bivalent micronutrients in a wide variety of biological functions.

This review focuses on the obtention of metal-peptide complexes and their effect on mineral bioavailability, including the mechanisms proposed for bioavailability enhancement with different minerals and different protein sources, as well as the possible decrease in the pro-oxidant effect of Fe through coordination with peptides.

Principle of peptide-metal complexation

The peptide linkage to metallic ions is based on the interaction between an electron donator group in the ligand surface (peptide) and a metallic ion (electron receptor) with one or more available coordination sites, ensuring that the metal becomes part of a biologically stable structure due to the coordinated linkages (Ueda, Gout, and Morganti 2003). Ligands can be considered as Lewis bases, which can share an electron pair with metals, in this case, taken as Lewis acids. Thus, the coordination compound is formed as a result of interaction between a Lewis acid and a Lewis base (Kratzer and Vohra 1986).

For the complexation reaction to occur, some conditions must be fulfilled: (i) the ligand must have functional groups capable of establishing a coordinate covalent bond; (ii) the complex must be sterically possible; (iii) the complexation reaction must be energetically possible (Ashmead 2001). Once complexed, the physicochemical features of the metal change. The components of the complex share some properties; however, maintaining their own properties (Bell 1977).

The specificity of complex formation is governed by the spatial arrangement of the ligands, i.e., which depends on where the functional groups are in the peptide sequence. The interactions can be increased or decreased by changing the amino acid residues in these sequences (Carlton and Schug 2011), and the peptide surface charge can influence their metal complex stability (Udechukwu, Downey, and Udenigwe 2018). The atomic structure, covalent or ionic bonds, as well as the linkages in the coordination spheres of the metal or ligand, and their oxidation states, can influence the mineral bioavailability (Cozzolino 2007).

Obtaining metal-binding peptides

Figure 1 depicts the main processes involved in the obtention and evaluation of metal-binding peptides and their obtained complexes. On the left are the main steps usually applied for purification and fractionation of peptides, including their sequencing and synthesis. On the right are the main assays used to evaluate bioaccessibility/

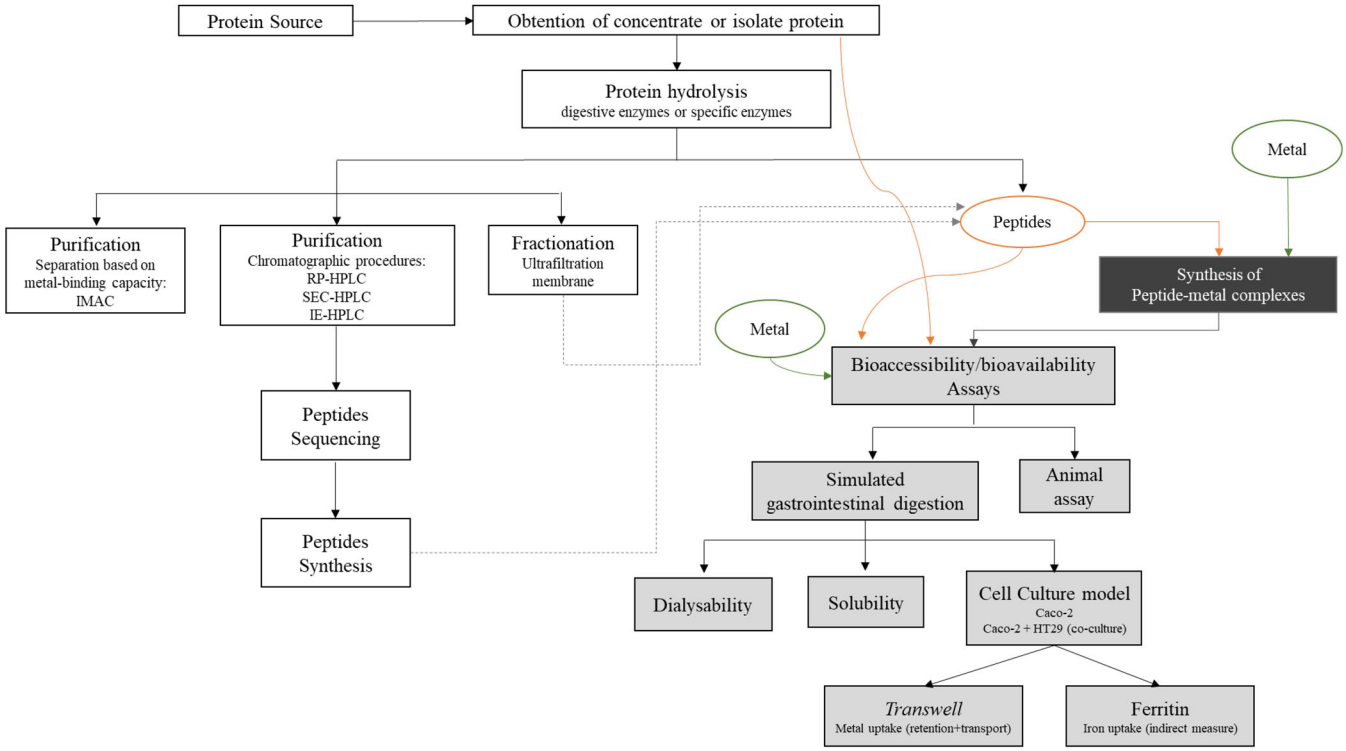

Figure 1. Obtention and evaluation of metal-binding peptides and their complexes.

bioavailability of metal along with peptides, or from previously formed peptide-metal complexes.

The main amino acids residues with mineral-binding affinity and their current protein sources

The choice of the protein source is of great importance since the amino acid sequence plays a crucial role on the metal-binding capacity of the peptides, and it is defined by the parent protein (Guo et al. 2014). The mineral-binding capacity of peptides also depends on other factors, such as peptide structure, steric effects, and molecular mass (MM) (Carrasco-Castilla et al. 2012).

In general, regardless of the protein sources, studies of peptide-Fe complexes have been showing that the main Fe-binding site corresponds, primarily, to the carboxylic groups, especially from Glu and Asp residues (Caetano-Silva et al. 2015; Chaud et al. 2002; Huang, Ren, and Jiang 2011; Lee and Song 2009), although the nitrogen of ε-amino group from Lys, guanidine group from Arg, and imidazole group from His can also be involved in the linkage (Chaud et al. 2002; Reddy and Mahoney 1995; Torres-Fuentes, Alaiz, and Vioque 2012). Li et al. (2019), studying the effect of peptides from duck egg white on Fe bioavailability in iron-deficiency anemia (IDA) rats´ model, reported that the residues of Glu, Asp, Lys, His, Ser, and Cys might play a crucial role in the chelating of the obtained peptides with Fe. Residues of Pro, although they do not participate directly in the Fe coordination, exert a crucial role promoting the peptide folding, favoring the formation of a ring structure with the Fe atom in its core (Eckert et al. 2016). Most proteins have plenty of Glu and Asp acid residues, the main functional sites for Fe-binding (Guo et al. 2014). Therefore, Fe-binding ability has been studied in a range of sources, such as casein (CN) (Chaud et al. 2002), muscular tissue from different meats (Storcksdieck, Bonsmann, and Hurrell 2007), porcine blood plasma (Lee and Song 2009), yak-casein (Wang, Li, et al. 2011), anchovy (Wu et al. 2012), egg (Palika et al. 2015), soybean (Lv et al. 2014; Zhang, Huang, and Jiang 2014), chickpea (Torres-Fuentes, Alaiz, and Vioque 2012), and barley (Eckert et al. 2016). Among the protein sources, the milk and soybean proteins have been largely studied for their Fe-binding peptides in the last years.

The Zn-binding sites are reported to be mainly His, Cys, and Met (Guo et al. 2014). Zinc readily binds to sulfur and nitrogen atoms in Cys and His residues of proteins and polypeptides (Cummings and Kovacic 2009). Protein from oyster (Chen et al. 2013), whey (Udechukwu, Downey, and Udenigwe 2018), yak-casein (Wang, Zhou, et al. 2011), sesame (Wang, Li, and Ao 2012), rapeseed (Xie et al. 2015), and wheat (Zhu, Wang, and Guo 2015) have been studied as sources of peptides with Zn-binding ability.

In Ca-binding peptides, both the oxygen atoms of the carboxyl group and the nitrogen atom of the amino group seem to be involved in the formation of chelate with the mineral. Ca-binding capacity has been largely studied in casein phosphopeptides (CPPs), which are phosphorylated CN-derived peptides that can be released by in vitro or in vivo enzymatic hydrolysis of αs1, αs2, and β-CN (Miquel, Gomez, et al. 2005). Most CPPs contain the cluster SpSpSpEE, a highly polar acidic sequence of 3 phosphoseryl groups followed by 2 Glu residues, which are a binding site for minerals such as Ca, Fe, and Zn, and play an important role in mineral bioavailability (Miquel, Alegria, et al. 2005; Miquel et al. 2006). The CPPs have also been studied by

their Zn-binding ability, as reviewed by Miquel and Farré (2007). The authors reported that Zn binds weakly to phosphoserine residues, which is quite relevant to nutrition, as the mineral can be released progressively in the intestinal lumen for absorption. The CPPs are commonly studied using matrices such as milk-based infant formulas (García-Nebot et al. 2010; García-Nebot et al. 2015; Miquel et al. 2006). Studies of Ca-binding ability also include peptides from whey (Zhao et al. 2014), Pacific cod bone (Zhang et al. 2018), Antarctic krill (Hou et al. 2018), soybean (Bao et al. 2008), and wheat germ (Wang et al. 2018).

Another source of great interest which has been studied for complexing different metals are proteins from byproducts of the food industry, which present diverse amino acid compositions, and are typically neglected and discarded. A number of bioactivities from byproducts and underutilized resources have been largely studied in the last years as a sustainable potential source of bioactive peptides. Among the sources of mineral-binding peptides, it is possible to highlight the marine byproducts, such as shrimp byproducts (Huang, Ren, and Jiang 2011; Huang, Ren, and Jiang 2014), Pacific cod skin gelatin (Wu et al. 2017), and Alaska pollock skin (Chen et al. 2017; Guo et al. 2015).

Since a specific amino acid residue can show affinity to bind with more than one metal, some matrices have been evaluated by their ability to bind different metals. For instance, Miquel, Alegria, et al. (2005) studied the Ca-, Fe-, and Zn-binding ability of the cluster sequence SpSpSpEE from CPPs in toddler milk-based formula, and Guo et al. (2015) studied the Fe and Ca-binding ability of Asp, Glu and Gly residues from Alaska pollock skin collagen following SGID.

The ability of peptides to bind Cu has also been evaluated using different matrices as peptide sources, such as ovotransferrin (Moon et al. 2015), phaseolin and lectin (Carrasco-Castilla et al. 2012), and fish skin collagen (Guo et al. 2015). Most of the studies evaluate the Cu-binding ability of peptides; however, to our knowledge, there is no study on peptide-Cu complexes structure and bioavailability evaluation, as can be found for Fe, Ca, and Zn. Therefore, the mineral Cu was not the focus of this review.

Enzymatic hydrolysis

The bioactive peptides generally have from 2 to 20 amino acid residues. Their potential bioactivity only arises when they are released from the sequence of the whole protein since the peptides are encrypted within this parent sequence. This process can be done by enzymatic hydrolysis using: (i) digestive enzymes such as pepsin, trypsin, and chymotrypsin; (ii) microbial / bacterial proteinases and peptidases during fermentation, or (iii) proteinases derived from microorganisms or plants, or a combination of all the above (de Castro and Sato 2015; Harnedy and FitzGerald 2011).

The enzymatic protein hydrolysis can improve the nutritional value of proteins and increase their physical, chemical, and functional properties (Ghosh, Prasad, and Saha 2017). Regarding the ability to chelate minerals, the hydrolysis process increases the availability of metal-binding sites by the peptide bond breakdown, which releases carboxylic groups, and the exposition of hidden binding sites in the protein structure.

The choice of proteolytic enzymes is critical to hydrolysis processes because enzyme specificity influences the peptide profile of the hydrolysates (Wang, Zhou, et al. 2011). Different enzymes yield peptides with different sequences – and thus different bioactivities. Some studies focus on evaluating the difference among the structure, sequence, and biological activity, i.e., the capacity of increasing mineral absorption, of peptides obtained using different enzymes (Cian et al. 2016; de la Hoz et al. 2014; Ou et al. 2010; Wang, Zhou, et al. 2011). For enzyme choice, one should consider the metal to be bond and the specificity of the protein source under study.

For this purpose, enzymes with endopeptidase activity have been used, such as Alcalase, neutrase, trypsin, and pepsin, or with exopeptidase activity, such as Flavourzyme. Enzymatic systems, such as pancreatin and Protamex, has also been studied, presenting both endo and exopeptidase activities.

The feasibility of the application of metal-binding peptides from the economic standpoint should be considered. In this sense, naturally occurring peptides, from gastrointestinal digestion, pose advantages when compared to the peptides produced by enzymatic hydrolysis, concerning their safety since they are naturally obtained as well as the low cost with no need of using other enzymes. When using peptides isolated from foods and, in the case of peptide-metal complexes, subjected to reaction with metal ions, one should consider that they are not single entities such as synthetic drug molecules, and the presence of different peptides in this mixture could represent beneficial additive or synergistic effects among different molecules. Thus, it is challenging to determine the quality assurance of these materials (Chakrabarti and Guha 2018).

Ultrafiltration

Several studies have presented the ultrafiltration process as an alternative to separate peptides of lower MM from the pool of peptides obtained through enzymatic hydrolysis since peptides with low MM are indicated as better ligands than the larger ones.

A membrane with cutoff 5 kDa has been used by Caetano-Silva, Cilla, et al. (2018) and de la Hoz et al. (2014), rendering two fractions, the retentate (MM $>$ 5 kDa) and the filtrate (MM $<$ 5 kDa). While the latter authors focused on the filtrate fraction, to study the Fe-binding capacity of isolated peptides, the former studied both fractions in a Caco-2 cell model, evaluating the Fe bioavailability of peptide-Fe complexes, after SGID. Budseekoad et al. (2018) ultrafiltered mung bean hydrolysate with 4 different MM cutoff (1, 5, 10, and 30 kDa). They observed that peptides with lower MM, i.e., from the permeate fraction, showed higher Ca and Fe-binding capacity than the larger ones, presented in the retentates fractions. Wang et al. (2018) also

used ultrafiltration aiming at purifying Ca-binding peptides. For that, the authors used a membrane with 3-kDa cutoff, but along with other chromatographic purification steps, anion-exchange, gel filtration, and reversed-phase high-performance liquid chromatography using the strategy described in the next topic.

Purification, sequencing, synthesis and in silico analysis of mineral-binding peptides

The studies on the composition, structure, behavior, and sequence of metal-binding peptides are generally accomplished by purification steps. Purified binding peptides from protein hydrolysates can be useful to obtain peptide-metal complexes. Bioactive peptides are usually purified by using a combination of chromatographic techniques (Megías et al. 2007). For metal-binding peptides, the purification is usually carried out by liquid chromatography, using different separation principles, such as gel filtration, ionic exchange, and reverse phase. In some studies, this purification is performed after a preliminary step of isolation by Immobilized Metal Affinity Chromatography (IMAC). This technique explores the interaction between electron donator species from the surface of the biomolecules in solution and metallic ions immobilized in a solid support (Bresolin, Miranda, and Bueno 2009). Proteins, peptides or other solutes added to the mobile phase are adsorbed mainly by the formation of coordinated bonds with remaining sites of the chelated metallic ions, apart from other involved forces, such as electrostatic forces, hydrophobic interactions, and Van der Waals forces (Ueda, Gout, and Morganti 2003).

Although the protein separation by IMAC is mainly based on interaction forces between their amino acid residues and the metallic ions bonded to the column, several other factors influence the formation of this complex and consequently the protein retention, such as pH, salt concentration, and elution buffer (Bresolin, Miranda, and Bueno 2009). Thus, the amino acids residues and the possibility of binding to the immobilized metal ions under the chromatographic conditions must be considered.

Argyri et al. (2009) obtained digested milk fractions from repeated chromatographic fractionations and subjected then to SGID and Caco-2 cell experiments. The authors observed that peptides of milk digested from the chromatographic fraction with MM between 1579 and 1000 Da, selected as the most potent in the formation of soluble iron, showed a positive effect on Fe absorption when compared to whole milk. However, in general, the chromatographic fractionation step is only used to determine the pool of peptides with the highest activity. After that, purification followed by identification using mass spectrometry are carried out, and then the synthesis of the most promising sequence (Figure 1). Thus, when a hydrolysate is purified with the aim to identify the peptides with high metal-binding capacity, the absorption studies, after structure characterization, are usually carried out with the synthetic peptide.

Chen et al. (2017) synthesized peptides previously identified as Ca- and Zn-binding peptides, after sequential purification steps, including IMAC and gel permeation chromatography (GPC) from Alaska pollock skin. The authors evaluated the effect of peptides on mineral uptake in a Caco-2 cell culture model. Eckert et al. (2016) synthesized a complex peptide-Fe with a synthetic peptide SVNPLY. This sequence was prominent in the most potent fraction from barley protein hydrolysates, after membrane fractionation (<1 kDa), separation of the most hydrophobic peptides (the last fraction to be eluted) by RP-HPLC (Bamdad and Chen 2013). The complex led to Fe uptake in Caco-2 cells 4-fold higher than $FeSO_4$ (Eckert et al. 2016). Cui et al. (2018) synthesized the heptapeptide NDEELNK, released during trypsin hydrolysis of sea cucumber ovum. The sequence was defined after separation and identification of peptides by UPLC by mass spectrometry. The authors screened out the potential Ca-binding peptide based on the molecular size and specific amino acids that closely associated with Ca binding. They observed that the NDEELNK-Ca complex could be conducive to Ca absorption across Caco-2 cell monolayers.

García-Nebot, Barberá, and Alegría (2013), studying CPPs from α- and β-CN, evaluated the synthetic phosphopeptides (αs1-CN(64–74)4P and αs2-CN(1–19)4P), and also the pool of peptides obtained through SGID, which were then identified by HPLC–ESI-MS/MS. Unlike the pool of CPPs, the authors observed that the synthetic peptides increased ferritin synthesis by Caco-2 cells. In a subsequent study, García-Nebot et al. (2015) showed that the CPPs origin (from α_s- or β-CN fractions) could influence Fe bioavailability depending on their structural properties and on the conformation of the CPP-Fe complex, which reinforces the importance of studying not only the pooled fraction but also the synthetic peptides. The results may provide a subsidy to proposing mechanisms and to better understand factors which can be crucial for mineral bioavailability.

A different way to identify potential mineral-binding peptides is the *in silico* analysis. Bioinformatic, or *in silico* analysis, is a computational method used to predict bioactivities of peptides from the known protein sequences (Kumar and Mann 2009). Bioinformatic tools can aid in the identification process of specific bioactivities, including mineral-binding capacity, in unique sequences previously identified and registered in a database. The potential sequences obtained through *in silico* enzymatic hydrolysis of a given protein can also be predicted using these tools. Among them, BIOPEP-UWM database has been largely used to predict bioactivities of specific peptides. This database contains biologically active peptide sequences and its program enables the construction of profiles of the potential biological activity of protein fragments (Minkiewicz et al. 2008). Some sequences, as an example, are listed as Zn-binding (HNAPNPGLPYAA and NAPLPPLKH), Fe-binding (SVNVPLY), or Ca-binding (YDT). Other sequences, such as most of the ones indicated in this review (Table 2), could be registered in these databases as well.

The *in silico* analysis can also aid in predicting the toxicity of the peptides involved in the formation of complexes. The bioinformatics tool called ToxinPred is an *in silico*

Table 1. Synthesis of metal-peptides complexes and its conditions of obtaining.

Metal	Ligand	Metal precursor	Peptide:metal ratio	Main conditions of complexation reaction	Method to separate the peptide-metal complexes	Complexes analysis	References
Fe	Whey protein and whey protein hydrolysate ultrafiltered (5 kDa) or not	$FeSO_4$ or $FeCl_2$	40:1 (w/w)	4% peptide and 0.1% Fe 25 ± 2 °C / 1 h; pH 7.0 under stirring	Centrifugation (5000g/ 20 min) ➔ supernatant freeze-drying	*In vitro* Fe bioaccessibility and bioavailability	Caetano-Silva, Cilla, et al. 2018
	Duck egg white hydrolysate	$FeSO_4$	2:1 (w/w)	4% hydrolysate, 0.5 g Fe/g peptide and 0.2 g ascorbic acid/g peptide 40 °C / 40 min; pH 5.5	Centrifugation (3800g/ 10 min) ➔ 95% ethanol ➔ centrifugation (10.000g/15 min, 4 °C) ➔ precipitate freeze-drying	*In vivo* (iron-deficiency anemia rats) iron bioavailability and structure characterization	(Li et al. 2019)
	Synthetic peptide from barley protein (SVNVPLY)	$FeSO_4$	0.25:1 to 2:1 (molar ratio)	25 °C / 2 h; pH 7.0	Centrifugation (2500g/ 10 min) ➔ supernatant freeze-drying	Biophysical and *in vitro* absorption studies	(Eckert et al. 2016)
	Synthetic peptide REE	$FeSO_4$	1:1 (molar ratio)	ultrasonic tank room temperature / 5 min under nitrogen	Not specified	Hematological test and Organ Coefficient in iron-deficiency anemia rats	(Xiao et al. 2016)
	α-lactoalbumin and β-lactoglobulin hydrolysates	$FeCl_3$	40:1 (w/w)	25 °C / 30 min; pH 7.0 under stirring	Centrifugation (3000g/ 20 min) ➔ supernatant freeze-drying	*In vitro* Fe absorption	(Wang et al. 2014)
	β-lactoglobulin hydrolysate	$FeCl_3$	40:1 (w/w)	25 °C / 30 min; pH 7.0 under stirring	Centrifugation (3000g/ 20 min) ➔ supernatant freeze-drying	Effect on haematological parameters of anemic rats	(Zhou et al. 2014)
	Yak casein hydrolysate	$FeSO_4$	Not specified	Not specified	Dialysis (cutoff 500 Da) ➔ retentate freeze-drying	Structure characterization and Fe-releasing percentage at different pH	(Wang, Li, et al. 2011)
Zn	Whey protein hydrolysate	$ZnSO_4$	10 mg/mL peptide; 50 µM Zn	Room temperature / 1 h; pH 7.0 (50 mM phosphate buffer); under stirring	Dialysis for 5 h (cutoff 500 Da) ➔ retentate freeze-drying	Structural and surface properties and *in vitro* gastric stability and bioaccessibility	(Udechukwu, Downey, and Udenigwe 2018)
	Yak-casein hydrolysate	Zn acetate	Not specified	37 °C / 40 min	Dialysis (cutoff 500 Da) ➔ retentate freeze-drying	Zn dialyzability and solubility under SGID and structure characterization	(Wang, Zhou, et al. 2011)
Ca	Synthetic peptide from Sea cucumber (*Stichopus japonicus*) ovum (NDEELNK)	$CaCl_2$	1:6 (molar ratio)	50 °C / 20 min; pH 8.0 under stirring	90% ethanol ➔ centrifugation (12.000g/ 5 min) ➔ precipitate freeze-drying	Ca-binding mode, *in vitro* digestion profile and Ca absorption	(Cui et al. 2018)
	Pacific cod bone hydrolysate	$CaCl_2$	11g $CaCl_2$ to 30 g hydrolysate	6% hydrolysate 50 °C / 1 h; pH 7.0 under stirring	absolute ethanol ➔ centrifugation (7.000g/ 15 min) ➔ precipitate freeze-drying	Structure characterization, *in vivo* (ovariectomized rat model) Ca bioavailability and anti-osteoporosis activity	(Zhang et al. 2018)
	Sea cucumber (*Stichopus japonicus*) ovum hydrolysate	$CaCl_2$	3:1 (w/w/)	50 mg/mL hydrolysate 50 °C / 20 min; pH 8.0 under stirring	85% ethanol ➔ centrifugation (12.000g/ 5 min) ➔ precipitate freeze-drying	Structure characterization and *in vitro* Ca absorption	(Sun et al. 2017)

Table 2. Metal-peptide complexation during or prior digestion and its effects on mineral bioavailability.

Metal	Formation of peptide-metal complexes	Protein source	Method to estimate bioaccesibility/ bioavailability	Identified/proposed peptide sequence or proposed amino acids or side groups involved	Effect on bioaccessibility/ bioavailability	Reference
Fe	Prior to digestion	Whey protein	Previous hydrolysis with pancreatin and complexes synthesis + SGID + Caco-2 cell culture model; ferritin synthesis	^{42}YVEELKPTPEGDLEIL^{57} and ^{12}4RTPEVDDEALEK^{135} of β-Lg ^{82}DDDLTDDI^{89} of α-La ^{11}FKDLGEEH^{18} and ^{106}KDDSPDLPK^{114} from BSA	Ferritin synthesis: Complexes of Fe-whey peptides (MM< 5 kDa) > $FeSO_4$	(Caetano-Silva et al. 2015; Caetano-Silva, Cilla, et al. 2018)
		Duck egg white	Previous hydrolysis with Neutrase and complexes synthesis + Animal iron-deficiency anemia (IDA) model	Pro-Val-Glu-Glu / Arg-Ser-Ser	Effects of IDA: Complexes of duck white egg peptides-Fe > $FeSO_4$	(Li et al. 2019)
		Barley protein	Peptide synthesis and complexes synthesis + SGID + Caco-2 cell culture model; ferritin synthesis	SVNVPLY	Ferritin synthesis: Complex Fe-SVNPLY > $FeSO_4$	(Eckert et al. 2016)
		Synthetic peptide complexed with iron: Fe-REE	Previous complexes synthesis + Animal IDA model	Specific synthetic peptide REE	Hematological parameters in IDA: Complex Fe-REE restored the normal levels	(Xiao et al. 2016)
		αs- and β-Casein (CN)	Previous SGID and complexes synthesis + HuH7 cells culture model; ferritin synthesis and soluble transferrin receptor contents	Phosphoseryl-Glu cluster SpSpSpEE	Ferritin synthesis or soluble transferrin receptor: Complexes of Fe-CPPs did not improve it	(García-Nebot et al. 2015)
		α-lactoalbumin and β-lactoglobulin	Previous hydrolysis with Alcalase and complexes synthesis + SGID + Caco-2 cell culture model; ferritin synthesis	*	Fe uptake: Complexes of Fe-peptides of β-Lg > $FeSO_4$	(Wang et al. 2014)
		β-lactoglobulin	Previous hydrolysis with Alcalase and complexes synthesis + Animal IDA model	*	Hematological parameters and Fe bioavailability: positive effects of Complex of Fe-β-Lg hydrolysate	(Zhou et al. 2014)
	Prior to Caco-2 cells assay	CPPs (β-CN(1–25)4P, αs1-CN(64–74)4P and αs2-CN(1–19)4P)	Specific CPPs resistant to SGID + $FeSO_4$ in Caco-2 cells; ferritin synthesis	caseinophosphopeptides (CPPs)	Ferritin synthesis: specific CPPs > $FeSO_4$	(García-Nebot, Barberá, and Alegría 2013)
	During digestion	Red Seaweed	Previous hydrolysis with alkaline protease and Flavourzyme + SGID + dialyzability	His and Ser	Fe solubility and bioaccessibility: maintained by red seaweed chelating peptides	(Cian et al. 2016)
		Egg protein	SGID + identification and synthesis of the released iron-binding peptide + Caco-2 cell culture model; ferritin synthesis and ^{59}Fe uptake	DKLPGFGDS(PO_4)IEAQ	Ferritin synthesis and ^{59}Fe uptake: Synthetic egg peptide (DKLPGFGDS(PO_4)IEAQ) > $FeCl_2$	(Palika et al. 2015)
		Sugar cane yeast	Previous hydrolysis with Alcalase, Viscozyme or Protex + SGID + dialyzability	His, Lys, and Arg	Fe Dialyzability: Peptides of sugar cane yeast obtained with Viscozyme > $FeSO_4$	(de la Hoz et al. 2014)
		Soybean protein	Previous hydrolysis with pancreatin or not + SGID + Caco-2 cell culture model; ferritin synthesis	*	Ferritin synthesis: Peptides released from previous soybean hydrolysis > whole protein	(Lv et al. 2014)
Zn	Prior to digestion	Whey protein	Previous hydrolysis with different proteases and complexes synthesis + SGID + dialyzability	Carboxylate ion and sidechain CO of Asp/Glu and Ser/Thr	Dialyzability and Zn releasing: Complexes of Zn-whey peptides improved Zn dializability and hindered Zn releasing	(Udechukwu, Downey, and Udenigwe 2018)
		Wheat germ protein	Previous hydrolysis with different proteases + synthesis of specific isolated peptide + complexes	HNAPNPGLPYAA	Zn bioavailability: HNAPNPGLPYAA-Zn complex > $ZnSO_4$	(Zhu, Wang, and Guo 2015)

(*continued*)

Table 2. Continued.

Metal	Formation of peptide-metal complexes	Protein source	Method to estimate bioaccesibility/ bioavailability	Identified/proposed peptide sequence or proposed amino acids or side groups involved	Effect on bioaccessibility/ bioavailability	Reference
			synthesis + SGID + Zn bioavailability estimated by Caco-2 cell			
		Yak-casein	Previous hydrolysis with different proteases and complexes synthesis + SGID + dialyzability and solubility	*	Zn dialyzability and solubility: Complexes of yak-casein hydrolysate-Zn > zinc acetate	(Wang et al. 2011)
	Prior to Caco-2 cells assay	CPPs (β-CN(1–25)4P, αs1-CN(64–74)4P and αs2-CN(1–19)4P)	Specific CPPs resistant to SGID + $ZnSO_4$ in Caco-2 cells; ferritin synthesis	caseinophosphopeptides (CPPs)	Zn retention, transport and uptake: αs1-CN(64–74)4P > $ZnSO_4$	(García-Nebot, Barberá, and Alegría 2013)
	During Caco-2 cells assay	Alaska pollock skin	Peptide synthesis + SGID; then synthesized peptide and Zn applied to Caco-2 cell culture model	GPAGPHGPPG	Zn transport: GPAGPHGPPG-Zn formed during digestion > $ZnSO_4$	(Chen et al. 2017)
Ca	Prior to digestion	Sea cucumber (*Stichopus japonicus*) ovum	Previous hydrolysis with different proteases and complexes synthesis + Caco-2 and HT-29 cell culture model; cytoplasmic calcium	Carboxyl oxygen and amino nitrogen atoms of Glu and Asp, and phosphoserine Residues	Ca solubility after SGID and Ca absorption: Complexes of Ca-sea cucumber hydrolysate > $CaCl_2$	(Sun et al. 2017)
		Pacific cod bone	Previous hydrolysis with trypsin and neutral protease and complexes synthesis + Animal (ovariectomized Wistar rat) model	*	Ca absorption and serum Ca: Complexes of Ca-pacific cod bone hydrolysate > $CaCO_3$ group	(Zhang et al. 2018)
		Sea cucumber (*Stichopus japonicus*) ovum	Peptide synthesis and complexes synthesis + SGD + Caco-2 cell culture model	NDEELNK	Ca absorption: NDEELNK-Ca complex > $CaCl_2$	(Cui et al. 2018)
	During Caco-2 cells assay	Alaska pollock skin	Peptide synthesis + SGID; then synthesized peptide and Ca applied to Caco-2 cell culture model	GPAGPHGPPG	Ca transport: GPAGPHGPPG-Ca > $CaCl_2$	(Chen et al. 2017)

*Nor identified or proposed. SGID: simulated gastrointestinal digestion; CPPs (caseinophosphopeptides).

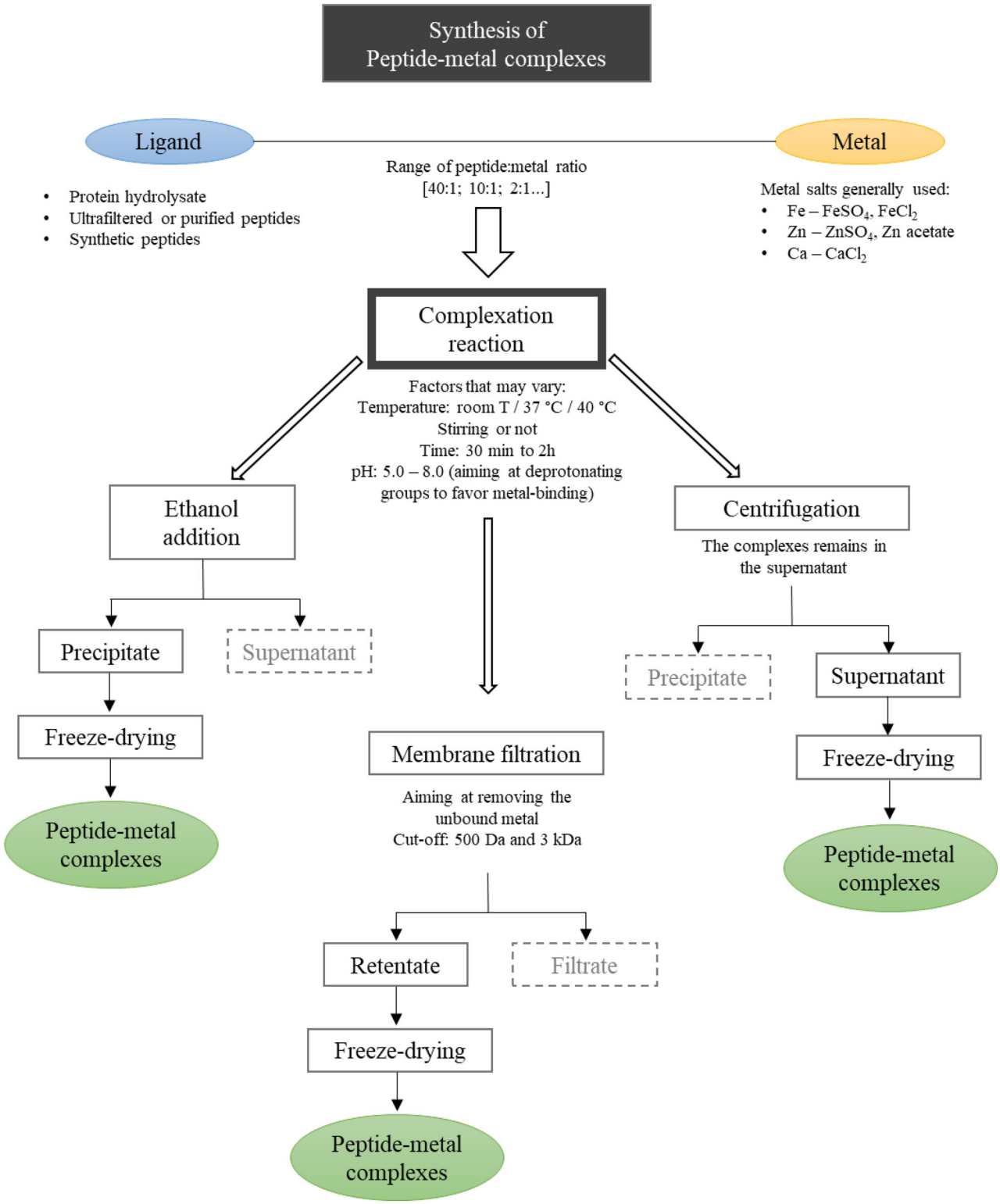

Figure 2. Flowchart of the synthesis of peptide-metal complexes.

method, which is developed to predict and design toxic/non-toxic peptides (Gupta et al., 2013). Using the available information, we can conclude that all the sequences cited in Table 2 were predicted to be nontoxic. Nevertheless, all the peptides identified to be used as metal coordinator should be evaluated by their toxicity.

Considering the great concern of food allergy in the development of novel and safe food products/ingredients, the allergenicity of the potential peptides involved in the complexation should also be addressed. The Algpred tool aids in predicting allergens based on the similarity of known epitope with any region of the protein (Saha and Raghava 2006), including the peptides involved in metal complexation. However, although it is important to address the allergenicity of these peptides, it can be highlighted that the complexation with metal ions can occupy some epitope

regions, preventing the allergen site recognition by a specific antibody.

Complexes formed prior to or during gastrointestinal digestion

Complexes can be formed during digestion or can be previously present in a fortifying compound. The interaction between the metal and other dietary components can form complexes, and the metal complexation by peptides can occur during the digestive process. On the other hand, a synthesized metal complex may protect the metal throughout the GIT, hindering its interaction with other components and increasing its absorption.

Several studies have proposed that proteins/peptides can act as mineral bioavailability enhancers, and hence the mineral uptake can be improved when the digestion occurs in the presence of proteins/peptides from different sources, such as fractionated human and bovine milk (Etcheverry, Miller, and Glahn 2004), peptides from whole milk obtained from SGID (Argyri et al. 2007), whey protein concentrate fortified with Fe (Nakano et al. 2007), whey protein concentrate hydrolyzed with different enzymes (Ou et al. 2010), soybean protein (Lv et al. 2014), or egg protein (Palika et al. 2015).

The intermediate products formed by the interaction of proteins/peptides and metal ions during digestion depend on the degree of protein digestion, the composition of peptides, and the amino acids of the protein, especially the presence of sulfur-containing amino acids such as Cys, in the case of Zn (Cámara and Amaro 2003). On the other hand, minerals not yet complexed with peptides can interact with dietary compounds, such as phytate and polyphenols, which yield the formation of insoluble complexes and thus decrease mineral bioavailability.

Ou et al. (2010) studied the effect of whey protein hydrolysates on Fe absorption in Caco-2 cells, by adding $FeSO_4$ to the hydrolysate's solutions upon the start of SGID. Consequently, the positive effect observed on Fe uptake was due to peptide-Fe interaction during digestion. Argyri et al. (2009), in turn, isolated two fractions of milk peptides released during SGID and evaluated their effect on Fe uptake in Caco-2 cells. As with the abovementioned authors, the Fe source was added at the moment of SGID, and hence the enhancement in Fe solubility was due to the metal interaction with the released peptides during the digestion process.

Chaud et al. (2002) studied the effect of simultaneous oral administration of peptides and $FeSO_4$ to Wistar rats and observed that increasing the free peptide concentration did not alter the profile of serum Fe, indicating that the complexation did not occur during the administration or during the gastrointestinal transit time. The authors reported that the addition of peptides along with previous formed peptide-Fe complexes did not improve the serum Fe profile.

During digestion, the pH varies from acid in the gastric to neutral in the intestinal phase. At acidic pH, the metal-binding sites will be protonated and hence the interaction between peptide and metal ions will be harder to occur, since coordinated binding is favored when the ionizable electron-donating groups of the amino acid residues are partially deprotonated, i.e., when the pH is higher than the pKa of binding groups (Porath 1990). At intestinal pH, two aspects are relevant: the peptide-metal binding is favored, but the mineral solubility sharply decreased, and the presence of dietary inhibitors can hinder their absorption. The reduction in Fe solubility by the neutral condition of the intestine is one of the main factors which contribute to the low Fe bioavailability (Scheers and Sandberg 2008) since, to be absorbed, the mineral has to be in the soluble form (Fidler et al. 2004). The other minerals might have the same behavior during the digestion. Therefore, the same scenario might be considered for them. Hence, the peptide-metal interaction should occur prior to the metal ion insolubilization. In this sense, the previous formation of peptide-metal complexes may be efficient to further increase the metal uptake compared to the addition of peptides and mineral at the moment of ingestion, i.e., complexes potentially formed during digestion.

Complexes formed with digestion-resistant peptides can potentially protect the mineral through the GIT, favoring its solubility and hindering its reactivity. However, only in the last few years, the studies regarding mineral bioavailability as peptide-metal complex synthesized prior to digestion have gained attention, either in vitro, through SGID (Caetano-Silva, Cilla, et al. 2018; Cui et al. 2018; Eckert et al. 2016; Sun et al. 2017; Udechukwu, Downey, and Udenigwe 2018) or in vivo (Xiao et al. 2016; Zhang et al. 2018; Zhou et al. 2014). In these studies, the complexes were synthesized under controlled conditions, with different metal:ligand ratios, to better understand and predict the mineral behavior since the ligand is well known.

Synthesis of peptide-metal complexes

The peptide-metal complexes can be obtained from a pool of peptides, i.e., from a hydrolysate obtained with different proteases, or from a synthetic peptide, such as the heptapeptides SVNVPLY from barley protein (Eckert et al. 2016) or NDEELNK from sea cucumber (*Stichopus japonicus*) ovum (Cui et al. 2018). Figure 2 depicts the flowchart of the synthesis of peptide-metal complexes, pointing out the main parameters that can be controlled, which are explained below.

Peptide: Metal ratio

In the peptide-metal complexation reaction, the peptides act as ligands, since they share electron pairs with the metal ion and stabilize it in a ring structure. Table 1 shows the peptide-metal ratios and other conditions involved in the synthesis of peptide-metal complexes in the last decade.

The peptide:metal ratio may vary substantially among studies, making it difficult to compare some results obtained from these different complexes. The peptide:metal ratio can be calculated in a molar (mol/L) basis when the reaction of

complexes is accomplished with synthetic peptides or compounds with known MM. In most cases, the ligand amount is lower or equal to the metal amount, due to the high affinity of all the ligand molecules.

On the other hand, if the ligand is a mixture of peptides from a hydrolysate, with a broad range of MM, the peptide:-metal ratio is generally used in a mass/mass ratio. In in vitro or in vivo assays, peptide:metal ratio may vary from 2:1 up to 40:1, depending on several factors, such as the type of ligand, the possible purification steps carried out before, and the bond strength of peptides. Generally, the ligand amount is higher than the metal amount to guarantee that all the metal ion sites will be fulfilled with peptides, whose average affinity tends to be lower than a specific synthetic peptide. Regardless of using molecular basis or mass/mass ratio, several studies have been yielding promising results, as discussed in the following sections.

Ligands and metal precursors

As presented above, the ligands for the formation of complexes can be a protein hydrolysate, ultrafiltered or purified peptides, or even synthetic peptides (Figure 2). Different metal precursors, i.e., the metal salts used as sources of ions to bind with peptides, have been used in the synthesis of the complexes. For Fe, it is more common to use ferrous sulfate, ferrous chloride, or ferric chloride, while Ca complexes are mostly synthesized with Ca chloride. The Zn source can also vary, in which case the sulfate or acetate salts are the most used. However, the effect of these precursors in the formation of the complexes has seldom been studied, although the role of different ions in the structure and interactions of peptides and proteins is well known (Zhang and Cremer 2006). Caetano-Silva et al. (2017) demonstrated that complexes synthesized with whey peptides and ferrous sulfate or chloride differ in structure, which, in a subsequent study, showed to be crucial to their absorption in Caco-2 cells (Caetano-Silva, Cilla, et al. 2018).

Complexation reaction

The complexation reaction generally consists of adding a specific quantity of metal salt to a peptide solution and incubating under specific time and temperature. The reaction medium can be stirred or not, and the pH, in most cases, is controlled since deprotonating groups favor metal-binding.

Regardless of the main objective (biological assays or structure characterization after complexation reaction), the peptide-metal complexes can be achieved by the same type of techniques. One of the most common is to centrifuge the reaction medium (generally 2500–5000 *g* during 10–20 min) and collect the supernatant, considering that the complexes are soluble (Caetano-Silva, Cilla, et al. 2018; Eckert et al. 2016; Wang, Wang, Wang, et al. 2014; Zhou et al. 2014). In other studies, a permeable membrane (generally cutoff 500 Da) is used after the reaction in order to remove the unbound ions (Udechukwu, Downey, and Udenigwe 2018; Wang, Zhou, et al. 2011; Wang, Li, et al. 2011). In this step, the free ions are dialyzed and the retentate is collected.

In some synthesis protocols, the last step consists of ethanol addition (85% to absolute ethanol), followed by centrifugation and collection of the precipitate. This step aims to precipitate the complexes formed and eliminate the free metal not bound to the peptides (Cui et al. 2018; Li et al. 2019; Sun et al. 2017; Zhang et al. 2018). However, there is no report concerning the possible influence of ethanol on the conformation of peptides after precipitation and their metal-ligand capacity. Chaud et al. (2002) also obtained the insoluble form of a peptide-Fe complex; but the precipitation was carried out by adjusting the pH to 3.5 and washing it with HCl to remove loosely bound Fe^{3+}.

In all cases (supernatant of centrifugation, retentate of dialysis, or precipitate by ethanol addition and centrifugation), the complexes are usually freeze-dried to continue the studies.

Peptide-metal complexes and bioavailability

Assessing the bioaccessibility is the first step to evaluate metal bioavailability. The bioaccessible fraction can be defined as the portion of a compound that is released from its food matrix in the GIT and thus becomes available for intestinal absorption. This definition, typically used for mineral bioavailability studies and based on in vitro procedures, refers only to digestion and release from the food matrix and does not consider the absorption in intestinal cells (Cilla, Barberá, and Alegría 2017). Some studies, based on this definition, used the soluble fraction obtained by SGID to determine bioaccessibility (Cian et al. 2016; Wang, Zhou, et al. 2011; Wang, Li, et al. 2011). The solubility of peptide-metal complexes can significantly enhance the metal bioaccessibility for uptake into the lumen (O'Loughlin et al. 2015), but this does not necessarily imply in minerals being effectively more bioavailable.

The bioavailability of a nutrient comprises its accessibility to the metabolic and physiological normal processes, although there is no universally accepted definition to this term, which has several working applications. From a nutritional standpoint, the bioavailable fraction can be defined as the portion of ingested component available for use in normal physiological functions, determined from in vivo assays (Alegría-Torán, Barberá-Sáez, and Cilla-Tatay 2015). However, some authors use the term in vitro bioavailability (Caetano-Silva, Cilla, et al. 2018; Etcheverry, Grusak, and Fleige 2012; García-Nebot, Barberá, and Alegría 2013; Lv et al. 2014; Zhu et al. 2009), referring to the fraction which is absorbed by the intestinal cells, or even transported across them, and available to be moved to the target tissue.

The mechanism by which the complexing agent improve the mineral utilization depends on the ligand capacity to sequester the mineral or to compete with other ligands in the GIT (Kratzer and Vohra 1986), even among other metal ions, since competitive interactions can occur between Zn and other minerals that have similar physical and chemical properties, such as Fe and Cu.

Peptide-metal complexes absorption route

Considering that the mineral has been protected through the GIT, it can be released from the ligand to be absorbed, or it can follow the usual route of the ligand absorption. Chaud et al. (2002) observed that the binding force must be strong enough to hinder metal complexation with other ligands, but, at the same time, weak enough to allow the metal mobilization by the carriers in the enterocyte's membranes. Ashmead (1991) proposed that amino acid-metal chelates could behave as a di- or tripeptide-like molecule through the GIT, following the same pathway of absorption of peptides. Nevertheless, when the mineral is in a peptide-metal complex, it is still unclear how it is absorbed. It could be released from the ligand in the vicinity of intestinal cells and transported across the intestinal membrane by the usual pathway to divalent metal ions, i.e., DMT-1. This *divalent metal transporter* is a transmembrane protein that transfers divalent metal ions into enterocyte across the apical membrane (Frazer and Anderson 2014). On the other hand, the metal could enter the intestinal cells by the peptides absorption route, hence still complexed with the organic molecules.

The literature presents different hypotheses concerning the peptide-metal complex absorption. The peptide resistance to the action of gastrointestinal enzymes seems to be of utmost importance for keeping the complexes intact during GIT. García-Nebot et al. (2010) observed that CPPs from whole CN added to milk-based fruit beverages could offer a good alternative for improving mineral bioavailability due to their resistance to SGID.

In the same way, the Fe-binding peptides studied by Caetano-Silva et al. (2015) were obtained from whey protein hydrolysis with pancreatin. The peptides, identified by mass spectrometry, were previously reported as extremely resistant to the SGID (Picariello et al. 2010); thus the authors suggest that these can exert a crucial role in Fe absorption since the complexes might remain intact during GIT. In a subsequent study, Caetano-Silva, Cilla, et al. (2018) suggest that peptide-Fe complexes synthesized with these peptides (with MM $<$ 5 kDa), enter the Caco-2 cells while remaining coordinated through a usual intestinal absorption route for peptides. This is because larger peptides (MM $>$ 5 kDa) with similar sequences and the same Fe-binding sites showed very low Fe uptake, suggesting that Fe ions were not released in the vicinity of intestinal cells and that these larger peptide-Fe complexes were also not absorbed. Thus, the authors hypothesize that, similarly, low MM peptides do not release Fe and are absorbed while still coordinated with Fe.

Chen et al. (2017) attributed the increase of Fe, Zn, and Ca uptake by Caco-2 cells to the presence of the SGID resistant decapeptide, GPAGPHGPPG, found in Alaska pollock skin. The four Pro residues make the peptide less susceptible to proteolytic enzymes (Vanhoof et al. 1995); therefore the protection enabled by this peptide sequence to minerals is not modified as a consequence of digestion.

It can be highlighted that, apart from the sequence composition, the chain length of the peptide may be another factor that influences the stability of peptides during gastrointestinal digestion (Chen et al. 2017), and, as a consequence, may also impact the stability of the peptide-metal complexes, formed either during digestion or before ingestion. The absorption of low MM peptides through the intestinal epithelium may be: (1) a carrier-mediated absorption by peptide transporter (PepT1) for di- and tripeptides, (2) transcytosis (intracellular vesicle-mediated transport system) for oligopeptides, and (3) paracellular passive diffusion for oligo and di-/tripeptides (Wada and Lönnerdal 2014). Potentially part of the Fe bounded with peptides may be taken up by one of these routes in Caco-2 cells, which is an alternate pathway to the usual for Fe salts (Caetano-Silva, Cilla, et al. 2018; Chen et al. 2017). In some cases, larger peptides can be absorbed intact through the intestinal barrier, as the 17-residue peptide from bovine β-CN, reported by Regazzo et al. (2010). Once complexed with a metal ion, this ion may be absorbed by the same route. García-Nebot, Barberá, and Alegría (2013) also proposed that Zn could be transported through Caco-2 cell membranes still bound to CPPs and attributed the positive influence of β-CN(1–25)4P on Zn uptake to a protective effect against insolubilization during SGID or interactions with other components in the gut.

On the other hand, the metal, such as Ca, can be released from peptide-Ca complexes when it reaches the small intestine prior to uptake (Guo et al. 2014). Lee, Noguchi, and Naito (1980) reported the increasing concentration of soluble Ca in the lumen of the distal ileum of rats, released from CPP-Ca complexes, enhancing the passive Ca absorption. The primary route of Ca transport across the small intestine and Caco-2 cell monolayers is the paracellular way, i.e., passive transport. In the same way, Eckert et al. (2016) suggested that Fe from the complexes with the synthetic heptapeptide (SVNVPLY) enters the common non-heme Fe pool and activates the same transporter DMT-1 on the cells (Eckert et al. 2016).

Another technique which has been studied as an alternative to also increase mineral bioavailability is encapsulation. This technique provides limits to the interaction of the metal with the food matrix and protects it against oxidation, rendering it more accessible for intestinal uptake (Bryszewska 2019). Bryszewska (2019) observed high Fe bioaccessibility of encapsulated ferrous sulfate with thermoresistant modified starch, measured as Fe content in the soluble fraction after SGID. However, the authors highlighted that the ferric ions do not pass through the intestinal membrane, and, from the speciation analysis, only 10-30% of Fe was in ferrous form after SGID when encapsulated. The potential mechanism of iron uptake in this case is by DMT-1, which depends on the metal ion state. Gupta et al. (2015), studying iron microcapsules, prepared with a blend of gum arabic, maltodextrin and modified starch, observed an increase of nearly 17% of in vitro iron bioavailability when comparing non-encapsulated and encapsulated iron.

To our knowledge, there is no study comparing the encapsulation technique with peptide-metal complexation. However, an important aspect should be highlighted: for encapsulation, the wall material is usually a gum, starch, or another polymer, which should provide a controlled release

of the mineral through the GIT. Thus, the mineral should be released before entering the intestinal cells, unlike some of the peptide-metal complexes, as reported in the literature.

Bioavailability assays with peptide-metal complexes

Table 2 shows a number of studies from the last decade on peptide-metal complexes obtained during or prior to gastrointestinal digestion and their effects on mineral bioavailability. Although the literature shows many studies regarding Zn and Ca complexes, Fe is the most common mineral. In most cases, the studies focusing on the metal-binding peptides suggest that the binding capacity or even the capacity to keep the metal soluble is an indication of higher bioavailability. In this sense, Wang, Li, et al. (2011) affirmed that the complex CN hydrolysate-Fe might be more bioavailable than ferrous sulfate due to its higher Fe solubility compared to the salt, under intestinal conditions. Wang, Zhou, et al. (2011) observed that Zn solubility and dializability were higher for the complex synthesized with yak-casein hydrolysate than with the whole protein. The solubility of Zn salt sharply decreased at intestinal pH, and the previous formation of a peptide-Zn complex improved the mineral solubility.

Udechukwu, Downey, and Udenigwe (2018) analyzed Zn dialyzability of whey peptides-Zn complexes submitted to SGID. The Zn complexes were stable to gastric conditions, releasing only 6.7–10.5% of Zn after peptic digestion, and showed a dializability from 38.8% to 46.5%. The authors presumed that the high affinity between Zn and peptides could limit Zn dialyzability, and further studies are necessary to understand the physiological fate of the released Zn and those bound to the peptides at the intestinal digestion phase.

In fact, in certain circumstances, the statement "higher bioaccessibility, higher bioavailability" might not be true, and studies on in vitro absorption using cell culture models after SGID aiming to test this hypothesis have gained attention in the last decades. Although the in vitro assays show several limitations to completely mimic the complexity of the digestive tract, they are often very useful in predicting outcomes of the digestion in vivo (Bohn et al. 2018).

For all cell assays, it is important to emphasize that cytotoxicity of the peptide-metal complexes should be evaluated, using an assay such as MTT (3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide) assay, which aims to evaluate if the compounds do not damage the integrity of the cells (Caetano-Silva, Cilla, et al. 2018). Different authors addressed the cytotoxicity of peptide-metal complexes and reported no significant influence in the cell's viability (Caetano-Silva, Cilla, et al. 2018; Eckert et al. 2016; Wang, Ai, et al. 2014).

A synthesized peptide (DKLPGFGDS(PO_4)IEAQ), previously identified in digested chicken egg white, was able to stimulate the Fe-induced ferritin synthesis in intestinal Caco-2 cells 2-fold higher than the control (Palika et al. 2015). The synthetic peptide, which is resistant to further digestion by gastric and intestinal enzymes, kept the ability to bind Fe during the digestion process and thus facilitated the absorption of the metal. Lv et al. (2014) demonstrated that peptides obtained from digested soybean proteins (SGID) significantly enhanced the in vitro bioavailability of intrinsic and added Fe, measured as ferritin content in Caco-2 cells. The authors, when analyzing the Fe bioavailability of added Fe (6 mg/L), observed that peptides with MM $<$ 1 kDa led to higher Fe uptake than the ones with higher MM, suggesting that the small peptides released during digestion can bind Fe free ions and facilitate their entry into intestinal cells.

Although, in the recent past, there was a lack of studies concerning complexes synthesized prior to digestion, more recent literature shows promising results using synthesized complexes. Caetano-Silva, Cilla, et al. (2018), studying Fe complexes synthesized with whey peptides with different MM and with the whole protein, observed that the Fe bioaccessibility (metal solubility after SGID) of all complexes was high, regardless of the ligand, with no statistical difference, whereas in vitro bioavailability, measured by ferritin synthesis in Caco-2 cells, sharply differed among them. The authors also evaluated peptide-Fe complexes obtained from different Fe sources, compared with compounds that are frequently used for Fe fortification, such as ferrous sulfate and ferrous bisglycinate, and observed higher in vitro Fe bioavailability for Fe from $FeCl_2$ complexed with low MM peptides compared to the Fe salt or even the commercial Fe complex ferrous bisglycinate.

Wang, Ai, et al. (2014) studied α-lactalbumin and β-lactoglobulin hydrolysates complexed with Fe and evaluated Fe in vitro absorption by measuring soluble Fe after SGID, Fe retention in cells, and Fe transported by Caco-2 cells, apart from the content of ferritin. The authors reported higher Fe absorption of synthesized complexes than a mixture of hydrolysate and Fe, corroborating the positive impact of obtaining the complexes before the digestion process.

Peptide-metal complexes can also be formed with protein which has previous chemical modification, in a way that after gastrointestinal digestion the chemical modification still influences the maintenance of the mineral complexes. This is the case of succinylation of caseins, as reported by Shilpashree et al. (2018). Introducing additional carboxylic groups to the protein chain (by succinylation process) enhances the cation mineral binding efficiency of proteins. Thus, mineral complexes with succinylated casein can lead to higher mineral uptake (Fe and Zn) by intestinal cells, when compared to mineral salts (Shilpashree et al. 2018).

Although Caco-2 cells human colon carcinoma cell line is the most used model for mineral bioavailability studies (Alegría-Torán, Barberá-Sáez, and Cilla-Tatay 2015), some studies utilized the HT-29 line, another type of human colon carcinoma cells, which also differentiates in mature intestinal cells, but as mucus-producing cells (Martínez-Maqueda, Miralles, and Recio 2015). Sun et al. (2017) evaluated the Ca in vitro absorption from sea cucumber (*Stichopus japonicus*) ovum hydrolysates-Ca complexes. The authors observed that the intracellular [Ca^{2+}] increased in both Caco-2 and HT-29

cells (between 25% and 37%) in comparison to the CN phosphopeptide-Ca complex. Moreover, the Ca complexes maintained the solubility of the mineral under SGID conditions, regardless of the presence of dietary components such as oxalate.

Non-intestinal cell models can also be used to evaluate Fe bioavailability. Human hepatoma cells (HuH7) have been used to assess the ferritin content and soluble transferrin receptor using complexes of Fe-CPPs from CN, without improving the Fe bioavailability (García-Nebot et al. 2015).

Mineral uptake can be estimated by the measure of mineral content in the apical and basolateral chambers of a polyester membrane chamber inserts, the Transwell system, or even the measure of mineral content in the cell suspension, i.e., the mineral which was transported across the apical membrane and remained inside the cell (Chen et al. 2017; Cui et al. 2018; Eckert et al. 2016). Chen et al. (2017) evaluated the effect of a chelating peptide derived from Alaska pollock skin on Ca, Zn, and Fe transport, uptake, and retention in Caco-2 cells in a Transwell system. As in most studies, the mineral content was determined by atomic absorption spectrophotometry. The authors observed that the synthetic peptide GPAGPHGPPG remained approximately 75% intact after SGID, and attributed the promotional effects on Ca (113%), Zn (32%), and Fe (21%) transport in Caco-2 cell monolayer to the complexation with the peptide via His residue or by formation of a cyclic structure in terms of dehydration. The authors stated that minerals might be transported through the transcellular pathway in the form of chelates.

Zhu, Wang, and Guo (2015) isolated and identified a novel Zn-binding peptide (HNAPNPGLPYAA, MW 1221 Da) from wheat germ hydrolysate and observed that the peptide-Zn complexes showed higher Zn absorption in Caco-2 cells than $ZnSO_4$. The His residue at the N-terminal of the peptide might play an important role.

In recent years, as the metal-binding peptides and, especially, the synthesis of peptide-metal complexes have gained more and more attention, it is now possible to find more in vivo studies on this topic, especially using rat models. Xiao et al. (2016), studying a synthetic tri-peptide complexed with Fe, Fe-REE, observed that this complex was effective to restore body weight, organ coefficients, and hematological parameters to normal level in IDA rats. Zhou et al. (2014) observed a positive effect of β-Lg hydrolysate-Fe complexes upon hematological parameters and Fe bioavailability, in a dose-dependent manner, in IDA female weaning Sprague–Dawley rats.

More recently, Zhang et al. (2018) synthesized complexes of Pacific cod bone hydrolysate-Ca and reported their positive effect in improving the intestinal Ca absorption and serum Ca, and anti-osteoporosis effects, in an ovariectomy-induced osteoporosis rat model. Li et al. (2019) observed that the gene expression of DMT-1 could be regulated by the duck egg white peptides-Fe chelate on Fe uptake in IDA rats. The authors reported that the supplementation with Fe chelates led to an increase in hemoglobin, hematocrit, red blood cells, mean corpuscular volume, serum iron, and serum ferritin to the normal levels. However, in vivo studies are still needed, especially regarding the effect of matrix on the mineral absorption.

Peptide-metal complexes to avoid pro-oxidant or other side-effects

Other than the bioavailability, another property of utmost importance can be affected by the peptide-metal complexation: the pro-oxidant effect of metals such as Fe can be dramatically reduced once complexed and stabilized. In food fortification, one of the great challenges to choose the fortificant compound is "bioavailability versus stability", since, in most cases, the more bioavailable the compound, the more reactive it will be. The soluble compounds of Fe, which are more bioavailable, generally promote the development of undesirable color and flavor, apart from causing rancidity in lipid products. Insoluble compounds, on the other hand, do not cause sensory changes but might be so poorly absorbed to the point of having no nutritional effect (Hurrell 2002).

The bivalent nature of Fe, i.e., the ability to change from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state, is one of the main characteristics responsible for the biological relevance of this mineral. However, this same property is intrinsically related to the formation of Fe-catalyzed reactive oxygen species (ROS), which may result in peroxidation of membrane lipids and damage to the DNA structure (MacKenzie, Iwasaki, and Tsuji 2008), which is related to cardiovascular and neurological diseases (Torres-Fuentes, Alaiz, and Vioque 2012), as well as side effects in the gastrointestinal system, such as heartburn, abdominal pain, nausea, and diarrhea (Mimura et al. 2008). The free Fe^{2+} also appears to be also responsible for damage to the gastrointestinal mucosa (Slivka, Kang, and Cohen 1986) and may intensify inflammatory disorders (Lih-Brody et al. 1996).

Also, ROS have a negative impact on flavor, texture, nutritional value, as well as the shelf life of food products. In fortified products, lipid oxidation is a crucial factor in quality and shelf life and the decomposition of lipid hydroperoxides can be associated with free Fe. Therefore, for food fortification, it is crucial that the added Fe does not cause undesirable sensory changes in the products, as well as side effects in the organism, while still being well absorbed. Thus, the efficacy of Fe fortification is still a challenge.

Some complexation processes may prevent the redox potential of Fe, and thus avoid it from participating in catalytic reactions which produce free radicals and/or ROS, for instance, via Fenton's reaction (Eckert et al. 2016). Thus, for food fortification, research has focused on the use of Fe in its complexed form in order to minimize its impact on lipid oxidation, both in food and in the organism, possibly preventing the side effects of free Fe.

Studies have shown the potential of milk proteins to reduce the Fe reactivity in supplemented milk and influence the oxidation process by complexing Fe in the system (Hegenauer, Saltman, and Ludwig 1979; Hekmat and McMahon 1998). More recently, the same potential of protein-iron complexes in synthetic emulsions has been

reported (Sugiarto et al. 2010; Ueno, Urazono, and Kobayashi 2014). Proteins dispersed in the continuous phase of oil-in-water emulsions can inhibit lipid oxidation by the mechanism of free radical scavenging or complexation with pro-oxidant metals within the system (Elias, McClements, and Decker 2005; Faraji, McClements, and Decker 2004; Guzun-Cojocaru et al. 2011). However, this antioxidant activity can be enhanced by the hydrolysis of these proteins (Díaz et al. 2003; Elias et al. 2006).

In addition to the capacity of peptides to neutralize lipid radicals, the previously formed peptide-Fe complexes can exert an indirect antioxidant capacity. The lipid oxidation of emulsions containing these complexes was significantly lower than those containing peptides with Fe salts, which indicates that the previous formation of a ring structure with the metal has a crucial role in protecting Fe from interacting with the lipid phase (Caetano-Silva, Mariutti, et al. 2018).

Unlike Fe, free Zn in the divalent cationic state (Zn^{2+}), does not trigger oxidation-reduction reactions under physiological conditions and is therefore relatively nontoxic (Cummings and Kovacic 2009). Carrasco-Castilla et al. (2012) observed that soy peptides with high Cu and Fe chelating activity, produced during SGID, showed antioxidant activity in Caco-2 cell model. Hence, the metal-binding capacity of peptides is a powerful antioxidant mechanism since the peptides prevent the metals from catalyzing oxidation reactions in the cell model.

Conclusion and perspectives

Considering that the minerals used in food fortification are commonly applied in their inorganic form, fortified products can therefore result in low bioavailability. Thus, in the last years, researchers have studied alternatives for more bioavailable Fe, Zn, and Ca compounds for food fortification. An exception to the rule of "the more bioavailable, the more reactive" might be the peptide-metal complexes, in which the mineral is protected via physical or chemical form. Thus, mineral complexation by peptides is an alternative to increase mineral bioavailability, apart from potentially minimizing the pro-oxidant effect, and these complexes have been identified as potential functional ingredients.

In the face of many results concerning the structure, composition, and sequence of mineral-binding peptides, and also the mineral bioaccessibility and bioavailability in in vitro or in vivo models, there is a huge need for the absorption to be evaluated in food matrices, in order to consider the effect of the matrix. The importance of these studies can be justified by the fact that metal ions can interact with dietary components and potentially the complexation with peptides may hinder this process.

Although some amino acid-mineral complexes are already used for food fortification, these still lack bioavailability studies from synthesized peptide-metal complexes in food matrices. The synthesis of purified and identified peptides is a possible tool to obtain the ligand for peptide-metal complexes which can potentially be used as food ingredients. Complexes obtained from a pool of peptides of protein hydrolysates can also be used as food ingredients, though the behavior of this mixture of peptides must be studied regarding their binding force, structure, mineral release, and absorption, especially in food matrices. Thus, we consider that there is still considerable time and research needed until these complexes can be effectively be applied to food products. The potential application of these complexes as food ingredients might be directed to niche products, i.e., targeted to anemic people, but not for replacing metal salts in the large-scale market of food fortification products consumed by the population.

In relation to the source of protein to obtain the peptide-metal complexes, the literature shows a number of possibilities, as presented in this review, and the alternatives have grown significantly in recent years. There are several studies concerning the quality of a non-conventional protein source, which can be considered a future trend that can contribute to the global food security: insect protein has presented potential application in food ingredients, as well as the peptides derived from these proteins (de Castro et al. 2018).

Latunde-Dada, Yang, and Vera Aviles (2016) evaluated the in vitro Fe bioavailability from edible insects using Caco-2 cells culture model and affirmed that commonly consumed insect species could be excellent sources of bioavailable Fe and also serve as an alternative strategy for increased mineral intake in the human diet. On the other hand, from the enzymatic hydrolysis of this protein, it is possible to obtain peptides with different bioactivities, such as anti-inflammatory and antioxidant bioactivities (Zielińska, Baraniak, and Karaś 2018). The insect protein after SGID can also yield peptides with Fe-binding capacity, as evaluated by Zielińska, Karaś, and Jakubczyk (2017). Considering that there are different metals with high affinity for different residues, although insect protein has not yet been applied to obtain peptide-metal complexes to study their bioavailability, it has great potential and we highlight that this protein source is a promising field of study.

Despite the "gold standard" label to the human or in vivo animal studies, most of the studies comprising mineral bioavailability apply the SGID process, due to its short time, simplicity and reduced bioethical restrictions (Shani-Levi et al. 2017). However, the number of different protocols has led to considerable variability in results which make it harder to compare results and increase the knowledge on this topic and also the application of these complexes. The use of the harmonized static in vitro digestion, standardized by Minekus et al. (2014) and subsequently improved by Brodkorb et al. (2019) in more studies could gradually change this scenario and, in the long term, could allow the SGID models to be extended to recreate various strata of the human population. The design of products with high efficacy, in this case high mineral bioavailability, depends on profoundly understanding the in vivo digestion conditions in different population groups and applying them to standardized and validated protocols (Shani-Levi et al. 2017).

Other potential benefits of Fe-complexes can be the positive effect on reducing the potentially pathogenic gut

bacteria growth since Fe chelation changes the gut microbiota profile and influences human gut microbial homeostasis (Parmanand et al. 2019). If peptide-Fe complexes allow the absorption of the metal at the brush border of enterocytes, as has been proposed, thus the mineral will not reach the colon to be used by the pathogenic gut bacteria.

In recent years, results in the literature regarding the peptide-metal complexes have increased, although their efficacy as food ingredients still needs to be proved. For instance, the safety evaluation of these peptide-metal complexes is really required so that they can be considered food ingredients. The regulatory regimens for protein hydrolysates may vary across different national and regional jurisdictions (Chakrabarti and Guha 2018). Also, the toxicity of the minerals should also be evaluated when they make up these complexes since metals such as Fe, Cu, and Zn can be toxic to the human body depending on the quantity, state, and source (Egorova and Ananikov 2017). The aim of addressing the safety of these compounds with all their components is always to protect the well-being of consumers and to prove the efficacy of such products in this translation of discoveries from laboratory to industry (Chakrabarti and Guha 2018). To the best of our knowledge, there is no study addressing the safety of peptide-metal complexes. Nevertheless, considering the promising results discussed here, we understand the value and relevance of further studies on these complexes.

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ORCID

Maria Elisa Caetano-Silva http://orcid.org/0000-0001-8650-6616

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