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Iron-caseinglycomacropeptide complexes: characterization and application in beverages

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Highlights

- Iron binding capacity of CMP was evaluated.
- CMP can bind ferrous iron in a 1:1.5 molar ratio.
- Acid residues of Glu, Asp and sialic acid would be involved in Fe-CMP binding.
- Fe/CMP complex remained stable in a wide pH range.
- No changes were observed in the colour of beverages fortified with Fe/CMP complex.

Abstract

Complexing iron with organic compounds has been considered an alternative strategy to mitigate the problems associated with the level of bioavailable iron and the acceptability of products supplemented with this mineral. CMP contains specific amino acids associated with iron binding. The present study aims to optimize the conditions of Fe/CMP complex formation and understand the molecular basis of interactions between CMP and iron ions. Results showed that CMP can bind ferrous iron in a 1:1.5 molar ratio, forming a stable peptide-iron complex, where CMP assembles in a tetrameric form. FTIR spectra indicated that iron binding altered the secondary structures of CMP. The iron-binding sites of CMP corresponded primarily to acid residues of Glu, Asp and sialic acid. Moreover, Fe/CMP complex remained stable in a wide pH range (2.0-6.5), suggesting the adequacy to be efficiently added in food or beverages and to keeping complexed in the digestion environment. Finally, Fe/CMP complex was added to a commercial beverage (2 mg of Fe per serving of beverage) and no changes were observed in their colour during storage. A model to explain the binding between CMP and iron is proposed. These results suggest a potential application of this peptide for iron fortification.

Keywords: caseinglycomacropeptide; iron fortification; beverage; interaction; particle size; complexes stability.

1. Introduction

Iron is an essential trace element in animal and human nutrition. It is a component of haem in haemoglobin and myoglobin, cytochromes and several enzymes. Moreover, it plays an important role in the transport, storage and utilization of oxygen (Chitturi, Baddam, Prasad, Prashanth, & Kattapagari, 2015; Conway & Henderson, 2019). Iron deficiency and iron deficiency anemia are classified as the nutritional disorders prevalent in the world by the World Health Organization (WHO), affecting more than 3.5 billion people in the world (ACC/SCN, 2000; Zimmermann & Hurrell, 2007).

In order to resolve this problem, the most used strategies are the provision of supplements and food fortification. However, the sources of iron used for fortification (ferrous sulfate, ferrous lactate, ferrous gluconate, and ferric ammonium citrate) often present the challenge of providing a significant level of bioavailable iron without affecting the taste and appearance of the final product (Hurrell, 2009).

Ferrous sulfate has been widely used to fortify diets; however, this salt may cause some side effects, such as heartburn, abdominal pain, nausea, and diarrhea (Mimura, Breganó, Dichi, Gregório, & Dichi, 2008; T. W. Wu & Tsai, 2016). Experimental evidence also suggests that ferrous sulfate may promote the formation of hydroxyl radicals, which can start the peroxidation of lipids from biologic membranes, enzyme inactivation, and DNA damage (Schümann, Ettle, Szegner, Elsenhans, & Solomons, 2007). Iron complexation with organic compounds has been considered an alternative strategy for mitigating these problems. The complexed mineral is more stable and less prone to interactions with the chemical environment (Miquel & Farré, 2007; Wu, Yang, Sun, Bao, & Lin, 2020).

Caseinphosphopeptides (CPP) are bioactive peptides derived from the digestion of milk proteins and have been reported to act as vehicles of different minerals, playing a very important role in their stability and bioavailability (Caetano-Silva et al., 2017; Clare & Swaisgood, 2000; Delshadian et al.,

2018; Miao et al., 2019; Miquel, Alegría, Barberá, & Farré, 2006; Miquel & Farré, 2007; Walters, Esfandi, & Tsopmo, 2018). However, besides the proved ability of CPP to protect and ensure iron bioaccesibility, their production at a commercial scale is limited by costs. These peptides contain polar amino acid sequences of three phosphoseryl groups followed by two glutamic acid residues, being binding sites for minerals (Vegarud, Langsrud, & Svenning, 2000). Some authors reported an improvement in the bioavailability of iron when it is bound to CPPs because CPPs would keep this mineral soluble in the digestive tract and would prevent the formation of oxidized forms that have a much lower absorption capacity (Ait-Oukhatar et al., 2002).

Regarding the mechanism of iron–peptide complexation, Reddy & Mahoney, (1995) suggested that the net charge, the side chain length and the functional groups of the amino acids and peptides seem to be directly related to the extent of complex formation with iron. Studies on iron–peptide complexes show that the major iron binding sites correspond primarily to the carboxyl groups (Chaud et al., 2002; Lee & Song, 2009), although the ε-amino nitrogen of lysine, the guanidine nitrogen of arginine, and the imidazole nitrogen of histidine may also have been involved in iron–peptide binding (Reddy & Mahoney, 1995, Li, Jiang, & Huang, 2017). Additionally, glycine and proline could also be involved in iron complexation (Storcksdieck, Bonsmann, & Hurrell, 2007).

Caseinglycomacropeptide (CMP) is a CPP of higher molecular weight, formed during cheese making and accounting for by about 20 - 25% of cheese whey proteins (Thöma-Worringer, Sörensen, & López-Fandiño, 2006; Wang & Lucey, 2003). It is also purified at a commercial scale for specific applications. CMP comprises the 64 amino acids in the hydrophilic C-terminal portion of κ-casein, and contains all the posttranslational modifications (glycosylation and phosphorylation) present in κ-casein that contribute to its marked heterogeneity (Mikkelsen et al., 2005). CMP contains many potential sites for iron binding: 8 glutamic acids, 2 aspartic acids, lysine and sialic acid, that is the main carbohydrate in the glycation sites that makes it very attractive as a potential peptide to complex iron. Thus, the aim of this work was to determine the capacity of CMP to complex ferrous

iron and to optimize the conditions of Fe/CMP complex formation. Additionally, the stability of Fe/CMP complex to critical pH and temperature conditions, and its effect on some physical characteristics of beverages will be also evaluated.

2. Materials and methods

2.1. Sample preparation

BioPURE caseinglycomacropeptide (CMP) was provided by Agropur (Le Sueur, MN, USA). The purity of CMP was 86.3 % and the extent of glycosylation is about 50%. The ferrous sulfate ($FeSO_4 \cdot 7H_2O$) was purchased from BIOPACK (Buenos Aires, Argentina). Ferrozine (3-(2- pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4 triazine, disodium salt) was acquired from Sigma-Aldrich Co. (Steinheim, Germany). Other chemicals employed were of analytical grade.

CMP solutions were prepared at concentrations between 1 and 7 % (w/w) in deionized water. The pH of the solutions after preparation was 6.5. Iron (Fe)/CMP solutions were prepared by the addition of $FeSO_4.7H_20$ salt to CMP solutions in order to produce final Fe/CMP ratios ranging from 1.1 to 8 mol/mol and maintained under agitation with an orbital shaker at 20 ºC overnight.

2.2. Iron binding capacity

Iron binding capacity of CMP was carried out according to the method described by Chang, Wu, & Chiang, (2007) with slight modifications. Ten microliters of Fe/CMP solutions previously prepared at different ratios were mixed with 890 µl of distilled water and 100 µl of $5x10^{-3}$ $mol.L^{-1}$ Ferrozine (Fz). After incubation at room temperature for 10 min, the absorbance of these mixtures was measured at 562 nm in a T70 UV/VIS Spectrophotometer (PG instrument Ltd.). A complex of Fe^{2+}/Fz has a strong absorbance at 562 nm, so a high ferrous ion binding capacity in the sample results in a low absorbance. Distilled water, instead of sample, was used as a control. Free iron (Fe_F) was calculated by:

$$Fe_F\ (\%) = (A/A_0) * 100$$

where, A is the absorbance of the sample and A_0 is the absorbance of the control.

2.3. Particle size and zeta potential determinations

Dynamic light scattering (DLS) was used to determine the particle size and zeta potential (ζ) of CMP and Fe/CMP solutions. Measurements were made with equipment Zetasizer Nano-Zs (Malvern Instruments, Worcestershire, United Kingdom), at a scattering angle of 173° for particle size determinations and of 17º for zeta potential measurements, and at 25 °C. One milliliter of solution of CMP (5 % w/w) and Fe/CMP complex at ratios: 0.4, 0.8, 1.2, 1.5, 2.0, 2.5, 2.8 and 3.2 was placed in a disposable polystyrene cuvette. In DLS, the sample is illuminated with a laser beam and the intensity of the resulting scattered light produced by the particles fluctuates at a rate that is dependent upon the size of the particles. Two approaches were applied to obtain size information: cumulative method, to find the mean average (z-average) which is the size of a particle that corresponded to the mean of the intensity distribution and CONTIN method, to analyze the data for percentile distribution of particle/aggregate sizes. The assay was performed in duplicate on two individual samples.

The solutions were placed into special folded capillary cells (DTS1060C, Malvern Instruments, Worcestershire, United Kingdom) for zeta potential determinations. Average values of two replicates per sample are reported.

2.4. Fourier transform infrared spectroscopy (FTIR) measurement

One milligram of freeze-dried CMP or Fe/CMP was mixed with 100 mg of dry KBr. The mixture was ground into a fine powder and compressed into a thin disc. All FTIR spectra were recorded by a FT-IR spectrometer (Shimadzu®, IRPrestige-21, Japan) over a wave number region between 4000

cm^{-1} and 400 cm^{-1} at a resolution of 2 cm^{-1}. The peak signals in the spectra were analyzed using OMNIC 8.2 software (Thermo Nicolet Co., Madison, WI, USA).

2.5. Stability of bound Fe in CMP/Fe complex

The effect of acidification using HCl 1 M or 0.1 M (up to pH 2, 3 and 4) and of heat treatment (45 ºC and 65 ºC for 30 min) on bound iron from Fe/CMP complexes were evaluated. After acidification or heat treatment, Fe_F was determined by the Ferrozine method described above. After heat treatment, the samples were incubated for 1 h at 25 ºC, before Fe_F determination.

2.6. *Incorporation of Fe/CMP complex to beverages*

Considering a Recommended Dietary Allowance (RDA) mean between different age groups as 10 mg/day, the commercial beverage (non-carbonated orange flavored beverage) was fortified with the Fe/CMP complex (molar ratio 1.5) in order to obtain 20 % of this RDA per serving. The pH values of beverages (3.5) were not modified by the fortification. Fortified beverages were stored in sextuplicates, in a chamber at 20 ºC. After fortification, colour of beverages was evaluated during 45 days (every 15 days, evaluated by duplicates) by using a spectrocolorimeter Minolta 508-d (Minolta Co. Ltd., Tokyo, Japan). A standard calibration with white and black references was performed and samples were placed in a circular cell of 2 cm diameter. Chromatic coordinates L*, a* and b* (of the chromatic space CIE L*a*b*) were obtained with the Illuminant D65 (corresponding to the sun light distribution spectrum) and an observer angle (determined by the visual field and the horizon line) of 2°. Colour measurements were performed in duplicated in two individual samples.

3. Results and discussion

3.1. Iron binding capacity

The ferrozine method (Chang et al., 2007; Hidalgo et al., 2015; Oloughlin, Kelly, Murray, Fitzgerald, & Brodkorb, 2015; Smialowska, Matia-Merino, & Carr, 2017) has been widely used for estimating the binding ability of peptides by determining the unbound or free iron that can be detected just when the maximum load of iron is reached. **Figure 1** shows the free iron occurrence when increasing the molar Fe/CMP ratio from 1.1 to 8.0 at pH 6.5. No free iron was assessed up to Fe/CMP ratio of 1.5 suggesting that all the iron was bound to CMP; after that free iron started to increase. This result indicates that 1 mol of CMP is able to complex 1.5 mol of iron. Sun, et al., (2017) determined by isothermal calorimetry that a small peptide from egg white protein hydrolysates could accommodate two iron atoms. Many other works also reports on the iron binding capacity of different peptides obtained from food protein hydrolysates, but these values are always expressed on weight basis or as a % of initial iron bound to the peptide, making difficult to know the binding capacity in terms of mol of ferrous iron that can accommodate one mol of peptide.

CMP exhibits a high degree of heterogeneity according to κ-casein genetic variant (mostly A and B) and post-translational modifications. Phosphorylation and glycosylation are the main modifications in CMP mainly occurring in the inner part of the peptide chain, in between aminoacids 16 and 44, being the sites of post-translational modifications threonine and serine **(Figure 2**). Fourteen glycosylated forms of CMP have been identified by Molle & Léonil (1995). In a recent work (Sunds, Poulsen, & Larsen, 2019) as much as 19 CMP isoforms were identified in a commercial caseinomacropeptide sample, of which 13 were glycosylated and 6 were non glycosylated. Molle & Léonil, (1995) found five potential glycosylation sites, a maximum of three are substituted with highly glycosylated carbohydrate chains which may contain up to six *N*-acetyl neuraminic acid (NeuNAc) residues per molecule. gCMP is rich in sialic acids which are substituted neuraminic acid derivatives, being the main representative forms of sialic acid NeuNAc and N-glycolylneuraminic acid (Salcedo et al., 2011). NeuNAc is the terminal residue of the carbohydrate chains.

The non-glycosylated CMP (aCMP) has a molecular mass between 6787 and 6755 Da depending on the genetic variant and the highest mass of up to 9631 corresponds to highly glycosylated CMP (gCMP) (Mollé & Leonil, 2005). The average molecular mass of total CMP is about 7500 Da. The last value was taken to calculate the number of CMP moles in **Figure 1**. This average molecular mass fits well with the fact that gCMP accounts for by 50 % of total CMP (Mollé & Leonil, 2005).

At pH 2, CMP exhibited a very low iron binding capacity, being free iron about 90 % of total iron (data not shown). These results indicate that pH plays a key role in iron binding. The pI of aCMP fraction is located at pH 4.15, but the pI of pure gCMP is in contrast 3.15, as the negative charge of the sialic acid residues (pK of sialic acid: 2.2) reduces the net charge of the peptide backbone (Kreuß et al., 2009). This negative charge would favor the binding with the ferrous ions. Contrarily at pH 2, below the pI, a positive charge of CMP would not favor the ferrous binding. Moreover, Farías, Martinez, & Pilosof, (2010) have shown that CMP self-assembles at room temperature at pH values less than 4.5. They proposed a first stage of hydrophobic self-assembly to form dimers which further interact through electrostatic bonds to form gels over time. Iron binding to these assembled CMP forms occurring at pH 2 would be hindered.

A similar effect of pH on iron binding to other milk proteins or peptides has been reported. Baumy & Brule, (1988) found that iron bound to β-lactoglobulin decreased from pH 6.6 to 5.0. Zhou et al. (2012) reported that the iron chelation capacity of β-lg hydrolysates was favored at neutral pH. Also, other authors reported a reduction of iron-releasing percentage with the increase of pH for whey protein concentrate (Shilpashree, Arora, Sharma, Bajaj, & Tomar, 2016) and egg white hydrolysates (Sun, Cui, Li, et al., 2017).

3.2. Particle size

As mentioned above, CMP is prone to self-assemble under specific conditions of pH. In order to evaluate the impact of iron binding capacity on the particle size of CMP, DLS measurements of

particle size of CMP upon iron binding were performed and compared with those of single CMP. Molar Fe/CMP ratios close to the saturation ratio were selected (Fe/CMP ratios between 0.4 and 3.2 mol/mol). All the solutions showed multimodal intensity size distributions (not shown), but from volume size distributions it was possible to deduce the relative proportion of the populations, showing that populations with higher size can be negligible and only the lower size peak was the predominant in all the solutions. The lower size peak for all the solutions is plotted in **Figure 3**. From the particle diameter corresponding to the maximum values of the lower size peaks in the intensity size distributions it could be estimated the molecular weight (MW) of the protein by a tool in the Zetasizer Nano-Zs software. From the MW, the predominant association state of CMP at each Fe/CMP ratio can be calculated **(Figure 4)** as a relation between the MW corresponding to the diameter of the maximum value of the lower size peak and the MW of CMP monomer (average MW 7500 Da). CMP showed a predominant population with a maximum value of hydrodynamic diameter at 2.3 nm, that corresponds to the monomeric form of CMP (Farías et al., 2010). As shown by Kreuß et al., (2009), above pH 6.5 when all acidic AA side chains are deprotonated, the zeta potential is approximately -20 mV for aCMP and - 30 mV for gCMP so that self-association would not occur due to the shielding by the strong negative charge.

By the addition of Fe at increasing Fe/CMP molar ratios the predominant lower size peak moved towards higher values, to 3.1 nm (dimeric form), to 6.8 nm (hexameric form) or higher forms as shown in **Figure 4**, indicating that CMP self-assembled. Concomitantly, the zeta potential of particles, that reflects their surface charge, decreased from –27.30 ± 1.73 to –6.24 ± 0.31 mV, revealing the negative charge neutralization by bound iron. In fact, iron binding sites correspond primarily to the carboxyl groups of aspartic and glutamic acids (Chaud et al., 2002; Lee & Song, 2009). Moreover, iron could also neutralize the negative charges occurring in sialic acid. Sialic acid residues could play a key role in the binding of iron as demonstrated by desialylation of human lactoferrin, an iron-chelating protein, with neuraminidase to remove sialic acid residues. This

reduced its ability to bind iron and caused release of iron already bound to the protein (Z. Li & Furmanski, 1995). As a result of a decrease in the negative charge of the CMP molecules and consequently of a decrease in the masking of the hydrophobic sites, an approach and interaction of monomeric Fe/CMP complexes could occur, forming dimeric structures. In fact, CMP monomers can interact by hydrophobic bonds to form dimers when the negative charge is decreased by lowering pH (Farías et al., 2010).

During this stage, pH slightly decreased from 6.5 to 6 (**Figure 4**), probably due to H^+ release from carboxyl residues that may be the primary sites of Fe binding. A further increase of Fe/CMP ratio to 1.5 – 2, promoted the formation of tetramers, involving more iron and probably further hydrophobic interactions. Moreover, during this stage pH kept constant (**Figure 4**), probably indicating that iron is getting bound on sites that do not liberate H^+, as nitrogen atoms. The molar ratio 1.5 is the observed saturation ratio in **Figure 1**. Excess of ferrous sulphate (ratio ≥ 2) promoted a further pH decrease because the occurrence of free iron. At this stage, free iron promoted a continuous CMP assembly forming growing particles as hexamers or more associated forms (**Figure 4**). However, the increase in CMP polymerization could not be explained solely by pH. In **Figure 5** the particle size and self-assembled forms of CMP and its Fe/CMP complex are compared at different pH values to point out that iron binding promoted CMP assembly to a higher extent than what can be expected from pH lowering. The increase in particle size would indicate that not only intramolecular interactions but also intermolecular interactions occur in the binding reaction between iron and CMP; therefore, more associated structures were formed, such as dimers, tetramers, polymers.

3.3. FTIR

Infrared spectroscopy technique was used to verify whether a peptide-mineral interaction occurred. Proteins and polypeptides show nine characteristics infrared absorption bands, amide A, B and I-VII. The two most prominent vibrational bands are the Amide I (1600-1700 cm^{-1}) and Amide II (1500-

1560 cm^{-1}) bands, which are mainly attributed to C=O stretching, and the movement of N-H vibrations and C-N stretching vibrations of the protein/peptide backbone, respectively (Kong, J., Yu, 2007).

Figure 6 shows FTIR spectra of CMP and Fe/CMP complex (Fe/CMP ratio = 1.5). According to Burgardt et al. (2014), the absorption band of CMP at 1655.2 cm^{-1} and 1545.1 cm^{-1} correspond to Amide I and II, respectively. The amide I and II vibration are hardly affected by the nature of the side chain. It depends, however, on the secondary structure of the backbone and is therefore the amide vibration that is most commonly used for secondary structure analysis. The sensitivity of the amide I vibration to secondary structure makes it possible to study protein folding, unfolding and aggregation with infrared spectroscopy (Barth, 2007).

Upon coordination with Fe^{2+} the Amide I band at 1655.2 cm^{-1} in CMP spectrum splits in two bands, at 1676.5 and 1631.5cm^{-1}, suggesting a change in the structure backbone of CMP due to bound ferrous iron. According to Barth (2007) the band at 1655.2 cm^{-1} is assigned to both α–helix or to a disordered structure. The three-dimensional structure of CMP cannot be determined, because a crystallisation is not possible, thus only structure prediction tools can be used to obtain estimates of the secondary structure of CMP. Different predictive models agree that CMP is largely a disordered peptide or random coil (Kreuß et al., 2009; Smith, Edwars, Palmano, & Creamer, 2002).

In **Figure 7**, the predicted 3D structure of CMP by the program QUARK, for template-free protein structure prediction (Xu, Yang, & Zhang, 2012) also corroborates that CMP is intrinsically disordered with small sections of β-strands. Therefore, band at 1655.2 cm^{-1} is revealing a disordered structure. The new bands at 1676.5 and 1631.5 cm^{-1} after iron binding correspond to turns and β-sheets, respectively (Barth, 2007), indicating an increase in secondary structure due to iron binding. Zhou et al. (2012) also found a structural transformation during binding between iron and β-lactoglobulin hydrolysates producing a significant amount of α-helix in the complex. Sun et al., (2017) also reported that ferrous ions might induce the loss of ramdom coil structure of a peptide

from egg white hydrolysates, studied by fluorescence and circular dichroism. Additionally, the band corresponding to the Amide II group shifted from 1545.1 cm^{-1} for CMP to 1537.7 cm^{-1} for Fe/CMP complex. Modifications in the amide I and/or amide II bands were reported in complexes of sea cucumber ova hydrolyzed with iron (Sun et al., 2017), in complexes of β-lactoglobulin hydrolysates with iron (Zhou et al., 2012), in complexes of WPI hydrolysate with iron (Oloughlin et al., 2015), that also showed an extensive reductions in peak intensities in the amide I and amide II and in complexes of casein hydrolysate with iron (Chaud et al., 2002) which could be attributed to the C-O-Fe binding.

The ionized form of the carboxyl groups provides a band at 1400-1420 cm^{-1} (Nakai, Li-Chan, Hirotsuka, 1994). The band corresponding to the carboxyl group (1400 cm^{-1}) for Fe/CMP complex has a low intensity compared to that of CMP. This could be related to the fact that the presence of negatively charged COOH groups in the side chains of Asp and Glu of CMP at pH 6.5, could be shielded due to their interaction with Fe^{2+}.

Additionally, sialic acid presents absorption bands at 3340, 2933 and 1655 cm^{-1} (Singh et al., 2020), therefore, the peaks at 3301.4 and 2933.4 cm^{-1} in CMP spectra (**Figure 6**) can be attributed to the glycosylation of CMP, mainly with sialic acid, and the low intensity of these bands in Fe/CMP complex could suggest the linkage between iron and sialic acid. The band at 1655 cm^{-1} overlaps with previous described band, so the shift of this band in Fe/CMP complex can be attributed both to change in Amide I and also to the interaction of iron with sialic acid.

On the other hand, the presence of sulfate groups in the Fe/CMP complex could interfere in some bands found in the area of 1045-1150 cm^{-1} because of sulfate overlapping the band at the same region (sulfate vibrational mode, as a very intense band at 1110 cm^{-1}) (O'loughlin et al., 2015). It was reported that the sulfate group could be an integrant part of the Fe complexes, either in the coordinated form - $[Fe(pept)SO_4]$ - or as a counterion - $[Fe(pept)]SO_4$ (Caetano-Silva et al., 2017).

Finally, the band at 984 cm^{-1} corresponds to the phosphate groups due to phosphorylated sites of CMP (Burgardt et al., 2014b; Carmona & Rodriguez, 1986). The Fe/CMP complex presented a modification in the area of the spectrum corresponding to the phosphate groups, moving to a higher wave number (997.8 cm^{-1} in the presence of Fe, instead of 983.9 cm^{-1} in the absence of Fe), and showing a more pronounced peak, which could be related to the possible interaction of iron with phosphate groups of CMP.

3.4. Model for Fe/CMP complex

Taking together the results discussed above regarding saturation ratio of CMP by the ferrous ion (molar ratio 1.5), the pH and particle size evolution close to that saturation ratio, the FTIR evidence, the structural characteristics of CMP and the pH or Fe dependent self-assembly of CMP, a schematic model for the formation of ferrous iron/CMP complex is proposed in **Figure 8.** In a first stage one mol of iron binds to primary –COOH sites in monomeric aCMP or gCMP (in this case also may bind to sialic acid), greatly decreasing the strong negative charge of CMP at neutral pH and thus promoting in a second stage the formation of CMP dimers by hydrophobic bonds (Farías et al., 2010). During these stages a pH decrease is occurring. Finally, in the third stage, those dimers get attached by the inclusion of two other moles of ferrous iron forming a tetrameric CMP structure of 4.8 nm in size. The fact that pH keeps constant in this stage suggests that this additional iron is binding to sites that do not involve H^+ exchange.

3.5. Stability of Fe/CMP complex

The stability of Fe/CMP complex for a potential incorporation in foods as well as its ability to protect iron during gastrointestinal digestion is very important to assess in order to evaluate its potential industrial application. Iron is considered a very challenging micronutrient to add to foods and beverages because it interacts with food constituents to produce undesirable changes (Ahmad &

Ahmed, 2019). Ferrous iron is known to be more stable in acidic aqueous solutions, where it exists in hydrated form, while at alkaline pH hydroxide is formed, which is considerably less soluble than hydrate (Smialowska et al., 2017; Sugiarto, Ye, & Singh, 2009). This hydroxide is usually poorly absorbed because it becomes insoluble in digestive lumen at alkaline pH (Pérès et al., 1999). The use of iron-fortified phosphopeptides has been reported to improve their solubility and digestive absorption compared to inorganic salts (Ahmad & Ahmed, 2019; Aït-Oukhatar et al., 1999; Pérès et al., 1999). In order to evaluate the stability of Fe/CMP complex, the amount of iron released from Fe/CMP complex under different pH values (2-6.5) and heat treatment (45 ºC and 65 ºC) conditions was determined.

Figure 9 shows the iron released from complexes with different Fe/CMP ratios up to 2, formed at pH 6.5, and then kept at pH 2, 3 and 4 by HCl addition. At pH 3 and 4 the complex was stable and at pH 2 a small amount of free iron was apparent (10 %). The iron release at pH 2 can be attributed to the release of iron bound to sialic acid that is hydrolyzed at this pH (Furuhata, 2004; Kreuß et al., 2009). No release of iron when decreasing pH indicates that iron is bound by coordinate bonds to and not by ionic bonds.

When submitting the complex (at the optimal Fe/CMP ratio 1.5) to heat treatment, at 45 and 65 °C, free iron was less than 20 %. These results indicate that the stability of complex is very high suggesting the adequacy to be efficiently added in food or beverages with a wide pH range that might be submitted to a thermal process such as pasteurization.

Additionally, the stability of Fe/CMP complex could prevent the interaction of iron with other components in foods and even in the digestion environment. In fact, the stability of the Fe/CMP complex at low pH, as observed in the human gastric environment, deserve an special comment since Fe/CMP could trough all the digestion steps keep the iron complexed. Therefore, Fe/CMP complex could prevent iron ions from forming precipitate and so the complex could be effectively reach duodenum.

Regarding the stability over time of Fe/CMP complex, the volume distribution for Fe/CMP complex at ratio up to the saturation value (ratio 1.5) is reported in **Figure 10**. The size of the complex did not change, indicating that tetramer, once formed, remains stable.

3.6 Stability of Fe/CMP complex *in beverages*

The colour of a food or beverage is the first sensory impression that a consumer experiences and which will determine its acceptability or not. **Figure 11** shows L* (luminosity), a* (red/green colour component) and b* (yellow/blue colour component) parameters for fortified orange beverage with Fe/CMP complex and $FeSO_4$, as a control, compared with the unfortified beverage upon storage time.

Beverages did not show great differences neither between them nor during storage, concerning to L* parameter (**Figure 11A**) which was about 40. Despite of some significant differences, no clear trend related to the impact of fortification was observed, and the differences were minimal.

All beverages showed negative values of a* parameter (**Figure 11B**). Beverage fortified with $FeSO_4$ showed the more negative values which could be attributed to the green colour provided by this salt. In fact, it has been reported the increase of green colour in an orange flavour sweet whey beverage (Hashimoto Rivera, 2010), and it was attributed to the oxidation of a certain percentage of ferrous ions to ferric ions. Beverage fortified with Fe/CMP complex showed more similar a* values than unfortified beverage, although with slightly more negative values. Storage time did not show a significant effect on this parameter either.

Finally, regarding b* parameter (**Figure 11C**), all beverages showed positive values, indicating a more intensity of the yellow colour. Again, despite of some significant differences, beverage fortified with Fe/CMP complex showed similar b* values than the unfortified beverage, while beverage fortified with $FeSO_4$ showed the lowest b* values. Similar to L* and a* parameters, no relevant changes were detected for b* values during storage time.

The addition of whey protein to foods or beverages could modify the yellow and/or green colours (Wherry, Barbano, & Drake, 2019); however, the presence of CMP in the complex did not show any significant change in a* or b* parameters. The results obtained for chromatic parameters indicate the potential of Fe/CMP complex to be used in fortified beverages, without substantial changes in the colour of them. However, these are preliminary results which should be complemented with sensory assays and others shelf life studies.

4. Conclusions

CMP can bind ferrous iron in a 1:1.5 molar ratio, forming a stable peptide-iron complex where CMP assembles in a tetrameric form of 4.8 nm. The main sites involved in iron binding would be the acid residues of Glu, Asp and sialic acid. Moreover, nitrogen atoms could also participate in forming the tetrameric complex that keeps stable and soluble under pH conditions relevant to the gastric digestion (pH 2-4) and intestinal digestion (neutral pH), thus ensuring the iron protection and hence its bioaccessibility. In addition, the Fe/CMP complex proved to be stable when added to some commercial beverages without modifying their colour over storage.

This suggests a potential application of Fe/CMP complex as potential iron supplement. Future studies on the performance under digestion will be done to assess its bioaccesibility under gastro-intestinal digestion.

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