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Stability and bioaccessibility of iron in pumpkin discs vacuum impregnated with ferrous gluconate, β-cyclodextrin and ascorbic acid

María Sabrina Lencina [a], Cristina dos Santos Ferreira [b], Diego Archaina [a,e], María Beatriz Gómez [a], María Florencia Mazzobre [c,d,e,*]

[a] Facultad de Bromatología, Universidad Nacional de Entre Ríos, Entre Ríos, Argentina
[b] Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Departamento de Química Orgánica, Ciudad Universitaria, Intendente Güiraldes, 2160, C1428EGA, Buenos Aires, Argentina
[c] Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Departamento de Industrias, Intendente Güiraldes 2160, Ciudad Universitaria, C1428EGA, Buenos Aires, Argentina
[d] CONICET - Universidad de Buenos Aires, Instituto de Tecnología de Alimentos y Procesos Químicos (ITAPROQ), Buenos Aires, Argentina
[e] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

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ABSTRACT

Present work evaluates the content and bioaccessibility of iron in vacuum-cooked pumpkin discs (VCPD) impregnated with ferrous gluconate (FeGlu) solutions added with β-cyclodextrin (BCD) and/or ascorbic acid (AA). The stability of Fe^{2+}, AA and color of the VCPD after refrigerated storage were also studied. Pumpkin discs treated with any of the FeGlu studied solutions reached a total iron content between 17 and 20 mg Fe^{2+}/100 g. Addition of BCD or AA to FeGlu solution improved Fe^{2+} of VCPD compared to control fortified only with FeGlu. After *in vitro* digestion, soluble Fe^{2+} was also significantly higher in pumpkins impregnated with BCD and AA solutions. Both BCD and AA probably exert their positive effect favoring the solubility of iron and/or the stability of the ferrous iron form, being the bioaccessibility of Fe^{2+} approximately 17 and 20% w/w respectively, and 10% for the FeGlu-impregnated discs. Although BCD did not have the impact observed for AA, a synergistic effect was observed in BCD-FeGlu-AA systems, where the proportion of Fe^{2+} was approximately 50% higher than in FeGlu after 21 days of refrigerated storage. The use of BCD and AA also allowed to better maintain the color of the fortified pumpkin discs after cooking and refrigerated storage.

1. Introduction

Food fortification is one of the main strategies to prevent iron deficiency, along with supplementation and diversification of the diet. Fortification is considered a feasible, cost-effective, and long-term method to prevent micronutrient deficiency (Shubham, 2020). Iron is an essential micronutrient in everybody's diet since it participates in practically all redox *in vivo* processes. This mineral plays a central role in the transport of oxygen and carbon dioxide, being a cofactor of a considerable number of critical proteins that regulate aspects of cell and body physiology. Its high redox potential, together with its facility to promote the formation of reactive compounds, determines that iron metabolism is controlled by a powerful regulatory system (Crichton, 2019). Its ability to gain or lose electrons affects its solubility thus conditioning its intestinal absorption. In the diet, iron is mainly present as non-heme iron derived from plants, animals and iron-enriched foods, while hem iron is only provided by foods of animal origin. While heme-iron is easily absorbed, non-heme iron is less well absorbed. Once released from food, most of the non-heme iron is present in the ferric form (Fe^{3+}) or cation iron (III), which is unavailable for absorption in the human gastrointestinal tract. Only the ferrous (Fe^{2+}) ions could be absorbed by the duodenal enterocytes. Therefore, ferric iron must first be reduced prior to absorption by divalent metal cation transporters in the duodenum (Blanco-Rojo & Vasquero; Shubham, 2020). Thus, it is important to promote and keep the reduced (soluble) form of iron in food to favor its absorption.

Unfortunately, iron deficiency in the diet is the cause of 50% of the cases of anemia in the world population. In addition to its low content,

* Corresponding author. Instituto de Tecnología de Alimentos y Procesos Químicos, Departamento de industrias, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Av. Intendente Güiraldes, 2620, C1428BGA, Ciudad Universitaria, Buenos Aires, Argentina.
E-mail address: florm@di.fcen.uba.ar (M.F. Mazzobre).

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the low bioavailability of iron present in many foods makes it not enough to meet the physiological needs of the body (WHO/FAO – Wordl Health Organization/Food and Agriculture Organization of the United Nations, 2004). Other problem with adding iron to food is its low stability against the conditions of handling, cooking and storage, therefore one of the main technological challenges of food fortification is to optimize the amount and stability of the added iron so that it can be absorbed at the intestinal level (Hurrell, 2002). Oxidation from ferrous to ferric state leads also to sensory changes that affect the acceptability of the product and the stability of other nutrients (Blanco-Rojo & Vasquero, 2018; Habeych, van Kogelenberg, Sagalowicz & Galaffu, 2016). Many investigations have reported that the addition of ascorbic acid (AA) has a beneficial effect on the absorption of iron, due to its ability to reduce and chelate iron compounds at low pH, increasing solubility and absorption at alkaline pH at the level of the duodenum (Akasapu, Ojah, Gupta, Choudhury, & Mishra, 2020; Hurrell, 2002; Shubham et al., 2020). However, under processing and storage conditions, the added AA decreases its content and can also cause sensory changes in the food vehicle (Hurrell & Venkatesh Mannar, 2018).

Fortification of processed foods with encapsulated nutrients emerges as an interesting strategy to improve iron stability, handling and protection against oxidation, preventing also sensory changes and potential interactions with other food ingredients (Gharibzahedi & Jafari, 2017, Shubham, 2020). In this sense, cyclodextrins (CDs) have been proposed as promising nanoencapsulation systems to stabilize or control the release of compounds of interest in processed food. CDs are cyclic oligosaccharides, derived from enzymatic degradation of starch, capable of forming inclusion complexes with hydrophobic or hydrophilic molecules and also with different ions (Dhiman & Bhatia, 2020) such as iron (Leite, Lino, & Takahata, 2003; Kapor et al., 2012). It is known that cyclodextrins are stable at high temperatures (Gouin, 2004) and that their ability to establish inclusion complexes have shown to improve the stability of labile compounds during thermal processing (dos Santos, Buera, & Mazzobre, 2011). Leite et al. (2003) studied inclusion complexes between β-cyclodextrin and ferrous lactate, showing that the cyclodextrin prevented the oxidation of ferrous ions in aqueous solutions. However, to our knowledge, there are no studies using CDs as nanoencapsulation systems to fortify foods with iron. In particular, ferrous gluconate has been recommended for oral iron administration, it has a high bioavailability in humans similar to ferrous sulfate and greater than ferrous lactate (WHO/FAO - World Health Organization/Food and Agriculture Organization of the United Nations, 2017). It was reported that ferrous sulfate is suitable for fortifying dry foods such as flour and powdered milk, but in liquid preparations it can precipitate forming insoluble complexes (Blanco-Rojo & Vaquero, 2019; WHO/FAO – Wordl Health Organization/Food and Agriculture Organization of the United Nations, 2004), whereas ferrous gluconate has been used successfully in fortified juices and liquid milk (Blanco-Rojo & Vaquero, 2019; Hurrell & Venkatesh Mannar, 2018). Compared to inorganic iron compounds that are usually poorly absorbed because they attach extensively to proteins, salts, and fat, organic compounds of iron (such as gluconate and lactate) absorb more easily to the water phase of food (Hendricks, Guo, & Kindstedt, 2001).

Vacuum impregnation (VI) is a useful technique to quickly introduce external liquids or desired compounds in the porous structure of animal or plant tissues through a hydrodynamic mechanism prompted by pressure changes (Fito et al., 2001; Soares et al., 2018). It is a useful and promising technique to improve food quality, introducing external liquids or desired compounds in a porus sample in a controlled and rapid way and it is recommended as an energy-saving pre-treatment before drying, freezing, or frying of different fruits and vegetables (Duarte-Correa, Granda-Rastrepo, Corte & Vega-Castro, 2019; Erihemu, Hironaka, Koaze, & Oda, 2014). The gas and liquid inside pores of the tissues is removed under vacuum conditions and exists a mass transfer process, replacing them with the VI solutions of cryoprotectants, antioxidants, salts, enzymes, vitamins and probiotics under restored atmospheric pressure. The composition of the matrix or food and the sensorial properties change as well as prolonged shelf life (Zhao et al., 2019, 2021).

On the other hand, the increase in consumer demands for minimally processed packed refrigerated foods with characteristics closer to that of the fresh products has led to a growth in the use of vacuum processing technologies to extend the shelf life and to keep the quality of fresh food. Cooking under vacuum or *"Cook vide process"* applies at reduced pressure and mild temperatures (65–95 °C), which generates less loss of thermolabile compounds than conventional treatments, improving the quality of the final product (Iborra-Bernad, García-Segovia, & Martínez-Monzo, 2015).

Pumpkin is a vegetable that provides few calories but high content of potassium and water, in addition to being an important source of β-carotenes that can enhance iron absorption. It should also be noted that pumpkin does not contain lignin or phenolic compounds, which are inhibiting factors for iron absorption. These nutritional properties of pumpkins are useful for the development of fortified foods, enriched with minerals such as iron (de Escalada Pla, Campos, Gerschenson, & Rojas, 2009; de Escalada Pla, Flores, & Genevois, 2020). However, no vacuum impregnation studies of pumpkins with cyclodextrin and iron solutions have been recorded. These vegetables improved in their composition can be a novel and interesting source of iron.

The objective of this work was to evaluate the effect of vacuum impregnation with ferrous gluconate solutions added with β-cyclodextrins (BCD) and/or ascorbic acid (AA), on the content and bioaccessibility of iron vacuum-cooked pumpkin discs (VCPD). The stability of iron, AA and color of the discs after cooking and refrigerated storage was also studied. Present work evaluates the content and bioaccessibility of iron in vacuum-cooked pumpkin discs (VCPD) impregnated with ferrous gluconate (FeGlu) solutions added with β-cyclodextrin (BCD) and/or ascorbic acid (AA).

2. Material and methods

2.1. Materials

The pumpkin (*Cucurbita Moschata*) was obtained from a local market. Ferrous Gluconate (FeGlu), Bio Ferroso A.A.s, was donated by Lipotech S.A. Buenos Aires, Argentina; (L+) ascorbic acid (AA) was provided by Jebsen & Jessen (China) and β-Cyclodextrin (BCD) was obtained from Roquette-Food (France). All reagents used were of analytical quality.

2.2. Preparation of the impregnation solutions

2.2.1. Ferrous gluconate solutions with and without ascorbic acid

Ferrous gluconate was used in present work as source of iron due to its high solubility in water and hence high bioavailability of the iron. The iron salt of gluconic acid have been widely used as a supplement for certain anemias, and also as an additive in the food industry to stabilize color (Codina, Ropciuc, Voinea, & Dabija, 2019; Kumar et al., 2020).

Ferrous gluconate solution (FeGlu) was prepared by dissolving FeGlu (6.200 g) in distilled water (1.00 L), to obtain a solution with 500 mg Fe/L. This concentration was selected based on previous studies, that used a FeGlu impregnation solution containing 500 mg of iron/L to obtain a vegetable matrix portion (100 g eggplant discs) with an iron content that covers almost 20% of the recommended iron daily intake for an adult (Fito et al., 2001). The same FeGlu solution was prepared in presence of ascorbic acid (AA) in a FeGlu/AA molar ratio 1:1.

2.2.2. β-cyclodextrin/ferrous gluconate solution (BCD/FeGlu)

BCD/FeGlu solution, with a 1:1 M ratio, was prepared following the co-precipitation method proposed by Kapor et al. (2012). First, a saturated solution of BCD was prepared (18.50 g BCD/L of distilled water) stirring at 50 °C until a clear and transparent solution was obtained. After cooling at 25 °C, 6.200 g of FeGlu were added and stirring was

continued at room temperature for 12 h (100 rpm) (MRC TOu-50N/120N). Stirring in these systems is critical to promote the interaction of ligands and CDs (dos Santos et al., 2011). Although clear solutions were obtained, the systems were filtered (0.45 μm, Sartorius) to avoid the potential presence of precipitated compounds.

2.2.3. β-cyclodextrin, ferrous gluconate, and ascorbic acid solution (BCD/FeGlu/AA)

A mass of 6.20 g of FeGlu and 2.50 g of AA were added to a saturated solution of BCD to obtain a Fe/BCD/AA solution with a molar ratio 1:1:1. Components were dissolved by stirring (100 rpm) at 25 °C for 12 h (MRC TOu-50N/120N). Although clear solutions were obtained, the systems were filtered (0.45 μm, Sartorius) to avoid the potential presence of precipitated compounds.

2.3. Preparation of fortified pumpkin discs

2.3.1. Vacuum impregnation

Pumpkins were carefully washed and peeled manually. Then, cylinders of 35 mm of diameter and 10 mm thickness (approximately 10 g), were cut from the mesocarp using a stainless steel cork borer. The pumpkin cylinders were immersed during 25 min in the corresponding impregnation solution (section 2.2) using a Gastrovac® equipment (International Cooking Concepts, Spain) set at a temperature of 25 °C and at a vacuum pressure of 800 mbar to promote the internal gas outlet of the product. The pumpkin/solution ratio used was 1:10. After the vacuum phase, atmospheric pressure was restored for 25 min to promote the entry of external liquid into the food structure.

The impregnated cylinders were drained on a wire rack during 1 min and dried with absorbent paper to remove the excess of liquid. Both the impregnated and non-impregnated samples used as controls were packed in heat resistant polyamide-polyethylene bags and sealed under vacuum (Vacuum Packing ICC, Barcelona, Spain).

2.3.2. Vacuum cooking of packaged samples

The packaged pumpkins were then subjected to a vacuum cooking process (*cook vide)* in the Gastrovac system (Gastrovac®, International Cooking Concepts, Spain), under the following operational conditions: 30 min at 80 °C and 600 mbar. Once the cooking process was completed, the pumpkins were stored at 3 °C during 21 days.

Cooked non-impregnated pumpkin discs were used as a control.

2.4. Physicochemical analysis

2.4.1. pH and moisture content

The pH was determined with a pH meter (ORION, model S.A 720, USA) in the pure homogenate of VCPD under constant stirring, according to the Association of Official Analytical Chemists (AOAC) 932.12.

The moisture content of the VCPD was determined with an infrared drying oven (Radwag Mag. 50/WH, Poland), heating the homogenized samples at a temperature of 105 °C during 30 min (AOAC 7.003–84). The value of moisture content was expressed in g H_2O/100 g cooked pumpkin (% w/w).

Both determinations were performed in triplicate, and the mean value was reported.

2.4.2. Iron content

2.4.2.1. Total iron content in pumpkin discs. The total iron content in pumpkin discs includes the iron naturally present in the tissue and the impregnated iron both in ferric and ferrous form. This total iron content was determined as Fe^{2+} by UV visible spectrophotometry (AOAC 944.02; 2005) in non-impregnated (control) and impregnated VCPD. after calcining the samples and reducing the Fe^{3+} to Fe^{2+}. Briefly, 5.00 g of VCPD were crushed and then calcined at 550 °C for 24 h. The obtained ashes were dissolved in 1 mL of 2 M HCl at 60 °C, and diluted to 50 mL in a volumetric flask with distilled water. Aliquots of 5 mL were taken and 1 mL of 6 M HCl solution was added. Then 1 mL of hydroxylamine 10% w/w was incorporated to reduce Fe^{3+} to Fe^{2+}, thus, the subsequent determination of Fe^{2+} corresponds to the total iron present. Finally, 5 mL of buffer solution at pH 4 (glacial acetic acid - sodium acetate) and 1 mL of o-phenanthroline were incorporated and diluted to 25 mL with distilled water. The absorbance at 510 nm was measured in aliquots of the soluble ash filtrates using an UV–visible spectrophotometer (Jenway Mod. 6505 UV/VIS™). The calibration curve was performed using standard solutions of ferrous ammonium sulfate (0, 0.05, 0.10, 0.20, 0.30, 0.40, 0.80, 1.00 and 2.00 mg of Fe^{2+}/L). All determinations were performed in triplicate.

2.4.2.2. Soluble iron content in treated pumpkin discs. The soluble iron was the content of iron both in ferric and ferrous form present in solution after acid treatment or *in vitro* digestion of the discs:

- Soluble iron determined in the impregnated VCPD after being treated under acid conditions: Briefly, each VCPD sample (4.00 g) was ground with 20.0 mL of an extraction solution (HCl 0.04N, pH 1.50). The suspension was homogenized under constant stirring at 25 °C during 30 min using a magnetic stirrer (Barnstead Thermoline), and then centrifuged for 1 min (Rolco Mod. 2036 ™ centrifuge). Aliquots (0.90 mL) of the supernatant were taken for the determination of soluble iron.
- Soluble iron after *in vitro* digestion of the impregnated VCPD was measured in the filtrates (0.90 mL) as describe in section 2.5.

The iron present in solution after both treatments (acid or *in vitro* digestion) was determined spectrophotometrically with addition of hydroxylamine (to reduce Fe^{3+} to Fe^{2+}) as it was described previously, and was expressed in mg Fe^{2+}/100 g VCPD.

2.4.2.3. Ferrous iron content. The extraction in HCl solution from the pumpkin ashes and the ferrous ion (Fe^{2+}) was carried out as detailed in 2.4.2.a and as described by other authors (Aruoma, Chaudhary Grootveld & Halliwell (1989)). An aliquot of 0.90 mL of the supernatant was added to a solution containing 5.00 mL of glacial acetic acid-sodium acetate buffer pH 4.00 (Merk) and 1.00 mL of o-phenanthroline (Panreac). It was diluted to 25.00 mL with distilled water and then the absorbance at 510 nm was measured in triplicate using the UV–visible spectrophotometer. Unlike the determination of total iron (2.4.2.a.), in this case hydroxylamine is not used so the determination does not include the Fe^{3+} present. The glacial acetic provides the adequate pH to maintain the iron in a ferrous state, but it does not have enough reducing power to reduce the Fe^{3+}.

To evaluated the degradation of ferrous ion during storage the relative ferrous content (RFe^{2+}) was determined in control and impregnated pumpkin (Eq. (1))

$$\left(RFe^{2+}\right)\% = \left(Fe^{2+}_{time} \,/\, Fe^{2+}_{initial}\right) \times 100 \quad (1)$$

where:

$\left[Fe^{2+}\right]_{time}$ is the ferrous iron content (Fe^{2+}) at different refrigeration storage times (14 and 21 days at 3 °C), express as mg Fe^{2+}/100 g of VCPD.

$\left[Fe^{2+}\right]_{init}$ is the initial ferrous content (time zero, t_0), express as mg Fe^{2+}/100 g of VCPD.

2.4.3. Ascorbic acid content (AA)

The AA content was determined spectrophotometrically using the 2,6-dichlorophenol indophenol technique (Rojas & Gerschenson, 1997). Briefly, samples of 4.00 g of VCPD were crushed with 25 mL of 1% (w/v) oxalic acid solution. Each homogenate was then centrifuged 3 min in a

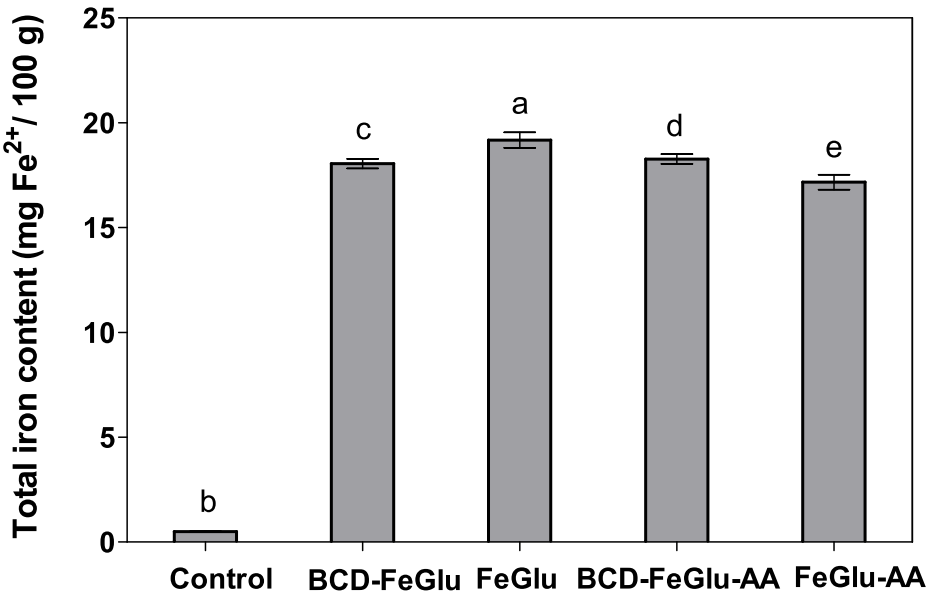

Fig. 1. Total iron content (mg Fe^{2+}/100 g) determined in VCPD impregnated with FeGlu and BCD and/or AA. Control: VCPD without impregnation. Different letters on the error bar indicate a significant difference between FeGlu and the other samples measurements ($p < 0.05$). Results are expressed as mean ± standard deviation (N = 3). Impregnation conditions: 25 min at 25 °C vacuum pressure of 800 mbar, pumpkin/solution ratio 1:10. After the vacuum phase, atmospheric pressure was restored for 25 min.

Rolco Mod. 2036 ™ centrifuge. The absorbance at 520 nm was measured in aliquots (0.90 mL) of the supernatant using a spectrometer. The results were expressed as mg of AA present in 100 g of VCPD.

2.4.4. Surface color

Color parameters were measured using a Minolta MiniScan EZ colorimeter (Minolta, Japan) with a D65 illuminant and a 10° standard observer in the CIELAB color space. The parameters L*, a* and b* were obtained and Chroma (Eq. (2)) and color difference ΔE (Eq. (3)) were calculated (Gliemmo, Latorre, Gerschenson, & Campos, 2009; Genevois, Flores, & de Escalada Pla, 2014).

$$\text{Chroma} = \sqrt{a^2 + b^2} \tag{2}$$

$$\Delta E = \sqrt{\left(L^* - L_0^*\right)^2 + \left(a^* - a_0^*\right)^2 + \left(b^* - b_0^*\right)^2} \tag{3}$$

where:

L*, a* y b* are the values measured for each sample after treatment.

L_0^*, a_0^* y b_0^* are the values measured for each untreated sample (control).

2.5. In vitro gastrointestinal digestion

The bioaccessibility of iron was determined using the gastrointestinal simulation model described by Genevois, de Escalada & Flores (2016). The gastrointestinal simulation procedure included three stages: oral, gastric and intestinal digestion. Briefly, 3.00 g of impregnated VCPD were mixed with 2.50 mL of artificial saliva solution for 2 min in a vortex. The gastric phase was started adding 15 mL of gastric solution (0.3% p/v porcine pepsin in 0.04N HCL), followed by incubation at 37.0 °C under constant shaking for 2 h (120 rpm in an MRC TOU-50N/120N incubator). Subsequently, 15.00 mL of the intestinal solution (0.6% w/v bile salts and 0.3% w/v pancreatin in phosphate buffer) were added, followed by incubation at 37.0 °C, under shaking at 120 rpm for another 2 h. After the three *in vitro* digestion steps, the samples were filtered and both soluble iron content and ferrous iron content were determined in triplicate in the filtrated as described in sections 2.4.2.b y 2.4.2.c.

The bioaccessibility, in percentage, of soluble iron and ferrous iron was calculated by the relationship between the total iron content of the untreated sample using Eqs. (4) and (5), respectively (Kapsokefalou, Kakouris, Makris, Galiotou- Panayotou & Komaitis, 2007).

$$\text{Bioaccesibility of soluble iron (\%)} = \left(Fe_{sol}D \;/\; Fe_S\right) \times 100 \tag{4}$$

$$\text{Bioaccesibility of ferrous iron (\%)} = \left(Fe^{2+}D \;/\; Fe_S\right) \times 100 \tag{5}$$

where:

Fe_sol _D: soluble iron content after *in vitro digestion*.

$Fe^{2+}D$: ferrous iron content after *in vitro digestion*.

Fe_S: total iron content of impregnates VCPD not subjected to *in vitro* digestion.

In both cases the results were expressed as mg of Fe^{2+} in 100 g of the VCPD.

2.6. Stability of iron, ascorbic acid and color in refrigerated pumpkin discs

Stability of iron, AA content and color parameters were studied in refrigerated VCPD treated with the different impregnation solutions. The samples were stored in a chamber at 3 °C for 21 days and the content of ferrous iron, AA, and the surface color were determined as indicated in section 2.4. All determinations were made in triplicate.

2.7. Statistic analysis

The results were statistically analyzed using an analysis of variance (ANOVA) to determine significant differences between means with a limit of significance at $p < 0.05$. A Tukey multiple-comparison test was performed using SPSS statistical software (SPSS 19.0, United Kingdom Ltd.).

3. Results and discussion

3.1. Total iron content of control and vacuum impregnated pumpkins

After pumpkins impregnation with the different studied solutions (FeGlu, FeGlu-AA, FeGlu-BCD, FeGlu-BCD-AA), packaging and vacuum cooking, the moisture content and pH values of the samples ranged between 89 and 91% w/w and 5.55 to 5.70, respectively. These values were not different to those determined for no impregnated cooked control pumpkins (moisture content 90.6% w/w and pH 5.97) indicating that vacuum impregnation did not affect significantly these parameters

Table 1
Bioaccessibility of Fe, expressed as soluble Fe^{2+} (mg Fe^{2+}/100 g) or soluble Fe, including Fe^{2+} and Fe^{3+} (mg Fe^{2+}/100 g) measured after digestion of VCPD impregnated with FeGlu solutions with BCD and/or AA, with respect to the amount of total iron in the sample (%).

Samples	Total Fe content	After *in vitro* digestion		Bioaccessibility	
	(mg/100 g)	Sol Fe (mg/100 g)	Fe^{2+} (mg/100 g)	Sol Fe (%)	Fe^{2+} (%)
FeGlu	19.17 ± 0.37[a]	11.90 ± 0.22[a]	2.07 ± 0.08[a]	62.08 ± 1.17[a]	10.90 ± 0.45[a]
FeGlu-AA	17.17 ± 0.36[c]	13.17 ± 0.98[a]	3.62 ± 0.37[c]	71.81 ± 5.65[a]	20.42 ± 2.09[c]
BCD-FeGlu	18.05 ± 0.23[b]	15.71 ± 0.23[b]	3.10 ± 0.28[b]	82.03 ± 7.43[b]	17.18 ± 1.56[b]
BCD-FeGlu-AA	18.27 ± 0.23[d]	13.10 ± 1.18[a]	3.68 ± 0.55[d]	71.72 ± 6.50[a]	20.19 ± 3.02[d]

Results are expressed as mean ± standard deviation (N = 3). Different letters in the same column indicate a significant difference between the sample impregnated with FeGlu and the rest of the samples ($p < 0.05$).

probably due to the use of VI solution with a pH of 5.50. The pH of a ferrous gluconate solution of 10% (w/v) is nearly 4–5,50.

Fig. 1 shows the total iron content determined for the pumpkin discs vacuum impregnated with the different solutions containing FeGlu that were packed and subjected to vacuum cooking process, compared with the control (cooked without VI). The control samples that were not impregnated, had a total iron content of 0.50 ± 0.01 mg of Fe^{2+}/100 g of VCPD. This content is in agreement with the values reported by Della Gaspera (2017), who obtained a total iron content of 0.58 mg/100 g of pumpkin samples. Regarding the samples treated with the different impregnation solutions, they presented a total iron content between 17 and 20 mg of Fe^{2+}/100 g VCPD. These results, evidence that the impregnation with the studied solutions increased more than thirty times the iron content in the pumpkin discs. de Escalada Pla et al. (2009) reported similar values of iron in pumpkin fortified with ferrous sulfate and ascorbic acid by the dry infusion method. Erihemu et al. (2014) found values of 4 mg of iron/100 g of potatoes impregnated under vacuum with ferric pyrophosphate solution.

Although all the studied systems showed a significant increase in the total iron content, when the samples were treated with FeGlu in presence of BCD or AA the total Fe content was somewhat lower. This is in accordance with the observations of de Escalada Pla et al. (2009) in fortified pumpkin with ferrous sulfate and AA. During the VI it is important the mass transfer of solutes and water, so this fact could be attributed to diffusion interferences where one of the compounds (BCD or AA) may interfere with the entry of the other (iron) into the pumpkin matrix. Even though, the high total iron content obtained for the impregnated pumpkins discs compared to pumpkins without VI treatment (control) indicates that the vacuum impregnation process was effective and allows obtaining a fortified vegetable product. This is in agreement with the results reported by Fito et al. (2001) and Erihemu et al. (2014), who used vacuum impregnation to fortify different vegetables (apples and potatoes).

The World Health Organization (WHO) recommend a daily iron intake of 29 mg/day for women between 19 and 50 years (WHO/FAO – Wordl Health Organization/Food and Agriculture Organization of the United Nations, 2004). In this sense, it is interesting to note that the consumption of a 100 g serving of impregnated VCPD could cover more than 60% of the recommended daily iron intake for this population.

3.2. Effect of BCD and AA on the bioaccessibility of iron

In these studies, the term bioaccessibility was considered as the release of the active compound from the food in the simulated digestive juices of the gastrointestinal tract (Versantvoort, Oomen, Van De Kamp, Rompelberg, & Sips, 2005). Table 1 shows the results of bioaccessibility of Fe, expressed as percentage of soluble Fe^{2+} or soluble Fe measured after digestion of VCPD impregnated with FeGlu BCD and/or AA solutions. Being pumpkin a simple food matrix, does not contain iron absorption inhibition factors, the iron could be released during the *in vitro* digestion. Anyway, the bioaccessibility was greater than 60% w/w for all cases. de Escalada Pla et al. (2009) reported soluble iron percentage values similar to those obtained in the present study for pumpkins treated with ferrous sulfate and ascorbic acid by the dry infusion method. In addition, Kapsokefalou, Kakouris, Makris, Galiotou-Panayotou, and Komaitis (2007) reported that ferrous gluconate in aqueous solution with and without phytates presented a percentage of total iron of approximately 15% w/v and 40% w/v respectively after *in vitro* digestion. Results indicate that ferrous gluconate as impregnation solution and a matrix like pumpkin with a high content of β-carotenes (antioxidants) and without iron absorption inhibiting factors, are adequate to achieve a product with a good bioaccessibility of iron.

Different studies have established that the evaluation of the content of ferrous iron is better for predicting the intestinal iron absorption than the soluble iron determination (Kapsokefalou & Miller, 1991; Argyri et al., 2011). After *in vitro* digestion, the content of soluble Fe^{2+} was significantly higher in impregnated VCPD with solutions containing BCD and AA compared to those treated only with FeGlu (Table 1). Both BCD and AA probably exert their positive effect by favoring the solubility of iron and/or the stability of the ferrous iron form, being the percentage of bioaccessibility of Fe^{2+} approximately 17 and 20% w/w, respectively. The solubility and stability of Fe^{2+}, depends largely on the changes of pH at the gastrointestinal tract (Carpenterz & Ummadi, 1995). It was observed that ferrous gluconate in aqueous solution or in phytate solution present a percentage of Fe^{2+} of approximately 15 and 4% w/v, respectively, after *in vitro* digestion (Kapsokefalou et al., 2007), these values are lower than those reported in the present study. Therefore, the obtained results show that compounds such as BCD and/or AA would act stabilizing ferrous cation during *in vitro* digestion of the VCPD. In the case of AA, it is known that this compound favors the reduction of ferric to ferrous form, enabling active transport by microvilli at the duodenum (Shubham et al., 2020). Argyri, Birba, Miller, Komaitis, and Kapsokefalou (2009) reported that the addition of ascorbic acid favors the formation of Fe^{2+} in different fortified systems, being the content after *in vitro* digestion of 0.36 mg ferrous iron/100 g of corn flakes and 0.60 mg of ferrous iron/100 mL of milk. Thus, ascorbic acid acts as a reducing and chelating agent, it has the ability to reduce non-heme iron and also forms soluble chelates or complexes with Fe^{2+} that allow maintaining solubility (Akasapu et al., 2020). On the other hand, Kurkov and Loftsson (2013) reported that BCD could increase the solubility and bioavailability of different active compounds in food systems. It was observed that for hydrophobic compounds such as terpineol, the solubility in water increases when it is associated with β-cyclodextrin (dos Santos et al., 2011). It was also observed that the formation of anthocyanin inclusion complexes with β-cyclodextrin improves its stability during the gastrointestinal tract (modeled through *in vitro* studies of human colon) compared to the free compound. Complexation with BCD allows anthocyanin to be released in the colon and to exert its potential health benefit (Flores et al., 2015). Cyclodextrins are cyclic oligosaccharides, they have a truncated cone shape with a hydrophilic exterior. The inner walls of CD are formed by the hydrophobic carbon backbones of glucopyranose monomers, making the interior somewhat hydrophobic. This structural feature predetermined the application of CD as a solubilizer for a wide range of poorly water-soluble compounds and also for different ions (Dhiman & Bhatia, 2020) such as iron (Leite et al., 2003; Kapor et al., 2012). Their ability to establish inclusion complexes through supramolecular interactions, giving rise to an equilibrium in solution, have shown to improve the aqueous solubility and/or stability of labile molecules (dos Santos et al., 2011). Also, numerous studies have been carried out to study the thermodynamic parameters

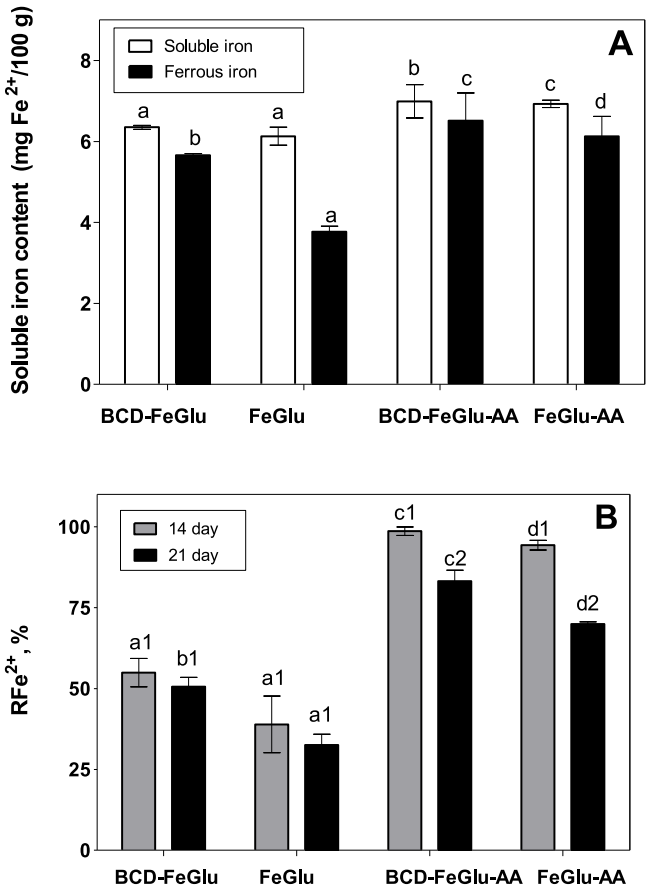

Fig. 2. Soluble iron content (mg Fe^{2+}/100 g) and ferrous content (mg Fe^{2+}/100 g) in VCPD impregnated with FeGlu solutions containing BCD and/or AA (t_0) (**A**). Relative percentage of ferrous iron (RFe^{2+}, %), calculated as Fe^{2+} after different refrigeration storage times (14 and 21 days at 3 °C) with respect to the initial ferrous content (time zero, t_0) (**B**). Determinations were carried out in HCl extraction solution. Results are expressed as mean ± standard deviation (N = 3).
Different letters indicate significant differences ($p < 0.05$) between the VCPD impregnated with FeGlu and with the other impregnated VCPD samples (**A**), 14 or 21 days under refrigeration (**B**). Different numbers on the error bar indicate a significant difference ($p < 0.05$) between the days in each system (**B**).

associated with this equilibrium using the phase equilibrium methodology proposed by Higuchi and Connors (1965).

3.3. Effect of BCD and AA on iron stability in vacuum-packed pumpkins during refrigerated storage

Soluble iron content (mg Fe^{2+}/100 g VCPD.) and ferrous content (mg Fe^{2+}/100 g VCPD) in impregnated pumpkin slices with FeGlu solutions containing BCD and/or AA (t_0) (**A**). Relative percentage of ferrous iron (RFe^{2+}, %), calculated as Fe^{2+} after different refrigeration storage times (14 and 21 days at 3 °C) with respect to the initial ferrous content (time zero, t_0).

Fig. 2A shows the soluble iron content and ferrous content of the vacuum impregnated pumpkins with FeGlu solutions containing BCD and/or AA after cooking (initial time, t_0). Fig. 2B shows the relative percentage of ferrous iron (RFe^{2+}, %), calculated as Fe^{2+} after the corresponding refrigeration storage time (14 or 21 days at 3 °C) with respect to the initial ferrous content (time zero, t_0). After the impregnation treatment, packaging and vacuum cooking, the content of ferrous iron was higher in any of the combined solutions ($p < 0.05$) than in FeGlu alone, particularly in BCD-FeGlu-AA (Fig. 2A). The presence of BCD or AA and in particular their combination seems to prevent to some extent the oxidation of iron in pumpkin discs during cooking. It is known that AA prevents the oxidation of Fe^{2+} to Fe^{3+}, however the stability of Fe^{2+} by redox modulation has limitations in foods with pHs greater than 5 (Mehansho, 2006), so the presence of BCD is of interest. In samples impregnated with both compounds, probably BCD inhibited not only iron oxidation but also the AA degradation.

Regarding the stability of iron during refrigerated storage (Fig. 2B), it was observed that in the pumpkins impregnated with FeGlu, the percentage of relative ferrous iron decreased approximately 60%, while when AA was added (FeGlu-AA), Fe^{2+} remained in a high proportion (higher than 70%) both at 14 and 21 days. The addition of BCD alone (FeGlu-BCD), improved the ferrous content (20%) compared to samples impregnated only with FeGlu. Although BCD did not have the impact observed for AA, a synergistic effect was achieved in the combined BCD-FeGlu-AA systems, where the proportion of ferrous was higher than in FeGlu-AA systems (retention percentage was approximately 80% after 21 days of refrigerated storage).

The AA content in the discs impregnated with BCD-FeGlu-AA and

Table 2
Chromatic coordenates L*, a*, b*, Chroma and color difference (ΔE) and (ΔL*) obtained in pumpkins discs impregnated with FeGlu solutions with BCD and/or AA, after cooking and storage under refrigeration for 21 days.

	Day	Samples				
		Control	FeGlu	BCD-FeGlu	FeGlu-AA	BCD-FeGlu-AA
L*	0	53.13 ± 0.41^{a1}	50.87 ± 1.03^{a1}	51.88 ± 1.45^{a1}	51.39 ± 0.58^{a1}	52.32 ± 0.68^{a1}
	14	55.41 ± 0.94^{1}	52.99 ± 0.42^{1}	54.45 ± 0.50^{2}	53.35 ± 1.02^{1}	53.65 ± 0.55^{1}
	21	55.94 ± 0.57^{2}	54.88 ± 0.99^{2}	56.57 ± 0.59^{3}	55.05 ± 0.94^{2}	53.98 ± 0.43^{1}
a*	0	25.51 ± 0.40^{a1}	24.59 ± 0.68^{a1}	28.51 ± 0.59^{b1}	28.28 ± 0.64^{c1}	24.41 ± 0.22^{a1}
	14	29.61 ± 0.65^{2}	27.01 ± 0.15^{2}	29.23 ± 1.39^{1}	29.45 ± 0.34^{1}	27.15 ± 0.94^{2}
	21	28.41 ± 1.41^{3}	27.71 ± 0.23^{2}	29.34 ± 1.50^{1}	28.70 ± 1.70^{2}	26.72 ± 1.06^{3}
b*	0	55.53 ± 0.43^{b1}	47.03 ± 1.79^{a1}	54.24 ± 0.84 ^{c1}	51.76 ± 0.72 ^{d1}	54.57 ± 1.61^{f1}
	14	53.88 ± 0.39^{1}	45.51 ± 1.23^{1}	52.15 ± 1.18^{1}	49.27 ± 1.58^{1}	52.25 ± 1.23^{1}
	21	54.99 ± 0.85^{2}	46.37 ± 0.61^{1}	46.32 ± 1.31^{2}	47.55 ± 1.55^{2}	51.69 ± 1.49^{1}
Croma	0	63.39 ± 1.24^{b1}	52.46 ± 0.27 ^{a1}	61.27 ± 0.80^{c1}	58.81 ± 2.40^{d1}	60.53 ± 0.19^{e1}
	14	60.63 ± 0.87^{2}	52.34 ± 0.73^{1}	59.30 ± 1.20^{1}	56.73 ± 0.88^{1}	58.94 ± 0.73^{1}
	21	59.19 ± 1.10^{3}	53.98 ± 0.60^{1}	54.71 ± 1.67^{2}	55.67 ± 2.16^{2}	58.50 ± 1.71^{1}
ΔL* (%)	0	–	5.62 ± 1.72^{a1}	3.73 ± 0.87^{b1}	3.32 ± 0.94^{c1}	1.22 ± 0.86^{d1}
	14	0.13 ± 0.53^{1}	−3.62 ± 0.23^{2}	−4.12 ± 0.96^{2}	−3.39 ± 1.42^{2}	−2.11 ± 0.20^{2}
	21	3.00 ± 0.71^{2}	−6.89 ± 1.93^{3}	−8.48 ± 1.13^{3}	−7.51 ± 1.84^{3}	−3.26 ± 0.81^{3}
ΔE	0	–	7.17 ± 0.32 ^{a1}	3.41 ± 0.52 b^{1}	5.78 ± 0.85^{c1}	3.02 ± 0.16^{d1}
	14	4.12 ± 0.93^{1}	3.58 ± 0.47^{2}	3.47 ± 0.47^{1}	3.90 ± 0.97^{2}	3.68 ± 0.87^{1}
	21	4.62 ± 0.92^{1}	4.81 ± 0.95^{1}	9.29 ± 1.22^{3}	5.52 ± 0.25^{1}	5.02 ± 0.25^{2}

Results are expressed as mean ± standard deviation (N = 3).
ΔE and ΔL* in day 0 were calculated as the difference between control VCPD values (without impregnation) and impregnated VCPD values, expressed in %. ΔE and ΔL* in days 14 or 21 were calculated as the difference between the value of impregnated VCPD system (without storage) and the value of the same system after refrigeration (14 or 21 days), expressed in %. Different letters in the same row indicate a significant difference ($p < 0.05$) between VCPD impregnated with FeGlu and the rest of the impregnated and control VCPD (without impregnation). Different numbers in the same column indicate a significant difference ($p < 0.05$) between the days in each system.

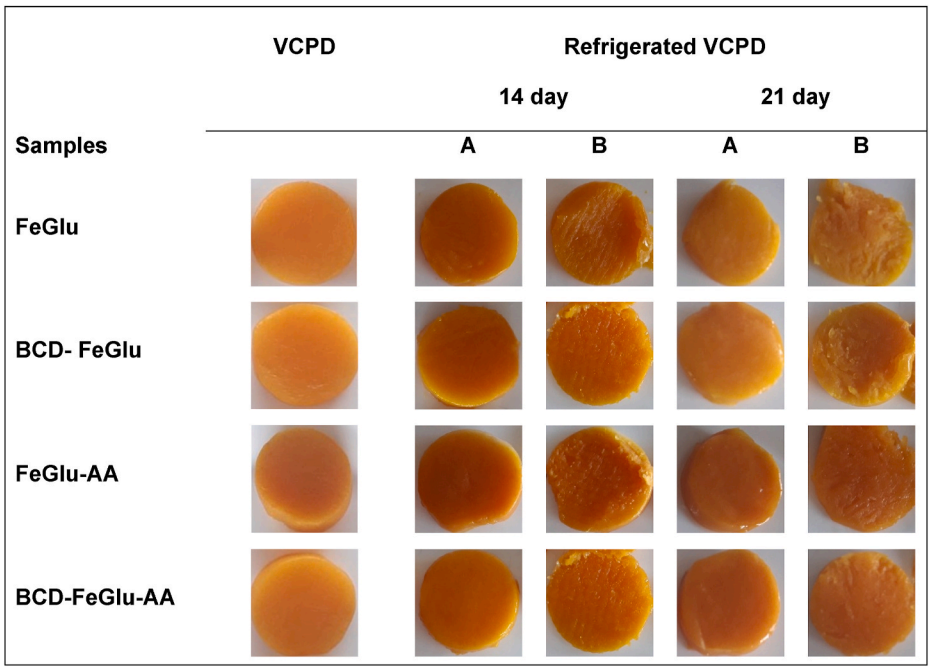

Fig. 3. Digital images of VCPD impregnated with FeGlu solutions (6.20 g/L) with BCD and/or AA (2.50 g/L) and stored under refrigeration (4 °C) for 21 days, external surface (A) and internal surface after a tangential cut of the discs (B).

FeGlu-AA was determined both after vacuum packaging-cooking and after refrigerated storage. After cooking, BCD-FeGlu-AA and FeGlu-AA contained average AA values of 73 ± 6 and 79 ± 3 mg/100 g of VCPD sample, respectively. The AA content decreased markedly after 14 days of storage, being the retention percentage of AA 50% w/w in the FeGlu-AA system (39.83 ± 0.18 mg AA/100 g VCPD.) and 55% w/w in BCD-FeGlu-AA (40.49 ± 2.79 mg AA/100 g VCPD). These values were maintained until 21 days of storage at 3 °C. These results were similar to those reported in a previous study for potatoes impregnated with AA and stored 14 days at 5 °C (Hironaka et al., 2011).

Possibly BCD could exert its positive effect by stabilizing iron in its reduced state, and in systems containing ascorbic acid, preventing AA oxidation. These results are in agreement with those reported by López-Nicolás and García-Carmona (2007), who demonstrated that CDs form complexes with substrates of enzymatic reactions (phenols) in pear juice which reduces browning, and could also prevent the oxidation of AA used as an antioxidant. López-Nicolás, Rodríguez-Bonilla, and García-Carmona (2014) reported that CDs would act as a secondary antioxidant, improving AA's ability to prevent browning due to the protection they offered against AA oxidation. On the other hand, Leite et al. (2003) studied the addition of β-cyclodextrin in aqueous solution of ferrous lactate and established that BCD reduces the oxidation of iron in the aqueous solution exposed to air. Other authors showed that the inclusion of ascorbic acid in β-cyclodextrin solutions increased the stability of the host molecule against oxidizing agents (Manzanares, Solis, & De Rossi, 1996). In this sense, it could be proposed that BCD prevents AA oxidation or its degradation in the studied pumpkin samples.

3.4. Effect of BCD on color stability during refrigerated storage

Table 2 shows the chromatic coordenates (L*, a* and b*), Chroma, ΔE and ΔL* obtained for pumpkins impregnated with FeGlu, BCD and/or AA solutions, after cooking and refrigerated storage during 21 days. The control corresponds to the values obtained for VCPD without impregnation.

The addition of iron with and without AA led to a decrease in the Chroma values in impregnated pumpkins compared to the sample without impregnation (Table 2), this was also observed by other authors (Genevois et al., 2014). The ferric cation (Fe^{3+}) generated during the oxidation reaction has a pro-oxidant effect (Habeych, van Kogelenberg, Sagalowicz, Michel, & Galaffu, 2016). This effect has been related to the loss of carotenoids and therefore of yellow color values (Rebellato, Bruna, Roger, & Pallone Lima, 2018).

It can be seen that no marked changes are observed on L* or a* values due to impregnation with any of the studied solutions. The main changes were observed on the b* parameter, which was minor than the control mainly in the systems impregnated with FeGlu and FeGlu-AA (Table 2). In the presence of BCD, the proportion of yellow in the samples was better maintained. This behavior was also evident in the Chroma values, which were lower than the control in the systems without BCD.

Regarding the effect of refrigerated storage, a slight increase in L* and a* values was observed after 14 and 21 days of treatment in all systems, both control and impregnated. In parallel, the b* values decreased during storage, especially in the impregnated systems (Table 2). These variations in the parameters account for changes in the stored pumpkins towards more reddish tones (greater a*) and less yellow (less b*), as can be seen in the images presented in Fig. 3. The inner and outer surfaces show a similar and uniform color, which could indicate a homogeneous impregnation throughout the slice. The FeGlu-AA pumpkin disc has a less yellow and darker color. This system in the presence of BCD (BCD-FeGlu-AA) showed a more intense coloration. The effect of ascorbic acid was previously observed by Gliemmo et al. (2009), who worked with pumpkin puree impregnated with ascorbic acid in polyvinylidene, packed in polyethylene bags and stored at 25 °C. They observed that AA protected the red color in pumpkin, but favored the degradation of the yellow color in the puree.

ΔE and ΔL values presented in Table 2 were calculated as the difference between cooked (without impregnation) and cooked impregnated systems after refrigeration. The positive initial values of ΔL* % indicated that the impregnation produced some darkening of the VCPD compared to the control VCPD (without impregnation). This behavior was less evident in the BCD-GluFe-AA system, which cleared less during storage than the systems treated with the other impregnation solutions. This darkening followed this order BCD-FeGlu-AA < BCD-FeGlu < FeGlu-AA < FeGlu. The increase in the L* parameter associated with the decrease in the b* value (Table 2), has been related to the occurrence of

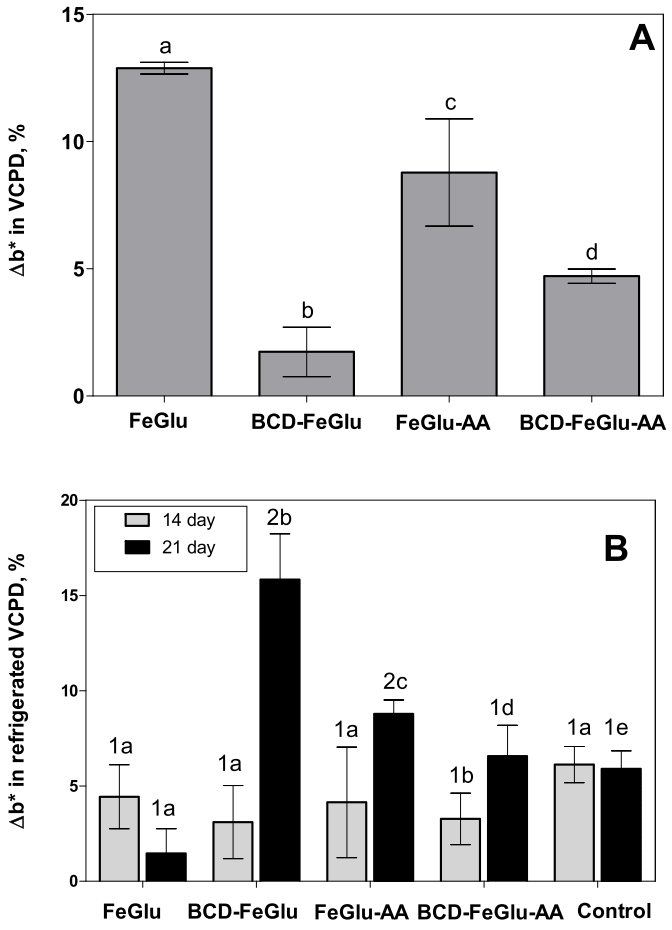

Fig. 4. Variation of b* chromatic coordinate in VCPD (**A**), and in refrigerated VCPD samples (**B**) impregnated with different impregnation solutions (FeGlu, BCD and/or AA).
Δb^* in VCPD, % = $(b_0 - b_{impregnated})/b_0$ x 100, with b_0: b* in non impregnated VCPD and $b_{impregnated}$: b* in impregnated VCPD. Δb^* in refrigerated VCPD = $(b_0-b_r)/b_0$ X 100, with b_0: b* in impregnated VCPD and b_r: b* in refrigerated impregnated VCPD (14 or 21 days at 3 °C). The results are expressed as the mean ± standard deviation (N = 3). Different letters indicate significant differences ($p < 0.05$) between the VCPD impregnated with FeGlu and with the other impregnated VCPD samples (**A**), 14 or 21 days under refrigeration (**B**). Different numbers on the error bar indicate a significant difference ($p < 0.05$) between the days in each system.

oxidation and isomerization reactions of β-carotenes (Gliemmo et al., 2009). As mentioned above, BCD could act as a secondary antioxidant, and synergistically with AA to prevent oxidation of iron in the food matrix.

Regarding the difference in color between samples (ΔE), it is generally considered that the differences are imperceptible for values $\Delta E \leq 3$ and very evident when $\Delta E \geq 5$ (Habeych et al., 2016). After the impregnation treatment and vacuum cooking, samples impregnated with FeGlu or FeGlu-AA (Table 2) presented the highest ΔE values. As expected, the heating during cooking affected the iron, promoting a loss of color in the pumpkin. During storage, ΔE was equal or greater than 5 in these systems, indicating that the change in color was detectable. The change in ΔE was mainly associated with the loss of yellow color (<b*). Regarding the presence of BCD, it was observed that in BCD-FeGlu and BCD-FeGlu-AA systems, color change compared to the control (without impregnation) was imperceptible ($\Delta E \leq 3$) up to 14 days of storage (Table 2).

As it is observed in Table 2, b* is the color parameter that changed most. Fig. 4 shows the changes in the b* parameter for the different cooked systems (Fig. 4A) and after cooking and refrigerated storage (Fig. 4B).

It should be noted that a higher value of Δb* indicates a greater decrease in b* in the system, that is, greater loss of yellow. Regarding the cooked impregnated samples (Fig. 4A), FeGlu showed the greatest change in b* (>Δb*). This indicates a greater loss of yellow color compared to the control during cooking. On the other hand, the lowest values of Δb* corresponded to the systems impregnated with BCD. This is in agreement with the lower values of ΔE determined for these systems (Table 2). However, an important variation in b * was observed in BCD-FeGlu refrigerated for 21 days (Fig. 4B), hence the effect of BCD was not maintained during the refrigerated storage.

4. Conclusions

The vacuum impregnation treatment using different solutions of ferrous gluconate, allowed to obtain a product (vacuum cooked pumpkin discs) fortified with a total iron content between 17 and 19 mg Fe^{2+} per 100 g VCPD portion, nearly thirty times higher than the not impregnated discs.

Impregnation with ferrous gluconate in presence of BCD or ascorbic acid improved the nutritional profile (considering the ferrous iron content) of cooked pumpkins compared to the control fortified only with FeGlu. Both compounds seem to act synergistically favoring the soluble form of iron (Fe^{2+}) during cooking and refrigerated storage of the fortified pumpkin discs. BCD encapsulation could probably act preventing oxidation of iron and of AA.

The studies of iron bioaccesibility performed by gastrointestinal "*in vitro*" simulation showed that the addition of BCD and ascorbic acid to the impregnation solution lead to a higher proportion of soluble iron and ferrous iron in cooked pumpkins compared to pumpkins fortified only with FeGlu. Possibly, both BCD and ascorbic acid, act preventing the oxidation of iron and promoting its solubility and bioaccessibility. Although some changes in chromatic parameters were observed associated with changes in the Fe^{2+} content, they were minimal considering that in most cases ΔE values < 5 were obtained.

Present results are novel and of great interest since they suggest that the use of β-cyclodextrin in FeGlu solutions could improve the stability and solubility of iron, keeping it in a ferrous state after *in vitro* digestion of the fortified cooked pumpkins.

CRediT authorship contribution statement

María Sabrina Lencina: Investigation, Methodology, Writing – original draft. **Cristina dos Santos Ferreira:** Writing – review & editing. **Diego Archaina:** Formal analysis. **María Beatriz Gómez:** Conceptualization, Resources. **María Florencia Mazzobre:** Project administration, Writing – review & editing.

Declaration of competing intrerest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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