



# Development of fortified bread using peptide-iron chelate: A perspective to prevent iron deficiency anemia

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## Abstract

The study entertained in the present research effort aims at evaluating the acceptability of bread fortified with peptide-iron chelate, by children of preschool age. The peptide-iron chelate was prepared from casein hydrolysate, and duly characterized through infrared spectroscopy, differential scanning calorimetry analyses, and X-ray diffraction spectroscopy. The iron content in the chelate, in the baking flour and in the loaves was determined by X-ray fluorescence spectroscopy. The breads were flavored and their (sensorial) acceptance by tasting was assessed by children in preschool age, using a hedonic scale. The results obtained clearly demonstrate the formation of the chelated compound. The iron content in the peptide-iron chelate was found to be 12.25 mg/Kg<sub>chelate</sub>, whereas in the breads it ranged from 0.222 to 0.253 mg/Kg<sub>bread</sub>. The rate of (sensorial) acceptance of the fortified breads was higher than 90%. The results indicated that breads fortified with peptide-iron chelate are an alternative and attractive form for the prevention of iron deficiency anemia.

**Keywords:** Iron deficiency anemia, foodstuff fortified with iron, iron bioavailability, peptide-iron chelate

## Introduction

Iron deficiency anemia (IDA) represents a serious health problem and affects about one third of the world population. Children from low- and middle-income countries are particularly vulnerable to develop iron deficiency and anemia, which can be prevented or controlled with different iron intervention strategies [1-4]. Anemia due to iron deficiency is the most common type of nutritional disorder, leading to irreversible delays in both physical and cognitive development of the children, together with the propensity to infectious diseases with increased rates of morbidity and mortality [5]. IDA is of multifactorial origin, having multiple determinants such as the socioeconomic conditions, health care assistance to the children's health, nutritional status of the children, the presence of comorbidities, food intake and biological factors [6]. IDA occurs when dietary iron reserves are depleted, due to a negative balance between intake, physiological needs and losses resulting from menstrual blood, intestinal parasites [7,8]. Food fortification is accepted as the best means of combating anemia in preschool children because, in principle, it does not imply changes in the eating

habits of the population, being socially accepted [9,10]. Overall, the research in food fortification with iron has been focused in order to establish which foodstuff should be fortified, the chemical form and contents of the element iron. The food product used as a vehicle for iron enrichment must be accessible to the portion of the population most vulnerable to iron deficiency. Additionally, the effectiveness of food fortification with iron depends on three main factors, viz. the iron source, the type and acceptance of food [5,11,12]. The enrichment of bread fortified with iron of high relative biological value, may be a useful strategy to combat iron deficiency and anemia. Bread, being a low-cost food consumed daily by individuals of all age groups, can contribute to the reduction of this nutritional disease, whose consequences are severe and permanent. The aim of the present research effort was to prepare breads fortified with iron chelated to casein peptides, and assess their acceptance by a group of children of 4-6 years old.

## Methods

All reagents were of pharmaceutical grade, PA or suitable for

human consumption, and were utilized without any additional purification.

### Production of peptide-iron chelate

The peptide-iron chelate (P-IC) was carried out according to the procedure described by Chaud et al [13]. Casein hydrolysate (CH) was dispersed in ultrapure water (10%w/v) by mechanical stirring device (1,000rpm). After 24h (26±1°C) the pH of the dispersion was gradually corrected with NaOH 1.0 M until the dispersion remained completely clear. Anhydrous FeCl<sub>3</sub> was added in the proportion of 1:2 (FeCl<sub>3</sub>:CH). The dispersion of FeCl<sub>3</sub>-CH was constant stirring (500 rpm), for a period of 12h (26±1°C) and maintained at rest under refrigeration. After 24h, the pH of the medium was measured and the supernatant discarded. The precipitate was then suspended in HCl 0.1 mM to remove non-chelated iron, and kept at rest to allow for decantation. This procedure was repeated three times. Drying of the precipitate was done by lyophilization. The samples were stored protected from light.

### Physical-chemical characterization of the peptide-iron chelate

FTIR spectra were gathered in the region of wavelengths between 400 cm<sup>-1</sup> and 4000 cm<sup>-1</sup> (Shimadzu model-8300, Japan). The samples (CH, ferric chloride, P-IC) were prepared by compression with 0.3g of KBr. X-RD studies of the samples with purified nickel and copper radiation were performed using an X-ray diffractometer (Siemens-model D5000, Germany). All analyses were performed with the diffraction at an angle of 2θ, varying from 5 to 70 degrees. DSC analyses carried out in the temperature range from 25°C to 350°C, under inert atmosphere, with a heating rate of 10°C/min and nitrogen flow of 50mL/min. For these thermal analyses, a DSC from Shimadzu (model DSC-50, Japan) was utilized. XFR analyses carried out in spectrometer by energy dispersion (Shimadzu-model EDX-700, Japan), constituted by a Rh tube and a semiconductor detector of Si(Li); Chemplex 1300 cells supported by Mylar® film (Chemplex 100) with 2.5µm thickness.

### Preparation of the fortified breads

The breads were prepared according to Good Food Handling Practices. All the ingredients were weighted according to the amounts indicated in the formulations (Table 1). The breads were prepared in the Laboratory of Patisserie and Baking of the University of Sorocaba, and followed the same technique except for the addition of the Peptide-Iron Chelate in the fortified breads (recipes II and III) and addition of flavoring in fortified breads (recipes IV and V). Briefly, wheat flour, powder milk, salt and sugar, were homogenized in a planetary mixer for 2 min, and Reinforce (mix of starch, soybean flour, inactive dry yeast, hemicellulose and lipase enzymes, mono and diacylglycerol emulsifiers, alpha-amylase flour improver, glucose oxidase, anti-oxidant) and Peptide-Iron Chelate (recipes II-V) were sequentially added. After homogeniza-

**Table 1.** “Milk-roll” type normal breads (I), peptide-iron fortified breads (II, III), onion flavored and peptide-iron fortified breads (IV, V).

Ingredients	Dough formulation				
	I	II	III	IV	V
Wheat flour (g)	625	625	625	645	645
White sugar (g)	75	75	75	75	75
Salt (g)	12.5	12.5	12.5	12.5	12.5
Reinforce® (g)	6	6	6	6	6
Powder milk (g)	19	19	19	19	19
Vegetable cream (g)	37.5	37.5	37.5	37.5	37.5
Eggs (g)	62.5	62.5	62.5	62.5	62.5
Biological ferment (g)	47	47	47	47	47
Iced water 2-8°C (mL)	250	250	250	250	250
P-IC (g)	--	6	9	6	9
Onion (g)	--	--	--	50	50

tion, the remaining formulation components (Table 1) were added and duly mixed until adequate texture. The onion used as flavoring (recipes IV and V), previously crushed, in a blender with cold water, before being added to the mixture. The dough was removed from the mixer, placed on a stainless steel bench, covered with plastic and allowed to stand. After 20 min., it was divided into portions (35g) and duly molded mechanically. The dough-breads were placed in the baking sheets and put in the Combined Oven (Rational-model/SCC61, Brazil) so as to complete the fermentation process. After 40 min, the breads were slightly moistened with water and baked (150°C/20 min). After production, the breads were transferred to an oven and maintained during until complete cooling (60 min) and individually weighed. The breads were packaged in plastic bags free from contamination, stored at room temperature, according to the parameters and criteria for the hygienic-sanitary control.

### Nutritional evaluation of the breads

The nutritional characteristics of the fortified breads were duly analyzed [14]. To calculate the nutritional characteristics of the breads, Eq. 1 was used, considering the caloric value, and the contents in carbohydrates, proteins, total fat and dietary fiber. The results were compared with an industrialized product possessing the same characteristics.

$$V_n = \sum_{i=1}^n \frac{VnI_i \times M}{100} \quad (\text{Eq. 1})$$

In Eq. (1), Vn is the nutritional value of the bread, VnI<sub>i</sub> is the nutritional value of ingredient *i*, and M is the mass (g) of the bread after baking.

### Determination of iron content in the fortified bread

The samples were analyzed by X-ray fluorescence (XRF) after

grinding in a porcelain mortar and standardized to 850 mm sieve. The voltage applied in the X-ray tube was of 50 kV, with 25% dead time of the detector. The spectra were gathered sequentially, with a resolution of 0.02 keV, from 0 to 40 keV. The irradiation time frame was of 120 s.

### Sensorial (acceptance) tests of the fortified bread

The sensorial (acceptance) tests were performed by a group of 60 children between 4 and 6 years of age attending a Municipal Preschool (EMEI) at Porto Feliz (São Paulo, Brazil). As exclusion criteria, one considered the existence of food intolerance to any of the components of the bread formulations, reported by the children's parents or guardians or yet by the spontaneous rejection of the child to consume the bread. The research study entertained herein was duly approved by the Ethics and Research Committee of the University of Sorocaba, receiving the decision 028/2011 and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. To assess the acceptability of the breads, the model of acceptability test suggested by the National Education Development Fund (FNDE) via 5-point facial hedonic scale was used [15]. The fortified breads were served with an interval of seven days, during the morning snack, as part of the school lunch menu. The responses were measured by a gradual scale of points, contemplating all the possibilities between non-acceptance (hated) and full acceptance (loved it). The breads were served with margarine and its accompaniments (tea or chocolate milk) and consumption was *ad libitum*. During the tests, the amount of bread consumed was registered. Next, the children were asked to fulfill the facial hedonic scale record, according to their perception relative to acceptance of the loaves.

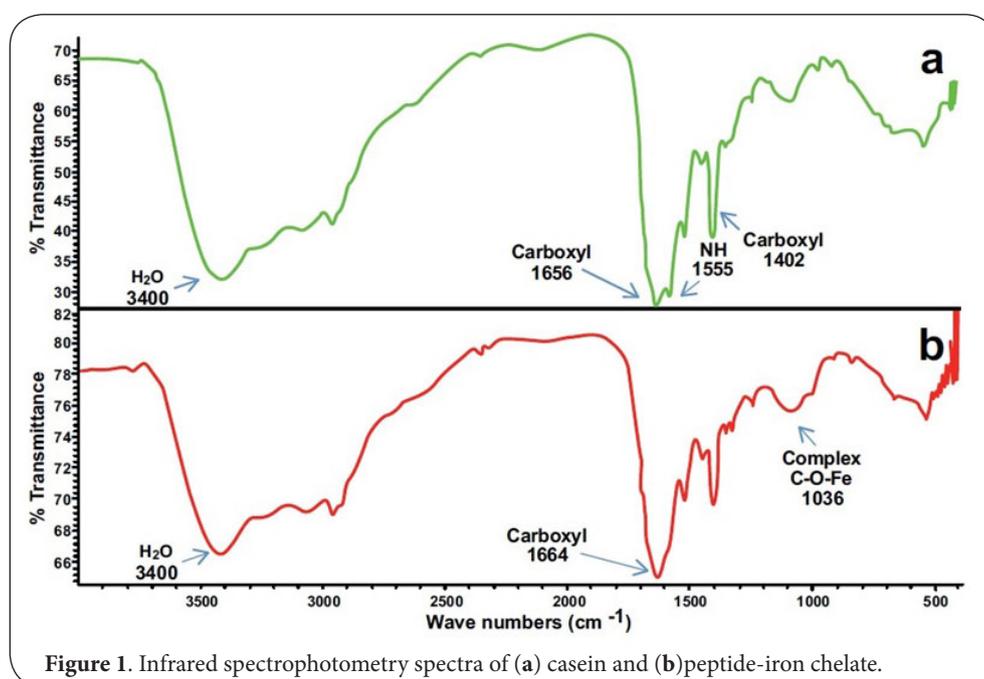
## Results and discussion

### Production of peptide-iron chelate

Production of the P-IC involves the determination of the isoelectric point (IP) of the CH, which can vary according to the peptides present in the hydrolysate. Freitas et al., [16] and Chaud et al., [13], identified the IP of CH between pH values of 7.5 and 7.8, but in the present research effort, the IP was obtained for a pH value of 9.6. At the IP, the aqueous dispersion of CH becomes completely clear, thus indicating obtaining an equilibrium state for the solubility. The difference in the IP may be related to the origin of the CH and with the size and amount of peptides present in the CH. At the IP, the overall solubilization of CH occurs, leading to the exposure of the carboxyl groups (COO<sup>-</sup>) due to the loss of hydrogen. The addition of FeCl<sub>3</sub> is performed quickly to allow coordination of the iron ion (Fe<sup>3+</sup>) to the COO<sup>-</sup> of the peptides. In the embodiment of FeCl<sub>3</sub>, the pH of the medium decreases (ca. 2.5) by the formation of HCl. The CH then precipitates with the iron ions coordinated in the carboxyl groups, thus forming the peptide-iron chelate (P-IC). According to Chaud et al., [13], the formation of an annular composite justifies the biological value of the P-IC found in the present research effort.

### Physicochemical characterization of the peptide-iron chelates

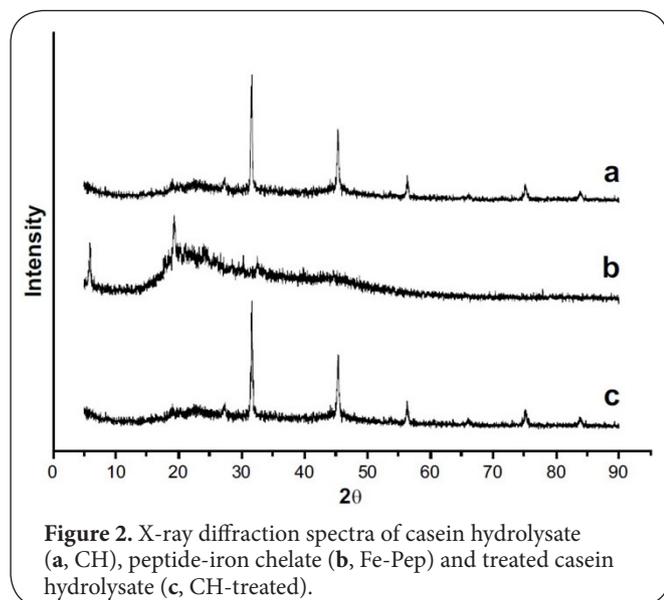
In **Figure 1**, the IR spectra of the CH (1a) and of the P-IC was observed (1b). The major contributions in the spectrum in **Figure 1a** can be observed at 1,656cm<sup>-1</sup> and 1,402cm<sup>-1</sup>, relative to the stretching of the COO group, and at 1,555cm<sup>-1</sup> relative to the absorption of NH bonds. In the region of 3,400 cm<sup>-1</sup>, the broader band related to the water of hydration. In the spectrum in **Figure 1b**, the largest contributions can be



**Figure 1.** Infrared spectrophotometry spectra of (a) casein and (b) peptide-iron chelate.

observed in the region of  $1664\text{ cm}^{-1}$ , and the appearance of a band in the region of  $1,036\text{ cm}^{-1}$  can be attributed to the complex C-O-Fe [13].

In **Figure 2**, the XRD spectrum of the P-IC was compared with the XRD spectrum of pure CH. CH exhibits diffraction angle peaks of  $2\theta$  for  $32.15$ ,  $45.06$ ,  $56.10$ ,  $75.20$  and  $84.05$ . Such series of peaks indicates the crystalline state of CH. While there is no benchmark to compare the spectrum, the peaks are probably related to the different peptides resulting from the hydrolysis of casein [17].

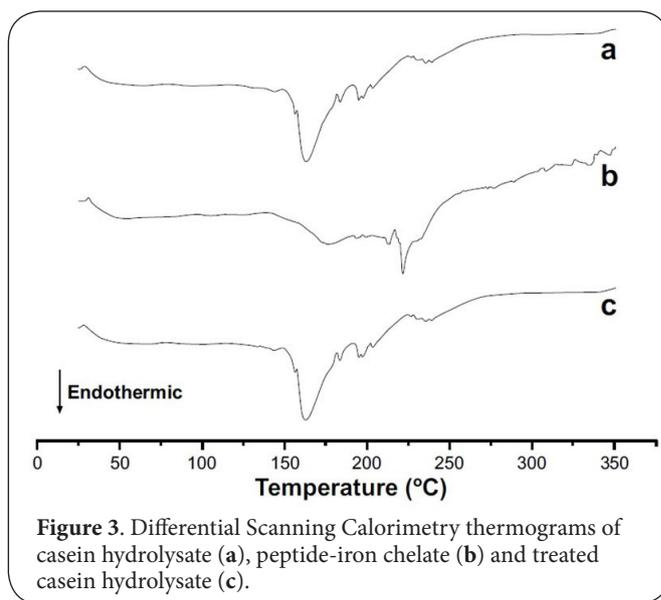


**Figure 2.** X-ray diffraction spectra of casein hydrolysate (a, CH), peptide-iron chelate (b, Fe-Pep) and treated casein hydrolysate (c, CH-treated).

The diffraction pattern of the P-IC(2-b) reflects a change in the crystalline structure of CH(2-a). This difference may be related to a chemical interaction between Fe ions and casein peptides. After dissolution of the casein hydrolysate (CH-treated, **Figure 2c**), a sample was withdrawn from the medium prior to the addition of  $\text{FeCl}_3$  and properly lyophilized, in order to evaluate the crystallinity profile of the sample after recrystallization when compared with the CH(2a) and the P-IC(2b). No change in the crystalline pattern of CH was found after recrystallization (CH-treated, 2c).

The DSC spectra provide information about the melting, crystallization or decomposition of crystalline substances. These analyses are useful to evaluate the physical-chemical status of pure raw material, as well as the interaction between different compounds. The CH exhibits (**Figure 3a**) at least 5 endothermic peaks corresponding, most likely, to the melting points of casein peptides.

Melting points can be related to the absorption angles of diffraction spectrum. Taking into account the sizes of the peaks in the diffraction spectrum and the energy spent to melt each peptide of the hydrolysate, the endothermic peak at  $156.7^\circ\text{C}$  can be correlated with the crystalline peak at  $32.15^\circ$ , since



**Figure 3.** Differential Scanning Calorimetry thermograms of casein hydrolysate (a), peptide-iron chelate (b) and treated casein hydrolysate (c).

these are the largest areas under the curve and are related to the mass of the compound. The remaining peaks at  $180^\circ\text{C}$ ,  $189.2^\circ\text{C}$ ,  $191.0^\circ\text{C}$  and  $198.7^\circ\text{C}$  correspond, respectively, to the angles  $45.06^\circ$ ,  $56.06^\circ$ ,  $75.20^\circ$  and  $84.05^\circ$  of the diffraction spectrum. The calorimetric spectrum of the P-IC shows two major endothermic events, the first at  $170^\circ\text{C}$  and the second at  $219.1^\circ\text{C}$ , and a secondary endothermic peak at  $215^\circ\text{C}$ . Keeping the postulated hypothesis of a relationship with the DRX angles, the endothermic events at  $170^\circ\text{C}$  and  $219.1^\circ\text{C}$  would correspond to the angles of  $19^\circ$  and  $6^\circ$ , respectively. On the other hand, the results from the DSC analyses confirm that there is an interaction between casein peptides and the  $\text{Fe}^{3+}$  ions. This interaction can be a coordination of the amine or carboxyl groups of the peptides with the  $\text{Fe}^{3+}$  ions, forming the P-IC, as can be seen in **Figure 3**. After the dissolution of the casein hydrolysate (CH-treated), a sample was withdrawn from the medium prior to the addition of  $\text{FeCl}_3$  and duly lyophilized, with the purpose of evaluating any changes in the characteristics of the CH and CH after recrystallization. By comparing the thermal events in the spectra of CH(3a) and CH-treated(3c), no changes whatsoever can be detected. This is a confirmation that the changes in the P-IC(3b) spectrum are due to the coordination of iron to casein peptides.

### Evaluation of the bread manufacturing process

Food fortification with iron may promote problems related to off flavor, color and product stability [18]. Aspects related to odor and flavor undermine the acceptance of food products. During the process of obtaining the dough, and especially at the times when the loaves were baking and still warm, a strong odor of casein was noticed, resulting from the use of P-IC. However, this odor became virtually imperceptible after the breads cooled down. The formulations were adjusted departing from a previous tasting conducted by both staff

and students at the Laboratory of Confectionery and Bakery of the University of Sorocaba (Sorocaba, Brazil). The minced onion was used to create both an optional recipe and to mask the taste of iron salts. This flavoring effect was successfully achieved without enhancing either the flavor or odor of the onions. Considering the iron content and the final taste of the breads, formulations III and V were selected for further analyses including the acceptance tastings.

### Nutritional content of the breads

The nutritional information of the fortified bread formulations is displayed in **Table 2**. As can be seen from inspection of the data displayed in this table, the nutritional values are similar when comparing the data for fortified breads with the data for a sample of industrialized bread of the type “milk-roll”.

**Table 2. Nutritional information of industrialized “milk-roll” type bread, peptide-iron fortified bread (dough formulation III) and onion-flavoured and peptide-iron fortified bread (dough formulation V).**

Bread unit (25g)	Industrialized “milk roll” type bread	Dough formulation III	Dough formulation V
Caloric value (kcal)	76.2	71.0	70.8
Carbohydrate (g)	15.6	11.85	11.86
Protein (g)	2.45	1.91	1.91
Total fat (g)	0.47	1.1	1.1
Food fibre (mg)	0.05	0.06	0.07

Source: TACO [17].

### Determination of the iron content in fortified breads

In **Table 3** is present the average contents in iron (mg/g) and associated standard deviations in samples of the flour utilized to manufacture the breads, in the breads without P-IC, in the breads fortified with the P-IC, and in the flavored bread fortified with P-IC.

The rates of iron recovery in the fortified breads and in the flavored and fortified breads were, 110% and 96%, respectively. The average iron content in the P-IC was 12.24 mg/g, and this result is twice that obtained by [13]. This fact might be explained by the change in the isoelectric point of the casein

**Table 3. Contents iron±SD in samples of chelate iron peptide to manufacture the breads, milk bread no chelate, in the milk breads with the peptide-iron chelate, and in the flavoured milk bread with peptide-iron chelate.**

Sample	Iron (mg/g)
Chelate	12.24±0.209
Milk bread no chelate	0.342±0.006
Milk bread+chelate 6g/Kg	4.592±0.022
Milk bread+chelate 9g/Kg	6.254±0.025
Milk bread Onion-flavoured+chelate 9g/Kg	5.915±0.023

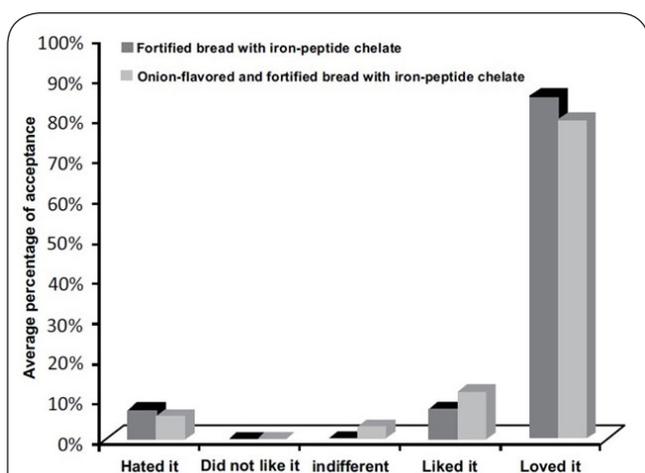
utilized with a consequent increase in the coordination of Fe<sup>3+</sup> to the COO- of hydrolyzed casein. In the wheat flour samples, the iron content is in agreement with the Brazilian standards for the enrichment of flours [19]. Considering the daily loss of iron, the recommended intake for children of preschool age, pregnant women, adult women and men is, respectively, 15, 27, 18 and 8 mg iron/day. The proportion of iron in the flour as a source to prevent iron deficiency remains below the levels needed, considering the average consumption and the bioavailability of iron in this type of food that is also rich in phytate. Food products fortified with chelates of iron and aminoacids exhibit a higher bioavailability when compared to those fortified with iron salts [18,19]. Therefore, considering the relative biological value of the P-IC [13], a daily intake of more than 10g of fortified breads would be enough to prevent the iron-deficiency in all age groups.

### Evaluation of the sensorial acceptability of the fortified breads

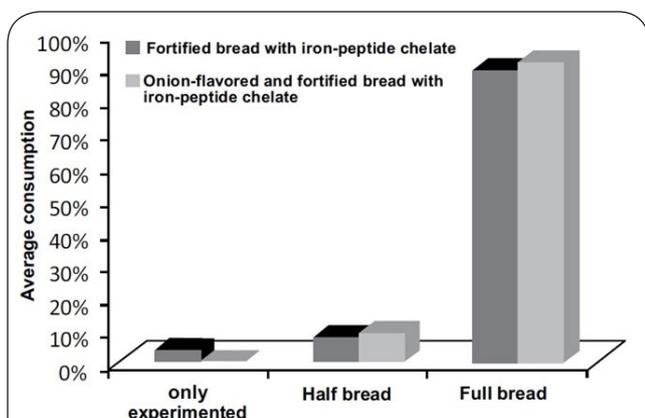
Sensorial analysis consists of a scientific method to identify sensorial properties of foods stuffs and, with this, evaluate the quality and acceptance of a food product by a population [20]. The acceptance test is an affective sensorial method that represents the sum of all sensorial perceptions and expresses the consumer’s judgement about the quality of the product [15]. In the case of food products destined at snacks, as part of school lunch menus, according to the *National Program of School Foods* (PNAE). The application of the acceptability test is mandatory whenever there is an introduction in the menu of an atypical food product with respect to the local food habits, or any other innovative changes, and the acceptability index must not be lower than 85% [15]. When comparing the results of the acceptability tests performed to both the fortified breads and the flavored and fortified breads (**Figure 4**), it was observed that the acceptance of both types of bread was similar. Considering the sum of the parameters “loved it” and “liked it” for the fortified breads and flavored and fortified breads was obtained, respectively, 92.6% and 91.2% of acceptance.

With respect to food consumption (**Figure 5**), 88.9% integral consumption of the bread fortified with P-IC and 91.2% integral consumption of the onion-flavored bread further fortified with P-IC were verified. However, repetition was significantly higher for fortified bread (80%) when compared with the onion-flavored fortified bread (58%). These results can be justified by considering that onion-flavored and fortified bread proved to be denser and, probably, promoted a quicker sensation of satiety.

According to the results presented in the research effort entertained herein, it may be concluded that both types of fortified bread complied with the standards of the *National Program of School Foods* (PNAE), presenting an index of acceptability higher than 85%, and can, therefore, be integrated in the snack offered as part of the school lunch. Not with stand-



**Figure 4.** Average percentage relative to the hedonic sensorial acceptance tests performed to the bread fortified with peptide-iron chelate and to the onion-flavored bread fortified with peptide-iron chelate, in children at preschool age (4-6 years old) (n=60).



**Figure 5.** Average percentage relative to the consumption of the bread fortified with P-IC and to the onion-flavored bread fortified with peptide-iron chelate, in children at preschool age (4-6 years old) (n=60).

ing the fact that preschool age children usually present some resistance to experiment and accept new foodstuff, the fortified breads had a high degree of acceptance by the children. Based on the results produced in the present research work, one may conclude that bread fortified with P-IC is a viable alternative in the solution of the serious health problem that constitutes iron deficiency anemia in nutritional risk groups. Additionally, the P-IC is an attractive alternative for the enrichment of other bakery products. The cost of the pilot-scale production of 1.1 Kg of P-IC is less than USD 77.3. Considering that 9g of P-IC is used for each 1.0 Kg of dough, the chelate represents a cost of USD 0.02 per fortified bread unit. The fortified bread recipe represents a cost of USD 1.16, which means that each fortified bread unit would cost in average USD 0.06.

The results obtained in the research effort presented herein, by using the analytical techniques of IRTF, X-ray diffraction and DSC showed that iron is bound to the peptides of CH in the form of a chelate, which indicates a good bioavailability of iron. The X-ray fluorescence technique allowed to quantify the iron content in both the P-IC and the fortified breads, and indicated a percentage of iron that permits to combat the deficiency in iron and to prevent iron deficiency anemia, by a daily up take of only 10 g of fortified bread. The enrichment of breads increases the nutritional value of this foodstuff, allowing to minimize the serious public health problems arising from iron deficiency or iron deficiency anemia. Inclusion of the P-IC in breads, in the amounts proposed, did not change in a perceptible way the organoleptic characteristics of the product. The acceptance of the fortified bread by the children was completely satisfactory; indicating that this product could be included in the morning snacks offered as part of the school lunch menus for preschool age children. The breads fortified with iron chelated to casein peptides were produced at pilot-scale, and this technology can be easily scaled-up to both public and private bakeries.

#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

Authors' contributions	TT	MCV	LS	PS	VB	JFS	TRA	JMO	MCV
Research concept and design	✓	✓	--	--	--	--	--	--	✓
Collection and/or assembly of data	✓	✓	✓	--	--	✓	✓	✓	✓
Data analysis and interpretation	✓	✓	--	--	✓	--	--	✓	✓
Writing the article	--	--	--	✓	--	--	--	--	✓
Critical revision of the article	--	--	--	✓	--	--	--	--	✓
Final approval of article	✓	✓	✓	✓	✓	✓	✓	✓	✓
Statistical analysis	✓	--	--	--	--	--	✓	--	✓

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