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ORIGINAL ARTICLE

Heme iron fortified flavored milk: quality and sensory analysis

Leite com sabor fortificado com ferro heme: análise sensorial e de qualidade

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Abstract

The fortification of dairy beverages is a widely developed strategy using non-heme or heme iron. Heme iron has a higher bioavailability. The investigation aimed to elaborate pasteurized milk with fortified chocolate flavor with heme iron that has good sensory acceptability. The preparation of the flavored milk was carried out based on the regulations and heme iron, obtained from a commercial source of whole blood of porcine origin, was added before the pasteurization process to achieve its complete dilution. The concentration of iron and chocolate flavoring was established as variables in order to evaluate the optimal formulation based on Sensory Acceptability (SA). The experimental design was a 3^2 factorial design in which eight formulations were established, which were sensory acceptability evaluated by a total of 35 school-age children, aged between 8 and 11 years using a five-point facial hedonic scale. The results of the analysis of variance and optimization of the response showed that SA was 4.71 (on a scale of 1 to 5) for a fortification of 6.76 mg Fe kg^{-1} sample and a chocolate concentration of 2.0 g kg^{-1} sample. The physicochemical characterization indicated a higher percentage of carbohydrates, a higher concentration of iron (9.3 mg Fe kg^{-1} sample) and vitamin C (349.0 mg kg^{-1} sample) with respect to fresh milk. According to the physicochemical and microbiological results, the approximate life time of the beverage was 5 days, which is in accordance with Peruvian regulations. These results showed a method of fortification of flavored milk that allowed the use of heme iron, whose content could contribute to the daily requirement of this mineral in children aged between 8 and 11 years old (8 mg of iron per day).

Keywords: Heme iron; Fortified flavored milk; Sensory acceptability; Chocolate; Factorial design; Physicochemical analysis.

Resumo

A fortificação de bebidas lácteas é uma estratégia amplamente desenvolvida, usando ferro não heme ou heme. O ferro heme tem maior biodisponibilidade. O objetivo da presente investigação foi elaborar um leite pasteurizado com sabor chocolate e fortificado com ferro heme que tenha boa aceitabilidade sensorial. O preparo do leite aromatizado foi realizado com base nas normas regulamentares do Peru e o ferro heme, obtido de fonte comercial de sangue de origem suína, foi adicionado antes do processo de pasteurização, para obtenção de sua diluição completa. As concentrações de ferro e sabor de chocolate foram estabelecidas como variáveis para avaliar a formulação ideal, com base na aceitabilidade sensorial. O delineamento experimental foi um esquema fatorial de 3^2, em que foram estabelecidas oito formulações, cuja aceitabilidade sensorial foi avaliada por um total de 35 crianças em idade escolar, com idades entre 8 e 11 anos, utilizando uma escala hedônica facial de cinco pontos. Os resultados da análise de variância e otimização da resposta mostraram que aceitabilidade senorial foi de 4,71 (em uma escala de 1 a 5) para uma amostra de fortificação de 6,76 mg Fe kg^{-1} e uma concentração de chocolate de 2,0 g kg^{-1} de amostra. A caracterização físico-química indicou maior percentual de carboidratos, maior concentração de ferro (amostra 9,3 mg Fe kg^{-1}) e vitamina C (349,0 mg kg^{-1} amostra), em relação ao leite *in natura*. De acordo com os resultados físico-químicos e microbiológicos, a vida útil aproximada da bebida láctea fortificada foi de cinco dias, o que está de acordo com a regulamentação peruana. Estes resultados mostraram um método de fortificação do leite aromatizado que permite a utilização de ferro heme, cujo teor poderia contribuir para suprir a necessidade diária desse mineral em crianças de 8 a 11 anos (8 mg de ferro por dia).

Palavras-chave: Ferro heme; Leite aromatizado fortificado; Aceitabilidade sensorial; Chocolate; Planejamento fatorial; Análises físico-químicas.

Highlights

- Chocolate flavored milk was fortified with heme iron
- Sensory acceptability was appreciated by school-age children between 8 and 11 years old
- The fortified milk had a concentration of 9.3 mg Fe kg^{-1} and vitamin C of 349.0 mg kg^{-1}

1 Introduction

Anemia is a condition that leads to an insufficient supply of oxygen to the cells and tissues of the body. Hemoglobin (Hb) is an iron-containing protein in red blood cells that binds to oxygen (Mohamed Saliq et al., 2020). World Health Organization (WHO) defines anemia as the decrease in the hemoglobin level standard as two standard deviations below normal for age and gender, for instance, in children from 6 months to 6 years below 11 g dL^{-1} and from 6 years to 14 years, 12 g dL^{-1} (World Health Organization, 2001). Approximately 30% of the world's population suffers from anemia, especially in developing countries. Iron deficiency anemia or iron deficiency can be reduced by dietary supplementation, dietary diversification, and iron fortification (Tang et al., 2015). According to the Demographic and Family Health Survey (*Encuesta Demográfica y de Salud Familiar* (ENDES)) prepared by the National Institute of Statistics and Informatics (*Instituto Nacional de Estatística e Informática* (INEI)) in Peru, in 2017 it was estimated that ~ 43.6% of the Peruvian population between 6 months and 3 years old suffers from anemia, however, in rural areas the percentage is higher (53.3%) than in urban areas of this country (40%) (Instituto Nacional de Estadística e Informática, 2018). Owing to this problem, the Peruvian state, through the Ministry of Health (*Ministerio de Salud* (MINSA)) and Social Programs, has been making efforts to eradicate anemia. For example, the QALIWARMA program aims to improve the nutritional status of children, and year after year it has been reinventing itself and demanding higher quality food (sensory, sanitary, etc.); consequently, the product developed in this work can be presented as an alternative and could be a candidate for inclusion in programs of this type.

Food fortification is a technique that has a significant influence in treating malnutrition with a wider and more sustained effect than supplementation. Fortification could also be used to improve the diet and its supplements (Mohamed Saliq et al., 2020). Among these foods, it can be found cow's milk, which is a nutritious food that provides energy to the human diet, contains essential nutrients such as proteins, vitamins, fats and minerals and is essential for children (Santos et al., 2017).

In recent work, A2 cow's milk fortified with lipoic acid nanocapsules (LANCPs) has been developed, which may act as a natural nutritional supplement that has antioxidant properties and maintains the red blood cells count through its redox mechanism (Mohamed Saliq et al., 2020). The literature also reports the preparation of flavored milk fortified with heme iron from bovine hemoglobin hydrolysates, which preserves sensory acceptance by consumers (Arias et al., 2018). Milk fortification has also been carried out with iron microcapsules by liposome, Fatty Acid Esters (FAE), freeze-drying and emulsification methods. Sensory analysis revealed that fortified milk by emulsification method containing 10 mg L^{-1} iron, resembled more to control (unfortified) milk when compared to others (Gupta et al., 2015a).

When fortification with iron microcapsules is carried out, these may be poorly soluble or insoluble in water, which makes their homogenization difficult; however, heme iron is a naturally protected compound, it has a high bioavailability (Arias et al., 2018; Toldrà et al., 2011) and can be obtained from bovine and / or pig blood. This is a by-product of the slaughter of these animals with high nutritional value, and that in many cases is discarded without taking advantage and whose inadequate dumping generates environmental pollution problems (Arias et al., 2018). The absorption of heme iron is two or three times greater than that of non-heme iron and is also less dependent on the other components of the food. However, its two main disadvantages to be used in food fortification are associated with its intense color which affects the sensory properties of food and with the difficulty to ensure the necessary hygienic conditions during the process of obtaining hemoglobin that allows its use as an input in food fortification (Boccio & Monteiro, 2004).

Therefore, the search for a fortification of heme iron with good sensory acceptability, a raw material for heme iron that meets the hygienic conditions and that is incorporated in an appropriate stage of the process, is necessary in the development of foods with improved nutritional quality. This work aimed to develop a flavored milk as a vehicle for heme iron, and to obtain a product with potential nutritional effect and nutritional acceptability appreciated by a group of children between 8 and 11 years old.

2 Material and methods

2.1 Materials

Homogenized milk was obtained from the Milk Pilot Plant of La Molina National Agrarian University. Whole blood powder of pig origin Aprosan^{TM} (188 mg Fe per 100 g of product), alkalized cocoa powder, ascorbic acid, carrageenan, vanilla, commercial sucrose and chocolate bit of Frutarom Peru were also purchased. All food additives were food grade.

2.2 Iron fortified chocolate flavored milk

Iron fortified chocolate flavored milk was made from fresh milk, to which sugar, flavorings, vitamin and authorized additives were added; and that was subsequently subjected to a heat treatment. The preparation was based on the Peruvian technical standard NTP 202.189.2020 (Instituto Nacional de Calidad, 2020), which indicates that flavored milk must have at least 85% of fluid milk in its composition. The fresh milk was pre-filtered and the Aprosan^{TM} was completely diluted in the cold filtered milk at 10 °C. A homogeneous mixture of sugar, cocoa, carrageenan and vitamin C was prepared and the milk was gradually heated to 30 °C and the cinnamon, cloves and anise were added. Heating continued up to 50 °C and the previously prepared homogeneous mixture was added and subsequently heated to 70 °C where the chocolate and vanilla flavorings were added. The fortified flavored milk was pasteurized, known as Low Temperature and Long Time (LTLT), at 75°C for ten minutes, using a stainless steel 100 liter jacketed pot. Finally, the fortified flavored milk was packaged and cooled to 4 °C (Figure 1). Table 1 shows the ingredients used in the formulation of chocolate flavored milk and their respective amounts. It was established to use the maximum amount of sugar allowed in the preparation of fortified flavored milk, according to the Law and Promotion of Healthy Eating for children and adolescents N°. 30021, which is 50 g per 1 L of milk (Peru, 2017).

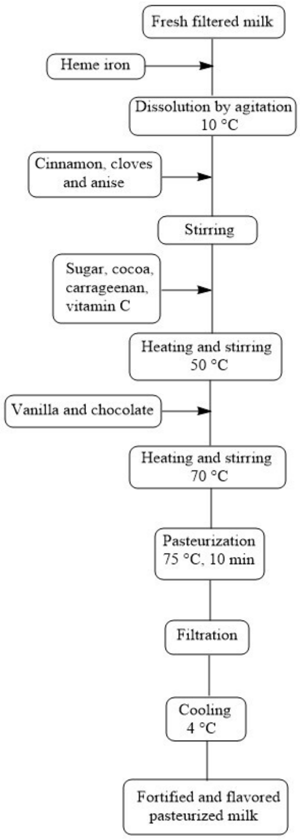

Figure 1. Process flow chart for the production of fortified chocolate flavored milk.

Table 1. Ingredients used in the formulation of fortified chocolate flavored milk.

Ingredients (g kg^{-1} sample)	Formulation
Sugar	50.0
Alkalized cocoa	6.0
Carrageenan	0.7
Vitamin C	0.7
Liquid vanilla	2.0
Chocolate	2.0-4.0
Cinnamon, aniseed and clove	1.0
*Aprosan™	3.40 -6.80

*Iron: 0.0064-0.0128 g kg^{-1}.

2.3 Sensorial analysis

The facial hedonic satisfaction test was performed in a total of 35 schoolers, untrained users, from a public school in the city of Lima in Peru, aged between 8 and 11 years. Samples were provided in 30 mL portions in letter-coded disposable containers in random order. The children also received an evaluation sheet like a consumer testing to understand the sensory perceptions of each formulation (Guinard, 2000). This test contained five (5) categories, which corresponded to: 5, I like it a lot; 4, I like it; 3, I do not like or dislike it; 2, I dislike it; 1. I really dislike it.

The researchers, teachers and parents accompanied and guided the children in filling out the survey. The children had a time close to 10 minutes to do it. The general appreciation value for each formulation was obtained from the sum of the acceptability of the respondents, divided by the number of children.

The children's participation in the study aimed to collect their opinions and help extrapolate preferences in similar populations, and potentially estimate the impact that these foods would have each time they are marketed on an industrial scale.

2.4 Statistical analysis

The factorial design 3^2 was used to investigate the effects of heme iron and chocolate flavoring concentrations to determine the optimum formulations. Three concentration levels (low, medium and high) were evaluated as variables (Table 2), for eight formulations included in the experimental design (Table 3). The choice of concentration levels is related to the purpose of supplementing the recommended daily intake for school-age children (8 mg Fe/day) who consume 250 g of milk daily (Institute of Medicine (US) Panel on Micronutrients, 2001). The low level represents 20% of fortification, the intermediate level of 30% and the high level 40% of fortification for 1 kg of milk. The ANOVA was perfomed using software Minitab Version 17 to determine the significance of the coefficients in the model at a confidence level of 95% ($p < 0.05$). The model fit was verified using R^2 value.

Table 2. Study factors of the statistical model.

Factors	Symbols	Levels		
		Low	Medium	High
Heme iron (mg kg^{-1} sample)	X_1	6.4 (20%)*	9.6 (30%)*	12.8 (40%)*
Chocolate flavoring (g kg^{-1} sample)	X_2	2.0	3.0	4.0

*Fortification for 1 kg of milk (Recommended dose, 8 mg Fe per day with a consumption of 250 g of milk).

Table 3. Variables and levels used for the experimental design.

Formulations	Heme iron (mg kg^{-1} sample)	Chocolate flavoring (g kg^{-1} sample)
F1	6.4	2.0
F2	9.6	2.0
F3	12.8	2.0
F4	12.8	4.0
F5	6.4	4.0
F6	9.6	4.0
F7	6.4	3.0
F8	9.6	3.0

2.5 Characterization

Physiochemical analyzes were carried out for fresh milk and fortified flavored milk based on Peruvian technical regulations. The percentage of carbohydrates was determined on (Collazos et al. 1993), fat percentage was obtained based on NTP 202.126:1998 (Instituto Nacional de Calidad, 1998a). The ash content and moisture percentage were obtained based on NTP 202.172(3.1): 1998 and NTP 202.118:1998, respectively (Instituto Nacional de Calidad, 1998b, 1998c). The protein content was carried out by the Kjeldahl total nitrogen determination method, NTP 202.119: 1998 (Instituto Nacional de Calidad, 1998d). Total energy, percentage of energy from protein, the percentage of carbohydrates and fats were also determined based on (Collazos et al., 1993).

The percentage of non-fat solids and acidity were analyzed using a milk analyzer MilkoScan™ FT1 (Foss Analytical). Viscosity was determined using a Brookfield viscometer. Relative density measurements were based on NTP 202.008:1998 (Instituto Nacional de Calidad, 1998e). Iron was analyzed according to AOAC 975.03 (Association of Official Analytical Chemists, 1988) and vitamin C determination was analyzed using the AOAC 967.21 (Association of Official Analytical Chemists, 1968).

The determination of the mesophilic aerobes and coliform were carried out based on (International Commission on Microbiological Specifications for Foods, 2000). The shelf life was evaluated from the microbiological analysis (mesophilic aerobes and coliform) and measurements of acidity, density, viscosity, pH and percentage of fat, protein and total solids during 14 days.

3 Results and discussions

3.1 Statistical analysis

Table 4 shows the results of the ANOVA for the significance test of the model coefficients. The factors and their combinations were: factor X_1: iron concentration; factor X_2: chocolate flavoring concentration.

Table 4. ANOVA data for factorial experimental design.

Factor	df	F-value	*p*-value
Model	6	3.43	0.048*
Sex	1	2.38	0.157^{ns}
X_1	1	0.03	0.862 ^{ns}
X_2	1	5.74	0.040*
X_1* X_1	1	1.38	0.27 ^{ns}
X_2* X_2	1	1.18	0.306 ^{ns}
X_1* X_2	1	9.3	0.014*
Error	9		
Total	15		

X1: heme iron; X2: chocolate flavoring. *significant $p < 0.05$. ^{ns}not significant $p > 0.05$. df: degrees of freedom.

The analysis indicated that effect of the concentration of chocolate flavoring (X_2) and X_1 *X_2 interaction (iron*chocolate flavoring concentration) were related to the factors with the greatest significance level ($p < 0.05$). According to the dimension of these coefficients in the model, the two variables were determinants in the sensory acceptability of fortified flavored milk. The effect represented by gender, according to the analysis of variance, did not influence sensory acceptability.

In Equation 1, the adjustment of the model for Sensory Acceptability (SA) is presented, whose coefficients were calculated by multiple regression. The model is the result of the exclusion of non-significant terms ($p > 0.05$), preserving the hierarchy of the model. The efficacy of the model was demonstrated by R^2 values of 0.6960, which suggests that the experimental and predicted data ensure a good fit of the model.

$$SA = 4.673 + 0.00053\,X_1 + 0.006\,X_2 + 0.01006\,X_1 * X_2 \quad (1)$$

The response optimization showed that the optimal values of the evaluated factors and the predicted SA were as following: %Fe fortification = 21.82% (6.76 mg Fe kg^{-1} sample); chocolate flavoring concentration = 2.0 g kg^{-1} sample; and SA = 4.7145 (scale from 0 to 5). According to the multiple response prediction, the 95% Confidence Index (CI) suggests that the found values of the levels for each factor evaluated are optimal working conditions for this system. Under this concentration of Fe, the sensory acceptability was optimal, considering that a higher percentage of fortification presents some limitations such as the metallic taste of Fe itself (Toldrà et al., 2011).

Therefore, a maximum sensory acceptability of 4.71 was obtained based on a scale of 0 to 5, working with a Fe concentration of 6.76 mg Fe kg^{-1} sample and a chocolate flavor concentration of 2.0 g kg^{-1} sample. Based on this iron fortification value, fortified chocolate-flavored milk can supplement part of the recommended levels for children from 8 to 11 years of age (8 mg of iron/day) whether 250 g of milk is consumed (Institute of Medicine (US) Panel on Micronutrients, 2001).

3.2 Characterization

The physiochemical characterization of fresh milk and chocolate milk is shown in Table 5. According to these results, the average fat content of fortified flavored milk was lower than the fresh milk, on the contrary, the protein content of fortified flavored milk was higher than the fresh milk. The amount of carbohydrates was higher in fortified flavored milk, probably due to the incorporation of the chocolate flavoring into the formulation.

Table 5. Results of the physiochemical analysis of fresh milk and fortified chocolate flavored milk.

Characterization	Values for fresh milk	Values for fortified flavored milk
Carbohydrates (g 100 g^{-1})	4.8	12.7
Fat (g 100 g^{-1})	3.5	2.6
Proteins (g 100 g^{-1})	3.0	3.5
Ash (g 100 g^{-1})	0.7	0.8
Moisture (g 100 g^{-1})	88.0	80.4
Total energy*	62.7	88.2
Kcal from protein (%)	19.1	15.9
Kcal from carbohydrates (%)	30.6	57.6
Kcal from fat (%)	50.3	26.5
Non-fatty solids (%)	8.85	15.6
Vitamin C (mg 100 g^{-1})	0.30	34.9

*Total Energy in kcal that corresponds to 100 g of sample.

The concentration of vitamin C was 0.3 mg 100 g^{-1} sample in fresh milk and for the fortified flavored milk it was 34.9 mg 100 g^{-1} sample, indicating an increase in this due to its incorporation into the formulation. The losses in the value of total vitamin C with respect to the value incorporated into the preparation (70 mg in 100 g of sample) may be due to the pasteurization process (Sharma & Lal, 2005). The incorporation of vitamin C into the formulation aimed at contributing to the absorption of heme iron and non-heme iron (Food and Agriculture Organization, 2002) found in other foods that are part of breakfast, such as cereals, and improving immunity against disease (Troise et al., 2016).

The iron concentration in fresh milk was 1.3 mg kg^{-1} sample and for fortified flavored milk it was 9.3 mg kg^{-1} sample, *i.e.*, this represents an increase of 715.38%. The total concentration of Fe represented the content in the fresh milk and its incorporation into the formulation, therefore, according to the results, there is no evidence of losses during the preparation. The value of 9.3 mg kg^{-1} of iron per milk sample was within the parameters established by the Institute of Medicine as accepted for human consumption and can supplement part of the recommended levels for children between 8 and 11 years of age (8 mg of iron/day) if they drink

250 g of milk (Institute of Medicine (US) Panel on Micronutrients, 2001), which in this study represents 2.33 mg of Fe in 250 g of fortified flavored milk.

Furthermore, the iron content in the fortified flavored milk in the present study had a lower value in comparison with some results of previous investigations, such as that of (Gupta et al., 2015b), who obtained concentrations of 25 mg L^{-1} of iron in a mixture of cow's and buffalo's milk in a 1: 1 ratio from the fortification with microcapsules prepared with iron salts. Kwak et al. (2003) fortified milk with iron coated with polyglycerol monostearate at a concentration of 100 mg L^{-1}. They evaluated the differences between encapsulated and non-encapsulated iron showing that the non-encapsulated iron had a stronger metallic taste even after one day of storage, however, with encapsulated iron it did not show a significant difference at 3 days of storage. On the other hand, Abbasi & Azari (2011) fortified microencapsulated iron in milk, showing good sensory acceptability and similarity to the control at low iron concentrations (7 mg L^{-1}).

According to the physicochemical analyzes of the sample during 14 days shown in Table 6, they indicated that these samples could remain relatively constant, except for the titratable acidity that increased gradually and may be related to the increase in the microbial load and therefore the decrease in the shelf life. From the results of the microbiological analysis shown in Table 7, the number of coliforms was below 3 MPN mL^{-1} during the 14 days and complies with what is allowed by the NTP 202.189.2004 (Instituto Nacional de Calidad, 2020) with respect to the microbiological requirement of a flavored milk, however, for the number of aerobic mesophilic, it could be seen that the useful life of the product was approximately 5 days because from that moment on the product exceeded the recommended number of 50000 CFU mL^{-1}.

Table 6. Results of physicochemical parameters for 14 days for fortified flavored milk.

Days	Acidity (°D)	Density (kg L^{-1})	Viscosity (cP)	pH	Proteins (g 100 g^{-1})	Fat (g 100 g^{-1})	Total solids (%)
0	11.75	1054.4	4.18	6.54	3.86	3.28	16.57
2	10.92	1053.6	4.24	6.68	3.82	3.28	16.55
5	13.31	1055.9	4.84	6.69	4.05	3.08	16.37
14	19.00	1053.4	5.22	6.62	4.07	2.72	16.13

°D: Dornic grades. cP: Centipoise.

Table 7. Microbiological analysis of fortified flavored milk.

Microbial Agent	Unit	NTP 202.189.2004	Day 0	Day 2	Day 5	Day 9	Day 14
Aerobic mesophilic	CFU mL^{-1}	50000	13000	30000	45000	90000	180000
Coliforms	MPN mL^{-1}	10	< 3	< 3	< 3	< 3	< 3

CFU: Colony Forming Units. MPN: Most probable number.

4 Conclusions

The sensory acceptability of fortified flavored milk was significantly influenced by the concentration of Fe incorporated into the formulation as well as the interaction between Fe concentration and chocolate flavor. The physicochemical characterization of the flavored fortified milk indicated a higher content of iron and vitamin C, seeing that the latter could influence a greater absorption of iron. In addition, the shelf life of the product was 5 days, influenced by an increase in the microbial load, according to the number of mesophilic aerobes. Therefore, this study may offer a flavored fortified milk alternative as a vehicle for heme iron, with good sensory acceptance and future availability of this type of product for children.

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