A Study of Factors Affecting Iron Uptake from a Functionalized Hibiscus Beverage

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Keywords: iron deficiency, Hibiscus sabdariffa, iron bioaccessibility

Abstract:

Iron deficiency accounts for over 50% of the world's anaemia burden and it is widely prevalent in low- and middle-income countries. In response to the menace of iron deficiency in Sub-Saharan Africa, a commonly consumed beverage, the vibrantly red aqueous extract of the calyces of Hibiscus sabdariffa, has been functionalized. To determine the conditions that could potentially result in the most iron uptake by consumers of the functional beverage, the present study evaluated the effect of the factors that could influence the bioaccessibility of its iron content in the gastrointestinal (GI) tract.

Hibiscus beverage was fortified to contain, 6 mg iron per 250 mL of the beverage, by adding O.358 mM solution of ferrous sulphate salt to top up the native iron content determined to be 0.93±0.19 mg/ 250 mL. Also, a competing chelating agent - disodium EDTA was added to increase the bioaccessibility of iron from the beverage. Previous results showed the feasibility of releasing iron from the iron-polyphenol complex formed during the digestion of plant "foods", with the addition of disodium EDTA.

The effect of changes in pH values of the beverage, to mimic different parts of the GI tract (pH 1.5 to 7.5 in incremental steps of 1), on the iron-polyphenol complex formed and the release of the iron with added disodium EDTA was investigated. Next, the molar concentration ratio of the iron to disodium EDTA was varied at 1:0, 1:1, 1:2 and 1:3 to evaluate the effect of the concentration of the competing chelating agent on the quantity of iron that was released. Absorbance peaks at 550 nm, corresponded to the iron-polyphenol complex formed. Complex formation that occurs at elevated pH was reduced by added disodium EDTA, indicating iron release, which made iron more bioaccessible. A 1:2 molar ratio of EDTA to iron gave the optimum increase in iron bioaccessibility from the Hibiscus beverage.

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ABSTRACT

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INTRODUCTION

Iron deficiency (ID) remains a very common nutritional deficiency around the world [1], and its prevalence is widespread among many vulnerable groups including children under the age of 5 and women of reproductive age, particularly those in low- and middle-income countries (LMICs) [1] [2] [3]. ID is a leading cause of anemia, it accounts for 50% of anemia cases globally [1] and in 2019, the prevalence of anemia worldwide was reported to be 22.8% (about 1.74 billion people) with Western and Central Sub-Saharan Africa (SSA) as well as South Asia regions baring most of the burden [4] [5]. This suggests that to meet the Global Nutrition Target 2: halve the prevalence of anemia among vulnerable groups by 2025 [4], there must be targeted interventions within these high-burden regions. Consequently, the target population for this study is Sub-Saharan Africa, more so because groups vulnerable to IDA remain disproportionately represented within the region and by extrapolation these numbers are only expected to grow over the years [3] [6].

The effects of iron deficiency anemia (IDA) on affected populations can be quite devastating, both IDA and ID are leading contributors to the global disease burden [2]. The consequences of ID and IDA include impaired cognitive development in children, unwanted pregnancy outcomes, higher incidences of infant and maternal mortality and decreased physical and mental work capacity which have serious implications on the economic situation of families and populations [2] [7] [8].

Among every population group, inadequate dietary iron intake has been identified as a primary cause for ID [9] however, in LMICs the prevalence of ID can also be attributed in part to reduced iron uptake from their predominantly plant-based diet [3] [8]. This diet is characterized by low iron absorption/ low iron bioaccessibility owing to the presence of inhibitors like phytates and polyphenols which are reportedly

abundantly present in the plants [9] that are the major ingredients in their staple foods. These inhibitors form chelation complexes with the non-heme iron associated with plant-food sources which makes them unavailable to be absorbed by the body [10]. Therefore, to successfully address the prevalence of ID and IDA in Sub-Saharan Africa, a region made up of LMICs, there is a need to tackle both issues: inadequate dietary iron intake and reduced iron uptake stemming from the dependence of most of the population on plants as their primary food source.

The Copenhagen consensus declared the fortification of stable foods as the most cost-effective strategy to curb the prevalence of micronutrient deficiencies in LMICs and thus improve the economies of these countries [11]. Consequently, there are many studies and interventions that have focused on addressing the challenge of inadequate dietary iron intake to combat ID and IDA through the large-scale fortification of staple foods in Sub-Saharan Africa [7] [12] [13]. There are also some studies that have focussed on increasing iron uptake from plant-based staple foods consumed within the region though most were not large-scale ID/ IDA interventions. However, there are very few studies that have focused on both inadequate iron intake and reduced iron uptake from these staple foods specifically for large-scale ID/IDA interventions in Sub-Saharan Africa [13]. In response therefore, the overarching objective of this study is to functionalize a Sub-Saharan African staple food so that it can provide 30% of the recommended daily allowance (RDA) for women of reproductive age: 18 mg/day, to consumers and ensure also that the iron in the food is bioaccessible during digestion.

Indeed the epidemiology of IDA within the Sub-Saharan African context is particularly unique due to factors such as overpopulation, dietary preferences, poor infrastructure, limited resources, conflict, HIV and limited access to health services which predisposes the region to this deficiency, other micronutrient deficiencies and indeed all forms of malnutrition [3] [6] [14] [15]. Consequently, as recommended in the report of an expert workshop – Iron for Africa in 2017, iron fortification initiatives which are adapted to the local context and consumption patterns of the targeted population groups could be a more successful approach to reduce the prevalence of ID within the region [15]. For this study, a commonly consumed beverage in Sub-Saharan Africa, the bright red aqueous extract of the calyces of Hibiscus sabdariffa, is considered for its potential as a viable, logical, and sustainable intervention strategy to cover gaps in other iron fortification initiatives within the target region.

The Food Vehicle

Hibiscus sabdariffa plant which is also known as roselle, is believed to be indigenous to West Africa but it can be found in many countries in the tropical and sub-tropical regions of the world [16] [17]. Recently, the plant has gathered some interest because of its economic potential, since almost every part of the plant is useful [17]. The calyces are considered its most useful part with beneficial products including food colorants, jellies, juices, and jam [17] [18]. The cold beverage which is extracted from the calyces is regarded as a staple in homes in many countries in Sub-Saharan Africa as well as other tropical countries [17] [19], it is also commercially produced as a substitute to conventional beverages because of its refreshing taste and the recent high demand for natural products [18]. The plant parts have demonstrated medicinal and nutritional characteristics [18] [19], and have even been considered as a potential source of iron to fight ID and IDA [20] [21]. However, like many of the staple foods in SSA, Hibiscus sabdariffa has also been reported to have iron inhibitors inherently present within it, the plant is rich in polyphenols which also impart therapeutic benefits to the plant [19] [22]. Consequently, the process of functionalizing the Hibiscus beverage offers an opportunity to address the two factors that were highlighted earlier as contributors to the prevalence of ID and IDA in Sub-Saharan Africa: low iron intake and reduced iron uptake from plant food sources. Furthermore, this process could then be adapted/adopted to fortify other plant-based foods that are rich in polyphenols with iron.

The Strategy

The Hibiscus beverage was functionalized by fortification to provide 6 mg/day of iron which is 30% of the targeted iron RDA of 18 mg/day. Thereafter, the beverage's functionality was further enhanced by increasing the bioaccessibility of its iron content (iron uptake). Some earlier studies reported an increase in iron bioaccessibility after a competing chelator was introduced into a fortified plant-food system [23], however, in these studies the focus was on the prevention of iron-phytate chelation complex formation not iron-polyphenol chelation complex formation. In a comparable study to the present study, McGee and Diosady (2017) demonstrated the effectiveness of using a competing chelator, disodium EDTA (Na_2EDTA), to increase the iron uptake from an iron fortified black tea with a very high polyphenolic content [10]. A similar approach is adopted in this study, however, in this study added iron as well as native iron are considered. Specifically, the objective in this paper is to study the factors that might influence iron uptake form the functionalized Hibiscus beverage system.

MATERIALS AND METHODS

Materials

Plant Samples

Hibiscus sabdariffa calyces were sourced from the National Horticultural Research Institute, Ibadan, Oyo State, Nigeria. Samples were air dried and milled from source, they were then kept in airtight containers pending use.

Reagents and Chemicals

For this study, unless otherwise stated, all chemicals used were reagent grade. The competing chelator, disodium EDTA, was purchased from BioShop Canada Inc. (Burlington Ontario, Canada). The gallic acid used for the model reaction and the fortificant, ferrous sulphate heptahydrate, were purchased from Sigma-Aldrich Canada Co. (Oakville Ontario, Canada). For pH adjustments, sodium hydroxide and hydrochloric acid (1N) were purchased from Caledon Laboratories (Caledon Ontario, Canada) and BioShop Canada Inc. (Burlington Ontario, Canada) respectively. Reverse osmosis purified (RO) water was used for all experiments.

Methods

Hibiscus Calyces Extraction Process

The following procedure had previously been confirmed by benchmarking as one that produced a sample closest in consistency and taste to a commercially available Hibiscus beverage in Nigeria, West Africa. Briefly, to make a 100 mL of the beverage, 12.5 g of the dried and milled calyces was dispersed in 87.5 mL of reverse osmosis (RO) water. Next, the mixture was brought to a boil in about 30 minutes. The sample was allowed to cool down before straining using a 3-inch mini-strainer. Collected samples were then vacuum filtered using a vacuum filtration setup which included a 250 mL Pyrex Buchner flask, a porcelain Buchner funnel to hold 11 mm filter papers and a set of filter cones to create an airtight sealed connection between the flask and the funnel. Filtrates were made up to 100 mL with RO water in a volumetric flask. Using a VWR scientific model 8000 pH meter the pH value of the Hibiscus beverage was determined to be 2.62± 03. The beverage samples were stored in a refrigerator pending analysis.

Iron Fortification by Direct Mixing

To meet the target of supplying 30 % of the recommended daily allowance (RDA) of iron for women of reproductive age, 6 mg was achieved by adding 5 mg (0.358 mM) of ferrous sulphate heptahydrate to in 250 mL of the beverage, since the Hibiscus extract naturally contained ~4 mgL^{-1} iron (~0.07 mM).

Iron-Polyphenol Complex Quantification

Adapting a traditional colorimetric assay test for phenolic compounds - the ferric chloride test [24], a Tecan infinite 200 pro microplate reader was used to measure the absorbance of prepared samples. NaOH and HCl (if required) were used to adjust all samples to pH 6.5 ± 0.2 to mimic the pH of the site of iron absorption in the small intestine. The absorption was scanned between 400 nm and 800 nm and the absorption of the iron-polyphenol complex peak was measured at 542 nm to 561 nm [25]. Iron-complex calibration curves were generated by preparing standards with a gallic acid concentration of 0.3 g/L to match the phenolic content of the beverage. Standards were adjusted to pH 6.5 ± 0.2.

There were 2 sets of samples, and each set of experiments was performed in triplicates.

Hibiscus beverage pre-fortification

Net absorbances were calculated by zeroing using water as a blank, and ferrous sulphate heptahydrate (used in place of ferric chloride) concentrations of from 0.01 mM to 0.05 mM in steps of 0.01 mM.

Fortified Hibiscus beverage

Similarly, a calibration curve was prepared using 0.1 M - 0.5 M in steps of 0.1 M, to encompass the expected concentration of 0.428 mM. iron in the beverage after fortification.

Effect of Different Na_2EDTA Concentrations on Iron Bioaccessibility

Previously, it had been demonstrated that disodium EDTA is able to release iron from the iron-polyphenol complex, making the iron available for absorption into the bloodstream, where it can potentially influence the iron status of the body. The effect of different concentrations of this competing chelator on the bioaccessibility of iron in the Hibiscus beverage was investigated.

Briefly, two sets of experiments were carried out as follows: aliquots of samples of the Hibiscus beverage before and aliquots of samples of the beverage after fortification were spiked separately with four different concentrations of Na_2EDTA salt to give final iron to Na_2EDTA molar concentration ratios 1:1, 1:2, 1:3 and 1:4.

For Hibiscus beverage with no added iron, the final concentrations of NA_2EDTA in the different aliquots were 0.07 mM, 0.14 mM, 0.21 mM and 0.28mM, while for the fortified beverage, the final NA2EDTA concentrations for the aliquots were 0.43 mM, 0.86 mM, 1.28 mM and 1.71 mM. Samples were analysed as described above.

From the absorbance scans generated, the effect of varying the concentration of the competing chelator – disodium EDTA on the iron-polyphenol peaks was evaluated.

Effect of pH Variation on Iron Bioaccessibility

Another factor of interest is the effect of pH on the bioaccessibility of iron in the beverage, as an indication of iron bioavailability in the GI tract

Four different samples were prepared, unfortified Hibiscus beverage, unfortified Hibiscus beverage with added disodium EDTA (x3 the molar concentration of the native iron), fortified Hibiscus beverage and fortified beverage with added disodium EDTA (x3 the molar concentration of the total iron content). The pH of an aliquot of each sample was sequentially adjusted using 0.1N NaOH, 1N NaOH, 0.1N HCl and 1N HCl as needed to a preselected pH between 1.5 and 7.5 in steps of 1. A Tecan infinite 200 pro microplate reader was used to measure the absorbance of each aliquot at 550nm. The absorbance of the unadjusted aliquot sample was also determined. The absorbance data were used to estimate the bioaccessibility of iron across the digestive system.

RESULTS AND DISCUSSION

Iron-Polyphenol Complex Quantification

Figures 1a. and 1b. below are samples of the absorbance scans of the unfortified Hibiscus beverage and the fortified Hibiscus beverages.

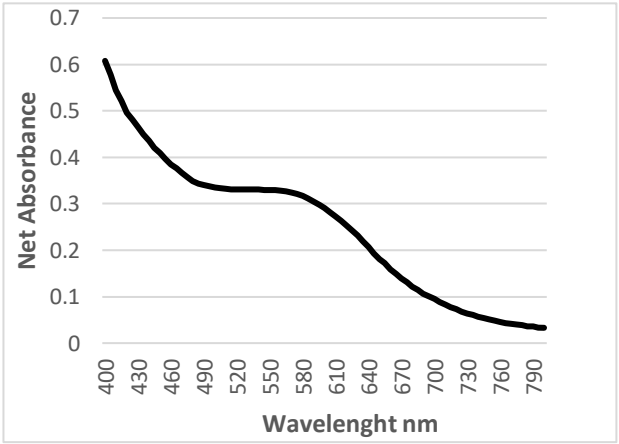

Figure 1a.: A sample absorbance scan of unfortified hibiscus beverage at pH 6.5

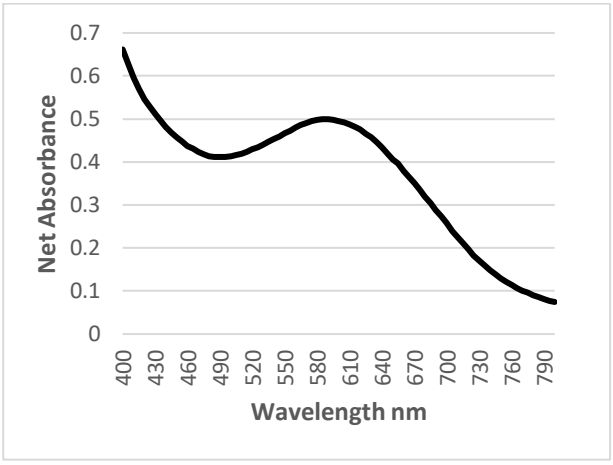

Figure 1b.: A sample absorbance scan of fortified hibiscus beverage at pH 6.5

The peak in Figure 1b. is more pronounced than the peak in Figure 1a. and this could be explained by the fact that the fortified beverage would have more of the iron-polyphenol complex formed than the unfortified beverage. Notably, there was a slight shift of the peak of the fortified beverage which only occurred after the absorbances were zeroed, however, this was similar to what was reported in a previous study [26]. A more simplified and direct form of the method developed by McGee (2018) was used to estimate quantities of the iron-polyphenol complexes formed by both the native and added iron: ferrous sulphate heptahydrate.

Using the calibration curves generated, as a measure of their absorbances, estimates of the iron-polyphenol complexes formed in 3 different samples of each beverage were reported as ferrous sulphate in gallic acid equivalents (mM Fe_2SO_4/GAE), see Table 1a. and Table 1b. below:

Table 1a: Iron-polyphenol complexes formed in Hibiscus beverage (pre-fortification)

Sample	Net Absorbance at 550nm	Iron-polyphenol Complex (mM Fe2SO4/GAE)
1.	0.3121	0.1829
2.	0.3363	0.1970
3.	0.3382	0.1981

Table 1b: Iron-polyphenol complexes formed in fortified Hibiscus beverage

Sample	Net Absorbance at 550nm	Iron-polyphenol Complex (mM Fe2SO4/GAE)
1.	0.5178	0.3118
2.	0.4452	0.2684
3.	0.4566	0.2752

The estimated values recorded in Table 1a. are well above the expected range of 0.07mM for the native iron, this suggests that there are other interactions besides the iron-polyphenol complex which also occur within the targeted wavelength range: 542 nm to 561 nm. However, the values recorded for the fortified beverage are much closer to the expected value of 0.358 mM, these estimates also suggest that since well over two thirds of the added iron is bound in the complex the iron would not be available for absorption. This further supports the need to prevent the interaction between iron and polyphenols. The readings for both beverages were recorded at the same wavelength to allow for better comparison.

Effect of Different Na_2EDTA Concentrations

The absorbances of both set of samples at a pre-selected wavelength of 550nm were used to generate Figures 2a. and 2b.

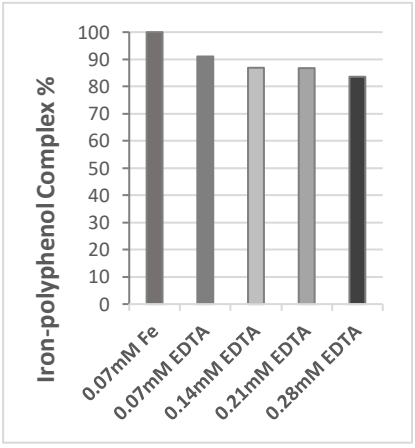

Figure 2a.: Relative percentages of iron-polyphenol complexes in Hibiscus beverage pre-fortification

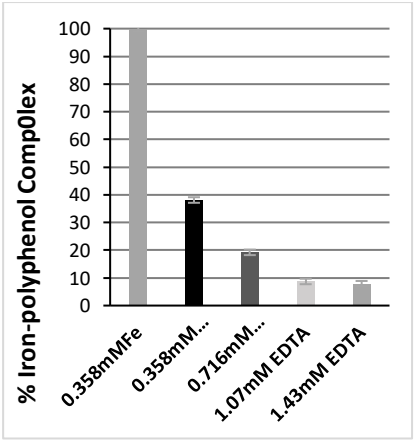

Figure 2b.: Relative percentages of iron-polyphenol complexes in Fortified Hibiscus beverage

Figure 2a. illustrates the effect of spiking the unfortified Hibiscus beverage sample with Na_2EDTA incrementally (1:0; 1:1; 1:2; 1:3 and 1:4). From this figure the most significant drop in the relative percentage of the iron-polyphenol complex present in the samples was at the molar ratio 1:1, thereafter, the drop, which represents iron release, wasn't as significant. It can also be inferred that by the molar concentration ratio: 1:2, equivalent to about 87%, most of the iron to be released had been released, the ratios 1:3 and 1:4 were equivalent to approximately 87% and 84% respectively.

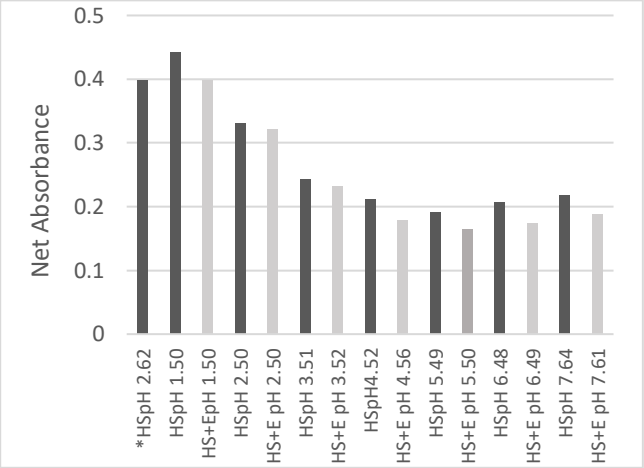

Figure 3a.: Absorbances of unfortified Hibiscus beverages with/without NA_2EDTA due to sequential pH variation at 550nm

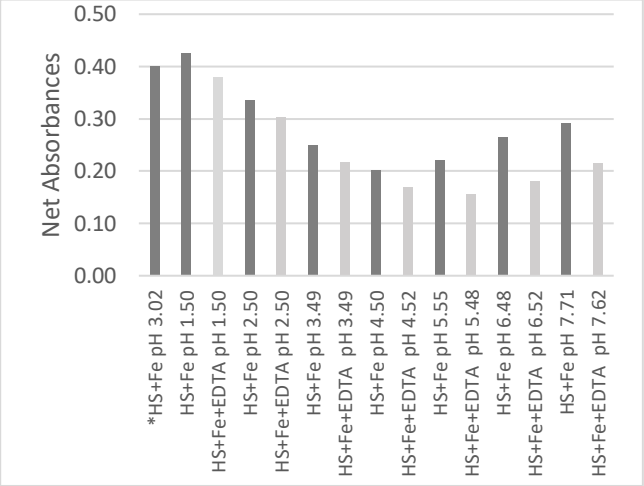

Figure 3b.: Absorbances of fortified Hibiscus beverage with/without Na2EDTA due to sequential pH variation at 550nm

The percentage of iron released is not as high as expected, with only 16% of the iron seemingly made bioaccessible. However, it must be recalled that earlier, it had been suggested that the model being adopted for this study might not accurately quantify the interaction between the native iron present in the beverage and the plant polyphenols. All estimated values are based on the assumption that the only interaction present is that between the native iron and polyphenols.

Figure 2b. shows a more promising result. After the initial spike, only at ratio 1:1, there was already a 62% decrease in the iron-polyphenol complex present which means a lot of iron is more bioaccessible. A continued increase in the molar concentration of Na_2EDTA produced further peak drops. However, from the figure, it can be inferred the relationship is not linear. From Figure 2b, it can also be seen that by adding Na_2EDTA it is possible to release up to 90% of the iron previously bound as iron-polyphenol complexes in the fortified beverage, suggesting that most of the added iron would be more bioaccessible.

Effect of Different Na_2EDTA Concentrations

As earlier stated, understanding the dependence of iron biocessibility is pertinent as this gives some insight into how the iron and the complexes formed might behave in the GI tract.

Figures 3a. and 3b. show a similar trend for both the fortified and unfortified beverage, including an obvious increase in absorbance after the pH value is dropped to 1.5. Thereafter a steady decrease is seen in the absorbances of all four samples within the more acidic pH range. Reportedly, a less acidic environment promotes the formation of iron-polyphenol complexes [10] [25] so it can be inferred that at the lower pH range, 1.5 – 4.5, the absorbances recorded are not as a result of iron-polyphenol complex formation. However, it is worth further investigation to know if the other interactions observed still influence the bioaccessibility of iron. The effect of disodium EDTA is more obvious in the less acidic range suggesting that it can compete more favorably for the iron within this range therefore, by inference, it would influence the bioaccessibility of iron better in this pH range: 5.5-7.5. Also, interestingly both graphs record similar absorbance values until they get to pH 5.5 which might suggest that the added iron doesn't contribute to whatever is happening in the lower pH range.

CONCLUSION

An increase in iron uptake is important to curb the prevalence of ID and IDA in Sub-Saharan Africa because majority of the populace are dependent on a plant-based diet. Therefore, there is the need to develop strategies that are sustainable within the Sub-Saharan African context as well as strategies that could possibly contribute to improving the iron status of the populace, particularly the most vulnerable to ID and IDA.

Functionalizing *Hibiscus sabdariffa* beverage has the potential to fill a gap yet to be filled by other iron-food fortification initiatives targeted at the Sub-Saharan African population. The study of the factors that influence the bio-accessibility of both the native and added iron, ferrous sulphate heptahydrate, has offered a technical understanding of how to prevent the inhibiting actions of the polyphenols inherently present in the Hibiscus beverage on its iron content.

Disodium EDTA added in excess relative to the iron content of the Hibiscus beverage has demonstrated promising results for improving iron bioaccessibilility. The 1:2 molar concentration ratio of iron to the competing chelator releases most of the iron previously bound in the iron-polyphenol complex formed, thus it provides the iron in an absorbable form at the site of iron absorption, the small intestine, during digestion. More statistical studies would be required to state this categorically, but this conclusion confirms the previous findings of McGee and Diosady.

The findings of the pH dependence of the bioaccessibility of the iron as the beverage travels through the GI tract provides information on future work to make the beverage more functional as a viable iron source for the target population.

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