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A rapid method for iron determination in fortified foods

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Abstract

The objective of this study was to develop and evaluate a rapid method for iron determination in fortified and unfortified foods. Method: samples were mixed with an iron-extracting solution (1.2 M HCl, 0.6 M trichloroacetic acid, and 0.7 M hydroxylamine hydrochloride) and heated in a boiling water bath for 15 min. The mixtures were cooled and filtered. The filtrate was mixed with a chromogen reagent (0.03% bathophenanthroline disulfonic acid in 3 M sodium acetate). Iron concentration was determined by measuring absorbance at 535 nm. The accuracy of the rapid method was validated by comparing results to a standard laboratory method for iron determination. Results: the rapid method produced accurate results for the majority of the food samples tested, including wheat flour fortified with $FeSO_4$, electrolytic iron, NaFeEDTA, Ferrochel® or ferrous fumarate; powdered drink mixes, and enriched rice. However, results obtained using the rapid method were significantly lower than results obtained using the standard method for the enriched cornmeal (30.04 vs. 33.16 μg Fe/g; $P=0.0118$) and the enriched flour (41.90 vs. 47.28 μg Fe/g; $P<0.0001$). Conclusion: The rapid method is simple, inexpensive, and suitable for monitoring iron concentrations in fortified foods.

Keywords: Iron; Analysis; Iron determination; Iron fortification; Colorimetric analysis

1. Introduction

Iron deficiency is a serious health problem affecting a large proportion of the world's population (MacPhail & Bothwell, 1992). Its most severe form, iron deficiency anemia, is reported to have a higher overall cost to society than any other disease except tuberculosis (UNICEF/WHO, 1999). Food fortification programs are cost effective means for reducing the prevalence of iron deficiency (Cook & Reusser, 1983; Yip 1997).

The effectiveness of a food fortification program depends on the consistent and uniform addition of iron compounds to appropriate food vehicles, such as flour and milk-based powders, which are widely consumed by the target population (Mejia, 1994). Established laboratory methods for accurately determining iron levels in foods often require expensive equipment and are time consuming and potentially hazardous. In many countries that lack a highly developed food processing industry, food fortification is the responsibility of small processing companies. These companies often lack the laboratory facilities and trained personnel required for monitoring fortification levels with standard laboratory methods. Thus, these companies can benefit from a rapid, reliable and simple method for determining the iron content of foods.

The goal of this study was to develop a rapid method for determining the iron content of fortified foods. Supporting objectives were: (1) to develop a safe, simple, and rapid method that allows for both visual and spectrophotometric readings; and (2) to validate the accuracy of this rapid method by comparing it to a standard laboratory method for iron determination.

The method was adapted from a spectrophotometric method used in the determination of serum iron concentration in human blood (International Committee for Standardization in Hematology, 1978). It measures the concentration of iron in extracts from foods. Food samples are heated in an iron-extracting solution, filtered, and mixed with bathophenanthroline disulfonic acid. Bathophenanthroline disulfonic acid, in the presence of a reducing agent, complexes with the extracted

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iron to produce a red color (McBride, 1980), thus, allowing for spectrophotometric or visual readings.

2. Materials and methods

2.1. Materials

Glassware was washed, soaked in 1 M HCl for at least 4 h, and rinsed with deionized water. Chemicals were obtained from Sigma Chemicals (St. Louis, MO), Fisher Scientific (Fairlawn, NJ) or Mallinckrodt Chemicals (Paris, KN) unless stated otherwise and were grade ACS or better. All water used in preparation of reagents was double de-ionized.

All flour, rice and cornmeal samples were purchased at local supermarkets in Ithaca, NY. The vanilla and chocolate powdered drink samples were collected in Lima, Peru. A list of food samples is shown in Table 1. The manufacturers and iron contents of the fortificants used for fortifying unenriched flour are shown in Table 2.

2.2. Preparation of fortified flour

Ferrochel®, NaFeEDTA, ferrous fumarate, ferrous sulphate or electrolytic iron was added to unenriched flour at a rate calculated to yield a total iron concentration of 44 mg Fe/kg flour. The flour and iron fortificant were mixed thoroughly in a glass mixing bowl with a wire (plastic-coated stainless steel) whisk. Each flour–iron mixture (iron fortified flour) was then stored in a Zip-Loc® bag at room temperature. On the day of analysis, each flour/bag was shaken for at least a minute to mix the fortified flour. Samples were withdrawn from different areas within the bag.

2.3. Reagents

2.3.1. Iron-extracting solution

In a 1-l volumetric flask, 50 g hydroxylamine monohydrochloride was first dissolved in water. Concentrated HCl (100 ml) and 100 g of trichloroacetic acid were added and the solution was brought to volume with water.

2.3.2. Chromogen solution

Bathophenanthroline disulfonic acid (BPDS; 300 mg) was dissolved in 10–15 ml of water in a 1-l volumetric flask. The solution was brought to volume with 3 M sodium acetate (Miller, Smith, Kanner, Miller, & Lawless, 1994). The solution was stored in an amber colored glass bottle to minimize lightcatalyzed deterioration.

2.3.3. Acids for standard laboratory method

Concentrated nitric acid (trace metal grade, Fisher Scientifics) was redistilled by evaporating at a sub-boiling temperature. A 50/50 mixture of concentrated nitric acid and 70% double-distilled perchloric acid (CAS. No. 7601-90-3, G Fredrick Smith Chemicals, Powell, OH) was prepared by mixing the two acids in equal volume. All acids were stored in Teflon bottles.

2.4. Development of the rapid method (heating time trial)

Samples of enriched cornmeal, enriched rice and enriched flour (1 g each) were placed in individual test tubes (16×100 mm; Fisher Scientific) and 10 ml of the

Table 1
Food samples analyzed in the study

Food samples (location of manufacture)	Location where samples were obtained
Uncle Ben's Converted enriched rice (Houston, TX)	Wegman's Stores Ithaca, NY
Unenriched long grain rice (China)	Win-Li Market Ithaca, NY
General Mills Gold Medal Enriched all-purpose flour (Minneapolis, MN)	Wegman's Stores Ithaca, NY
New Hope Mills unenriched, unbleached bread flour (Moravia, NY)	P&C Store Ithaca, NY
Quaker Oats enriched cornmeal (Chicago, IL)	Wegman's Stores Ithaca, NY
New Hope Mills unenriched yellow cornmeal (Moravia, NY)	P&C Store Ithaca, NY
Fortified, powdered drinks containing dry milk powder, soy flour, rice flour, and added vitamins and minerals (Lima, Peru)	Lima, Peru

Table 2
Iron content and manufacturers of fortificants

Iron Fortificant	mg Fe/g compound	Manufacturer
$FeSO_4 \cdot 7H_2O$	192	Sigma Chemicals (St. Louis, MO)
Electrolytic iron	967	ADM Arkady (Olathe, KS)
NaFeEDTA	128	Sigma Chemicals (St. Louis, MO)
Ferrochel®	204	Albion Laboratories (Clearfield, Utah)
Ferrous Fumarate	333	Sigma Chemicals (St. Louis, MO)

iron-extracting solution was added. The test tubes were then covered with Parafilm® and thoroughly mixed using a vortex mixer. Once mixed, the Parafilm® was removed. A boiling water bath was constructed using a 600 ml beaker and 150–200 ml of water. Glass boiling beads were added to smooth the boiling of the water. The hot plate was set to high and the water was brought to a rapid boil. The test tubes containing the food samples in iron-extracting solution were weighed on a balance and their weight recorded. The test tubes were then placed in the boiling water bath for 5, 10, 15, 30, or 45 min. A zero-time sample, not placed in the boiling water bath, was also included. Timing did not begin until the water returned to boiling. Care was taken to ensure that water, from the water bath, did not enter the test tubes. After the allotted time, the test tubes were removed from the boiling water bath and placed in a beaker containing water at room temperature. The test tubes were allowed to cool for 15 min. After cooling, the exterior of the test tubes was wiped dry and the test tubes were weighed individually on a balance. Initial volumes of the test tubes were restored by adding water. The test tubes were covered with Parafilm® the samples were mixed gently on a vortex mixer. An aliquot of each sample was filtered into a clean test tube using filter paper (Cat. No. 1001 110, Whatman INC, Clifton, NJ). Iron standards were prepared, in duplicate, by diluting a stock iron solution (atomic absorption standard, 1000 μg Fe ml in 1% HCl; Sigma Chemicals) with iron-extracting solution to achieve the following concentrations of iron: 0, 1, 2, 3, 4, 5, 8, and 10 μg/ml. A 1-ml aliquot of the iron standard or the sample filtrate was mixed with 3 ml of the chromogen solution. After standing at room temperature for 15 min, absorbance was measured spectrophotometrically at 535 nm. A standard curve was constructed with the iron standards using linear regression and the food sample readings were plugged into the regression equation to determine their iron concentrations. A filtrate blank was also prepared for each food sample by adding 3 ml of 3 M sodium acetate without the chromogen reagent (BPDS) to 1 ml of the food sample filtrate. The reading obtained from the blank was then subtracted from the reading obtained from the corresponding food sample filtrate containing the chromogen reagent.

2.5. Validation of the rapid method

Food samples listed in Table 1 and the fortified flours were analyzed with the rapid method and a standard laboratory iron determination method. Sample sizes were adjusted based on the expected sample iron concentration. After mixing with the extracting solution, samples were heated in the boiling water bath for 15 min. Visual and spectrophotometric readings were taken for all samples. Visual readings were accomplished by holding the sample/chromogen solution next to the set of iron standard/chromogen solutions. The iron concentration was determined by matching the color of the sample to the color of the iron standard.

The standard laboratory method was a modification of an AOAC method (AOAC: 14.013, 1984). Food samples (0.5 g) were placed in 25×150 mm (50 ml) Pyrex® test tubes (Corning, NY). One to two milliltres of concentrated HNO_3 was dispensed into each tube and the tubes were covered with plastic wrap. The tubes were placed in a fume hood and samples were allowed to partially digest overnight at room temperature. The tubes were then placed in a digestion block located in a $HClO_4$-rated fume hood. The temperature of the digestion block was raised to between 50 and 120 °C and samples were heated until dry. Once the samples had dried, another 1–2 ml of HNO_3 was added. The heating continued until the second aliquots of HNO_3 had completely evaporated. At this point, the temperature of the block was adjusted to 150 °C. The previous HNO_3 digestion step was repeated until the heated samples no longer gave off red brown fumes of nitrous oxide, and the samples were light brown to yellow in color. Then, 0.5 to 1.0 ml of a 50/50 (vol./vol.) mixture of HNO_3/$HClO_4$ acids was added to the samples. The temperature of the digestion block was increased to 180 °C and the color of samples was monitored for about 1 h. If samples began to darken, the addition of HNO_3/$HClO_4$ was repeated and the samples were allowed to digest for an additional 1–2 h at 180 °C.

When the digested samples became clear to light yellow in color, the temperature of the digestion block was increased to 240 °C and samples were heated to dryness. The tubes were then removed from the block and allowed to cool to room temperature. The residue in each tube was dissolved in 0.5 ml of concentrated HCl and diluted to 10.0 ml with 5% HNO_3. The iron contents of the residues were analyzed with an inductively coupled plasma/atomic emission spectrophotometer (ICAP 61E Thermal Jarrell Ash Trace Analyzer, Jarrell Ash Co. Franklin, MA).

2.6. Experimental design

All experiments were repeated eight times. The data obtained for the heating time trial were plotted and the optimum heating time was determined. Measurements obtained spectrophotometrically using the rapid method were compared to measurements obtained using the standard laboratory method. The experimental design for comparing the rapid method to the standard laboratory method was a balanced factorial design. When comparing visual readings to spectrophotometric readings with the rapid method, a balanced factorial design with blocking for experimental day was employed. Blocking was necessary because each sample within an

experimental day was subjected to both visual and spectrophotometric readings and different replicates were performed on different days. Tukey's multiple comparison was performed ($\alpha = 0.05$) when the ANOVA was found to be significant.

3. Results

3.1. Development of rapid method (heating time trial)

Fig. 1 illustrates iron concentrations calculated from the rapid method using the filtered extracts obtained at the different heating times for enriched flour, enriched cornmeal, and enriched rice. Large variations in iron concentration measurements in samples heated for 5 min were observed for enriched flour or rice. Iron concentrations in all three samples reached a maximum between 10 and 15 min of heating. Browning of the extracts became excessive at 30 min and beyond. Therefore, 15 min of heating was determined to be sufficient for optimum iron extraction for these samples.

3.2. Validation of rapid method

3.2.1. Rapid method (spectrophotometric reading) vs. standard laboratory method

Iron content of food samples was determined by measuring the iron concentration in the filtered extracts after 15 min of heating. Table 3 shows the iron content of Peruvian powdered drink samples, cereal products and fortified flours analyzed with the rapid method and the standard laboratory method. The rapid method (spectrophotometric reading) produced accurate results that were not significantly different from the standard method for the majority of the food samples tested. However, results obtained using the rapid method were significantly lower than results obtained using the standard method for the enriched cornmeal (30.04 vs. 33.16 μg Fe/g; $p = 0.0118$) and the enriched flour (41.90 vs. 47.28 μg Fe/g; $P < 0.0001$). Both of these samples were enriched with reduced iron.

3.2.2. Visual vs. spectrophotometric reading

The chromogen reaction in the rapid method produced a color that allowed for two methods of iron quantification, i.e. visual and spectrophotometric readings. Table 3 lists the means and standard deviations of the iron concentration of food samples determined with the two methods of iron quantification. For the powdered drink samples, the spectrophotometric readings were presented with and without correcting with a filtrate blank. The visual readings of iron concentration were not significantly different from the spectrophotometric readings for most of the samples with the exception of the Peruvian powdered drinks. The visual readings of all three Peruvian powdered drinks were significantly higher than filtrate blank-corrected spectrophotometric readings (vanilla powdered drink No. 38, P-value $= 0.0046$; vanilla powdered drink No. 47, $P = 0.0001$ and chocolate powdered drink No. 67, $P < 0.0001$). Mean iron concentrations determined visually were plotted against mean iron concentrations determined spectrophotometrically and a linear regression was performed (Fig. 2). The slope of the regression line and r^2 equaled 1.07 and 0.9986, respectively.

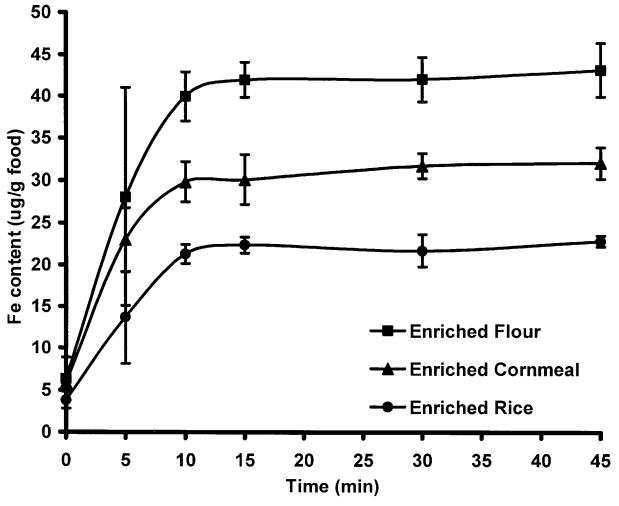

Fig. 1. Effect of heating time on measured iron concentrations in commercially enriched cereal products using the rapid method. Values on the y axis were calculated from absorbance readings of extract–chromogen mixtures. Error bars represent ±1 standard deviation, $n = 8$.

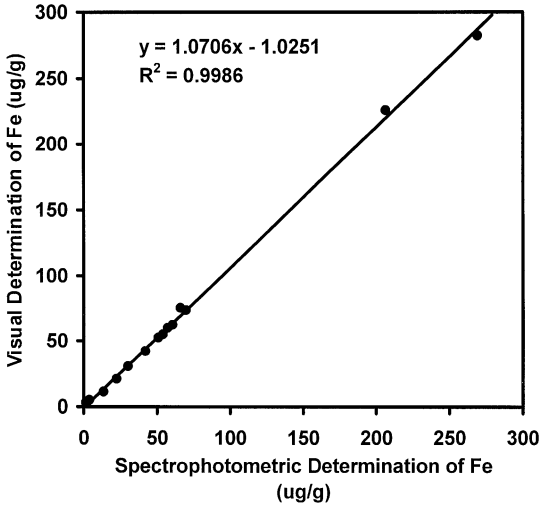

Fig. 2. Correlation of food iron concentrations determined by rapid visual and spectrophotometric methods. Mean iron concentration obtained visually was plotted against mean iron concentration obtained spectrophotometrically and a linear regression was performed. All foods listed in Table 3 are included.

Table 3
Comparison of iron concentrations in food samples determined by rapid (spectrophotometric and visual) and standard methods

Food Samples	Iron concentration (μg/g)[a]		
	Standard Method	Rapid Method	
		Spectrophotometric	Visual
No. 38 Vanilla powdered drink	66.1±0.92	66.0±2.76 (77.3±2.96)[c]	75.6±4.44[b]
No. 47 Vanilla powdered drink	266.6±6.73	269.1±6.41 (283.5±7.01)[c]	282.5±2.90[b]
No. 67 Chocolate powdered drink	209.6±4.04	206.4±9.65 (229.1±9.16)[c]	225.8±2.25[b]
Flour + NaFeEDTA[d]	59.3±11.57	60.6±13.83	62.6±14.96
Flour + electrolytic Fe[d]	57.5±9.70	57.2±2.03	60.1±0.34
Flour + ferrous sulphate[d]	62.4±38.17	69.7±54.61	73.9±56.28
Flour + ferrous fumarate[d]	48.1±9.30	50.7±3.79	52.6±4.72
Flour + Ferrochel®[d]	47.6±6.75	53.9±10.19	55.1±10.77
Flour, unenriched	13.9±0.97	13.5±1.05	11.3±3.55
Flour, commercially enriched	47.3±3.41[e]	41.9±2.11	42.2±2.52
Cornmeal, unenriched	3.6±0.38	3.8±0.16	4.9±0.05
Cornmeal, commercially enriched	33.2±1.52[e]	30.0±2.89	30.7±1.81
Rice, unenriched	1.4±0.09	1.5±0.20	3.1±2.56
Rice, commercially enriched	22.0±1.30	22.3±0.95	21.0±2.10

[a] Values are means ± standard deviations ($n=8$).
[b] Significantly higher than with the spectrophotometric reading of the rapid method. (Tukey's multiple comparison; $\alpha=0.05$).
[c] Spectrophotometric readings without subtraction of filtrate blanks
[d] New Hope Mills unenriched wheat flour fortified with the indicated form of iron fortificant.
[e] Significantly higher than the spectrophotometric reading of the rapid method. (Tukey's multiple comparison; $\alpha=0.05$).

4. Discussion

In the present study, a comparison was made between a rapid method and an established laboratory method for measuring iron concentrations of fortified and unfortified food products. In addition, different forms of iron fortificants added to unenriched flour were tested, including flour fortified with ferrous sulphate, ferrous fumarate, Ferrochel®, NaFeEDTA, or elemental iron. Three flavored Peruvian powdered drinks collected during a field trial were also included. These fortified powdered drinks were distributed to school children and members of the community as part of a Peruvian governmental food program.

It was necessary to determine the optimum heating time for the method since heating affects the accuracy of the rapid method. Heating the sample for an insufficient amount of time resulted in low iron extraction in some samples. Heating the sample for a prolonged period of time caused browning of the solution. This made accurate visual readings increasingly difficult as the browning increased. The browning of the samples may be attributed to caramelization. Caramelization is a nonenzymatic reaction that can occur when sugars are heated in the presence of acidic or basic catalysts (Miller, 1998). The rate of the caramelization can increase with increasing temperature and pH (BeMiller & Whistler, 1996). Heat treatment of the samples for 5 min resulted in much higher iron concentration readings than without heat treatment of the samples (Fig. 1). However, large variations in readings obtained from a 5-min heat treatment were observed in the rice and flour samples. Thus, it was concluded that an accurate reading could not be obtained with 5 min of heating. Readings obtained from samples after heating for 10 min began to approach the expected levels of iron content. The variation between readings taken at 10 min was considerably less than for readings taken at 5 min. The iron concentration of the samples began to level off and remained relatively constant after 10 min. Excessive browning of the samples began to occur at 30 min. Blanks were used to correct for browning in samples measured with the spectophotometer but were not helpful with the visual measurements. Fifteen minutes of heating was sufficient to extract all of the iron in most samples. Moreover, browning was not excessive after 15 min of heating.

Once the optimum heating time was determined, a variety of food samples were tested using the rapid method. Results obtained from the rapid method were compared to results obtained from the standard laboratory method. The rapid method yielded accurate values for most foods with the exception of commercially enriched flour and cornmeal. The rapid method underestimated the iron content of enriched flour and cornmeal but not the unenriched counterparts. This

might be due to the slow dissolution of the elemental iron fortificants in dilute acids (Hurrell, 1997). While the objective of this work was to determine total iron content of food samples regardless of their iron bioavailabilities, the incomplete dissolution of reduced iron under our assay conditions suggests that the bioavailability of this iron will be low. Presumably, the particle size of elemental iron plays an important role in the rate of its dissolution during the gastric phase of digestion (Shah, Giroux, & Belonje, 1977). Despite the slightly lower than expected readings from the enriched cornmeal and flour, the rapid method is sensitive enough to determine whether or not a food sample is fortified.

Some variations of iron content were observed within individual fortified flours regardless of the method of analysis (Table 3). The variation was especially large in the flour fortified with ferrous sulphate. However, since the variation was equally large in both the rapid method and the standard laboratory method, this variation was probably not a result of measurement error with the rapid method, but most likely a problem with mixing the fortificants with the flour. The large crystal size of the ferrous sulphate ($FeSO_4 \cdot 7H_2O$) made it difficult to evenly disperse the salt throughout the flour. In commercial fortification programs, exsiccated ferrous sulphate ($FeSO_4 \cdot 1.5\ H_2O$), not $FeSO_4 \cdot 7H_2O$, is used. Exsiccated ferrous sulphate is a fine powder (Nalubola & Nestel, 2000). Thus, uniform dispersion in commercially enriched flour should be more easily achieved.

The visual readings of the Peruvian powdered drinks were about 10% higher than the spectrophotometric readings. This was due to browning during the heating step. The visual readings matched closely with spectrophotometric readings when the spectrophotometric readings were not corrected using filtrate blanks (Table 3).

5. Conclusion

The rapid method described here provides an accurate measure of iron content in most fortified and unfortified foods when compared to a standard laboratory method. Quantification can be accomplished either visually or with a spectrophotometer. The r^2 value (0.99986) and 1:1 ratio (slope = 1.07) illustrates the close relationship that exists between the spectrophotometric and visual readings (Fig. 2). However, because of possible interference from browning due to heating, it is advisable to use a spectrophotometer whenever possible in order to obtain the most accurate results. Finally, the method takes a little over an hour to complete while most of standard laboratory methods for iron determination can take days to complete.

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