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# The Role of Iron and the Factors Affecting Off-Color Development of Polyphenols

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Iron deficiency affects over two billion people worldwide (Lotfi, M.; Venkatesh Mannar, M. G.; Merx, R. J.; Naber-van den Heuvel, P. *Micronutrient Fortification of Foods: Current Practices*, *Research*, *and Opportunities*; Micronutrient Initiative: Ottawa, Ontario, Canada, 1996). However, fortifying foods with highly bioavailable iron is technically challenging because of off-color and off-flavor development, catalytic degradation of vitamins, and oxidation of lipids. The role of highly bioavailable iron in the off-color development of foods and beverages is not well-understood. The goal of this research was to examine the interaction of iron with simple phenolics and polyphenols. Factors that may affect off-color development, such as pH, oxygen, temperature, and reducing and chelating agents, were evaluated as a model for food products. Our results demonstrated that the iron that reacts with the simple phenolic, catechol, to develop off-color must be in the oxidized state, and the iron is reduced in the presence of catechol. Because this is an oxidation/reduction reaction, the redox potential of all of the components is critical to the color development. Ferrous iron sources with low redox potentials and ferric iron sources with high redox potentials caused off-color development with catechol. Only polyphenols that contain *ortho*-hydroxyl groups cause off-color development with iron. All of the factors tested affect off-color development and redox potential of the system. Low pH, low oxygen, high temperature, and the presence of reducing and chelating agents inhibited off-color development. To confirm the model, foods that contained these polyphenols were evaluated for off-color development when iron was added. The foods tested reacted similarly to the models of polyphenols with iron. Off-color development was caused by oxidation−reduction interactions between ferric iron and polyphenols that contained *ortho*-dihydroxyl groups. Ferrous iron needed to be oxidized to participate in off-color development. In addition, methods identified in the models to prevent off-color development were effective in most of the food products examined. Using the ferrous form of iron and maintaining it in its reduced form by lowering pH, removing oxygen, and including reducing agents, it was possible to fortify foods with highly bioavailable iron.

**KEYWORDS: Iron fortification; color development; polyphenols; oxidation/reduction reactions; redox potential**

## INTRODUCTION

The world's most common nutritional disorder is iron deficiency anemia. Iron deficiency affects over two billion people worldwide (*1*). In developing countries, about half of all children and women suffer from anemia. Iron deficiency also affects numerous people in developed countries. In the United States, for example, 7.8 million teenage girls and women of childbearing age are iron deficient, as well as 700 000 toddlers, 7% of older children, and 7% of people over 50 years old (*2*).

The United States Recommended Daily Intake for iron is 15 mg (*3*). Although the body contains a relatively small amount of iron, iron is present in every cell of the body and is critical for normal function. The main function of iron is the transport and storage of oxygen (*4*). The iron-containing heme group is active in the oxidation of nutrients to release energy. Iron is an ideal compound to act as a cofactor in many other biochemical reactions because of its flexible oxidation state, oxidation/reduction potential, and electron spin state (*4*). Iron is involved as a cofactor in electron transport needed for respiration (*5*), deoxyribonucleic acid (DNA) and ribonucleic acid (RNA)

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formation, transport of fatty acids, vitamin A formation from $\beta\beta$-carotene, production of neurotransmitters, and collagen synthesis (*3*).

Iron deficiency anemia results in about a 30% decrease in work capacity and impaired intellectual and behavioral development (*6*). Studies repeatedly demonstrate that anemic children have impaired motor skills and mental development, decreased attention spans, perceptual difficulties, and lower scores on aptitude tests (*7*−*9*). Iron deficiency results in decreased resistance to infection (*10*). In cold temperatures, iron deficient people have difficulty regulating their body temperature. Impaired temperature maintenance is one of the key symptoms of iron deficiency. During pregnancy, iron deficiency increases the chance of prenatal and perinatal infant loss, maternal mortality, and premature birth. Iron deficiency of the mother also leads to low birth weight infants, preeclampsia, and intrauterine growth restriction (*11*). The need for iron is well-established; however, technical challenges for fortifying foods with iron still exist.

One of the major challenges of iron fortification is undesirable off-color development. The color of food can strongly influence the perception of the taste of that food (*12*). Many consumers use the color of a food product to indicate the quality of the product (*13*). When using highly bioavailable iron, foods and beverages can dramatically change color. Very short storage times for iron-fortified foods have been recommended due to undesirable color changes that occur (*14*). Studies have shown that adding iron to milk (*15*), soy infant formula (*16*), baby cereals (*17*), and chocolate milk (*18*, *19*) causes undesirable color changes to occur. When sugar that was fortified with ferric ethylenediaminetetraacetic acid (EDTA) was added to tea, the beverage turned black (*20*). Similar results were found when adding iron-fortified sugar to coffee (*21*). Work has been done in India to fortify salt with iron (*22*). However, the limiting problem is color changes that occur when the fortified salt is added to foods, especially those containing vegetables (*22*, *23*). Iron-fortified maize porridge develops an undesirable color (*20*). It has been suggested that polyphenols may be involved in the off-color development of iron-fortified foods (*24*). Metal ions may interact with polyphenols in wine, causing browning of the wine (*25*).

The goal of this research was to evaluate the off-color development of various simple phenolics and polyphenols with highly bioavailable iron. In addition, environmental factors that may influence off-color development, such as pH, temperature, oxygen, redox potential reducing agents, and chelating agents were studied. Finally, validation of these models in commercial food products was performed.

## MATERIALS AND METHODS

**Materials.** All of the chemicals used were purchased from Sigma Chemical Company (St. Louis, MO). The ferrous sulfate used was the hydrate form. The concentrations of catechol and iron used were equimolar, 28 mg of catechol and 50 mg of ferric sulfate in 100 mL of deionized water. All other samples tested were in equimolar concentrations to catechol or iron, unless otherwise noted. All of the food products tested were purchased from a Kroger grocery store (Cincinnati, OH). The apple juice was Mott's Natural 100% apple juice (Stamford, CT). The hot chocolate mix was Carnation fat free brand (Glendale, CA). The green tea was Lipton 100% Natural Green Tea Bags (Englewood Cliffs, NJ). The black tea was Tender Leaf brand black tea bags (Cincinnati, OH). Coffee was made using Folgers instant coffee (Cincinnati, OH). The banana-flavored instant baby cereal was Beechnut brand (Canajoharie, NY). All products were prepared according to package directions, using deionized water unless otherwise noted. All analyses were performed in triplicate.

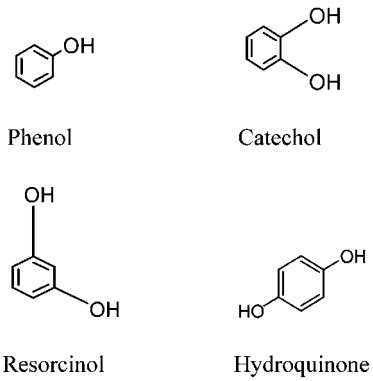


**Figure 1.** Structures of simple phenolics tested as model compounds.

**Spectrophotometric Measurement.** The absorbance of each sample was scanned (400−900 nm) using a Unicam model UV-1 double beam spectrophotometer (Cambridge, England) after an hour incubation period. Each sample was run in triplicate using a deionized water reference, unless otherwise noted. The spectrophotometer was calibrated using a calibration cell supplied by the manufacturer. Data were collected using Vision software (Unicam, Cambridge, England).

**Measurement of Iron Oxidative State.** Using a modified method, 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triaine (ferrozine) was used to determine the valence state of iron (*26*). Ferrozine in an aqueous solution is clear, but it produces a fuchsia color when it reacts with ferrous iron. No reaction occurs with ferric iron. A 1 mL aliquot of the test sample was combined with the ferrozine solution (10 mg of ferrozine in 25 mL of deionized water), and the absorbance was read at 560 nm. Each sample was tested in triplicate. As standards, the iron source alone in water was used, as well as a solution of the iron source with excess citric acid and excess ascorbic acid to ensure that all of the iron was reduced.

**Measurement of Oxidation−Reduction Potential.** Redox potentials were measured in triplicate using a Corning meter, model 340, with a Corning redox combo probe, model 476516 (Corning, NY) using the autoread function. The probe was calibrated with Corning brand Redox Probe Calibration solution.

**pH Determination.** The pH was measured in triplicate using a Corning meter, model 340, with a Corning pH probe. The meter was calibrated using pH 4 and pH 7 buffers. The pH of the samples was adjusted to 2, 4, 6, and 8 using either 0.1 N hydrochloric acid or 0.1 N sodium hydroxide.

**Effect of Oxygen on Off-Color Development.** Oxygen content of the samples was measured with an Orion Dissolved Oxygen Meter (model 862A, Beverly, MA). The meter was calibrated by bubbling air through a sample of deionized water for 1 h. To determine the effect of oxygen on the samples, oxygen was removed by bubbling the water with nitrogen for 30 min, before adding any chemicals. In these reduced samples, reduced iron (ferrous sulfate) was used. Samples were oxidized by adding the chemical to the water first and then bubbling with air for 30 min. Every combination of reduced and oxidized iron and polyphenol was scanned for absorbance.

**Effect of Temperature on Off-Color Development.** The samples used for testing the effect of temperature were prepared by heating deionized water in a microwave or cooling in an ice bath until the desired temperature was reached. Samples were evaluated at approximately 32 (0 °C), 73 (23 °C), 142 (61 °C), and 212 °F (100 °C). Iron and polyphenols were added after the temperature was adjusted.

**Hunter Colorimeter Measurements.** The color of the food products was measured using a Hunter Colorimeter, Color Quest II model (Reston, VA). Each sample was measured four times, and the average was reported. The baby cereal and the hot chocolate were evaluated using the absorbance measurement, while all of the other samples were evaluated using the transmittance measurement. Using the Hunter *L*, *a*, *b* scale, $L = 100$ is white, $L = 0$ is black, positive *a* is red, negative *a* is green, positive *b* is yellow, and negative *b* is blue.

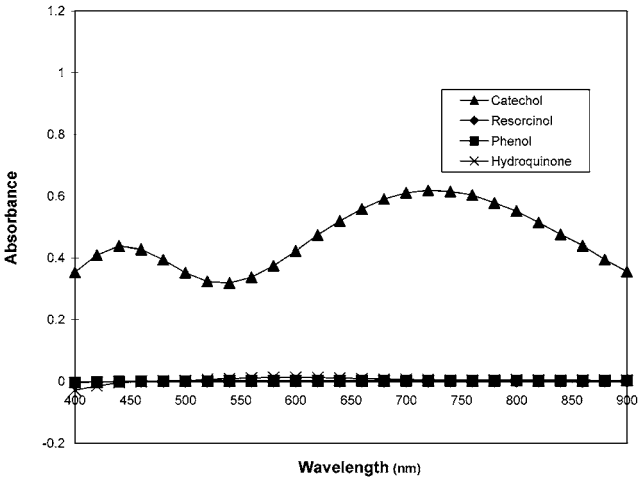


**Figure 2.** Absorbance spectra of ferrous sulfate combined with simple phenolics using ferrous sulfate in water as reference.

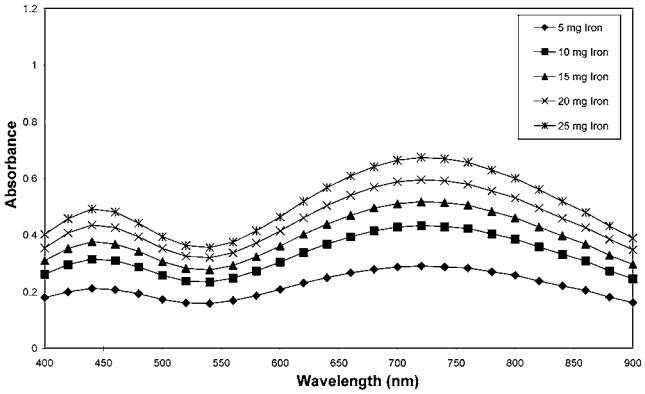


**Figure 3.** Effect of iron concentration on absorbance spectra of ferrous sulfate combined with catechol.

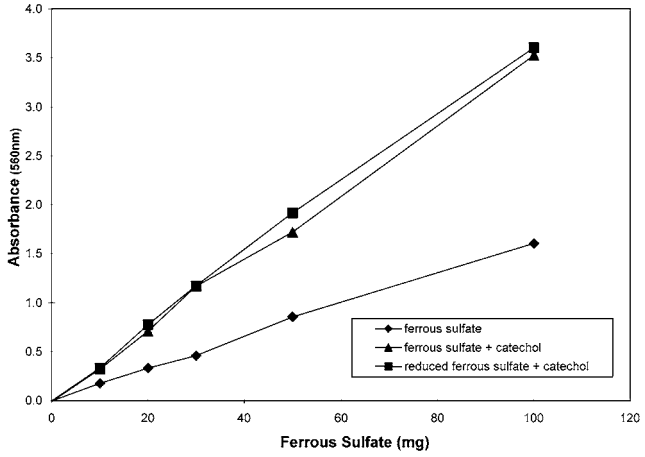


**Figure 4.** Oxidative state of iron in a solution of ferrous sulfate with catechol as a function of ferrous sulfate concentration.

**Statistical Modeling.** The effect of environmental factors was statistically analyzed using JMP software (SAS Institute, Cary, NC). Using coded data, a linear regression model was run. Terms were eliminated that had $p$ values higher than 0.3. The model was run again until all terms had $p$ values $< 0.3$. Once this equation was obtained, it was run again using uncoded units for reporting purposes.

## RESULTS AND DISCUSSION

**Role of Iron in Off-Color Development with Simple Phenolics.** The two most common iron sources used in fortification are ferrous ($Fe^{2+}$) and ferric ($Fe^{3+}$) sulfate. Ferrous sulfate was the first iron source tested because it is considered the "gold standard" for iron fortification. It is highly bioavailable and used as the standard for means of comparison. The four simple phenolics, phenol, catechol, resorcinol, and hydroquinone, were tested for off-color development with iron using ferrous sulfate as an iron source (**Figure 1**). The absorbance as

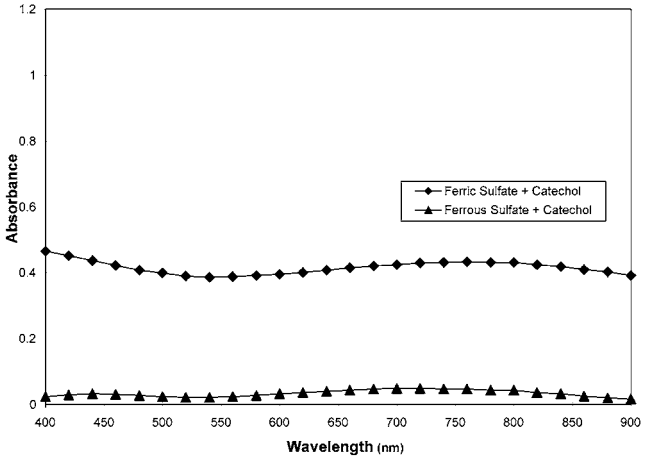


**Figure 5.** Absorbance spectra of ferrous sulfate combined with catechol vs ferric sulfate combined with catechol.

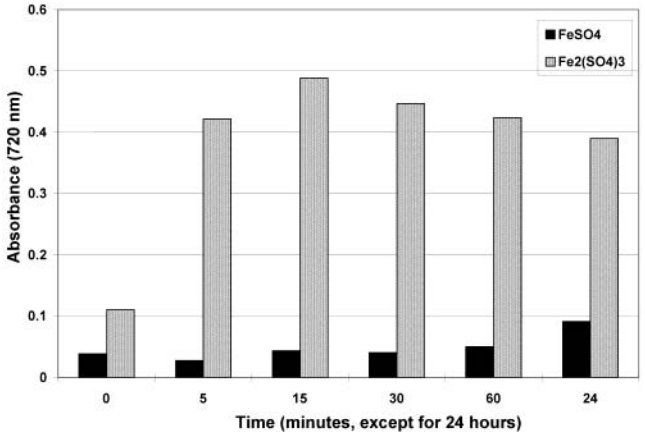


**Figure 6.** Intensity of off-color development of catechol combined with either ferrous sulfate or ferric sulfate over time (significantly different at $p < 0.002$).

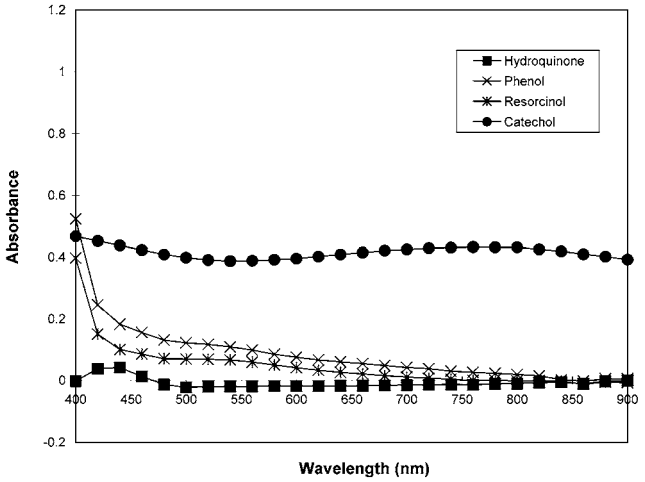


**Figure 7.** Absorbance spectra of ferric sulfate combined with simple phenolics.

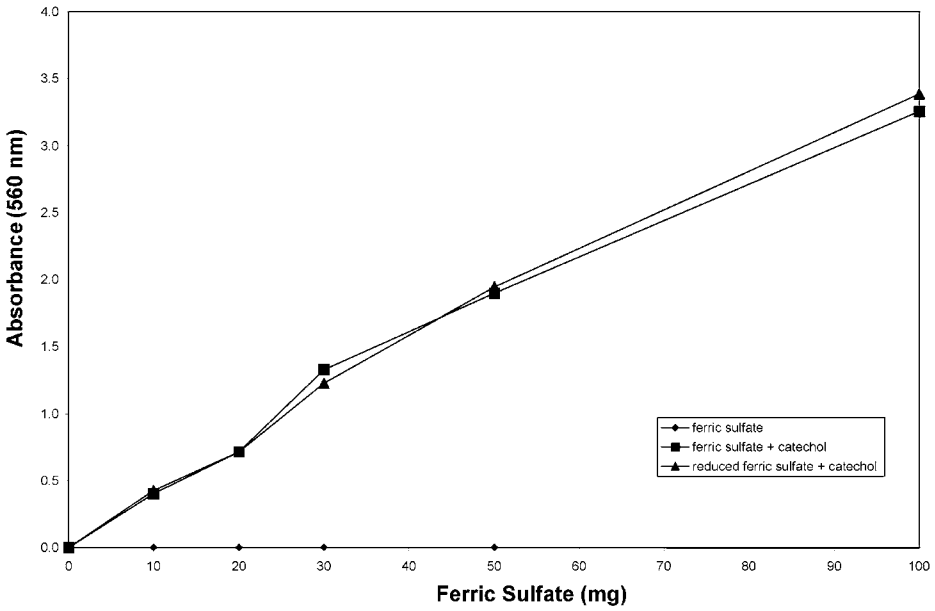


**Figure 8.** Oxidative state of iron in solution of ferric sulfate combined with catechol.

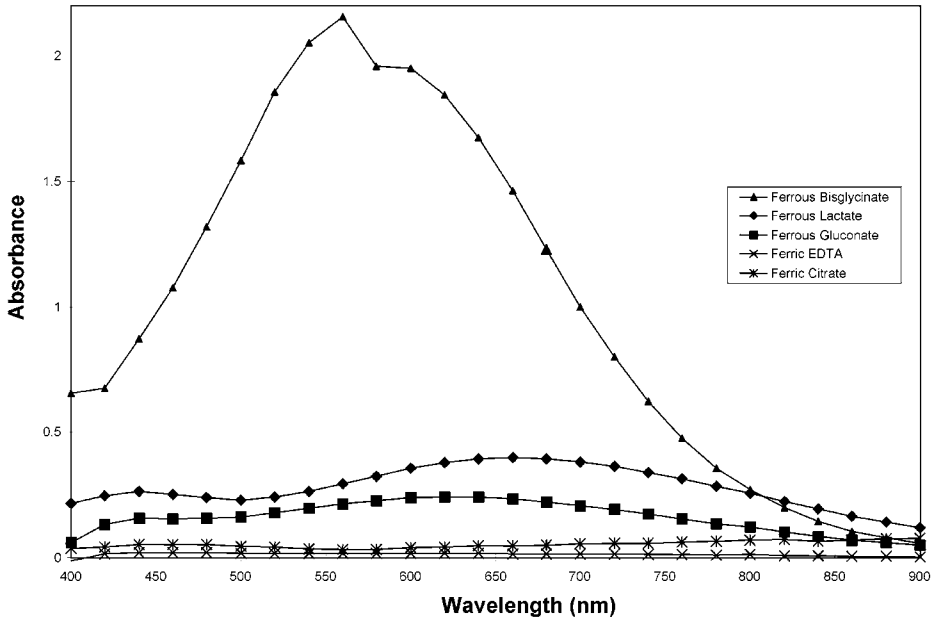


**Figure 9.** Absorbance spectra of catechol combined with iron sources other than ferrous or ferric sulfate.

a function of wavelength is shown in **Figure 2**. Although each of the phenolics and ferrous sulfate (separately) were clear in aqueous solutions, catechol combined with ferrous sulfate was the only one that developed a dark green color. Maximum absorbances were observed at 440 and 720 nm (**Figure 2**). This may indicate that there are two species that result from the combination of iron with catechol. Results showed that the ortho position of the two hydroxyl groups is critical for color development to occur. These results confirm that the ortho hydroxyl groups are needed for off-color development and iron absorption inhibition to occur (*27*). In addition, the color development was proportional to the ferrous sulfate concentration (**Figure 3**).

The oxidative state of iron in the dark green ferrous sulfate−catechol solution was evaluated using the ferrozine method. These results showed that all of the iron in the solution was in the reduced (ferrous) state (**Figure 4**). These results dispute the suggestion that there is a short-lived ferrous iron center that quickly oxidizes back to ferric iron when combined with catechol (*28*). In addition, these results disagree with the suggestion that iron forms ferric complexes with proanthocyanidins (*29*).

Because all of the iron from ferrous sulfate remained reduced when combined with catechol, the effect of ferric sulfate was tested with catechol to see if color development occurred the same with ferric iron as with ferrous iron. The same amount of iron from ferric sulfate was tested as the iron from ferrous sulfate. These results showed that the color development of ferric iron with catechol was approximately 10 times greater than that of ferrous iron (**Figure 5**).

To help quantify the difference between ferrous sulfate and ferric sulfate with catechol, the absorbance over time was measured. These results showed that the off-color development caused by ferric sulfate occurred very quickly, within the first 5 min (**Figure 6**). The off-color that resulted from the addition of ferrous sulfate to catechol developed more slowly and was less intense, with the absorbance increasing slowly over 24 h.

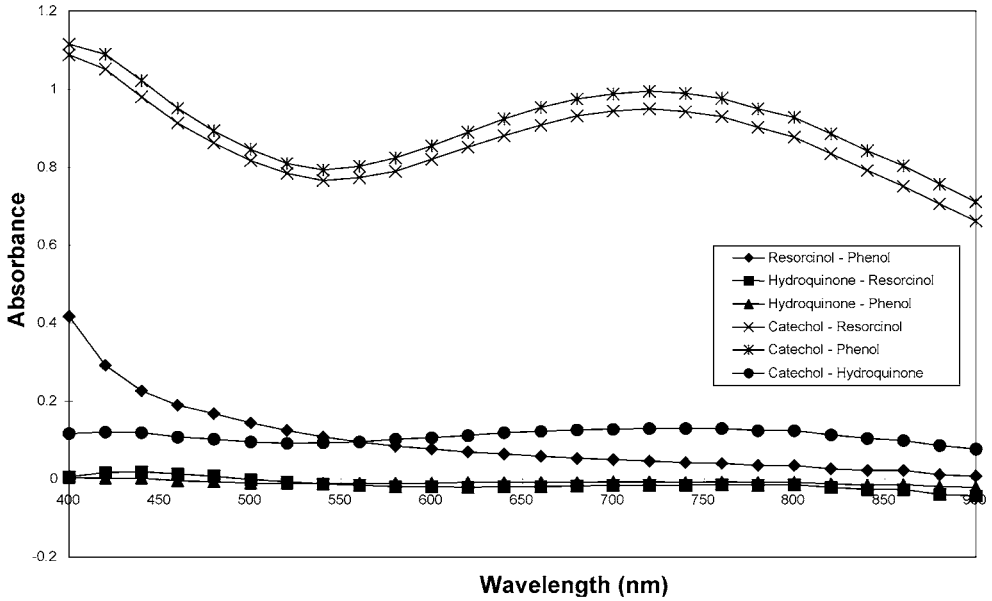


**Figure 10.** Effect on color development of the interaction of two simple phenolics combined with ferric sulfate.

**Table 1.** Redox Potentials of Metals and Simple Phenolics (mV)

| compound | redox potential (mV) | compound | redox potential (mV) |
|---|---|---|---|
| ferrous sulfate | 309 | ferrous gluconate | 290 |
| ferrous bisglycinate | 38 | ferrous lactate | 226 |
| ferric sulfate | 625 | ferric EDTA | 329 |
| ferric citrate | 405 | | |
| nickel sulfate | 293 | cuprous bromide | 242 |
| manganese chloride | 277 | cupric bromide | 273 |
| cuprous chloride | 231 | cobalt sulfate | 207 |
| cupric chloride | 245 | manganese sulfate | 251 |
| catechol | 267 | resorcinol | 333 |
| hydroquinone | 205 | phenol | 366 |

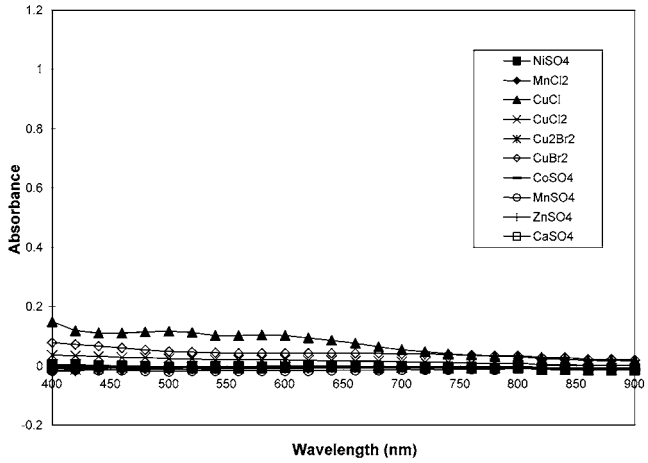


**Figure 11.** Effect on color development of metals other than iron combined with catechol.

These results illustrated that the color development with catechol was much more rapid and intense with ferric iron vs ferrous iron, suggesting that ferric is the reactive form of iron in the development of off-color with catechol.

The absorbance of ferric sulfate with the other simple phenolics was measured to confirm that the reactions are the same as with ferrous sulfate. The results shown in **Figure 7** demonstrated that ferric sulfate, like ferrous sulfate, developed color only with catechol and not the other simple phenolics. This again showed that the ortho position of the two hydroxyl groups was needed for the iron-mediated off-color development.

The results from the ferrozine assay showed that the oxidative state of the iron from ferric sulfate with catechol was the reduced state (**Figure 8**). These results were similar to that from ferrous sulfate (**Figure 4**). These results suggested that (i) the reactive iron species was ferric and it was reduced to ferrous during the process of color development and (ii) the iron-mediated off-color development was due to an oxidation−reduction reaction. Because this was happening only in the presence of catechol, it was likely that catechol was acting as a reducing agent. The color development of ferrous sulfate with catechol was slow, because it was likely that it was oxidized to ferric iron before it reacted with catechol to cause color development.

Oxidation−reduction reactions are dependent on redox potential, which is a measurement of the oxidation or reduction potential of a solution. The redox potentials of the various iron sources that are commonly used for fortification and the simple phenolics were measured (**Table 1**). The results showed that there was a large difference in redox potential among the various iron sources. Furthermore, there was a correlation between the redox potential of the iron source and the intensity of the color development (**Figure 9**). However, with the ferrous compounds, low redox potential was correlated with increased reactivity (strong color), whereas with the ferric compounds, those with higher redox potential were the most reactive. Although ferric EDTA and ferric citrate may also chelate iron, an off-color still developed when combined with catechol. The spectrum of the interaction of ferrous bisglycinate with catechol had the maximum at a different wavelength because there was evidence that ferrous bisglycinate maintained its bond between iron and glycine and also caused color development with catechol (unpublished data). Also, the simple phenolics had different redox potentials. Both catechol and hydroquinone had low redox potentials. This suggested that they were relatively strong reducing agents. However, only catechol developed off-color with iron since it contains ortho hydroxyl groups. The results from the redox potential measurements strongly supported the conclusion that the iron-mediated color development was an oxidation−reduction reaction. Among the ferric iron sources,

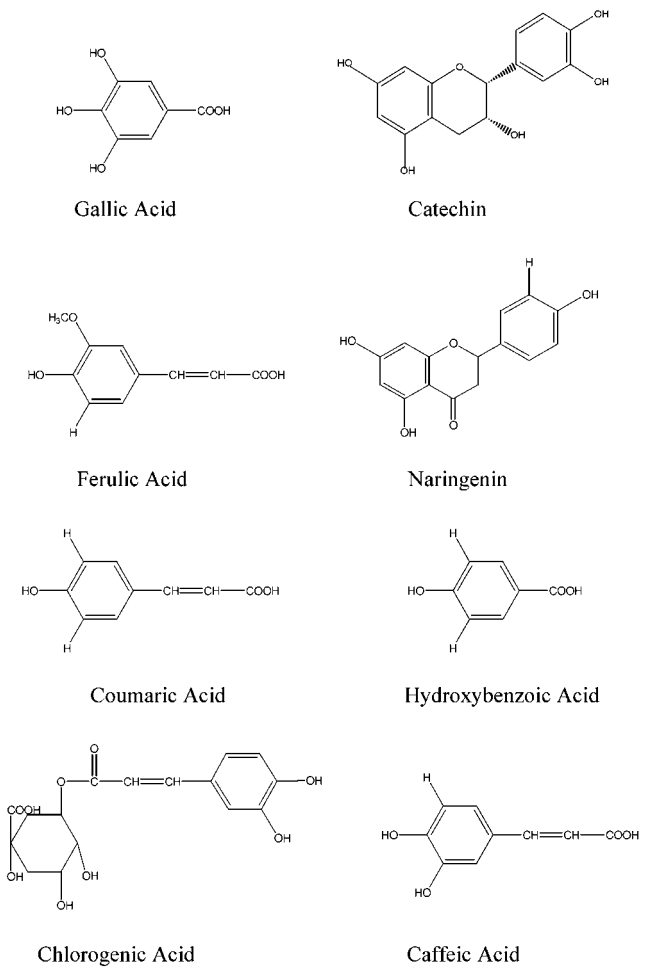


**Figure 12.** Structures of monomeric polyphenols commonly found in foods and beverages.

ferric sulfate, which had the highest redox potential (indicator of a strong oxidizing agent), developed the strongest color. It was easily reduced to ferrous iron in the presence of catechol, which was low in redox potential (indicator of a strong reducing agent). This is consistent with the redox potential data where catechol acted as a reducing agent in the development of off-color. These data may be useful as a bioavailability screening tool, since the least reactive iron soucres are generally the least absorbed iron sources.

The effect of the interaction of simple phenolics (**Figure 1**) on the color development with ferric sulfate was evaluated (**Figure 10**). These results showed that the only samples that developed color with iron were those that contained catechol. Even though the spectra of the catechol-containing samples were similar to that of catechol alone with ferric sulfate, the added phenolics had different effects on the intensity of the absorbance. This phenomenon of color intensity was dependent on the redox potential of the phenolics. Hydroquinone has a lower redox potential than catechol; thus, it acted as the strongest reducing agent among the simple phenolics (**Table 1**). As a result, hydroquinone reduced much of the ferric iron before it could react with catechol to form color. Similarly, resorcinol reduced a lesser amount of the ferric iron, due to its higher redox potential than catechol, allowing more oxidized iron to react with catechol to form the colored species. Phenol, which had the highest redox potential among the simple phenolics, had the least effect on the color development of catechol with ferric sulfate. These data support the conclusion that the iron-mediated off-color development was dependent on the reducing ability of the phenolics and the ortho positioning of the two hydroxyl groups. Furthermore, the data consistently demonstrated that during the off-color development the ferric iron was reduced while the catechol was acting as a reducing agent.

Other metals were analyzed for color development with catechol, especially those that undergo oxidation/reduction. None of these metals developed any off-color with catechol (**Figure 11**), since none of the oxidized metals, such as cupric chloride with a redox potential of 245 mV, had a very high redox potential, and none of the reduced metals, such as cuprous chloride with a redox potential of 231 mV, had a very low redox potential (**Table 1**).

**Off-Color Development of Iron with Polyphenols.** The monomeric polyphenols that commonly occur in foods and beverages (**Figure 12**) were analyzed for color development

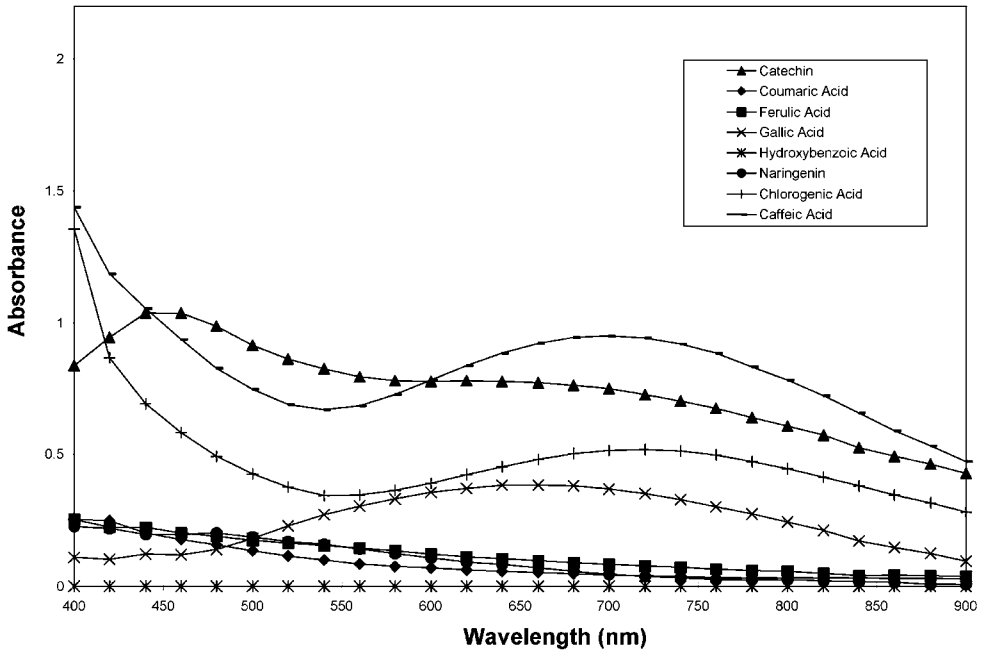


**Figure 13.** Effect of various dietary monomeric polyphenols on color development with ferric sulfate.

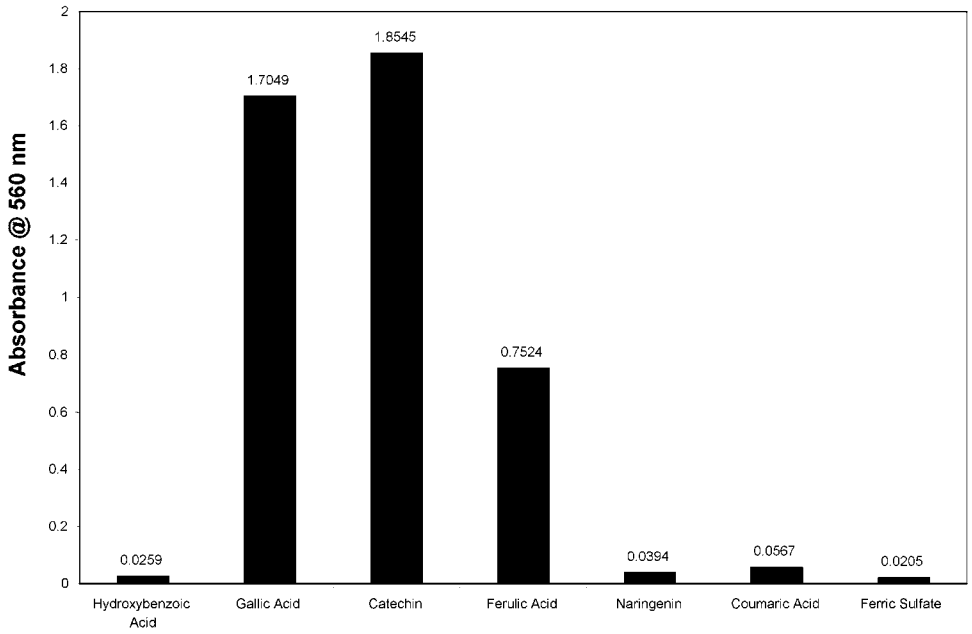


**Figure 14.** Oxidative state of iron from the monomeric polyphenols combined with ferric sulfate.

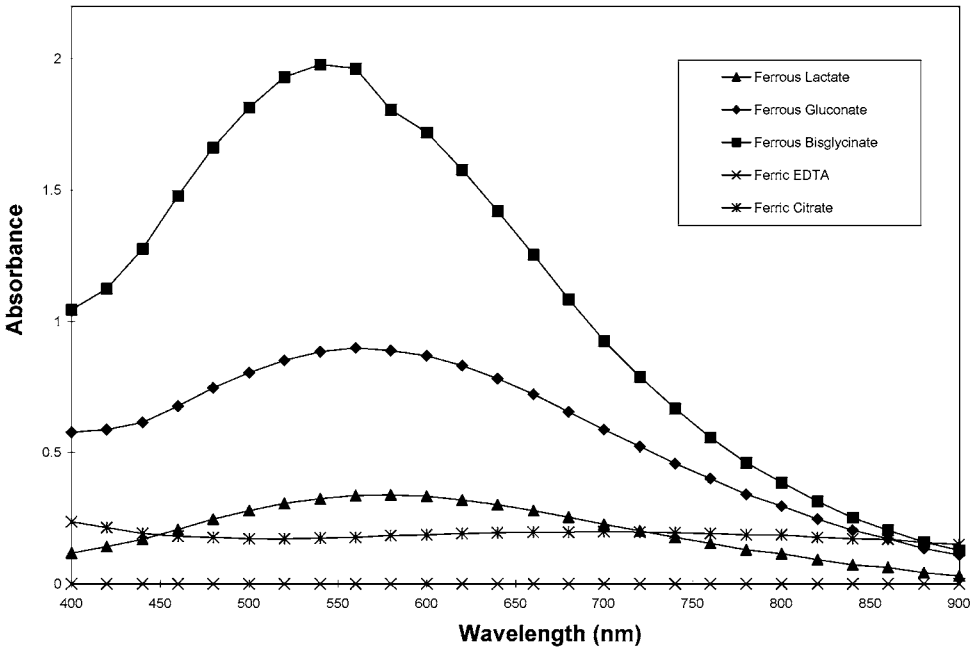


**Figure 15.** Absorbance of gallic acid with various iron sources.

with ferric sulfate. Of those tested, only gallic acid, catechin, caffeic acid, and chlorogenic acid produced color when ferric sulfate was added (**Figure 13**). These polyphenols are the only ones that contain ortho hydroxyl groups. This is consistent with the results obtained using the simple phenolics as a model and that reported in the literature (*27*). The measurement of oxidative status showed that ferric sulfate was reduced to ferrous iron by the polyphenols, which caused the color development (**Figure 14**). This was consistent with the conclusion from the simple phenolics that an oxidation−reduction reaction was occurring during color development. The redox potentials of the different monomeric polyphenols are shown in **Table 2**. The polyphenols that have relatively lower redox potentials were most easily oxidized, causing reduction of the iron and off-color development.

The polyphenols that developed off-color with ferric sulfate were evaluated for color development with other iron sources that are commonly used for iron fortification. **Figure 15** demonstrates the results of the color development of gallic acid

**Table 2.** Redox Potentials of Polyphenols with and without Ferric Sulfate

| | redox potential (mV) | |
|---|---|---|
| compound | alone | with ferric sulfate |
| hydroxybenzoic acid | 409 | 583 |
| salicylic acid | 431 | 560 |
| gallic acid | 341 | 321 |
| catechin | 258 | 353 |
| ferulic acid | 383 | 475 |
| naringenin | 384 | 592 |
| coumaric acid | 399 | 572 |
| chlorogenic acid | 299 | 382 |
| caffeic acid | 230 | 432 |

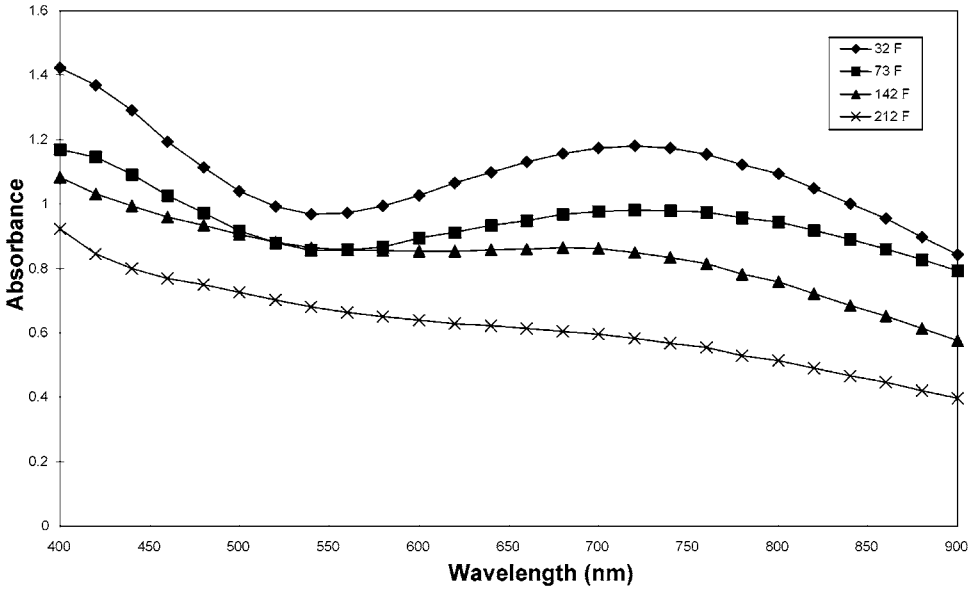


**Figure 16.** Effect of temperature on color development of ferric sulfate combined with catechol.

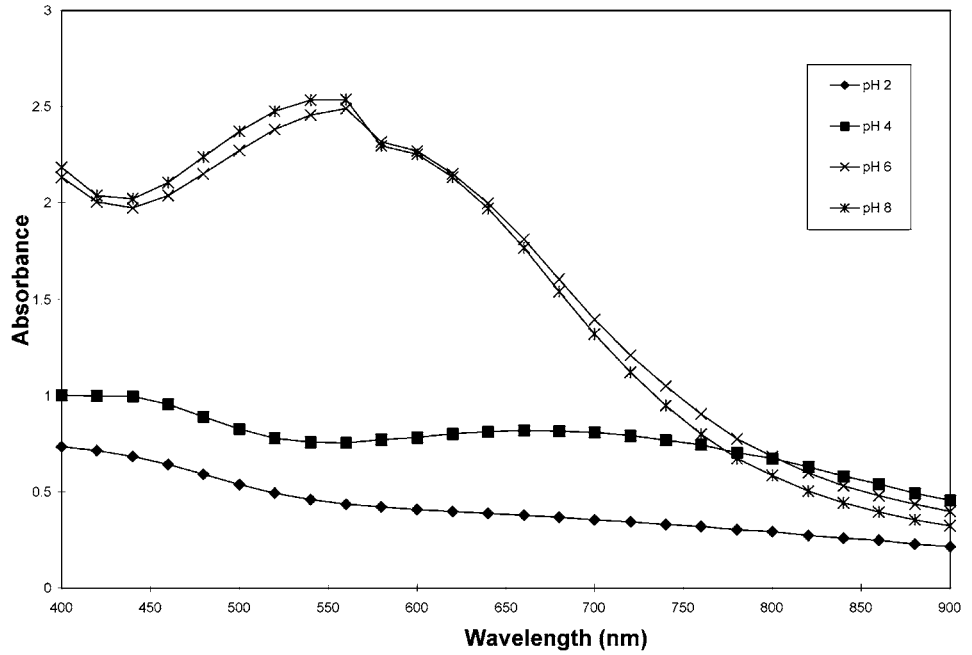


**Figure 17.** Effect of pH on the color development of catechin combined with ferric sulfate.

with different iron sources. Ferrous iron sources that had low redox potential (e.g., ferrous bisglycinate) were easily oxidized to ferric iron and produced significant color development with polyphenols. Ferric iron sources with high redox potentials (e.g., ferric sulfate) were easily reduced to ferrous iron and also developed off-color with the polyphenols. Consistent with the model of simple phenolics, these data demonstrated that off-color development is the result of an oxidation−reduction reaction, thus dependent on the redox potential of the iron used for fortification.

Results demonstrated that the off-color development that occurs when iron was added to catechol or polyphenols was dependent on the oxidative state and the redox potential of the iron source used for fortification. Ferrous iron sources with low redox potentials and ferric iron sources with high redox potentials caused off-color development with catechol and polyphenols containing ortho hydroxyl groups. Thus, it is likely that factors that influence the redox potential in the system will have a significant influence in the development of color development of iron-fortified foods and beverages.

**Role of Environmental Factors in Iron-Mediated Off-Color Development.** Compounds that formed off-color with ferric sulfate, including catechol, gallic acid, catechin, caffeic acid, and chlorogenic acid, were evaluated in different environments that may affect the redox potential of the system. All of these compounds reacted similarly; therefore, figures shown here are examples indicative of all of the reactive compounds.

The temperature at which the aqueous solution of ferric sulfate and polyphenol was prepared had an inverse effect on the absorbance. **Figure 16** demonstrates that as the temperature increased, the color development decreased. Higher water temperatures depleted the dissolved oxygen in the water, which was needed to oxidize any iron that had been reduced. The oxidized iron was needed for off-color development to occur. In addition, the temperature had an effect on the redox potential

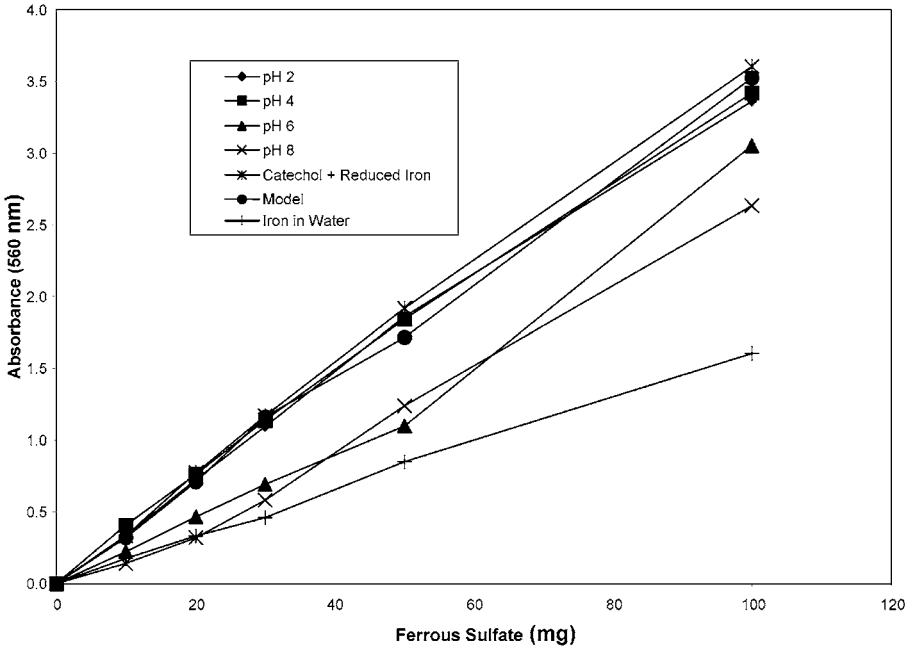


**Figure 18.** Effect of pH on the oxidative state of iron from ferric sulfate combined with catechol.

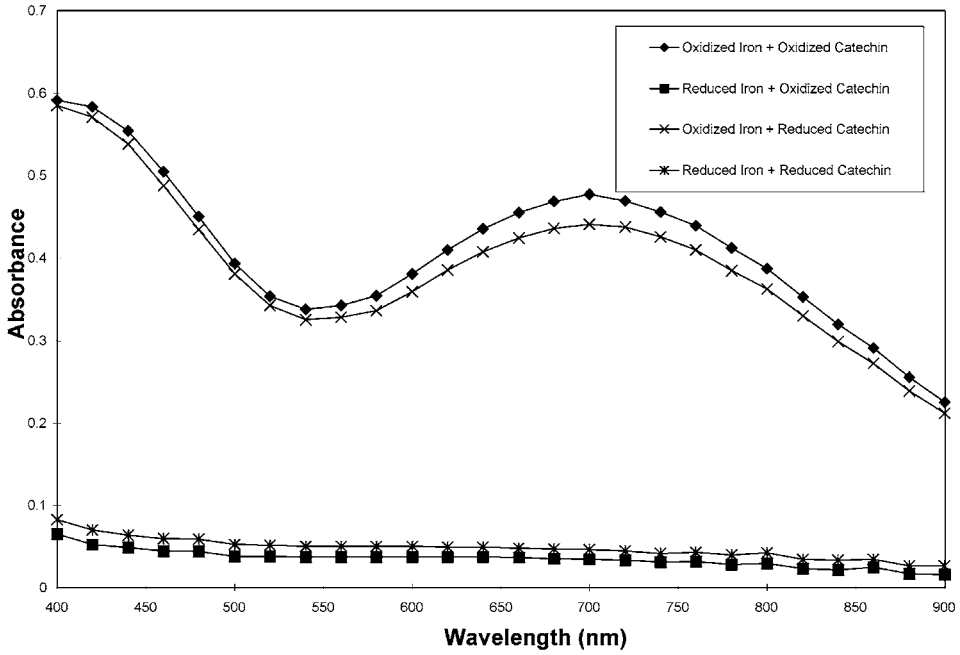


**Figure 19.** Effect of oxygen on the color development of catechin combined with ferric sulfate.

of the solutions. At lower temperatures, the redox potential was more favorable for the polyphenol to remain reduced. At higher temperatures, it is possible that the colored components were degraded. It is possible that the phenolic rings stack on top of each other to form the colored species. In this case, the lower temperature would facilitate the ordering of the molecules to develop greater color than at higher temperatures.

The pH of the system had an effect on the absorbance of polyphenols with ferric sulfate. **Figure 17** demonstrates that the color development increased as the pH increased. This is consistent with the data that indicated that ferric iron was required for color development with polyphenols. The pH had an effect on the oxidative state of the iron, since redox potential was greatly affected by pH. At low pH, most of the iron species is in the ferrous form. As the pH increased, the redox potential was more favorable for ferric iron to be present. The oxidative state of iron with polyphenols at different pH levels was evaluated using the ferrozine method. The amount of iron in the ferric form increased as the pH increased (**Figure 18**). These results were consistent with previous research, which showed that the antioxidant activity of catechins was reduced by lowering the pH (*30*).

The presence of oxygen had an effect on color development of iron with polyphenols. The samples that contained ferrous iron did not develop color with polyphenols when oxygen was removed (**Figure 19**). Only the oxidized iron samples developed color with polyphenols. The data showed that oxygen is needed for ferrous iron to oxidize to ferric iron. This was consistent with the data demonstrating that ferric iron was required for color development with polyphenols.

When reducing agents, such as ascorbic acid or sodium bisulfite, were added to the dry powders before water was added,

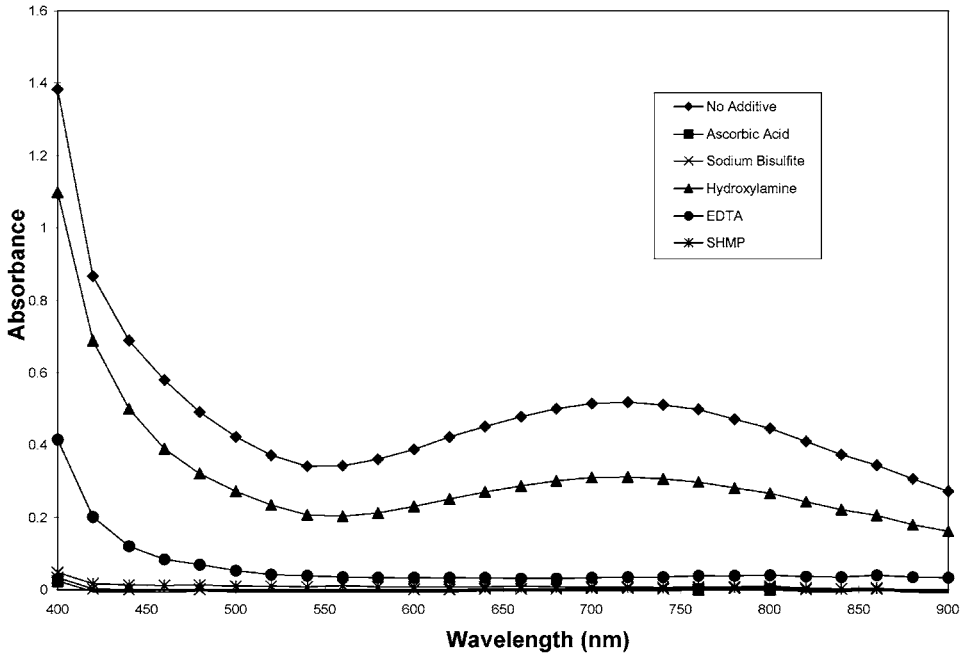


**Figure 20.** Effect of reducing and chelating agents on the color development of chlorogenic acid combined with ferric sulfate.

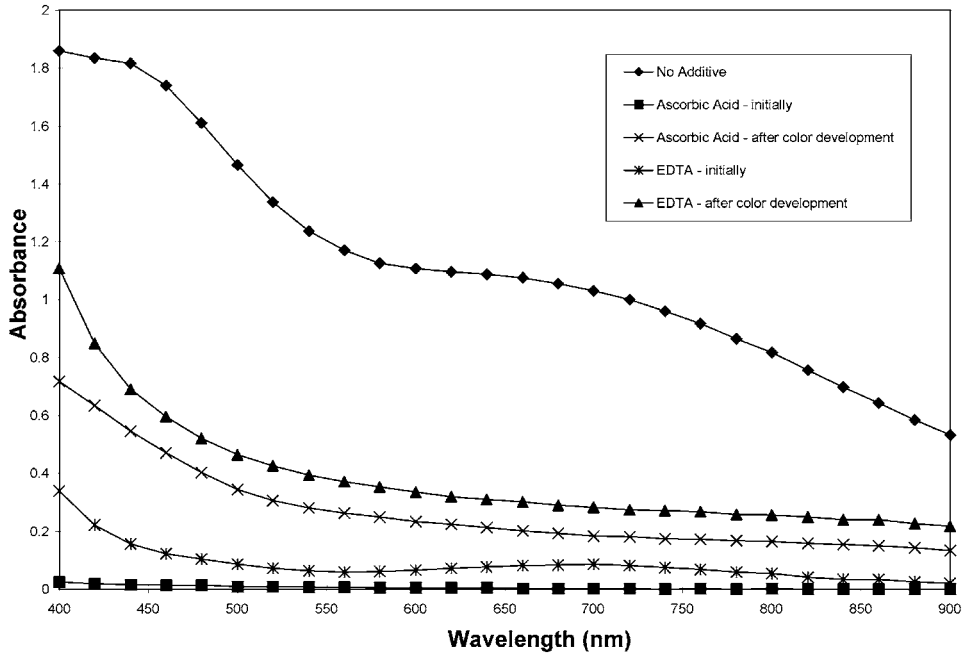


**Figure 21.** Reversibility of color development of catechin combined with ferric sulfate.

off-color development was prevented (**Figure 20**). Hydroxylamine was not quite as effective as ascorbic acid or sodium bisulfite in reducing iron. This is apparent in **Figure 20** since hydroxylamine with ferric sulfate and polyphenol demonstrated some absorbance. The samples that contained ascorbic acid and sodium bisulfite showed very little absorbance. These reducing agents reduce the iron and prevent it from being oxidized to the ferric state. In addition, some reducing agents, such as ascorbic acid, also can act as oxygen scavengers. Once the iron is in the reduced state, ascorbic acid removes the oxygen needed for ferrous iron to oxidize to ferric iron to develop off-color with polyphenols.

Chelating agents had an effect on preventing off-color development of iron with polyphenols when added as dry powders before the water was added (**Figure 20**). Chelating agents may allow the iron to participate in redox chemistry, which is needed for the off-color development. Sodium hexametaphosphate (SHMP) seemed to be effective in preventing the off-color development of ferric sulfate with polyphenols. EDTA was not as effective; however, it reduced the absorbance and changed from a dark green solution to a yellow solution.

Because reducing agents and chelating agents can prevent the off-color development when added before water, these compounds were evaluated to determine if they were effective in reversing the color development of iron with polyphenols. After an aqueous solution of ferric sulfate with a polyphenol was made, ascorbic acid or EDTA in excess was added. After they were stirred, the solutions turned from dark green to yellow, indicating that there was some change in the structure of the colored species; however, color formation was not reversed by the addition of ascorbic acid or EDTA (**Figure 21**).

**Table 3.** Units Used To Run Linear Regression Model

| factor | variable | uncoded unit | coded unit |
|---|---|---|---|
| oxidation state | ferrous iron | –1 | –1 |
| | ferric iron | 1 | 1 |
| oxygen | no oxygen | –1 | –1 |
| | with oxygen | 1 | 1 |
| reducing agents | none | 0 | –1 |
| | ascorbic acid | 1 | –0.33 |
| | sodium bisulfite | 2 | 0.33 |
| | hydroxylamine | 3 | 1 |
| chelating agents | none | 0 | –1 |
| | EDTA | 1 | 0 |
| | SHMP | 2 | 1 |
| temperature | 32 | 32 | –1 |
| | 73 | 73 | –0.544 |
| | 142 | 142 | 0.544 |
| | 212 | 212 | 1 |
| pH | 2 | 2 | –1 |
| | 4 | 4 | –0.33 |
| | 6 | 6 | 0.33 |
| | 8 | 8 | 1 |
| redox potential | ferrous sulfate | 309 | –0.077 |
| | ferrous lactate | 226 | 0.359 |
| | ferrous gluconate | 290 | –0.141 |
| | ferrous bisglycinate | 38 | –1 |
| | ferric sulfate | 625 | 1 |
| | ferric citrate | 405 | 0.25 |
| | ferric EDTA | 329 | –0.077 |

**Table 4.** Redox Potential and pH of Commercial Food and Beverage Products with and without Ferric Sulfate

| sample | color | pH | redox (mV) |
|---|---|---|---|
| apple juice | yellow | 3.66 | 260 |
| apple juice + $Fe_2(SO_4)_3$ | yellow | 3.56 | 255 |
| black tea | light brown | 5.01 | 177 |
| black tea + $Fe_2(SO_4)_3$ | black | 4.03 | 210 |
| green tea | yellow | 5.12 | 133 |
| green tea + $Fe_2(SO_4)_3$ | dark green | 4.34 | 169 |
| coffee | dark brown | 5.12 | 68 |
| coffee + $Fe_2(SO_4)_3$ | dark black/green | 4.90 | 56 |
| hot chocolate | brown | 6.65 | 42 |
| hot chocolate + $Fe_2(SO_4)_3$ | gray/brown | 6.54 | 51 |
| banana baby cereal | yellow | 5.65 | 73 |
| banana baby cereal + $Fe_2(SO_4)_3$ | gray | 5.65 | 70 |

A model was developed using JMP Software to describe the effect of environmental factors on the off-color development of iron with polyphenols:

$$A_{725} = -0.049x_1 + 0.262x_2 - 0.333x_3 - 0.348x_4 + 0.131x_5 + 0.001x_6 - 0.766$$

where $A_{725}$ is the absorbance at 725 nm, $x_1$ is ferric vs ferrous iron, $x_2$ is pH, $x_3$ is the presence of chelating agents, $x_4$ is the presence of reducing agents, $x_5$ is the presence of oxygen, and $x_6$ is the redox potential of iron. **Table 3** outlines the codes used for running the linear regression model. This model suggested that the presence of reducing agents had the greatest effect on the absorbance of polyphenols with ferric sulfate, followed by the presence of chelating agents, the pH of the solution, the presence of oxygen, and the state of the iron. The redox potential of the iron source also had a significant effect on the absorbance. Other factors were determined to be less important in off-color development using this model. The $R^2$ for this linear regression model was 0.67. This model may be useful in designing iron-fortified food products that contain polyphenols with minimal off-color development by understanding the impact of each of the environmental factors tested.

**Table 5.** Color Development of Foods with Various Iron Sources

| | Hunter *L* | Hunter *a* | Hunter *b* |
|---|---|---|---|
| apple juice | 54.09 | 11.78 | 30.15 |
| apple juice + $Fe_2(SO_4)_3$ | 50.42 | 11.40 | 28.49 |
| black tea | 39.24 | 30.35 | 25.14 |
| black tea + $Fe_2(SO_4)_3$ | 3.23 | 4.04 | 1.95 |
| black tea + ferrous bisglycinate | 8.47 | 8.20 | 5.13 |
| black tea + ferrous lactate | 7.91 | 7.15 | 4.75 |
| black tea + ferrous gluconate | 8.52 | 7.37 | 5.21 |
| black tea + ferric EDTA | 41.02 | 27.66 | 26.03 |
| black tea + ferric citrate | 25.77 | 19.46 | 16.19 |
| green tea | 78.83 | –0.28 | 36.67 |
| green tea + $Fe_2(SO_4)_3$ | 8.36 | 1.25 | 3.42 |
| green tea + ferrous bisglycinate | 8.54 | 3.39 | 4.33 |
| green tea + ferrous lactate | 11.04 | 3.09 | 5.56 |
| green tea + ferrous gluconate | 12.25 | 2.56 | 6.27 |
| green tea + ferric EDTA | 60.83 | 6.83 | 33.58 |
| green tea + ferric citrate | 43.64 | 5.77 | 23.57 |
| coffee | 18.51 | 24.58 | 11.84 |
| coffee + $Fe_2(SO_4)_3$ | 3.27 | 4.85 | 1.90 |
| coffee + ferrous bisglycinate | 8.30 | 9.39 | 5.29 |
| coffee + ferrous lactate | 10.74 | 11.71 | 6.88 |
| coffee + ferrous gluconate | 12.39 | 13.45 | 7.92 |
| coffee + ferric EDTA | 25.05 | 26.10 | 16.18 |
| coffee + ferric citrate | 19.62 | 21.28 | 12.62 |
| hot chocolate | 28.25 | 6.13 | 5.09 |
| hot chocolate + $Fe_2(SO_4)_3$ | 25.90 | 2.94 | 2.79 |
| hot chocolate + ferrous bisglycinate | 24.81 | 2.17 | 1.96 |
| hot chocolate + ferrous lactate | 25.34 | 2.73 | 2.46 |
| hot chocolate + ferrous gluconate | 26.07 | 3.45 | 3.11 |
| hot chocolate + ferric EDTA | 26.92 | 4.68 | 3.98 |
| hot chocolate + ferric citrate | 26.70 | 4.44 | 3.79 |
| banana baby cereal | 54.24 | –0.42 | 6.46 |
| banana baby cereal + $Fe_2(SO_4)_3$ | 48.24 | –0.89 | 3.93 |
| banana baby cereal + ferrous bisglycinate | 50.45 | –0.88 | 4.68 |
| banana baby cereal + ferrous lactate | 56.39 | –0.39 | 6.79 |
| banana baby cereal + ferrous gluconate | 55.59 | –0.35 | 7.01 |
| banana baby cereal + ferric EDTA | 57.58 | 0.08 | 8.81 |
| banana baby cereal + ferric citrate | 57.58 | 0.54 | 9.09 |

These results indicated that the color development depended on the oxidative state of the iron and the structure of the polyphenols. Furthermore, maintaining iron in its reduced state by controlling the redox potential of the environment prevented iron off-color development with polyphenols. Factors that contribute to the state and reactivity of the iron can be controlled to influence off-color development, including pH, oxygen, reducing agents, and chelating agents.

**Off-Color Development of Iron with Food and Beverage Products.** Many commonly consumed food and beverage products, such as tea, sorghum, vegetables, fruits, and cocoa, contain polyphenols that are of high molecular weight (*31*). Repeated studies have shown that addition of highly bioavailable iron sources causes the development of undesirable color (*17*, *18*, *20*, *32*, *33*). To determine the validity of the previous models using the simple phenolics and the monomeric polyphenols, the effect of iron on off-color development of foods and beverages was evaluated. Hot chocolate mix was used to test the gallic acid model, apple juice was used to test the caffeic acid model, coffee was used to test the chlorogenic acid model, and green tea was used to test the catechin model (*31*). Black tea and instant banana-flavored baby cereal also were evaluated as examples that contain the polymerized polyphenols. **Table 4** describes the pH and redox potential of the food products with and without ferric sulfate (added at 30% U.S. RDI per serving). All of the food products drastically changed color with the addition of ferric sulfate except for apple juice (**Table 5**). Of the products measured, apple juice had the lowest pH and the

**Table 6.** Effect of pH, Temperature, Oxygen, and Reducing and Chelating Agents on Color Development in Polyphenol-Containing Foods Combined with Ferric Sulfate

| | Hunter *L* | Hunter *a* | Hunter *b* |
|---|---|---|---|
| black tea | 39.24 | 30.35 | 25.14 |
| black tea, pH 2 | 30.46 | 20.47 | 19.20 |
| black tea, 32F | 16.12 | 12.67 | 10.01 |
| black tea, no oxygen | 64.22 | 2.11 | 22.70 |
| black tea + ascorbic acid | 38.49 | 22.96 | 24.53 |
| black tea + EDTA | 40.13 | 27.03 | 25.76 |
| green tea | 78.83 | –0.28 | 36.67 |
| green tea, pH 2 | 65.75 | 5.78 | 33.11 |
| green tea, 32 °F | 20.10 | 2.60 | 10.68 |
| green tea, no oxygen | 56.25 | –1.06 | 13.95 |
| green tea + ascorbic acid | 80.00 | –0.12 | 33.51 |
| green tea + EDTA | 75.43 | 1.32 | 32.28 |
| coffee | 18.51 | 24.58 | 11.84 |
| coffee, pH 2 | 21.07 | 22.72 | 13.57 |
| coffee, 32 °F | 11.66 | 12.60 | 7.45 |
| coffee, no oxygen | 16.02 | 18.62 | 10.28 |
| coffee + ascorbic acid | 22.69 | 24.04 | 14.66 |
| coffee + EDTA | 24.17 | 25.83 | 15.63 |
| hot chocolate | 28.25 | 6.13 | 5.09 |
| hot chocolate, pH 2 | 28.28 | 5.44 | 5.24 |
| hot chocolate, 32 °F | 27.05 | 4.42 | 3.95 |
| hot chocolate, no oxygen | 29.48 | 4.21 | 4.64 |
| hot chocolate + ascorbic acid | 28.68 | 4.86 | 4.73 |
| hot chocolate + EDTA | 28.63 | 5.94 | 5.26 |
| banana baby cereal | 54.24 | –0.42 | 6.46 |
| banana baby cereal, pH 2 | 58.07 | 0.13 | 9.74 |
| banana baby cereal, 32 °F | 51.55 | –0.18 | 7.22 |
| banana baby cereal, no oxygen | 60.21 | 0.33 | 8.44 |
| banana baby cereal + ascorbic acid | 53.85 | 0.54 | 6.79 |
| banana baby cereal + EDTA | 59.06 | 1.08 | 11.48 |

highest redox potential, both of which are known to inhibit off-color development.

Other iron sources that are used commonly for fortification, besides ferric sulfate, were analyzed for off-color development in the food products that are rich in polyphenols (**Table 5**). Intensity of color development was measured by using a Hunter Colorimeter. According to the results presented in **Table 5**, ferrous bisglycinate caused the most off-color development in the products, followed by ferrous lactate, ferrous gluconate, ferric citrate, and ferric EDTA, which had the least effect on color development. These results were consistent with those from the models using simple phenolics and monomeric polyphenols, as well as the literature (*34*, *35*).

Using the monomeric polyphenolic compounds, methods to reduce off-color development with iron have been identified. These methods were tested in the commercial products (**Table 6**) using ferric sulfate as an iron source. Lowering the pH to 2.0 was effective in preventing off-color development. Lowering the temperature to about 32 °F provided only slight inhibition of color formation. The addition of reducing agents, such as ascorbic acid, or chelating agents, such as EDTA, maintained the color of the food product. However, the flavor of these altered products should be evaluated.

These results from the interaction of iron with polyphenol-rich foods and beverages were in agreement with data from the simple phenolic and monomeric polyphenolic models. The iron-mediated off-color development in foods and beverages was due to the oxidation−reduction-dependent interactions with the polyphenols present in foods. This reaction process was influenced by the redox potential of both the iron source and the polyphenol. The ferric iron was the form of iron that participated in the color development. In the presence of polyphenols that have ortho-dihydroxyl groups, the ferric iron was reduced to ferrous iron. Even though the data are not available, the polyphenol was changed from the reduced to the oxidized state. Note that for ferrous iron to participate in the off-color development, it had to be converted to ferric iron by the oxidizing agent, oxygen. Thus, by using the ferrous form of iron and maintaining it in its reduced form by lowering pH, removing oxygen, and including reducing agents, it was possible to fortify foods with highly bioavailable iron.

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