Use of transferrin and EPR to probe the in vitro digestive chemistry of iron

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The study of the chemistry of iron in food materials undergoing digestion can increase our knowledge of the basis of iron bioavailability. Apotransferrin has been used as a probe of iron reactivity following the in vitro *enzymatic digestion of pinto beans at pH 3.0 and pH 5.0 in the presence and absence of additives such as ascorbic acid, citric acid, a combination of ascorbic and citric acids, phosvitin, tea, and orange juice. Electron paramagnetic resonance spectroscopy was used to monitor and quantitate the exchange reactions. The effects of additives were similar when either the digestive slurry or its supernatant was reacted with apotransferrin. Although citric acid caused a two-fold increase in the digestive solubilization of pinto bean iron when compared to control, the amount of iron donated to transferrin was similar. The addition of ascorbic acid did not increase digestive solubilization of iron, however the iron exchange to transferrin increased 1.6 times. A maximum reactivity of pinto bean iron was observed when orange juice or ascorbic plus citric acids were included in the digestion. A strong inhibitory effect of tea on iron reactivity was seen. The order of additives in terms of their enhancement of the reaction of pinto bean supernatant with apotransferrin is: ascorbic plus citric acids > orange juice > ascorbic acid > citric acid > control > phosvitin > tea. This order parallels that expected for the effect of the additives on human iron bioavailability.*

Keywords: iron; bioavailability; beans; transferrin

Introduction

Iron deficiency and iron deficiency anemia continue to be major nutritional problems in the world, affecting hundreds of millions of people in both developed and developing countries.[1] The problem, in general, is not the iron content of the meals because most diets contain well in excess of the amount required to be absorbed each day. The limiting factor is primarily one of a low iron bioavailability. In Third World diets, heme, which has a high bioavailability, is often a negligible fraction of the total iron content.[2] Hereafter, the term "iron" specifically refers to nonheme iron.

The amount of iron absorbed by the intestinal mucosa is dependent on the iron status of the individual, the quantity of iron in the meal and the composition of the meal, especially with regard to enhancers (meats, ascorbic acid) and inhibitors (phytate, tannins, phosphate, and some proteins). Evidence from several lines of research support the concept that Fe^{2+} is better absorbed than Fe^{3+}.[3,4]

While human iron absorption provides the definitive measure of iron bioavailability in humans, the in vitro methods that have become popular in recent years offer some major advantages. First, if one allows certain assumptions, they provide rapid and inexpensive estimates of the iron bioavailability of food and beverage combinations.[5-7] Second, unlike the human studies, in vitro methods allow direct chemical examination of the digestive mass relative to binding, adsorption, and redox phenomena.[6-8]

The basic approaches to in vitro iron bioavailability studies have involved using dialysis to estimate low molecular weight iron concentrations[5] and the measurement of Fe^{2+} and Fe^{3+} content of the supernatants obtained from centrifugation of digestive mixtures.[6-8] The in vitro technique has recently been reviewed by Miller and Berner.[9]

In this paper we introduce a novel in vitro approach

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based on the reactivity of iron in the digestive mixture with a ligand. The protein serum transferrin was chosen as an iron acceptor because it seems a likely analog for the mucosal receptor site and it will bind reactive Fe^{3+} and Fe^{2+}. The latter is rapidly converted to the trivalent state by reaction with molecular oxygen.[10] In addition, Fe^{3+}-transferrin has a distinct electron paramagnetic resonance (EPR) spectrum, which can be used to monitor the formation of the complex even in turbid digestive slurries. Pinto beans were chosen as a food iron source because they have a high iron content that varies in its bioavailability according to the nature of the meal.[6,7]

Materials and methods

Chemicals

Ferrozine [3(2-pyridyl)-5,6-Bis (4-phenylsulfonic acid)-1,2,4-Triazine], phosvitin (from egg vitellin), Hepes (N-2-hydroxyethylpiperazine N-2-ethanesulfonic acid), sodium bicarbonate, nitrilotriacetic acid, and the enzymes used for digestion were obtained from Sigma Chemical Co. (St. Louis, MO USA). EDTA, citric acid, and sodium dithionite were obtained from Fisher Scientific (Fair Lawn, NJ USA). Iron from phosvitin and pepsin was removed by dialyzing against 50 mmol/L EDTA at pH 4.25 and 50 mmol/L citrate at pH 6, respectively. EDTA and citrate were subsequently removed by dialysis against buffer. $NaHCO_3$ solution was prepared fresh daily. Distilled deionized water was used throughout the study. Human apotransferrin (Calbiochem, San Diego, CA USA) was dissolved in a small volume of Hepes buffer at pH 7.4 and dialyzed three times against the same buffer. The concentration of transferrin was determined by measuring the UV absorption at 280 nm. A small amount of iron, which was already present in the protein, was determined by measuring visible absorption at 470 nm.

Determination of total soluble iron

Pinto beans were digested in vitro using the method reported earlier.[8] At the end of in vitro digestion, half of the digestive mixture was centrifuged to separate the supernatant and residue fractions. The other half of the digestive mixture was left in the slurry form. The soluble iron was estimated by adding 0.1 mL (0.1 M) of sodium dithionite and 0.25 mL of ferrozine (10 mmol/L, pH 6.0) to 0.5 mL of the supernatant. The visible absorption of Fe^{2+}-ferrozine complex was measured at 563 nm using a Cary 118C spectrophotometer. For all the samples, both the supernatant and the slurry were reacted with apotransferrin.

Chemical reactivity of iron with apotransferrin

Because the digestive slurries contained solid residue materials, EPR spectroscopy (Bruker model ER200D Bruker Instruments Inc., Billerica, MA) was used to monitor the Fe^{3+}-transferrin complex formation. A Beer's law type plot for both spectrophotometric absorbance and EPR intensity was obtained for Fe^{3+}-transferrin in the following manner. To apotransferrin in 25 mmol/L $NaHCO_3$, various amounts of Fe^{3+}-nitrilotriacetic acid (NTA) were added and the increase in absorbance was measured at 470 nm. The Fe^{3+}-NTA was prepared at a 4:1 Fe^{3+} chelator to iron ratio according to the method described by Graham and Bates.[11] Samples were also frozen in liquid nitrogen to obtain an EPR spectrum. Fe^{3+}-transferrin-CO_3^{2-} has a distinct doublet signal centered near a magnetic field value of 1525 gauss (*Figure 1*, point a). Iron bound to citric acid and to other small molecular weight ligands (including the pinto bean components) interferes with measurement of the signal intensity of Fe^{3+}-transferrin at that magnetic field. However, Fe^{3+}-transferrin also has shown a strong signal at 1639 gauss (*Figure 1*, point b), a point at which iron bound to other ligands shows a minimum interference. Therefore, a magnetic field value of 1639 gauss was chosen to quantitate and monitor the Fe^{3+}-transferrin complex formation during the exchange reactions.

Because the EPR spectra change with the nature of additives present in the bean digestae, plots of EPR intensity versus concentration (Beer's law type plots) were obtained for each additive digested with the beans. The total iron was estimated in the cooked pinto beans using wet digestion and atomic absorption spectroscopy.[12] Bean supernatants and the digestive slurries were adjusted to pH 6.7. The variable amounts of iron required for the plot were obtained by diluting the samples with water. The samples were frozen in liquid nitrogen and the EPR signal intensities at 1639 gauss were measured. Slopes of EPR intensity versus concentration for transferrin and bean supernatant iron were determined using a linear regression program. Although these experiments were repeated with similar results, the data presented are from only one run of each additive.

To measure the reactivity of soluble iron, apotransferrin at pH 6.7 was reacted with the pinto bean supernatant in the presence of 25 mmol/L $NaHCO_3$ and 50 mmol/L Hepes buffer. A similar reaction was performed for bean slurries. Aliquots were frozen at 2, 5, 10, 15, 20, and 30 min during exchange reactions. The EPR signal intensities were measured for each. Using the slope obtained from the EPR signal intensity plots for transferrin and for pinto bean iron bound to various ligands, the amount of iron exchanged from beans to apotransferrin was calculated using equation 1:

$$TI = \frac{EI - (BC \times I)}{TC - BC}$$

where TI is the concentration of Fe^{3+}-transferrin, I is the total system iron concentration, EI is the EPR intensity at 1639 gauss, TC is the Fe^{3+}-transferrin EPR molar intensity coefficient, and BC is the bean iron EPR molar intensity coefficient.

Results

A typical EPR spectrum of Fe^{3+}-transferrin is shown in *Figure 1*. Fe^{3+}-transferrin has a distinctive spectrum

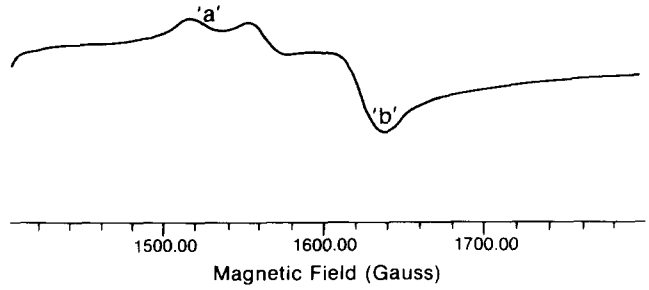

Figure 1 EPR spectrum of Fe^{3+}-transferrin-CO_3^{2-} at a microwave frequency of 9.3 GHz. Points "a" and "b" represent the signal intensities at field strength values of 1523 gauss and 1639 gauss, respectively.

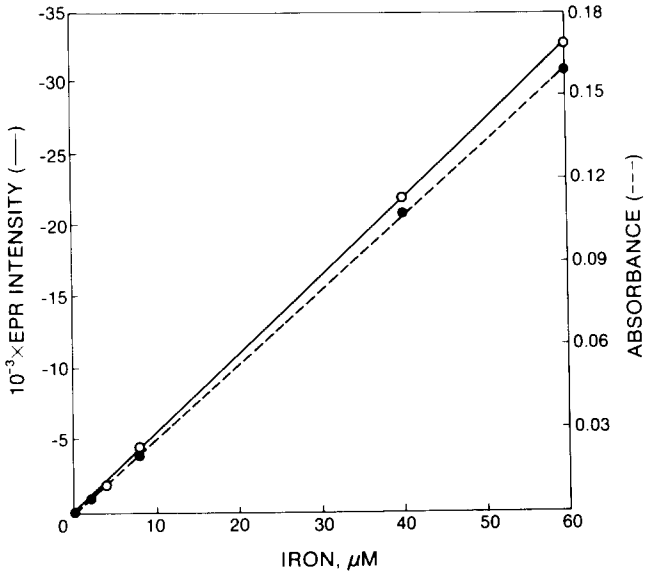

Figure 2 The EPR signal intensity plot for Fe^{3+}-transferrin-CO_3^{2-}. Variable concentrations of Fe^{3+}-NTA were added to apotransferrin at pH 7.4 in the presence of 50 mmol/L Hepes buffer at 25 mmol/L $NaHCO_3$. The visible absorbance at 470 nm and the EPR signal intensity at 1639 gauss were measured. The difference in slopes is simply a matter of choice of ordinate scales.

showing a doublet signal at field strength value centered near 1525 gauss and has a negative signal intensity at 1639 gauss at a microwave frequency of 9.3 GHz. *Figures 2 and 3* show the EPR signal intensity (Beer's law type) plots for Fe^{3+}-transferrin and pinto bean supernatants, respectively. The EPR intensity at 1639 gauss exhibits a linear dependence on the amount of iron present in the mixtures. Apotransferrin was reacted with bean iron in the presence of 25 mmol/L $NaHCO_3$ and 50 mmol/L Hepes at pH 6.7. The change in the EPR spectrum of the pinto bean supernatant (control) at various times during the iron exchange reaction is shown in *Figure 4*. The time-dependent formation of Fe^{3+}-transferrin as the reaction proceeds indicates the iron exchange from the supernatant iron complexes to apotransferrin.

The amount of iron solubilized during the digestion procedure and the amount of iron reacted with apotransferrin at 30 min are presented in *Table 1*. The amount of iron reacted with transferrin is based on 100 g of cooked pinto beans. Pinto bean supernatants containing no additives donated 74% of the iron, or approximately 17 μmol/L. Interestingly, ascorbic acid mobilized almost the same amount of iron as the control, but it donated 100% of its iron (26 μmol/L) to transferrin. Although citric acid caused a two-fold increase in the mobilization of pinto bean iron when compared with the control, the concentration of iron exchanged to transferrin (18 μmol/L vs. 17 μmol/L) was similar in both samples. Ascorbic plus citric acids and orange juice-containing samples exchanged about the same amount of iron, which is 1.7 times more than the control. When tea was added to pinto beans, 33%

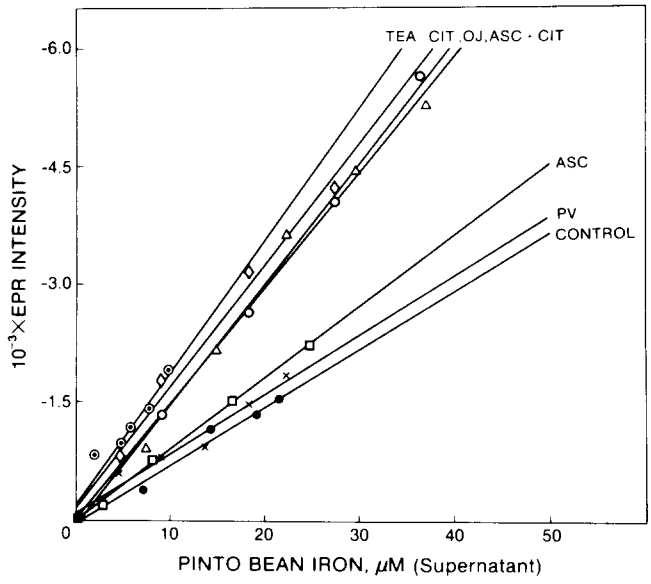

Figure 3 The EPR signal intensity plot for pinto bean supernatants at pH 6.7. Pinto beans were subjected to subsequent pH 3 and pH 5 in vitro digestions. The additives included were ascorbic acid (ASC), citric acid (CIT), a combination of ascorbic plus citric acids (ASC + CIT), tea, and dialyzed phosvitin (PV). EPR intensity values are much less than those shown in *Figure 2* for comparable concentrations of Fe^{3+}-transferrin.

Table 1 Reactivity of soluble pinto bean iron with apotransferrin*

Additives	Total Fe mobilized at pH 5†	Iron reacted with apotransferrin in 30 min at pH 6.7		
	(μmol/L)	(μmol/L)	(%)	(mg)‡
Ascorbic & citric acid	46.6	28.2	61	0.74
Orange juice	54.7	27.8	51	0.73
Ascorbic acid	25.0	25.7	100	0.68
Citric acid	44.4	18.2	41	0.48
Control	22.3	16.5	74	0.44
Phosvitin	26.0	13.4	52	0.35
Tea	9.8	3.3	33	0.09

*The in vitro digestion was carried out at pH 3 and pH 5 as described. The reaction with apotransferrin was carried out at pH 6.7 in the presence of $NaHCO_3$ (25 mmol/L) and Hepes (50 mmol/L) buffer.
†Values represent the iron concentrations in the supernatants of pinto beans following the pH 5 in vitro digestion.
‡Iron estimated to be reactive with apotransferrin from 100 g cooked pinto beans.

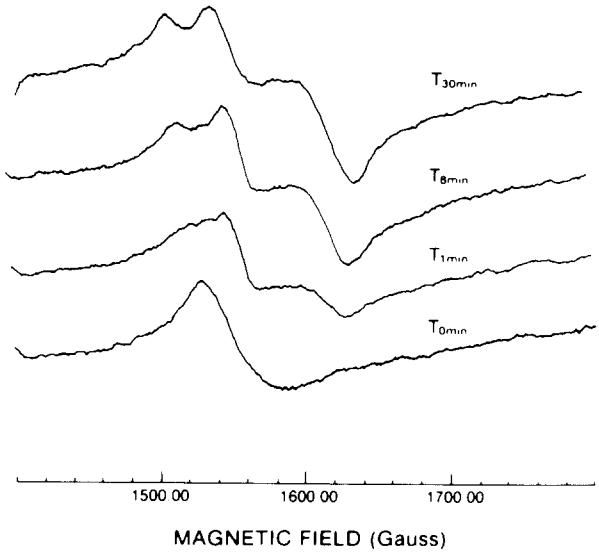

Figure 4 Change in the EPR signal of pinto bean supernatant containing no additives as a function of time during the reaction with apotransferrin at pH 6.7 in the presence of 50 mmol/L Hepes buffer and 25 mmol/L $NaHCO_3$.

of the soluble iron reacted with apotransferrin, however it was 80% lower than the control. Of the iron mobilized with phosvitin, 52% reacted with transferrin.

Table 2 shows the results of iron exchange when the pinto bean slurries (no prior centrifugation) were reacted with apotransferrin. The concentration of iron in the slurry is the same in all the samples, but the fraction of the iron associated with solid and soluble fractions varied. In all cases, the concentration of iron that reacted with apotransferrin in 30 min is higher than was observed in the supernatants. This indicates the possibility of residue-bound iron reacting with transferrin. A maximum of about 46% of the slurry iron is reactive. The remainder of the iron that is bound to the residue apparently cannot be mobilized or exchanged.

Again, the positive effect of ascorbic acid-containing additives and the negative effect of phosvitin and tea is clearly seen in terms of iron reactive with transferrin. The iron exchange with transferrin from the sample containing citric acid (25 μmol/L) is similar to the sample containing no additives (28 μmol/L).

Discussion

It is convenient to envision iron bioavailability in terms of a competition for iron between a mucosal-bound iron receptor protein and food-derived ligands in the digestive milieu. We chose to use transferrin as a model iron receptor because that is a part of its role in the serum and it reacts with Fe^{3+} complexes and Fe^{2+}, which is bound and then oxidized by molecular oxygen.[10] While a role for luminal transferrin in iron absorption was suggested,[13] this unlikely possibility has been reviewed by Osterloh et al.[14]

We investigated the effect of selected foods and additives on iron bioavailability in terms of reactive iron. In vitro digestion of pinto beans was carried out with consecutive enzymatic digestions at pH 3.0 and 5.0. Iron exchange reactions were carried out at pH 6.7 because it is a favorable pH for transferrin to sequester iron and also is within the normal duodenal pH range. By using a similar in vitro procedure, we found earlier[8] that at pH 5, in the presence of ascorbic acid, 85–92% of the pinto bean soluble iron was in Fe^{2+} state. In the present study, when the pH of the supernatant was raised to pH 6.7 for exchange reactions, no Fe^{2+} was detected in any samples. It has been shown earlier[15] that ascorbic acid at pH 6.6 can prevent the precipitation of ferric hydroxides in aqueous solution but cannot prevent the oxidation of Fe^{2+}.

We reported previously that much of the soluble Fe^{3+} in the pinto beans (digested without any additives) is chelated to low molecular weight ligands and the iron exchange to transferrin is rapid.[16] The present study confirms our earlier findings that a major fraction of the soluble iron bound to the intrinsic factors of pinto beans is reactive with apotransferrin. However, it should be noted that only 22% of the iron was found to be soluble after in vitro digestion when no additives were present in the pinto beans.

The exchange of iron to transferrin (from the slurry or supernatant form of the pinto beans) did not improve by the addition of citric acid. This would suggest that citric acid at 10 mmol/L concentration and at pH 6.7 may not have much iron donating capability. It has been reported[17] that the iron donation to transferrin is dependent on the pH and the concentration of citric acid. In the present study based on reactive iron, we can predict that consuming citric acid-containing foods with pinto beans may not improve the iron absorption. The studies on the effect of citric acid on human iron absorption are conflicting and inconclusive. Gillooly et al.[18] demonstrated the enhancement of iron uptake in humans from vegetables in the presence of citric acid. On the other hand, Hallberg and Rossander[19] showed a one-third reduction in iron absorption in humans when 1 g of citric acid was added to Latin American

Table 2 Reactivity of iron present in the pinto bean slurry with apotransferrin*†

Additives	Iron reacted with apotransferrin in 30 min at pH 6.7		
	(μmol/L)	(%)	(mg)‡
Ascorbic & citric acid	35.3	46	0.93
Ascorbic acid	33.7	44	0.89
Orange juice	30.6	40	0.81
Control	27.6	36	0.73
Citric acid	25.3	33	0.67
Phosvitin	16.9	22	0.45
Tea	4.6	6	0.12

*The reaction was carried out at pH 6.7 in the presence of 25 mmol/L $NaHCO_3$ and 50 mmol/L Hepes buffer.
†In each slurry 76.6 μmol/L iron was present.
‡Iron estimated to be reactive with apotransferrin from 100 g cooked pinto beans.

meals. While the effect of citric acid on iron absorption in humans is unclear, it is possible that citric acid competes with the mucosal receptor site.

Interestingly, the supernatant sample containing ascorbic acid donated 100% of its iron to apotransferrin in less than 30 min. Ascorbic acid enhanced the iron reactivity by 1.6 times over the control. Ascorbic acid is very effective in mobilizing pinto bean iron at gastric pH and keeping the iron in Fe^{2+} form.[6-8] When the pH increases to 5.0, ascorbic acid at 1.1 mmol/L concentration competes less effectively with the pinto bean residue in solubilizing iron. However, it retains a transient reducing capability. At pH 6.7, the pH at which the iron exchange reactions were carried out, the soluble iron even in the presence of ascorbic acid was 100% in the Fe^{3+} state. As indicated by Clydesdale,[20] Fe^{3+}-ascorbate has a higher stability constant than Fe^{2+}-ascorbate, yet a much lower stability constant than Fe^{3+}-transferrin.[17] Hence, we can expect a high iron donation to apotransferrin when iron is bound to ascorbic acid in either of the oxidation states. The enhancement of iron absorption by ascorbic acid that was reported in many studies[19,21] is clearly due primarily to reduction because simple Fe^{3+} chelators have little effect.

Similar iron solubilization was observed with the combination of ascorbic plus citric acid or citric acid alone, however, the combination was 1.6-fold (28 μmol/L versus 18 μmol/L) more effective in donating iron to apotransferrin. Sixty-one percent of the soluble iron was reacted with apotransferrin when ascorbic and citric acids were present together. When this mediated iron exchange process is carried out with ascorbic acid or citric acid alone, 100% or about 40% of the soluble iron would have been reacted with apotransferrin, respectively. The citric acid thus inhibits the reactivity found with ascorbic acid. This most likely reflects an inhibition of the rapid reaction of apotransferrin with Fe^{2+},ref.10. In the presence of ascorbate redox cycling between Fe^{3+} and Fe^{2+} would be expected to occur. The presence of citric acid would diminish the rate of redox cycling.

Tea, as anticipated, resulted in an approximate 80% decrease in the reactivity of soluble or slurry fraction of pinto bean iron with transferrin. The strong inhibitory effect of tea, with respect to low iron solubility, was shown previously by other researchers.[7,8,22,23] The polyphenols present in tea, complex iron and precipitate it, inhibiting the iron exchange to transferrin.

Eggs have been shown to reduce iron absorption because of the egg yolk protein, phosvitin.[24] The phosphorylated serine residues of phosvitin are known to be responsible for iron binding.[25] Sato et al.[26] indicated that the inhibitory effect of phosvitin on iron absorption might be due to the formation of insoluble phosvitin-iron complexes. However, we found that 16% more iron was solubilized when phosvitin was digested with the pinto beans versus the control. It should be noted that iron concentration observed in the supernatant is merely from the pinto beans because the phosvitin-bound iron was removed by dialysis. The iron exchange between the phosvitin containing sample and transferrin was 52%, but the amount of transfer was 20% lower than the control. The extent of iron exchange between transferrin and phosvitin is known to be a pH-dependent process.[27] Phosvitin has the capability to enhance the oxidation of Fe^{2+}, and its affinity for iron reportedly decreases as the pH increases from pH 6 to 7.2,[27] whereas the iron binding capacity of transferrin increases as the pH increases. These results are consistent with the observation that phosvitin, to a lesser extent than tea, can inhibit the iron absorption in humans.

When the slurry was included in the exchange reactions, the pattern of iron exchange was similar. The iron exchanged from pinto bean slurry to transferrin was higher than with the supernatant alone. It is possible that as the iron exchange reaction progresses the iron chelators mobilize more iron from the solid residues. About half of the iron appeared strongly bound to the solid residue such that it is neither exchanged nor solubilized under the conditions we used.

With the additives we used, the order of predicted iron bioavailability in terms of soluble iron reactivity is ascorbic and citric acids > orange juice > ascorbic acid > citric acid > control > phosvitin > tea. Our results are in close agreement with a similar study reported by Gorman and Clydesdale in which ascorbate donated its iron to apotransferrin faster than citrate.[28] EPR proved to be useful in examining the iron exchange reactions in the presence of solid materials and in quantitating the low amounts of iron. Our findings in the study indicate that iron reactivity approach with transferrin is consistent with the known effect of enhancers and inhibitors on iron bioavailability of complex meals. Further research is needed to validate the application of these reactivity studies to human nutrition.

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