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Influence of dietary phenolic acids on redox status of iron: Ferrous iron autoxidation and ferric iron reduction

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Abstract

We investigated the effect of dietary phenolic acids on the oxidation of Fe^{2+} caused by molecular oxygen. All phenolic acids bearing 3,4-dihydroxy (catechol) or 3,4,5-trihydroxy (galloyl) moiety formed chelates with ferric iron and significantly increased the rate of Fe^{2+} autoxidation. The carboxylate group and catechol substitution instead of galloyl moiety facilitated the ferrous ion oxidation more effectively. Caffeic acid and protocatechuic acid, the strongest accelerators of Fe^{2+} autoxidation, were able to facilitate autoxidation at concentrations lower than 1% of the initial amount of Fe^{2+}. Therefore chelates of these catecholic acids with iron displayed ferroxidase-like activity. Conversely, when we started from ferric ions, catechols partially formed ferrous ions in the presence of ferrozine. Thus, catecholic acids formed stable chelates with iron, in which ferric ion is the dominant species, but the redox cycling of iron between Fe^{2+} and Fe^{3+} in chelates probably plays a crucial role in the catalysis of ferrous iron autoxidation. Interestingly, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid and vanillic (4-hydroxy-3-methoxybenzoic) acid protected ferrous ions from autoxidation as effectively as ascorbic acid and cysteine. These monophenolic acids, differently from ascorbic acid and cysteine, were not able to reduce ferric ions. Syringic (3,5-dimethoxy-4-hydroxybenzoic) acid did not alter the redox state of iron, only in a large excess over metal, syringic acid slightly inhibited ferrous ions autoxidation and partially reduced ferric ions. Therefore, the effects of syringic acid at high concentration were similar but much lower to those of ascorbic acid and cysteine. The biological importance of ferroxidase-like activity of polyphenols, especially the influence on iron absorption, is also discussed.

Keywords: Iron; Phenolic acids; Polyphenols; Ferrous ion; Ferric ion; Autoxidation; Ferric iron reduction; Metal chelation; Ferroxidase-like activity; Ferrozine

1. Introduction

Polyphenols are plant secondary metabolites that are frequently components of human diet. Their daily intake in the Western diet was recently estimated to be about 1 g (Manach, Scalbert, Morand, Remesy, & Jimenez, 2004). Some epidemiological studies have suggested an association between the consumption of polyphenol-rich foods or beverages and the prevention of cardiovascular diseases and cancer. These pathological conditions are connected with excessive free radical production. Many studies confirmed the ability of plant polyphenols to scavenge free radicals, demonstrated their potential act as chain-breaking antioxidants (Pietta, 2000; Rice-Evans, Miller, & Paganga, 1996).

There is a general agreement that transition metals, above all iron, can cause formation of free radicals *in vivo*. Iron may easily oscillate between the ferrous (Fe^{2+}) and ferric (Fe^{3+}) states. Whereas ferric ions are the relatively biologically inactive form of iron, ferrous ions are responsible for formation of a part of reactive oxygen species that can lead to lipid peroxidation, nucleic acid or protein damage. Superoxide anion is readily produced through the one-electron reduction of molecular oxygen by ferrous ions. Hydrogen peroxide, which is produced

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by some enzymes or by dismutation of superoxide, is converted to extremely reactive hydroxyl radicals by Fenton reaction, which needs reduced transition metals, including ferrous ions. Ferrous iron may also bind to molecular oxygen and form very reactive perferryl ion (Fe^{2+}–O_2). Ferrous ion induces peroxidation of lipids, which causes the desired flavour of food and lipid peroxidation is an important toxic mechanism *in vivo* (Hippeli & Elstner, 1999).

Some plant polyphenols possess a remarkable transition metal chelating activity and form redox stable complexes with transition metal ions (Brown, Khodr, Hider, & Rice-Evans, 1998; Hider, Liu, & Khodr, 2001; Psotová, Lasovský, & Vičar, 2003; van Acker et al., 1996). Therefore, plant polyphenols can interfere not only with the propagation reactions of the free radicals by means of scavenging them, but also with the formation of radicals by chelating the transition metal ions.

Numerous authors have investigated the free radical scavenging activity of plant polyphenols, e.g. Rice-Evans et al. (1996) and Yokozawa et al. (1998). Several reports have been made to elucidate the iron or copper ions chelating ability of polyphenols, e.g. van Acker et al. (1996) and Brown et al. (1998), kinetics and mechanisms of reactions of polyphenols with ferric ions (El Hajji, Nkhili, Tomao, & Dangles, 2006; Hynes & O'Coinceanainn, 2004), superoxide radical scavenging by polyphenol iron complexes (Zhao, Khan, & O'Brien, 1998), chemoprotective effect (Chlopčíková et al., 2004) and the binding constants of complexes (Andjelkovic et al., 2006), although the interaction of plant polyphenols with transition metal ions has not been fully resolved. In this study we focused on the influence of plant polyphenols, above all phenolic acids, on the redox state of iron at the physiological pH. Phenolic acids were chosen mainly for two reasons. The first one is that phenolic acids commonly occur in foods and form approximately one third of the total intake of plant polyphenols, the most abundant antioxidants in human diet (Manach et al., 2004). Moreover, simple phenolic acids may be also formed by colon microflora from ingested flavonoids, the main class of polyphenols occurred in our diet (Pietta, 2000). The second reason is that simple structure of phenolic acids with one aromatic ring allows easy interpretation of relationships between the various substitutions of an aromatic ring and the metal chelation activity.

2. Materials and methods

2.1. Materials

Methanol (HPLC gradient grade), caffeic acid, gallic acid, 4-hydroxybenzoic acid, 3-hydroxybenzoic acid, EDTA, $FeSO_4 \cdot 6H_2O$, $FeCl_3 \cdot 7H_2O$ and ferrozine were obtained from Fluka. Chlorogenic acid, protocatechuic acid, vanillic acid, syringic acid, L-cysteine, Hepes and quercetin were purchased from Sigma (St. Louis, USA). Methylgallate was from Aldrich, catechol was from ICN Biomedicals (Aurora, USA) and ascorbic acid was from Tamda (Olomouc, Czech Republic). The spectrophotometric measurements were performed using a Shimadzu UV-1601 spectrophotometer in 1 cm quartz cuvettes. Temperature was controlled at 25 ± 0.1 °C by means of thermostatic bath.

2.2. Absorption spectra

Stock solutions of each polyphenol (5 mmol l^{-1}) were prepared in methanol. Working solutions of polyphenols were prepared by dilution of stock solutions with deionised water. Then 0.2 ml of solutions of polyphenols (300 μmol l^{-1}) was mixed with 0.067, 0.1, 0.2, 0.3 or 0.4 ml of solution (300 μmol l^{-1}) of $FeSO_4$ or $FeCl_3$. Then 1.8 ml of Hepes buffer (final concentration 10 mmol l^{-1}, pH 7.4) was added to a cuvette and the absorption spectra were recorded between 200 and 800 nm at 15 min after the Hepes addition. Scans with polyphenols and iron ions were compared with those of the polyphenol alone. The reversibility of the complexes formed was assessed by the addition of a fivefold excess of EDTA or ascorbic acid over polyphenol (0.1 ml of 3125 μmol l^{-1}). The absorption due to iron complex with EDTA was subtracted from the spectra of the mixtures of polyphenol with iron and EDTA.

2.3. Determination of Fe^{2+} autoxidation

The concentration of Fe^{2+} was determined using ferrozine that binds evidently only to Fe^{2+} in the presence of Fe^{3+} (Welch, Davis, & Aust, 2002). Ferrous sulfate stock solutions were prepared in deionised water under argon. Then Fe^{2+} solution (final concentration 42 μmol l^{-1}) was added to 3.6 ml of Hepes buffer (final volume 4.8 ml, final concentration 10 mmol l^{-1}, pH 7.4) containing polyphenols. At specific time points 0.5 ml aliquots were removed and mixed with 0.5 ml of 1 mmol l^{-1} ferrozine in cuvette. After 1 min, the content of Fe^{2+} was quantified by measuring absorbance at 562 nm.

2.4. Determination of Fe^{3+} reducing ability

We added gradually into the test tube 0.5 ml of polyphenol, 1.8 ml of the Hepes buffer (the final concentration 10 mmol l^{-1}) and 0.1 ml $FeCl_3$ (the final concentration 42 μmol l^{-1}). One minute after the addition of $FeCl_3$ 0.5 ml of the mixture was transferred to the cuvette with 0.5 ml of the ferrozine solution (1 mmol l^{-1}). The amount of resulting Fe^{2+} was quantified by measuring the absorbance at 562 nm. Each analysis was accompanied by a sample blank with water instead of ferrozine reagent. Values were calculated by subtracting sample blank values recorded in the absence of ferrozine. For some ligands, when ferrozine was added to the mixture, a biphasic increase in absorbance was observed (Figs. 9a–9c). Rapid increase in absorbance of the Fe^{2+}–ferrozine complex was evaluated after 5 min and expressed as the percentage of

the Fe^{2+} formed from the initial amount of Fe^{3+} (Table 1). The following slow increase in absorbance was expressed as percentage of the Fe^{2+} formed from the initial amount of Fe^{3+} during the following 60 min (Table 2).

Table 1
Reduction of Fe^{3+} by different concentration of ligands determined for the initial rate (first 5 min)

Ligand	Fe^{2+} formed (%) after 5 min[a]		
	Ligand to Fe^{3+} molar ratio		
	0.5	5	50
Ferrozine only	0.6 ± 0.8	0.6 ± 0.8	0.6 ± 0.8
Ascorbic acid	4.9 ± 3.3	9.3 ± 2.0	85.2 ± 0.9
Cysteine	3.1 ± 1.0	6.8 ± 0.7	55.2 ± 3.0
Catechol	13.1 ± 3.0	0.2 ± 0.7	−0.2 ± 1.2
Methylgallate	0.1 ± 0.5	0.2 ± 0.1	0.5 ± 0.1
Gallic acid	1.0 ± 0.4	−0.9 ± 0.6	−0.4 ± 0.6
Caffeic acid	0.7 ± 0.3	−0.8 ± 0.9	0.3 ± 0.7
Chlorogenic acid	−0.2 ± 0.7	0.9 ± 0.7	n.d.[b]
Protocatechuic acid	0.5 ± 0.8	−0.1 ± 0.2	0.4 ± 0.6
Vanillic acid	1.8 ± 0.5	−0.8 ± 0.7	2.4 ± 0.5
4-Hydroxybenzoic acid	3.5 ± 0.6	4.8 ± 0.7	5.6 ± 0.7
3-Hydroxybenzoic acid	4.1 ± 0.3	2.9 ± 0.3	5.0 ± 0.5
Syringic acid	0.0 ± 0.3	−0.2 ± 0.2	2.9 ± 1.0

[a] Reaction mixtures contained 42 μmol l^{-1} Fe^{3+} and different concentration of ligands in 10 mM Hepes buffer, pH 7.4. Determination of resulting Fe^{2+} was performed by ferrozine as outlined under Section 2. Results are shown as percentage of initial amounts of Fe^{3+} formed in the initial "fast" phase of reduction (first 5 min). The results were calculated by subtracting the absorbance of blank (without ferrozine) from absorbance of sample (with ferrozine). Results are expressed as means ± SD of three experiments.
[b] Not determined.

Table 2
Reduction of Fe^{3+} by different concentration of ligands determined during 60 min after the initial phase (first 5 min)

Ligand	Fe^{2+} formed (%) during 60 min[a]		
	Ligand to Fe^{3+} molar ratio		
	0.5	5	50
Ferrozine only	0.9 ± 0.3	0.9 ± 0.3	0.9 ± 0.3
Ascorbic acid	10.7 ± 0.5	16.1 ± 0.4	11.4 ± 1.0
Cysteine	11.2 ± 3.4	14.7 ± 1.5	8.8 ± 1.1
Catechol	5.6 ± 0.2	13.9 ± 0.7	4.5 ± 0.2
Methylgallate	0.3 ± 0.1	0.0 ± 0.1	−0.5 ± 0.1
Gallic acid	6.1 ± 0.3	5.5 ± 1.3	11.3 ± 2.0
Caffeic acid	7.2 ± 0.2	6.3 ± 0.6	9.8 ± 0.7
Chlorogenic acid	2.2 ± 0.3	0.3 ± 0.3	n.d.[b]
Protocatechuic acid	0.1 ± 0.2	4.3 ± 0.2	1.3 ± 0.1
Vanillic acid	0.1 ± 0.3	0.5 ± 0.4	0.4 ± 0.3
4-Hydroxybenzoic acid	−0.2 ± 0.1	−0.2 ± 0.1	0.0 ± 0.1
3-Hydroxybenzoic acid	−0.7 ± 0.2	−0.1 ± 0.1	0.0 ± 0.2
Syringic acid	−0.2 ± 0.2	0.0 ± 0.1	7.8 ± 0.2

[a] Reaction mixtures contained 42 μmol l^{-1} Fe^{3+} and different concentration of ligands in 10 mM Hepes buffer, pH 7.4. Determination of resulting Fe^{2+} was performed by ferrozine as outlined under Section 2. Results are shown as percentage of initial amounts of Fe^{3+} formed per 60 min during "slow" phase of Fe^{2+} formation after the "fast" initial phase (first 5 min). Results are expressed as means ± SD of three experiments.
[b] Not determined.

3. Results

3.1. Absorption spectra of phenolic acids with iron

The formation of the complexes of the plant polyphenols with iron ions (Fe^{2+} or Fe^{3+}) at pH 7.4 was determined by UV–Vis spectroscopy. The structures of phenolic acids tested are shown in Fig. 1. The spectroscopic studies indicated that only phenolic acids containing the 3,4-dihydroxy (catechol) substitution in aromatic ring (caffeic acid, chlorogenic acid, protocatechuic acid, gallic acid, catechol and methylgallate) formed the complexes with ferric ions. Addition of ferric ions to phenolic acid with catechol moiety produced a strong bathochromic shift. New absorption band in visible part of spectra with a law absorbance was also detected. For example, interactions of caffeic acid with ferric ions are shown in Fig. 2. Addition of ferric ions at equimolar ratio induced red shifts in band from 311 nm to 341 nm, which was connected with a small increase in absorbance. This was accompanied by the formation of new broad band around 590 nm with a low absorbance.

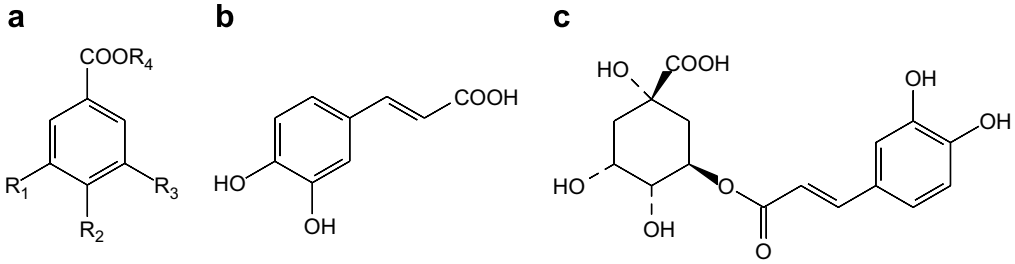
The spectrum of ferric ions with gallic acid, which contains additional hydroxyl group at 5′ position, differed from that of phenolic acids with catechol moiety (Fig. 3). The wavelength of absorbance maximum of gallic acid around 260 nm did not change after the addition of ferric ions. However, absorbance of this band increased indicating the formation of complexes with iron. The addition of ferric iron also enhanced the absorbance in the visible region, but no clear peak was observed in this case. The addition of EDTA to chelates regenerated the original spectrum of gallic acid (Fig. 3).

Under similar conditions, hydroxybenzoic acids with 3-methoxy-4-hydroxy (vanillic acid), 3,5-methoxy-4-hydroxy (syringic acid) or hydroxy substitution (3-hydroxybenzoic acid and 4-hydroxybenzoic acid) did not show any change in spectra after the addition of ferric ions.

We further investigated interactions of phenolic acids possessing catechol group with ferrous ions. For example, the spectrum of caffeic acid with Fe^{2+} is plotted in Fig. 4. Surprisingly, the spectrum of caffeic acid obtained with ferrous ions (Fig. 4) was closely related to that found after addition of ferric ions (Fig. 2). EDTA regenerated the original spectra of phenolic acids with Fe^{2+} only partially. In view of the similarities of both spectra, these observations suggest that catechols bond in complexes either ferrous or ferric ions. Therefore, we further explored the influence of phenolic acids on redox state of iron.

3.2. Ferrous ions autoxidation

We determined the rate of Fe^{2+} autoxidation in the presence of polyphenols. The remaining Fe^{2+} ions were quantified by ferrozine, a reagent for spectrophotometric determination of Fe^{2+}. The rate of Fe^{2+} autoxidation was significantly affected by the buffer. The phosphate



Benzoic acid derivatives	R_1	R_2	R_3	R_4
3-hydroxybenzoic acid	-OH	-H	-H	-H
4-hydroxybenzoic acid	-H	-OH	-H	-H
vanillic acid	$-OCH_3$	-OH	-H	-H
protocatechuic acid	-OH	-OH	-H	-H
syringic acid	$-OCH_3$	-OH	$-OCH_3$	-H
gallic acid	-OH	-OH	-OH	-H
methylgallate	-OH	-OH	-OH	$-CH_3$

Fig. 1. Chemical structures of (a) benzoic acid derivatives, (b) caffeic acid, and (c) chlorogenic acid.

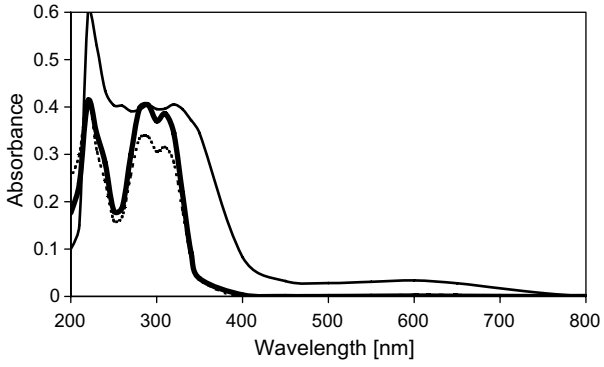


Fig. 2. The effect of EDTA on the Fe^{3+} complex of caffeic acid. Absorbtion spectra of caffeic acid (25 μmol l^{-1}), thick solid line; caffeic acid (25 μmol l^{-1}) + Fe^{3+} (50 μmol l^{-1}), thin solid line; caffeic acid (25 μmol l^{-1}) + Fe^{3+} (50 μmol l^{-1}) + EDTA (125 μmol l^{-1}), the absorption due to Fe^{3+}–EDTA complex was subtracted from the spectrum of caffeic acid + Fe^{3+} + EDTA, dotted line.

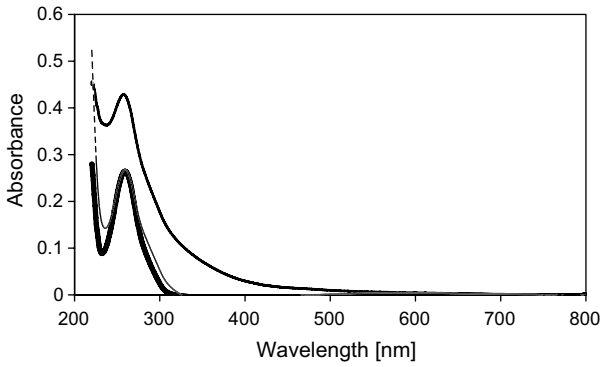


Fig. 3. The effect of EDTA on the Fe^{3+} complex of gallic acid. Absorbtion spectra of gallic acid (25 μmol l^{-1}), thick solid line; gallic acid (25 μmol l^{-1}) + Fe^{3+} (50 μmol l^{-1}), thin solid line; gallic acid (25 μmol l^{-1}) + Fe^{3+} (50 μmol l^{-1}) + EDTA (125 μmol l^{-1}), the absorption due to Fe^{3+}–EDTA complex was subtracted from the spectrum of gallic acid + Fe^{3+} + EDTA, dotted line.

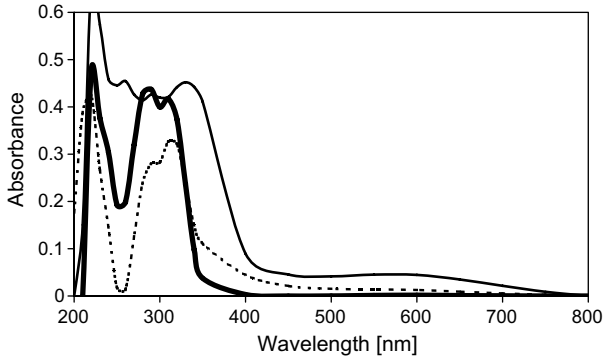


Fig. 4. The effect of EDTA on the Fe^{2+} complex of caffeic acid. Absorbtion spectra of caffeic acid (25 μmol l^{-1}), thick solid line; caffeic acid (25 μmol l^{-1}) + Fe^{2+} (50 μmol l^{-1}), thin solid line; caffeic acid (25 μmol l^{-1}) + Fe^{2+} (50 μmol l^{-1}) + EDTA (125 μmol l^{-1}), the absorption due to Fe^{2+}–EDTA complex was subtracted from the spectrum of caffeic acid + Fe^{2+} + EDTA, dotted line.

buffer considerably increased the rate of Fe^{2+} autoxidation. It is known that the piperazine based buffers (Hepes, Mops, and Mes) all have the same and low effect on Fe^{2+} autoxidation (Welch et al., 2002). Therefore, we used Hepes buffer for all experiments.

The rate of Fe^{2+} autoxidation in the absence of any ligands was low in the first 5 min and gradually increased till the 10th minute (Fig. 5a). This augmentation in the rate of Fe^{2+} oxidation can be explained by the increase in the concentration of Fe^{3+}, which speeds up the course of autoxidation (Welch et al., 2002). The speed of autoxidation slows down from the 20 min as the result of the decrease of the substrate (Fe^{2+}) concentration. The autoxidation of half of the initial Fe^{2+} amount without the presence of ligands took about 10 min at pH 7.4 (Fig. 5a). This is in a good agreement with the data already published (Welch et al., 2002).

We determined the effect of various phenolic compounds and reducing agents on Fe^{2+} autoxidation. Gener-

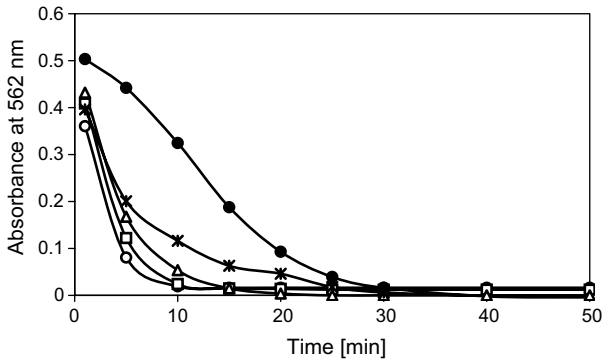


Fig. 5a. The effect of catechols (ligand to Fe^{2+} molar ratio 0.125) on Fe^{2+} (42 μmol l^{-1}) autoxidation in 10 mM Hepes buffer pH 7.4. Without ligands (●), caffeic acid (○), protocatechuic acid (□), catechol (△), chlorogenic acid (⚹). Aliquots were removed at a specific time and assayed for the amount of Fe^{2+} remaining as described under Section 2.

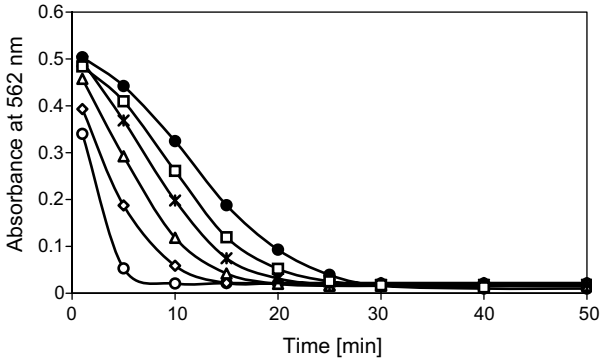


Fig. 6. The effect of caffeic acid on Fe^{2+} (42 μmol l^{-1}) autoxidation in 10 mM Hepes buffer pH 7.4. Without ligands (●), caffeic acid to Fe^{2+} molar ratio 0.0039 (□), caffeic acid to Fe^{2+} molar ratio 0.0156 (⚹), caffeic acid to Fe^{2+} molar ratio 0.0625 (△), caffeic acid to Fe^{2+} molar ratio 0.25 (◇), caffeic acid to Fe^{2+} molar ratio 0.125 (○). Aliquots were removed at a specific time and assayed for the amount of Fe^{2+} remaining as described under Section 2.

ally, some phenolic acids increased the rate of oxidation of Fe^{2+}; on the other hand other phenolic acids slowed it down. All polyphenols with catechol and pyrogallol moiety significantly increased the rate of Fe^{2+} autoxidation. The stimulatory effect of ligands with catechol moiety in the ratio Fe^{2+}/ligand of 8 is presented in Fig. 5a. The influence of autoxidation by ligands bearing pyrogallol moiety and the effect of quercetin is shown in Fig. 5b. The stimulatory effect of ligands on autoxidation falls in the order caffeic acid > protocatechuic acid > catechol ~ gallic acid > chlorogenic acid > quercetin > methylgallate. A dose-dependent increase of autoxidation caused by caffeic acid is plotted in Fig. 6. Surprisingly, the increase of the rate of autoxidation was observed also in the case, when the concentration of polyphenols was substantially lower than the initial concentration of Fe^{2+}. The impact of caffeic and protocatechuic acid, the most efficient catalysts of Fe^{2+} autoxidation, was evident at least up to the concentration that corresponds to 0.4% of the initial amount of Fe^{2+}!

To find out the influence of the carboxylic group of the phenolic acids we have compared the effect of protocatechuic acid with catechol, and effect of gallic acid with its methyl ester. Protocatechuic acid was more effective in speeding up the autoxidation than catechol, the product of its decarboxylation. Similarly, the effect of methylgallate in comparison to gallic acid was mild and contradictory. It was obvious only at almost equimolar level of methylgallate to Fe^{2+}. Methylgallate increased the rate of autoxidation in dose dependent manner from the beginning to approximately its half time of the reaction (Fig. 7). However, the effect of methylgallate changed with increasing incubation time from speeding up the reaction to its slowing down. Moreover, in the presence of methylgallate a part of ferrous ions remained in the solution for more than an hour (not shown). Phenolic acids increased the rate of autoxidation more than the corresponding esters or phenols without carboxylic group. This confirms that the negative charge of the conjugated bases of phenolic acids or carboxylate group itself, which are formed in the buffer with pH 7.4, accelerates the rate of autoxidation.

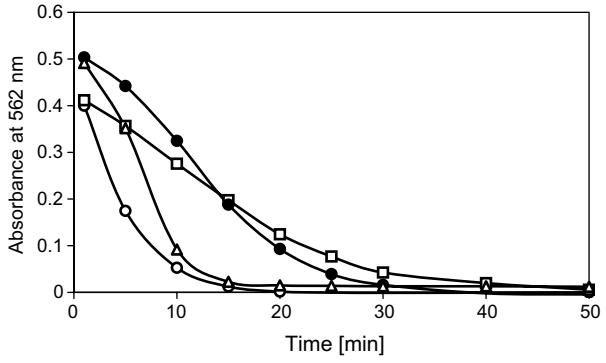


Fig. 5b. The effect of gallic acid derivatives and quercetin (ligand to Fe^{2+} molar ratio 0.125) on Fe^{2+} (42 μmol l^{-1}) autoxidation in 10 mM Hepes buffer pH 7.4. Without ligands (●), gallic acid (○), methylgallate (□), quercetin (△). Aliquots were removed at a specific time and assayed for the amount of Fe^{2+} remaining as described under Section 2.

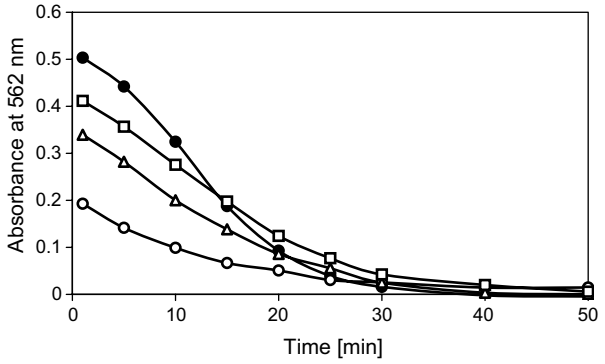


Fig. 7. The effect of methylgallate on Fe^{2+} (42 μmol l^{-1}) autoxidation in 10 mM Hepes buffer pH 7.4. Without ligands (●), methylgallate to Fe^{2+} molar ratio 0.125 (□), methylgallate to Fe^{2+} molar ratio 0.25 (△), methylgallate to Fe^{2+} molar ratio 0.5 (○). Aliquots were removed at a specific time and assayed for the amount of Fe^{2+} remaining as described under Section 2.

Among phenolic acids, caffeic, protocatechuic and gallic acid were the most powerful accelerators of autoxidation. The difference between the effect of caffeic acid and protocatechuic acid was not conclusive. This shows that the effect of hydroxybenzoic acids was similar to that of hydroxycinnamic acid with the same substitution on the aromatic ring. However, protocatechuic acid at all concentration induced Fe^{2+} oxidation more quickly as compared with gallic acid. The presence of the third phenolic hydroxyl on the aromatic ring of the phenolic acid unambiguously decreases the stimulating effect on autoxidation.

To explore the influence of partial methylation of phenolic hydroxyls and impact of monohydroxyphenolic acids on the rate of autoxidation, we further investigated the effect of vanillic, syringic, 3-hydroxybenzoic and 4-hydroxybenzoic acid (Fig. 8). Surprisingly, vanillic acid, contrary to protocatechuic acid, inhibited the rate of Fe^{2+} autoxidation as is shown in Fig. 8. Syringic acid was substantially a weaker inhibitor of Fe^{2+} autoxidation than structurally similar vanillic acid. Syringic acid slightly reduced the rate of autoxidation only in 20-fold over the Fe^{2+} concentration (Fig. 8). The impact of both hydroxybenzoic acids (3-hydroxybenzoic acid and 4-hydroxybenzoic acid) was very similar to that of vanillic acid. Thus, methylation or dehydroxylation of one phenolic hydroxyl of protocatechuic acid or methylation of two hydroxyls of gallic acid reversed the effect of phenolic acids on Fe^{2+} autoxidation. Interestingly, vanillic acid and both hydroxybenzoic acids were approximately as effective in diminution of rate of Fe^{2+} autoxidation as ascorbic acid and cysteine (Fig. 8). The comparison of effects of phenolic acids led us to the conclusion, that substantially higher concentrations of ligands were needed to inhibit Fe^{2+} autoxidation than were the effective concentrations of ligands that increased the rate of autoxidation.

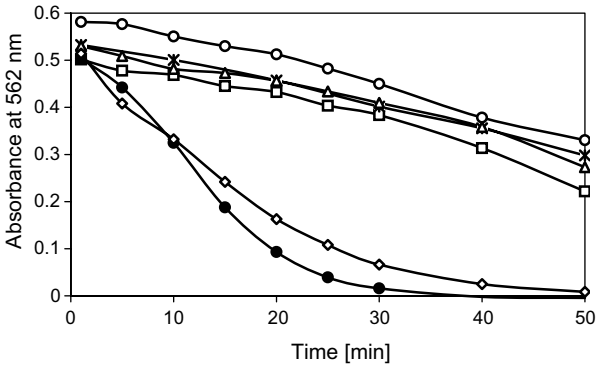


Fig. 8. The effect of ligands (ligand to Fe^{2+} molar ratio 10) on Fe^{2+} (42 μmol l^{-1}) autoxidation in 10 mM Hepes buffer pH 7.4. Without ligands (●), ascorbic acid (○), cysteine (⚹), 4-hydroxybenzoic acid (△), vanillic acid (□), syringic acid (◇). The effect of 3-hydroxybenzoic acid, which was approximately the same as 4-hydroxubenzoic acid, was omitted for clarity. Aliquots were removed at a specific time and assayed for the amount of Fe^{2+} remaining as described under Section 2.

3.3. Reduction of ferric ions

To further assess the effect of phenolic acids on redox state of iron, we also determined the ability of 10 polyphenols, ascorbic acid and cysteine to reduce Fe^{3+} to Fe^{2+}. The amount of Fe^{2+} formed was measured with the help of ferrozine.

The addition of ferrozine to the solution of Fe^{3+} in the Hepes buffer in the absence of any ligands did not produce Fe^{2+}–ferrozine complex during the first 5 min, because the range of Fe^{3+} reduction was $0.6 \pm 0.8\%$, as we can see from Table 1. During the following 60 min only a very small amount of Fe^{2+} ions was formed, corresponding to $0.9 \pm 0.3\%$ of the starting concentration of Fe^{3+}. This result implies that the Hepes buffer as well as ferrozine is practically not able to reduce Fe^{3+} at pH 7.4. This confirms the known fact, that ferrozine can be really used to measure the concentration of Fe^{2+} beside Fe^{3+}. Formation of a very small amount of the Fe^{2+}–ferrozine complex, approximately 1% of the initial Fe^{3+} over a period of 60 min, could be explained either by a low affinity of the Hepes buffer to the Fe^{2+} ions or by the action of ferrozine, which through its affinity to Fe^{2+} ions gradually shifted the balance towards Fe^{2+}.

We examined effects of polyphenols on Fe^{3+} in the presence of ferrozine. We evaluated the time course of the formation of Fe^{2+}–ferrozine complex with molar ratios ligand/Fe^{3+} of 0.5, 5, and 50 (Figs. 9a–9c). Only for several compounds, the amount of Fe^{2+} ions formed within 5 min was high enough that there were no doubts about their ability to reduce ferric ions (Table 1). We observed repeatedly a gradual increase in absorbance at 562 nm (the absorption maximum of the complex of ferrozine with Fe^{2+} ions). Therefore, we evaluated not only the amount of Fe^{2+} bound in a complex with ferrozine 5 min after the addition of a ligand, but also the rate of formation of Fe^{2+}–ferrozine complex in the course of the next 60 min (Table 2). To find out, if the increase in absorbance was

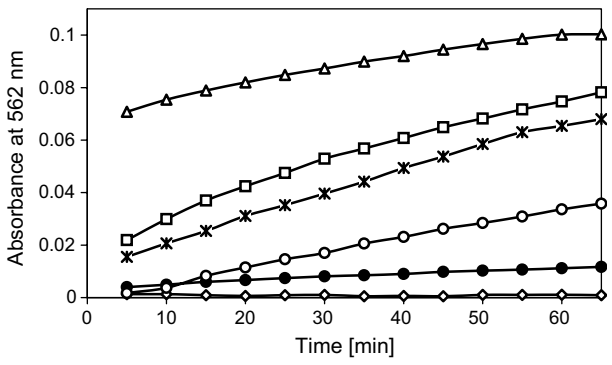


Fig. 9a. Time course of Fe^{3+} reduction at ligand to Fe^{3}+ molar ratio 0.5 determined in 10 mmol l^{-1} Hepes buffer pH 7.4. Representative ligands: without ligands (●), ascorbic acid (□), cysteine (⚹), catechol (△), gallic acid (○), syringic acid (◇). Samples containing ligand (21 μmol l^{-1}) and Fe^{3+} (42 μmol l^{-1}) were preincubated for 60 s before ferrozine addition as described under Section 2.

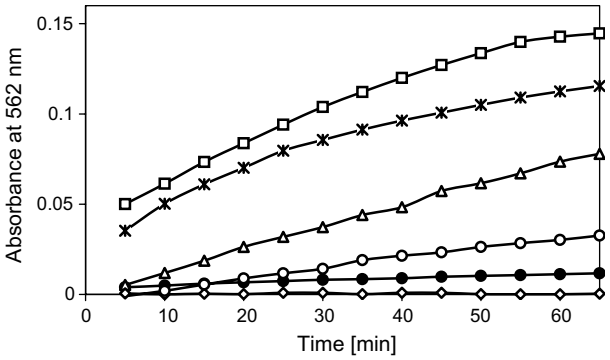


Fig. 9b. Time course of Fe^{3+} reduction at ligand to Fe^{3+} molar ratio 5 determined in 10 mmol l^{-1} Hepes buffer pH 7.4. Representative ligands: without ligands (●), ascorbic acid (□), cysteine (✳), catechol (△), gallic acid (○), syringic acid (◇). Samples containing ligand (210 μmol l^{-1}) and Fe^{3+} (42 μmol l^{-1}) were preincubated for 60 s before ferrozine addition as described under Section 2.

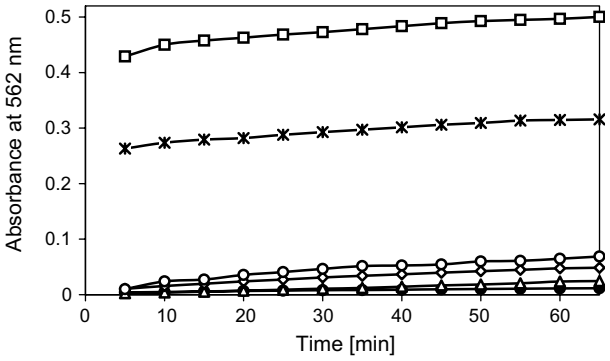


Fig. 9c. Time course of Fe^{3+} reduction at ligand to Fe^{3+} molar ratio 50 determined in 10 mmol l^{-1} Hepes buffer pH 7.4. Representative ligands: without ligands (●), ascorbic acid (□), cysteine (✳), catechol (△), gallic acid (○), syringic acid (◇). Samples containing ligand (2.1 mmol l^{-1}) and Fe^{3+} (42 μmol l^{-1}) were preincubated for 60 s before ferrozine addition as described under Section 2.

not caused by the formation of a complex between polyphenol and iron ions, the same experiments were carried out using water instead of ferrozine reagent. The results were calculated by subtracting the values recorded in the absence of ferrozine and are presented in Tables 1 and 2 as percentage of the Fe^{2+} formed from the initial amount of the Fe^{3+} ions.

It was not surprising that both ascorbic acid and cysteine effectively reduced Fe^{3+} in a dose-dependent manner (Tables 1 and 2). Ascorbic acid and cysteine reduced Fe^{3+} ions even in the lowest concentration tested. The reducing effect of ascorbic acid seems to be stronger than that of cysteine.

Some polyphenols with catechol moiety were able to partially reduce Fe^{3+} ions, but they were much less effective as ascorbic acid or cysteine. These polyphenols reduced less than 15% of the initial amount of Fe^{3+} within one hour even when assayed at a large excess over the metal.

The most effective reduction among polyphenols was observed in the case of catechol; the effect was more evident at the low catechol/Fe^{3+} ratio. The formation of Fe^{2+}–ferrozine complex was relatively low at the large catechol/Fe^{3+} ratio, apparently demonstrating a competition between catechol and ferrozine for the chelation of Fe^{2+}. Methylgallate elevated substantially the absorbance after addition of Fe^{3+} and ferrozine, but the same increase in absorbance was observed in the absence of ferrozine, indicating that the increase in absorbance was caused by the formation of complexes between methylgallate and iron, from which ferrous iron is not released by ferrozine.

Both, gallic acid and caffeic acid were able to reduce Fe^{3+}, but the effect was apparent only after prolonged incubation (Figs. 9a–9c). From the results shown in Tables 1 and 2, it was clearly evident that both gallic and caffeic acid caused the formation of Fe^{2+}–ferrozine complex from the low molar ligand/iron ratio. The effect of caffeic acid was approximately equal to that of gallic acid. Protocatechuic acid and chlorogenic acid have little, if any, influence on the Fe^{3+} reduction.

Atypical results were obtained for 3-hydroxybenzoic and 4-hydroxybenzoic acid. After the addition of ferrozine, the absorbance increased rapidly proportionally to the concentration of these phenolic acids. The rapid increase in absorbance caused by 3-hydroxybenzoic and 4-hydroxybenzoic acid, corresponded to 5–15% and 4–11% of the initial amount of Fe^{3+}, respectively (results are not shown). After 5 min the absorbance decreased to 2.9–5.6% of the initial amount of Fe^{3+} (Table 1) and did not change during the following one hour (Table 2). All ligands, which quickly reduce Fe^{3+}, also gradually increased the absorbance during one hour incubation, this was not observed in the case of 3-hydroxybenzoic and 4-hydroxybenzoic acid. Furthermore, the slow reduction of Fe^{3+} caused by ferrozine itself was completely abolished by both hydroxybenzoic acids in all concentrations tested (Table 2). It could be interpreted, that both hydroxybenzoic acids displayed either a very slight reduction of Fe^{3+} or more probably do not reduce Fe^{3+}. The unambiguous results were obtained with vanillic acid, which did not reduce Fe^{3+}. Moreover, vanillic acid decreased the reduction of Fe^{3+} in all tested solutions, caused by the ferrozine in the Hepes buffer itself (Table 2). Thus, vanillic acid stabilizes Fe^{3+} ions; this is a very interesting result, because in the case of autoxidation, vanillic acid inhibited the Fe^{2+} oxidation.

The effect of syringic acid till the fivefold of the concentration of Fe^{3+} was very similar to that of vanillic acid. However, at the highest molar ratio (syringic acid/Fe^{3+} = 50), syringic acid unambiguously slowly reduced Fe^{3+} ions (the reduction of 8% of the initial amount of Fe^{3+} within one hour, Table 2).

4. Discussion

In our study, we have selected a number of dietary phenolic acids together with their analogs and known iron reducing agents, ascorbic acid and cysteine, to explore their influence on the redox state of iron. The most common

oxidation states of iron in biological systems are Fe^{2+} and Fe^{3+}; iron may easily oscillate between them. Generally, ferric ion is more stable and relatively inactive form of iron, whereas ferrous ions are responsible for the formation of reactive oxygen species. It was found by Minotti and Aust (1992) and reviewed recently by Welch, Davis, Van Eden, and Aust (2002) that both Fe^{2+} and Fe^{3+} must be available to initiate lipid peroxidation with optimum activity occurring as the Fe^{2+}/Fe^{3+} ratio approaches unity. Intestinal iron absorption depends on the redox state of iron. Non-heme iron occurs in food largely as ferric iron. Some part of ferric dietary iron is reduced by dietary constituents to ferrous iron, which is more soluble at neutral pH than ferric iron. The reduction agents as ascorbic acid increase the amount of ferrous iron, rendering it available for absorption.

It is known that the autoxidation of ferrous iron is affected by pH, buffers and iron chelation (Welch et al., 2002; Yang & Chasteen, 1999). Low molecular weight iron is never being "free" under physiological condition and ligands bound to iron considerably influence on the reactivity and redox state of iron. The rate of Fe^{2+} oxidation is significantly affected by the buffer used. It is well documented that oxidation of ferrous iron is substantially facilitated by the phosphate buffer. Conversely, Hepes buffer, which we used in all experiments, has low effect on Fe^{2+}autoxidation (Welch et al., 2002).

Our data shown in Fig. 5a indicate that in the presence of 10 mM Hepes (pH 7.4) was an initial lag phase in the Fe^{2+} autoxidation, which is consistent with previous studies. After lag phase, the rate of autoxidation increased, because ferric ions promote the oxidation of ferrous iron (Tadolini, 1987; Welch et al., 2002; Yang & Chasteen, 1999).

The rate of Fe^{2+} autoxidation was significantly affected by the ligands used in our experiments. We have found that polyphenols bearing catechol or galloyl moiety strongly increased the rate of ferrous iron oxidation. At physiological pH, catechols form as bidentate ligands thermodynamically stable chelates with iron. It was shown that catechols are capable chelating both ferrous and ferric iron (Andjelkovic et al., 2006; Hider et al., 2001; Moridani, Pourahmad, Bui, Siraki, & O'Brien, 2003). Our results show that independently to initial redox state of iron, UV–Vis spectra catecholic acids with ferrous and ferric iron are the same as is shown for example with caffeic acid in Figs. 2 and 4. However, the strong acceleration of ferrous ion oxidation by molecular oxygen in the presence of catecholic acids clearly demonstrated that phenolic acids bearing catechol moiety did not form chelates with ferrous ions, but formed chelates with ferric ions. Our results are in a good agreement with those obtained by Paiva-Martins and Gordon (2005) and Andreu et al. (2005).

In one study, Lodovici, Guglielmi, Meon, and Dolara (2001) did not observe any interaction between catecholic acids (caffeic acid and 3,4-dihydroxybenzoic acid) and ferric ions contrary to ferrous ions. We can speculate that the lack of formation of complexes with Fe^{3+} in that study might be explained by a very low solubility of ferric iron at neutral pH. In our experiments concerned to the interaction of phenolic acids with ferric ions, we initially incubated ligands with freshly prepared solution of ferric chloride before addition of slightly alkaline buffer to avoid the precipitation of ferric ions in slightly alkaline buffer. Our results are also different from those obtained by Yoshino and Murakami (1998). They concluded that nonflavonoid polyphenols such as protocatechuic acid and chlorogenic acid show a potent iron reducing ability and protected completely Fe^{2+} from autoxidation. Nevertheless, our results presented here are in a good agreement with findings that most catechols form chelates with ferric ions (Kawabata, Schepkin, Haramaki, Phadke, & Packer, 1996; Zhao et al., 1998; Zolgharnein, Abdollahi, Jaefarifar, & Azimi, 2002).

The oxidation of ferrous ions was more effectively facilitated by phenolic acids bearing catechol group than their counterpart with galloyl moiety, as was obvious from comparison of the effects of protocatechuic and gallic acid. Phenolic acids were much effective than the corresponding phenols without the negative charge of carboxylate group. For example, protocatechuic acid and gallic acid were more effective than catechol and methylgallate, respectively (Figs. 5a and 5b). Thus, the negative charge of carboxylate group of phenolic acids should help stabilize the highly charged ferric state. This is in good agreement with the theory that ferric state formation is favoured by the strengthening of electrostatic attraction (Hider et al., 2001).

Methylgallate was the least effective among accelerators tested, showing an increase of rate of Fe^{2+} autoxidation only at almost equimolar level to Fe^{2+}. Therefore, the rapid step at the beginning of the reaction (Fig. 7) can be elucidated by the fast formation of complex of methylgallate with iron ions. Because this complex decreased the Fe^{2+} autoxidation rather than accelerated it, we may assume that most of the iron is in the Fe^{2+} state. Contrary to catecholic acids, methylgallate does not display ferroxidase-like activity.

Acceleration of ferrous ion oxidation by polyphenols containing the catechol moiety was not surprising, because, generally, oxygen ligands that favour Fe^{3+}, i.e. EDTA, nitrilotriacetic acid, citric acid, deferoxamine and phosphate, facilitated the autoxidation (Harris & Aisen, 1973; Welch et al., 2002). Interestingly, we found that this effect is evident from a very low catecholic acid/ferrous ion ratio. Phenolic acids bearing catechol moiety accelerated the ferrous ions oxidation at concentrations lower than 1% of the initial amount of Fe^{2+}. For this reason, the effect of catecholic acids at pH 7.4 appears to be due to not only stochiometric, but catalytic amounts of these agents. Therefore, according to our results, catecholic acids displayed ferroxidase-like activity. Since these phenolic acids form stable chelates with iron under the reaction condition, ferroxidase mimics is based on the action of these chelates. It has been found previously, that catecholic iron chelates

effectively scavenge superoxide radicals and these complexes were more potent scavengers than uncomplexed catechols. The dismutation of superoxide radicals by catecholic iron complexes were explained by the redox cycling of iron bound in complexes (Moridani & O'Brien, 2001; Moridani et al., 2003; Zhao et al., 1998). It means that iron is still catalytically active in these complexes. Since ferric ions itself facilitated the ferrous iron autoxidation (Welch et al., 2002; Yang & Chasteen, 1999) and iron remains catalytically active in catecholic iron complexes, the redox cycling of iron between Fe^{2+} and Fe^{3+} in such complexes probably play a crucial role in the catalysis of ferrous iron autoxidation.

Indeed, when we added ferric iron to the solution of catechols in the presence of ferrozine, a specific ferrous ion chelator, the slow and incomplete reduction of ferric ions was observed (Tables 1 and 2). The iron reduction was detected not only in the excess of catechols, but also at the catechol/iron ratio equal to 0.5. It supports the hypotheses that a small amount of ferrous ion is present in the complexes. The prolongation of incubation of catecholic acids (caffeic and gallic acids) with ferric ions and ferrozine in Hepes buffer pH 7.4 resulted in slow and incomplete formation of Fe^{2+}–ferrozine chelates (Table 2). In this experiment, ferric ions are partially reduced by catecholic acids to ferrous ions, which are bound tightly with ferrozine. Then the balance between Fe^{2+} and Fe^{3+} shifts slowly to ferrous ions, because ferrozine binds only to ferrous iron. The ability of catecholic acids to reduce ferric ions in complexes by electron transfer reaction was undoubtedly confirmed in the case of gallic acid (Hynes & O'Coinceanainn, 2001), caffeic and chlorogenic acid (Hynes & O'Coinceanainn, 2004). However, this rapid reduction of Fe^{3+} was carried out in strongly acidic pH where Fe^{3+} is a potent oxidant. In the ferric reducing antioxidant power (FRAP) asaay, which is performed at acidic pH 3.6, ferric iron is largely reduced by catecholic acids or other plant polyphenols. These polyphenols with multiple phenolic groups are more active in the FRAP assay than simple antioxidants as ascorbic acid (Ozgen, Reese, Tulio, Scheerens, & Miller, 2006). Our experiments were carried out at pH 7.4 where ferric ions are dominant species. Because of formation of stable chelates with Fe^{3+}, iron reducing capacity of phenolic acids, unlike cysteine or ascorbic acid, substantially decreases at slightly alkaline pH.

Interestingly, Lopes, Schulman, and Hermes-Lima (1999) discovered that tannic acid (penta-*m*-digalloyl-glucose), effectively reduced ferric ions at pH 7.2. Our results show that at neutral pH, catecholic acids quickly bind to iron and form stable chelates with ferric ion, which is the dominant species in chelates, but chelates are able to undergo an internal electron transfer reaction to generate low amount of Fe^{2+} and semiquinone in complexes. The redox cycling of iron and catechols thus allows the ferrous ion autoxidation.

Surprisingly, vanillic acid (4-hydroxy-3-methoxybenzoic acid), a methyl derivative of protocatechuic acid, conversely to protocatechuic acid, protected ferrous ion from oxidation by aerial oxygen (Fig. 8). Essentially, the same effect was observed when 3-hydroxybenzoic acid or 4-hydroxybenzoic acid was used as ligand. Surprisingly, vanillic, 3-hydroxybenzoic and 4-hydroxybenzoic acids used in our experiments inhibited the ferrous ion autoxidation as effective as ascorbic acid and cysteine. It means that methylation of protocatechuic acid by catechol-*O*-methyltransferase completely reverses the acceleration of autoxidation caused by this catecholic acid. The relatively strong inhibition of autoxidation of ferrous ion by monophenolic acids is somewhat surprising due to the theory that oxygen and negatively charged ligands generally prefer Fe^{3+}. On the other hand, it has been found previously that ferrous ion autoxidation is inhibited by some oxygen ligands as mannitol and sorbitol (Tadolini, 1987), tannic acid (Lopes et al., 1999) and pyruvic acid (Welch et al., 2002). Differently from ascorbic acid and cysteine, these monophenolic acids were not able to reduce ferric ions. Moreover, we found that vanillic acid completely abolished the slow reduction of ferric ions caused by ferrozine itself (Table 2).

Effect of syringic (3,5-dimethoxy-4-hydroxybenzoic) acid differed from that of other phenolic acids. Syringic acid did not alter the redox state of iron; only in a large excess over metal, syringic acid appeared to weekly inhibit the Fe^{2+} autoxidation (Fig. 8) and reduced ferric ions (Fig. 9c and Table 2). Therefore, its effect at a large syringic acid/iron ratio was generally similar, although considerably weaker, to that of ascorbic acid and cysteine.

Considering these results, the inhibition of Fe^{2+} oxidation by monophenolic acids could be related to their binding via phenolic hydroxyl rather than carboxylate group, because syringic acid, compared with other monophenolic acids, displayed a substantially lower inhibition of Fe^{2+} autoxidation. Both methoxy groups of syringic acid can create a steric hindrance for the interaction of phenolic hydroxyl with iron and thus make this interaction more difficult.

In conclusion, in this study was shown that phenolic acids bearing *ortho*-dihydroxy group on the aromatic ring significantly increased the rate of ferrous ion autoxidation in two ways. First, because their strong chelating ability towards ferric ions. Another way was apparently attributed to the ferroxidase-like activity of chelates of catecholic acids with ferric ions, since catecholic acids were able significantly accelerated the ferrous ion autoxidation from a very low phenolic acid/iron ratio. Through this fact, catecholic acids might display the ferroxidase-like activity not only *in vitro*, but also during food processing or at physiological conditions, e.g. or in the gastro-intestinal tract. After food ingestion a significant amount of "free" iron is mobilized from food because of the catabolism of endogenous ligands and strong acidic pH in stomach. Then the widespread interaction of plant polyphenols or their metabolites with iron ions can take place in gastro-intestinal tract. Polyphenols can not only form chelates with

ferric ions and therefore increase of the solubility of ferric state, as is the case of catechols, but can also influence on the redox state of iron and its reactivity. The daily intake of dietary polyphenols bearing catechol or galloyl moiety can be extraordinary high. For example, one cup of instant coffee contains 50–150 mg of chlorogenic acid (Clifford, 1999). It is well known that food and beverages rich on plant polyphenols, such as tea (catechins) and coffee (chlorogenic acid) substantially decrease of nonheme iron absorption (Hurrell, Reddy, & Cook, 1999). The main postulated mechanism of the diminution of iron absorption is chelation of iron by polyphenols. We have shown here that chlorogenic acid and other polyphenols bearing catechol or galloyl moiety catalyse the ferrous ion oxidation to less soluble ferric state. Both ferrous and ferric ions are absorbable, but the ferrous being more readily so. Importance of the ferric ions reduction in nonheme iron absorption is evident from the fact that ascorbic acid is the most potent enhancer of dietary iron absorption. Conversely, compounds, which would be able to decrease of the Fe^{2+}/Fe^{3+} ratio, thus could potentially decrease the iron absorption. Therefore, the acceleration of ferrous ion oxidation mediated by dietary polyphenols bearing catechol or galloyl moiety should participate in the inhibition of iron absorption.

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