

Iron-chelation properties of phenolic acids bearing catechol and galloyl groups

Mirjana Andjelković^{a,b}, John Van Camp^{b,*}, Bruno De Meulenaer^b, Griet Depaemelaere^a, Carmen Socaciu^d, Marc Verloo^c, Roland Verhe^a

^a Department of Organic Chemistry, Faculty of Biosciences Engineering, Ghent University, Belgium

^b Department of Food Safety and Food Quality, Faculty of Biosciences Engineering, Ghent University, Belgium

^c Department of Applied Analytical and Physical Chemistry, Faculty of Biosciences Engineering, Ghent University, Belgium

^d Department of Chemistry & Biochemistry, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania

Received 17 March 2005; received in revised form 24 May 2005; accepted 27 May 2005

Abstract

In this study, the capacity of seven phenolic acids and hydroxytyrosol for complex formation with iron was quantified. A metal-chelation mechanism was described by means of spectrophotometry and calculating the binding constants of the complexes. The influence of phosphate buffer, Hepes buffer, Tris buffer and water on this mechanism was investigated. UV–Vis absorption spectroscopy showed that the absorption of phenolic acids changes upon the addition of Fe^{2+} , which resulted in several shifts of their spectra. These bathochromic shifts were analyzed and evaluated by calculating binding constants. Furthermore, in the presence of different concentrations of EDTA (0–1 mM), a reduction of the constants was observed. However, not all of the phenolic compounds assessed here showed complex formation, those not bearing catechol or galloyl moiety like vanillic acid, syringic acid and ferulic acid, did not show any complex formation in our study. The ability of the phenolic compounds which chelate iron have been ranked in line with the binding constants in ascending order rendering the protocatechuic acid (1.43 M^{-1}) the weakest chelator, followed by hydroxytyrosol (2.66 M^{-1}), gallic acid (4.78 M^{-1}), caffeic acid (8.12 M^{-1}) and chlorogenic acid (20.13 M^{-1}) as the strongest chelator.

© 2005 Elsevier Ltd. All rights reserved.

Keywords: Hydroxytyrosol; Phenolic acids; Antioxidant properties; Metal chelation capacity; Ferrous ion; Binding constant

1. Introduction

Phenolic compounds are secondary plant metabolites that are naturally present in almost all plant materials, including food products of plant origin. These compounds are thought to be an integral part of both human and animal diets. They can enhance the oxidation stability of oil, especially in olive oil (Psoyiadou &

Tsimidou, 2002). Recently, they have even been detected in chocolate (Arts, Hollman, & Kromhout, 1999; Wollgast & Anklam, 2000). Since they exhibit antioxidative properties, they have attracted considerable interest. It has been reported that they have antimutagenic and anticarcinogenic (Kampa et al., 2004), cardioprotective (Caccetta, Croft, Beilin, & Puddey, 2000), and antimicrobial (Friedman & Jurgens, 2000; Wen, Delaquis, Stanich, & Toivonen, 2003) properties.

Polyphenols are cyclic derivatives of benzene with one or more hydroxyl groups associated to the aromatic ring. The main classes of polyphenols are defined

* Corresponding author. Tel.: +32 9 264 62 08; fax: +32 9 264 62 15.
E-mail address: john.vancamp@ugent.be (J. Van Camp).

according to the nature of their carbon skeleton: phenolic acids, flavonoids, stilbenes (Scalbert & Williamson, 2000); and lignans which have recently been recognized as phytoestrogens (Tapiero, Tew, Ba, & Mathe, 2002).

Phenolic acids are by their structure simple phenols and include two major subgroups: hydroxybenzoic acids and hydroxycinnamic acids. Phenolic acids are commonly present under two principal forms in all plant-derived foods: a free and a bound form. The latter is found more frequently and occurs in the form of esters, glycosides and bound complexes. In olives and olive oil one of the major component is simple phenolic hydroxytyrosol, (3,4-dihydroxy-phenylethylalcohol) which is a catechol derivative of oleuropein, the main component in olives.

Antioxidant activity of phenolic acids and their derivatives depends on the number and position of hydroxyl groups bound to the aromatic ring, the binding site and mutual position of hydroxyl groups in the aromatic ring, and the type of substituents (Masella et al., 1999; Rice-Evans, Miller, & Paganga, 1996; Sanchez-Moreno, Larrauri, & Saura-Calixto, 1998; Sroka & Cisowski, 2003). The highest antioxidant potency was found for *ortho*-diphenols (Rice-Evans et al., 1996).

It is recognized that polyphenols can act as antioxidants by radical scavenging (Lodovici et al., 2001; Re et al., 1999; Sroka & Cisowski, 2003), singlet oxygen quenching (Foley et al., 1999) and metal chelation (Brown, Khodr, Hider, & Rice-Evans, 1998). A free radical can either obtain an electron by removing it from another molecule; or it can bind itself to another molecule forming adduct; or it can also be donated with a hydrogen atom to stabilize the delocalization of the radical. On the other hand, quenching of singlet oxygen is the deactivation of the excited state of the molecule by either a physical or a chemical mechanism (Min & Boff, 2002). All these reactions provoke inhibition or reduction in the formation of free radicals, they interrupt the propagation of free radical chain reactions, or they delay the start or reduce the reaction rate. Furthermore, metal chelation can result in prevention of metal redox cycling, occupation of all metal coordination sites, formation of insoluble metal complexes, steric hindrance of interactions between metals, and formation of lipid intermediates (Hider, Liu, & Khodr, 2001; Moridani, Pourahmad, Bui, Siraki, & O'Brien, 2003; Rice-Evans et al., 1996).

Transition metals have a major role in the generation of oxygen free radicals in living organisms. Iron exists in two distinct oxidation states – ferrous and ferric ions. The ferric ion (Fe^{3+}) is the relatively biologically inactive form of iron. However, it can be reduced to the active Fe^{2+} , depending on the conditions, particularly pH (Strlic, Radovic, Kolar, & Pihlar, 2002), and oxidized back through Fenton type reactions, with production of hydroxyl radicals; or Haber–Weiss Cycle reactions

with superoxide anions (Kehrer, 2000; Wong & Kitts, 2001). The production of these radicals can lead to lipid peroxidation, protein modification and DNA damage. Chelating agents may inactivate metal ions and potentially inhibit the metal-dependent processes (Finefrock, Bush, & Doraiswamy, 2003).

There are some ambiguous results in literature concerning metal-chelation properties of polyphenols. Since they may act as antioxidants and pro-oxidants this may lead to a reduction in their antioxidant properties (Kececi & Gordon, 2002; Moran, Klucas, Grayer, Abian, & Becana, 1997). Metal chelation is recognized by some authors as a minor mechanism in some polyphenols (Rice-Evans et al., 1996), yet the contribution of free radical scavenging or of metal ion chelation to the antioxidative effect of polyphenols is not fully specified (Sugihara, Ohnishi, Imamura, & Furuno, 2001). The metal chelating ability of polyphenols is related to the presence of *ortho*-dihydroxy polyphenols, i.e., molecules bearing catechol or galloyl groups (Khokhar & Apenten, 2003; Moran et al., 1997). Since metal chelation can occur at physiological pH it has a physiological significance.

Evaluation of metal-chelation properties of phenolic acids is commonly done by means of UV–Vis absorption spectroscopy, analysing the shifts of UV bands I and II, which characterize polyphenolic spectra (Asakura, Nakamura, Inoue, Murata, & Homma, 1990; Iwahashi, 2000; Khokhar & Apenten, 2003; Moridani et al., 2003; Rice-Evans et al., 1996; van Acker et al., 1996). There are some investigations involving mass spectrometry for elucidation of this mechanism (Fernandez et al., 1998) and results are in agreement with spectrophotometric methods.

The majority of the studies dealing with antioxidant activity of polyphenols, focus on the action of flavonoids. Nevertheless, simple phenolic acids occur greatly in nature. Only few studies exist on metal-chelation properties of phenolic acids (Hider et al., 2001; Iwahashi, 2000; Rice-Evans et al., 1996). In the present work, the capacity of phenolic acids bearing catechol or galloyl groups (caffeic acid, gallic acid, protocatechuic acid, and chlorogenic acid) compared to those without these groups (ferulic acid, syringic acid, and vanillic acid) and together with an important component from olive oil, hydroxytyrosol, were investigated for forming a complex with ferrous iron at physiological pH (7.4). In an attempt to confirm these capacities, the spectrophotometric characteristics of phenolic acids and their complexes with iron were evaluated. The linear relationship of absorbance (A) and molar concentration was employed to calculate binding constants and evaluate the capacity of compounds to bind the iron. Based on these data, we developed a method that allows the ranking of phenolic acids according to their metal chelating capacities.

2. Materials and methods

2.1. Materials

All phenolic acids used in this study (caffeic acid, chlorogenic acid, gallic acid, protocatechuic acid, vanillic acid, syringic acid, and ferulic acid), shown in Fig. 1, as well as the reagents: caffeine, ferrous sulfate heptahydrate, and dimethyl sulfoxide [DMSO], were obtained from Sigma–Aldrich (Belgium). Trihydroxyl amino methane (Tris) buffer, Hepes buffer and EDTA were from Janssen Chimica (Belgium). Reagents for the preparation of phosphate buffer were: potassium phosphate dibasic, HK_2PO_4 (Chem Lab, Belgium) and sodium phosphate monobasic dihydrate $\text{H}_2\text{NaPO}_4 \cdot 2\text{H}_2\text{O}$ (Janssen Chimica, Belgium). Distilled water was de-aerated to prepare all solutions used.

Hydroxytyrosol (3,4-DHPEA) was synthesized in the laboratory following the procedure of Baraldi, Simoni, Manfredini, and Menziani (1983) and characterized by ^1H and ^{13}C NMR spectra, at 300 and 75 MHz, respec-

tively, using acetone as a solvent and tetramethylsilane as a reference compound.

2.2. Stock solutions

The modified method of van Acker et al. (1996) was used to prepare oxygen free solutions of phenolic compounds and phenolic compounds with ferrous iron. All stock solutions were freshly prepared before measuring. Distilled water and buffer were subjected to sonification for 10 min in an Ultrasonic bath (Elma, Transsonic 460/H). This was followed by continuously purging the solution with nitrogen gas in a closed atmosphere for minimum 1 h. Phenolic acids were solubilized in 5% DMSO and added to continuously purged 50 mM Tris buffer to a final concentration of 0.001% w/v and left for 1 more hour of purging. This solution was diluted with oxygen free buffer prepared together with other stock solutions used, to the target concentration prior to the measurements. The 4 mM iron (ferrous form) and EDTA solutions were prepared in oxygen free distilled

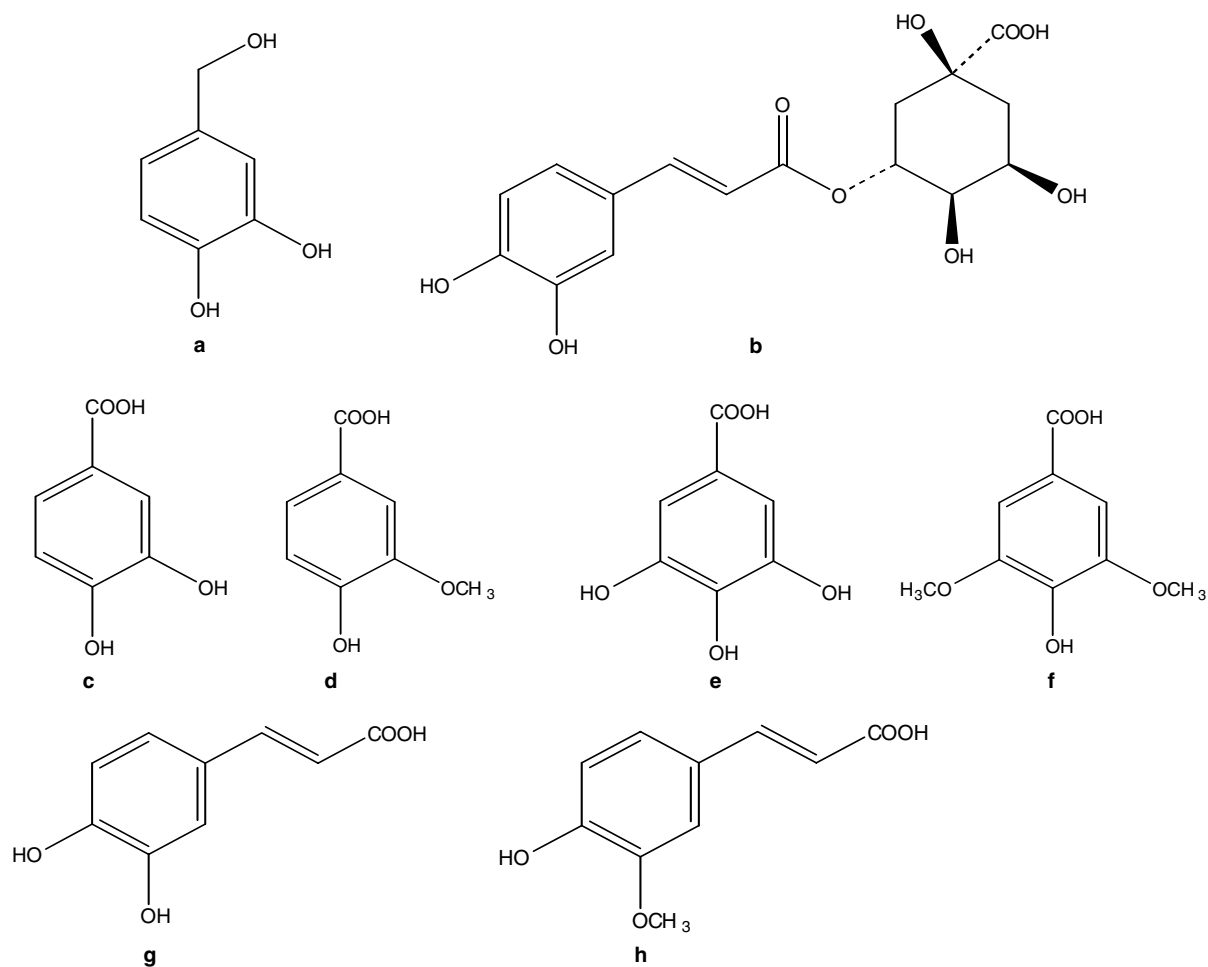


Fig. 1. Chemical structures of (a) hydroxytyrosol, (b) chlorogenic acid, (c) protocatechuic acid, (d) vanillic acid, (e) gallic acid, (f) syringic acid, (g) caffeic acid, and (h) ferulic acid.

water. After the addition of reagents to buffer or water, flasks were covered in order to protect them from light.

The stability constant of phenolic compound–iron complex was determined according to the method used by Kolayli, Ocak, Küçük, and Abbasoglu (2004) with some modifications. A dilution of Fe^{2+} in Tris buffer was prepared prior to measurement in order to attain final concentrations of 0.01–1.0 mM in solutions when added to phenolic acid or hydroxytyrosol. The phenolic compounds were at a final concentration 0.0005% w/v which remained the same throughout all of the experiments.

2.3. Effects of solvent

In order to study the effect of the solvent on phenolic acids chelation of Fe^{2+} the following solvents were used: phosphate buffer, Hepes buffer, Tris buffer and water. All solvents tested were prepared under the same conditions as explained above, at pH 7.4 and 50 mM concentration.

2.4. Absorbance spectra

The absorption spectra of the phenolic acids and hydroxytyrosol, and the modifications of them when combined with iron and upon addition of EDTA were recorded on a Varian UV–Vis Spectrophotometer (Cary 50 BIO). UV spectra were measured in the wavelength range of 200–800 nm vs. a reagent blank containing the buffer, DMSO and EDTA at specified concentrations.

Absorbances of phenolic compounds were determined at phenolic acid – specific maxima wavelengths while the absorbances of their mixtures with ferrous ion were measured at the band corresponding to the absorbance maxima of the iron–phenolic compound complex after mixing.

2.5. Complex capacity

The binding constant of phenolic compounds with ferrous ion is defined as

$$k = \frac{[\text{phenolic compound} \cdot \text{Fe}^{2+}]}{[\text{phenolic compound}] \cdot [\text{Fe}^{2+}]}$$

According to the method of Kolayli et al. (2004) this binding constant, $k[\text{M}^{-1}]$ can be calculated as

$$k = \frac{\text{intercept}}{\text{slope}},$$

where intercept and slope are parameters of a linear relationship between $1/(\text{total ferrous ion concentration})$ and $1/(\text{absorbance of complexed ion})$. The binding constant of caffeine with iron reported by Kolayli et al. (2004) was used as a positive control.

2.6. Complex stability

In order to ascertain and verify the stability of the complexes between phenolic acids or hydroxytyrosol and iron, EDTA was used for comparison. Dilutions of EDTA were prepared and added to phenolic compound–iron mixtures in final concentrations of 0.01, 0.05, 0.1, 0.5, and 1 mM.

2.7. Statistics

Experiments were performed in threefold and obtained data were presented as the arithmetic means \pm SD. Experimental data were analyzed statistically with Excel 2002 and SP-plus 6.1. To calculate the binding constants the linear regression lines were calculated and fitted.

3. Results

3.1. Effects of solvent

It has been shown that some buffers can chelate iron and interfere with iron binding affinities and assays (Welch, Davis, & Aust, 2002; Wong & Kitts, 2001). Therefore, several solvents were tested under the same conditions as explained above, for minimal binding effect on Fe^{2+} .

In this study, the phosphate buffer showed complexing behaviour which resulted in an extra band in the UV–Vis spectra. The maximum absorbance for the iron–phosphate complex was 280 nm. This peak appears in the area characteristic for phenolic acid absorption which impedes the deduction of the amount of iron complexed exclusively by phenolic compounds.

The impact of different concentrations of iron (0.01–1 mM) on complexing capacities of phenolic compounds was studied. When water was used as a solvent, the pH of the mixture was not stable. Along with each addition of iron in higher concentration the pH of the solution decreased.

When Hepes and Tris buffer were used the interferences were not so pronounced as for phosphate buffer. Essentially, when iron was present at 0.5 mM concentration and lower it has been observed some complex formation with Hepes buffer. This did provoke interference with our assay. On the other hand, a minor ability of Tris buffer to chelate iron was observed only when iron was added in concentrations higher than 0.5 mM.

Ultimately, Tris buffer was chosen for conducting further experiments in this study.

3.2. Absorbance spectra

The UV–Vis spectra of gallic acid, caffeic acid, protocatechuic acid, and chlorogenic acid showed two solid

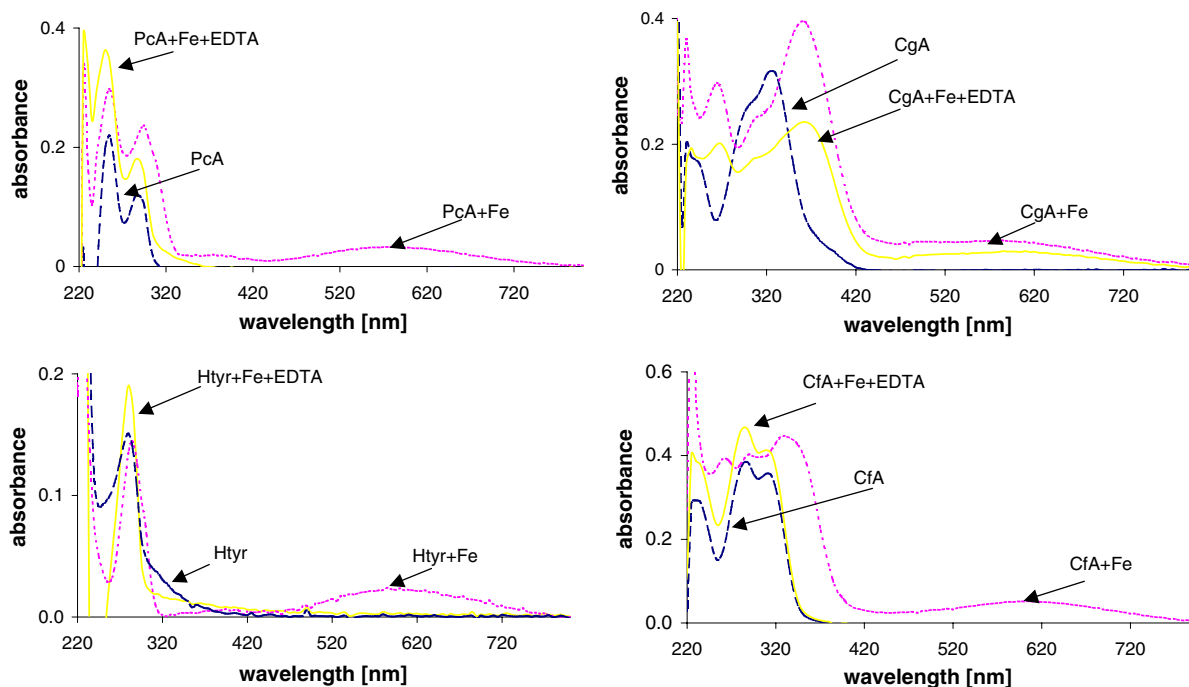


Fig. 2. Changes in absorption spectra for phenolic compounds after addition of Fe^{2+} and EDTA: CgA, chlorogenic acid; CfA, Caffeic acid; Htyr, hydroxytyrosol; PcA, protocatechuic acid.

absorbance maxima in the area 220–350 nm. With the addition of Fe^{2+} an additional low band in the range 450–650 nm with a maximum at 550–600 nm, depending on phenolic acid species, was detected (Fig. 2). This increase in absorbance indicates that phenolic acids chelated iron and produced complexes that gave a visible colour. Furthermore, in the region 220–350 nm, bathochromic shifts of the characteristic absorbance bands were observed upon addition of iron. In addition to this, an increase in absorption in these shifted bands over an ascending concentration range of iron was found. Upon addition of EDTA to mixtures of phenolic compound and iron the bands indicating the formed complexes with the absorbance in the range 450–650 nm did not remain, rendering the EDTA a stronger chelator than phenolic compounds. Additionally the shifted bands in the UV region returned to their initial position. On the other hand, phenolic acids without catechol or galloyl moiety did not exhibit any extra peaks in the higher wavelength range or any alterations in the area of their characteristic absorbance. Vanilic acid, syringic acid, and ferulic acid exhibited only their distinctive bands at 265 and 295; 225 and 260; and 295 and 325, respectively.

3.3. Complex capacity

The interaction of iron with some of the tested compounds produced bathochromic shifts of the two characteristic bands for which the increase in absorbance was

related to the increase in iron concentration. As mentioned above the additional band above 450 nm appeared. This absorbance band was not observed for the solutions without Fe^{2+} indicating complex formation between phenolic acids and iron. A wide range of iron binding capacities was found for different phenolic acids (Table 1).

In order to quantify the binding capacities of different phenolic acids and hydroxytyrosol, the binding constants were obtained as a linear relationship of total iron concentration and complexed iron concentration. They were calculated as a ratio of intercept and slope of linear curves (Fig. 3). These values revealed their various range of binding capacity towards iron.

Chlorogenic acid revealed the best complex formation capacity as it is shown in Table 1. Hydroxycinnamic acids (caffeic acid and chlorogenic acid) revealed better complex formation capacity than hydroxybenzoic acids (gallic acid and protocatechuic acid); and among the latter group gallic acid with galloyl moiety being a stronger chelator than protocatechuic acid bearing a catechol moiety. Hydroxytyrosol showed a higher chelating capacity than protocatechuic acid. Caffeine performed somewhat better than gallic acid (data not shown).

3.4. Complex stability

In this study, EDTA at concentrations between 0.001 and 1 mM reduced the specific absorbance bands of

Table 1
Binding constants [M^{-1}] for the iron–phenolic compound in the presence of 0–1 mM EDTA

Phenolic compound	Wavelength (nm)		EDTA		
	Characteristic	Shifted to	0 mM	0.01 mM	1 mM
Protocatechuic acid	250, 289	300	1.81 ± 0.10	$1.18^{***} \pm 0.06$	$0.25^{***} \pm 0.01$
Hydroxytyrosol	280	280	2.66 ± 0.24	$2.00^* \pm 0.18$	$1.10^{**} \pm 0.10$
Gallic acid	220, 260	230, 290	4.78 ± 0.25	4.54 ± 0.23	$0.51^{***} \pm 0.03$
Caffeic acid	230, 290, 310	285, /, 330	8.12 ± 0.94	$4.36^{**} \pm 0.50$	$0.77^{**} \pm 0.09$
Chlorogenic acid	255, 325	270, 350	20.13 ± 3.28	$12.00^* \pm 1.96$	$2.95^* \pm 0.48$

Absorbances were recorded at the shifted wavelengths. The values for binding constants of mixtures with and without EDTA present were determined in threefold and SDs calculated with significant difference within a row: $^*0.1 < p < 0.05$; $^{**}0.05 < p < 0.01$; $^{***}0.01 < p$.

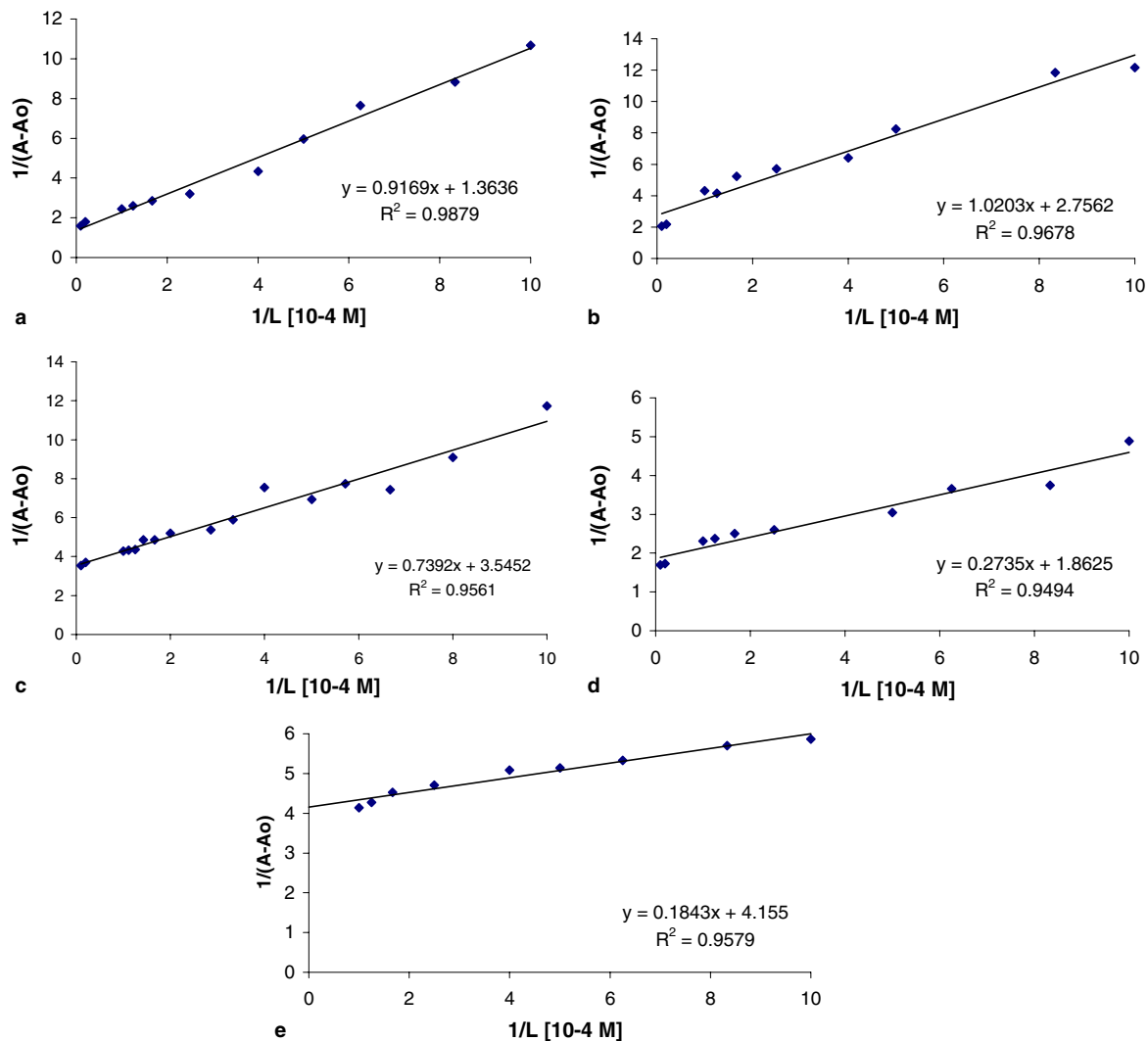


Fig. 3. Linear relationship between $1/(\text{absorbance of complexed ion})$ vs. $1/(\text{total iron concentration})$ for: (a) protocatechuic acid; (b) hydroxytyrosol; (c) gallic acid; (d) caffeic acid; (e) chlorogenic acid. A_0 is the initial absorbance of the phenolic compound at the shifted wavelength and A is the recorded absorbance at varying iron concentrations at the same wavelength.

mixtures containing Fe and phenolic compounds presumably by chelating Fe and making it unavailable to bind with the phenolic compound. It can be stated that the ability of phenolic compounds to chelate iron and produce complexes were far lower than that of EDTA. In Table 1, data are shown for stability of complexes be-

tween phenolic compounds and iron when EDTA was not present (0 mM) and for EDTA in concentrations 0.01 and 1 mM. Only in the case of gallic acid, the presence of EDTA in 0.01 mM concentration did not have significant influence ($p < 0.1$) on the value of binding constant.

4. Discussion

In order to evaluate iron chelation abilities of seven phenolic acids and hydroxytyrosol the effect of different buffers was assessed. The choice of a buffer is important when investigating the metal chelating abilities of phenolic compounds, since solvent can interfere with the spectrophotometric results and therefore with iron binding assays. The buffers tend to bind metal ions more weakly than strong chelators. However, they can still have a significant effect on the metal behaviour in solution. Some of the buffers are widely used, like phosphate buffer (Welch et al., 2002) which can affect the state of iron (Reinke, Moore, Rau, & Mccay, 1995). Ferrous ion is highly reactive, and its auto-oxidation can proceed depending on the conditions, such as pH. In solutions of pH 7.4, it is possible to expect two iron species: Fe^{2+} , and $\text{Fe}(\text{OH})_3$ depending on the electron activity in the solution. Concerning the autoxidation rate of Fe^{2+} in Tris buffer, it has been shown to be very slow, especially when compared to phosphate buffer and to Hepes buffer (Welch et al., 2002).

The bands appearing after addition of iron and disappearing after addition of EDTA can be seen as an indication of complex formation between phenolic compounds and iron (Mira et al., 2002). This is a reliable indication since these bands cannot be observed for the phenolic compounds alone. The same occurred in the case of the seven phenolic acids tested here. These outcomes were also in agreement with results reported by Iwahashi (2000). Gallic acid, caffeic acid, and chlorogenic acid in phosphate buffer demonstrated characteristic absorbance bands around 550 nm. Likewise, the chlorogenic acid exhibited two peaks at 488 and 640 nm whereas gallic acid at 552 nm and caffeic acid at 591 nm. The results of this study revealed in phosphate buffer similar peaks: for chlorogenic acid 500 nm and 590 nm, gallic acid 550 nm and caffeic acid 600 nm. Additionally, chlorogenic acid revealed the best complex formation capacity. This could be attributed to a more complex structure of this phenolic acid which is a caffeic acid ester linked to quinic acid. Furthermore, results obtained in Tris buffer have shown comparable behaviour of phenolic compounds and iron in this wavelength range.

Chelation of metal ions can be a prevention mechanism against free radical reactions (Lopes, Schulman, & Hermes-Lima, 1999; Sugihara et al., 2001). With the intention of demonstrating the structure dependency of the metal complexing behaviours of these seven phenolic acids and hydroxytyrosol, they were analyzed in two groups in the present study: on one hand phenolic acids bearing either galloyl or catechol groups, and on the other hand phenolic acids without these groups. As it was reported earlier (Rice-Evans et al., 1996; Rice-Evans, Miller, & Paganga, 1997) among three possible metal

chelating domains in flavonoids, one is the 3',4'-dihydroxy group on the B-ring which corresponds to the structure of phenolic acids (Fig. 1). The activity of this group is due to its electron-donating abilities. Moreover, the functionality of this domain was proven here since no reaction was observed for components lacking it.

To estimate the iron chelating abilities of these phenolic acids and hydroxytyrosol, the bathochromic shifts of their specific absorption bands were described. Evaluating the shifts, the binding constants were calculated from a linear relationship of total and chelated iron concentrations (Table 1) relating them to the chemical structure of the compounds. The values of the binding constants confirmed that hydroxycinnamic acids are better ligands for iron than hydroxybenzoic acids. Distinction between these two categories of phenolic acids is the ethylene group included between the benzene ring and carboxylic group in the case of hydroxycinnamic acids. The presence of this group may have an influence on the chelation capacity, as well as on scavenging activity (Sroka & Cisowski, 2003). Whereas, in the case of hydroxybenzoic acids, the carboxylic group directly linked to the benzene ring has a somewhat reducing effect on this mechanism. In addition, within the group of hydroxybenzoic acids, structures with galloyl moiety, namely gallic acid, scored better than those of the catechol type, corresponding to protocatechuic acid in this study. These results could be attributed to the number and position of hydroxyl groups whereby the relation between number of hydroxyl groups and antioxidant activity of the compounds was shown to be linear (Cao, Sofic, & Prior, 1997; Hider et al., 2001). Yet, gallic acid regardless, of its trihydroxy-benzoic structure, exhibited lower iron binding capacity than some other dihydroxy compounds. It appears that a galloyl group alone was less capable of chelating iron and the presence of this group had reducing effect on iron-binding (Khookhar & Apenten, 2003). By ab initio quantum studies was confirmed that the presence of the third OH group stabilize the electronic delocalization process within the ring structure (Fiuza, Van Besien, Milhazes, Borges, & Marques, 2004). Some other studies with flavonoids support the importance of this group particularly in conjunction with hydroxyl groups in the A and C ring (Pannala, Chan, O'Brien, & Rice-Evans, 2001; Rice-Evans et al., 1996; Silva et al., 2002; van Acker et al., 1996). Likewise, the enhancement of the antioxidant potential of compounds with the third OH group was reported for radical scavenging abilities (Pannala et al., 2001; Salah et al., 1995). On the other hand, compounds without galloyl or catechol groups did not show any complex formation. Even though these compounds have one OH group in position 3, iron chelation did not occur, probably due to a lower negative charge density on the chelation site. Presence of a methoxy group (vanillic acid, syringic acid, and ferulic acid) hindered their

chelation capacities contradictorily to their radical scavenging effectiveness. Methoxy groups could stabilize phenoxyl radicals due to its electron-donating abilities (Danilewicz, 2003), but did not have additional effect of chelation abilities. The iron chelation of hydroxytyrosol was assessed to be similar to those of hydroxybenzoic acids, namely protocatechuic acid and gallic acid. This was not surprising, in view of its *ortho*-dihydroxy structure.

In our study, the binding constant of caffeine for iron was 5.9 M^{-1} , which is lower than 59.3 M^{-1} reported by Kolayli et al. (2004). There may be several reasons for this discrepancy; first the different solvent used, i.e., water, whereas in our study Tris buffer was used. Also, we could not observe any reaction using the lowest concentration of iron (0.001 mM) reported by these authors. In our study, the lowest iron concentration was 0.01 mM .

The complex stability of the phenolic acids and hydroxytyrosol was compared with those of a known chelator, EDTA which will chelate iron making it unavailable for phenolic compounds. As reported earlier, the binding constant of EDTA for its complex with iron is $4.9 \times 10^8 \text{ M}^{-1}$ (Kolayli et al., 2004). The complexing abilities of phenolic compounds tested were far lower than that of EDTA (Table 1).

Phenolic acids and hydroxytyrosol have been recognized as antioxidants in vitro. However, a number of studies have been undertaken to evaluate their effects in in vivo conditions (Caccetta et al., 2000; D'Angelo et al., 2001; Miro-Casas et al., 2003; Olthof, Hollman, & Katan, 2001; Tuck & Hayball, 2002; Visioli, Borsani, & Galli, 2000). The intake of phenolic compounds is necessary to strengthen the defensive system of the human body, although endogenous antioxidant systems already exist in the human body. Iron, as a transition metal is involved in different biochemical oxidation reactions (autoxidation of biomolecules, DNA oxidation, protein oxidation, lipid oxidation, etc.) (Welch et al., 2002). Beyond the control of iron-mediated oxidation reactions, a variety of diseases and disorders may occur, like Alzheimer's disease (Finefrock et al., 2003). Therefore, it is needed to improve an understanding of initial reaction of metals in pathological processes as well as additional innate protection mechanisms. Even though very little is known about the biological properties of phenolic acids and their conjugates, the appreciation of antioxidative effects and particularly, metal chelation capacity, in terms of binding, inactivating, or eliminating reactive species and forms of iron from solutions, can imply a significant role of phenolic acids and hydroxytyrosol in metabolism.

In conclusion, for this study a relatively simple method for measuring metal chelating abilities of phenolic compounds in vitro was developed. This method allows comparison of the complex strength and chelating abil-

ities for iron of different phenolic compounds as expressed by binding constants. The structure–activity relationship was emphasized with an accent on the *ortho*-dihydroxy group.

Acknowledgements

This research is supported by the Special Research Fund (BOF) of Ghent University, Belgium and also by the Special Research Fund (BOF) for bilateral cooperation between universities, of Ghent University.

References

- Arts, I. C. W., Hollman, P. C. H., & Kromhout, D. (1999). Chocolate as a source of tea flavonoids. *Lancet*, *354*, 488–488.
- Asakura, T., Nakamura, Y., Inoue, N., Murata, M., & Homma, S. (1990). Characterization of zinc chelating compounds in instant coffee. *Agricultural and Biological Chemistry*, *54*, 855–862.
- Baraldi, P. G., Simoni, D., Manfredini, S., & Menziani, E. (1983). Preparation of 3,4-dihydroxy-1-benzeethanol – a reinvestigation. *Liebigs Annalen der Chemie*, 684–686.
- Brown, J. E., Khodr, H., Hider, R. C., & Rice-Evans, C. A. (1998). Structural dependence of flavonoid interactions with Cu^{2+} ions: implications for their antioxidant properties. *Biochemical Journal*, *330*, 1173–1178.
- Caccetta, R. A. A., Croft, K. D., Beilin, L. J., & Puddey, I. B. (2000). Ingestion of red wine significantly increases plasma phenolic acid concentrations but does not acutely affect ex vivo lipoprotein oxidizability. *American Journal of Clinical Nutrition*, *71*, 67–74.
- Cao, G. H., Sofic, E., & Prior, R. L. (1997). Antioxidant and prooxidant behavior of flavonoids: structure–activity relationships. *Free Radical Biology and Medicine*, *22*, 749–760.
- D'Angelo, S., Manna, C., Migliardi, V., Mazzoni, O., Morrica, P., Capasso, G., et al. (2001). Pharmacokinetics and metabolism of hydroxytyrosol, a natural antioxidant from olive oil. *Drug Metabolism and Disposition*, *29*, 1492–1498.
- Danilewicz, J. C. (2003). Review of reaction mechanisms of oxygen and proposed intermediate reduction products in wine: central role of iron and copper. *American Journal of Enology and Viticulture*, *54*, 73–85.
- Fernandez, M. T., Silva, M. M., Mira, L., Florencio, M. H., Gill, A., & Jennings, K. R. (1998). Iron and copper complexation by angiotensin-converting enzyme inhibitors. A study by ultraviolet spectroscopy and electrospray mass spectrometry. *Journal of Inorganic Biochemistry*, *71*, 93–98.
- Finefrock, A. E., Bush, A. I., & Doraiswamy, P. M. (2003). Current status of metals as therapeutic targets in Alzheimer's disease. *Journal of the American Geriatrics Society*, *51*, 1143–1148.
- Fiuza, S. M., Van Besien, E., Milhazes, N., Borges, F., & Marques, M. P. M. (2004). Conformational analysis of a trihydroxylated derivative of cinnamic acid – a combined Raman spectroscopy and ab initio study. *Journal of Molecular Structure*, *693*, 103–118.
- Foley, S., Navaratnam, S., McGarvey, D. J., Land, E. J., Truscott, T. G., & Rice-Evans, C. A. (1999). Singlet oxygen quenching and the redox properties of hydroxycinnamic acids. *Free Radical Biology and Medicine*, *26*, 1202–1208.
- Friedman, M., & Jurgens, H. S. (2000). Effect of pH on the stability of plant phenolic compounds. *Journal of Agricultural and Food Chemistry*, *48*, 2101–2110.
- Hider, R. C., Liu, Z. D., & Khodr, H. H. (2001). Metal chelation of polyphenols. *Flavonoids and Other Polyphenols*, *335*, 190–203.

- Iwahashi, H. (2000). Some polyphenols inhibit the formation of pentyl radical and octanoic acid radical in the reaction mixture of linoleic acid hydroperoxide with ferrous ions. *Biochemical Journal*, *346*, 265–273.
- Kampa, M., Alexaki, V. I., Notas, G., Nifli, A. P., Nistikaki, A., Hatzoglou, A., et al. (2004). Antiproliferative and apoptotic effects of selective phenolic acids on T47D human breast cancer cells: potential mechanisms of action. *Breast Cancer Research*, *6*, R63–R74.
- Keceli, T., & Gordon, M. H. (2002). Ferric ions reduce the antioxidant activity of the phenolic fraction of virgin olive oil. *Journal of Food Science*, *67*, 943–947.
- Kehrer, J. P. (2000). The Haber–Weiss reaction and mechanisms of toxicity. *Toxicology*, *149*, 43–50.
- Khokhar, S., & Apenten, R. K. O. (2003). Iron binding characteristics of phenolic compounds: some tentative structure–activity relations. *Food Chemistry*, *81*, 133–140.
- Kolayli, S., Ocak, M., Küçük, M., & Abbasoglu, R. (2004). Does caffeine bind to metal ions? *Food Chemistry*, *84*, 383–388.
- Lodovici, M., Guglielmi, F., Casalini, C., Meoni, M., Cheynier, V., & Dolara, P. (2001). Antioxidant and radical scavenging properties in vitro of polyphenolic extracts from red wine. *European Journal of Nutrition*, *40*, 74–77.
- Lopes, G. K. B., Schulman, H. M., & Hermes-Lima, M. (1999). Polyphenol tannic acid inhibits hydroxyl radical formation from Fenton reaction by complexing ferrous ions. *Biochimica et Biophysica Acta-General Subjects*, *1472*, 142–152.
- Masella, R., Cantafora, A., Modesti, D., Cardilli, A., Gennaro, L., Bocca, A., et al. (1999). Antioxidant activity of 3,4-DHPEA-EA and protocatechuic acid: a comparative assessment with other olive oil biophenols. *Redox Report*, *4*, 113–121.
- Min, D. B., & Boff, J. M. (2002). Chemistry and reaction of singlet oxygen on foods. *Comprehensive Reviews in Food Science and Food Safety*, *1*, 58–72.
- Mira, L., Fernandez, M. T., Santos, M., Rocha, R., Florencio, M. H., & Jennings, K. R. (2002). Interactions of flavonoids with iron and copper ions: a mechanism for their antioxidant activity. *Free Radical Research*, *36*, 1199–1208.
- Miro-Casas, E., Covas, M. I., Farre, M., Fito, M., Ortuno, J., Weinbrenner, T., et al. (2003). Hydroxytyrosol disposition in humans. *Clinical Chemistry*, *49*, 945–952.
- Moran, J. F., Klucas, R. V., Grayer, R. J., Abian, J., & Becana, M. (1997). Complexes of iron with phenolic compounds from soybean nodules and other legume tissues: prooxidant and antioxidant properties. *Free Radical Biology and Medicine*, *22*, 861–870.
- Moridani, M. Y., Pourahmad, J., Bui, H., Siraki, A., & O'Brien, P. J. (2003). Dietary flavonoid iron complexes as cytoprotective superoxide radical scavengers. *Free Radical Biology and Medicine*, *34*, 243–253.
- Olthof, M. R., Hollman, P. C. H., & Katan, M. B. (2001). Chlorogenic acid and caffeic acid are absorbed in humans. *Journal of Nutrition*, *131*, 66–71.
- Pannala, A. S., Chan, T. S., O'Brien, P. J., & Rice-Evans, C. A. (2001). Flavonoid B-ring chemistry and antioxidant activity: fast reaction kinetics. *Biochemical and Biophysical Research Communications*, *282*, 1161–1168.
- Psofiadou, E., & Tsimidou, M. (2002). Stability of virgin olive oil. 1. Autoxidation studies. *Journal of Agricultural and Food Chemistry*, *50*, 716–721.
- Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, *26*, 1231–1237.
- Reinke, L. A., Moore, D. R., Rau, J. M., & Mccay, P. B. (1995). Inorganic-phosphate promotes redox cycling of iron in liver-microsomes – effects on free-radical reactions. *Archives of Biochemistry and Biophysics*, *316*, 758–764.
- Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine*, *20*, 933–956.
- Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1997). Antioxidant properties of phenolic compounds. *Trends in Plant Science*, *2*, 152–159.
- Salah, N., Miller, N. J., Paganga, G., Tijburg, L., Bolwell, G. P., & Riceevans, C. (1995). Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. *Archives of Biochemistry and Biophysics*, *322*, 339–346.
- Sanchez-Moreno, C., Larrauri, J. A., & Saura-Calixto, F. (1998). A procedure to measure the antiradical efficiency of polyphenols. *Journal of the Science of Food and Agriculture*, *76*, 270–276.
- Scalbert, A., & Williamson, G. (2000). Dietary intake and bioavailability of polyphenols. *Journal of Nutrition*, *130*, 2073S–2085S.
- Silva, M. M., Santos, M. R., Caroco, G., Rocha, R., Justino, G., & Mira, L. (2002). Structure-antioxidant activity relationships of flavonoids: a re-examination. *Free Radical Research*, *36*, 1219–1227.
- Sroka, Z., & Cisowski, W. (2003). Hydrogen peroxide scavenging, antioxidant and anti-radical activity of some phenolic acids. *Food and Chemical Toxicology*, *41*, 753–758.
- Strlic, M., Radovic, T., Kolar, J., & Pihlar, B. (2002). Anti- and prooxidative properties of gallic acid in Fenton-type systems. *Journal of Agricultural and Food Chemistry*, *50*, 6313–6317.
- Sugihara, N., Ohnishi, M., Imamura, M., & Furuno, K. (2001). Differences in antioxidative efficiency of catechins in various metal-induced lipid peroxidations in cultured hepatocytes. *Journal of Health Science*, *47*, 99–106.
- Tapiero, H., Tew, K. D., Ba, G. N., & Mathe, G. (2002). Polyphenols: do they play a role in the prevention of human pathologies? *Biomedicine & Pharmacotherapy*, *56*, 200–207.
- Tuck, K. L., & Hayball, P. J. (2002). Major phenolic compounds in olive oil: metabolism and health effects. *Journal of Nutritional Biochemistry*, *13*, 636–644.
- van Acker, S. A., van den Berg, D. J., Tromp, M. N., Griffioen, D. H., van Bennekom, W. P., van der Vijgh, W. J., et al. (1996). Structural aspects of antioxidant activity of flavonoids. *Free Radical Biology and Medicine*, *20*, 331–342.
- Visioli, F., Borsani, L., & Galli, C. (2000). Diet and prevention of coronary heart disease: the potential role of phytochemicals. *Cardiovascular Research*, *47*, 419–425.
- Welch, K. D., Davis, T. Z., & Aust, S. D. (2002). Iron autoxidation and free radical generation: effects of buffers, ligands, and chelators. *Archives of Biochemistry and Biophysics*, *397*, 360–369.
- Wen, A. M., Delaquis, P., Stanich, K., & Toivonen, P. (2003). Antilisterial activity of selected phenolic acids. *Food Microbiology*, *20*, 305–311.
- Wollgast, J., & Anklam, E. (2000). Polyphenols in chocolate: is there a contribution to human health? *Food Research International*, *33*, 449–459.
- Wong, P. Y. Y., & Kitts, D. D. (2001). An iron binding assay to measure activity of known food sequestering agents: studies with buttermilk solids. *Food Chemistry*, *72*, 245–254.