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Limitations of the Tetramethylmurexide Assay for Investigating the Fe(II) Chelation Activity of Phenolic Compounds

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Limitations of the colorimetric assay involving tetramethylmurexide (TMM) to determine the extent of complex formation between metal ions and phenolic compounds have been studied. Older literature reports using this method to determine bound Fe(II). Our study shows the TMM assay is inadequate when determining the Fe(II) chelation activity of phenolic preparations rich in tannin constituents on account of the high absorbance values derived by control samples (i.e., those that do not contain the TMM reagent). Phenolic test samples comprising the TMM reagent, iron ions, and tannins could not yield meaningful absorbance data on Fe(II) chelation activity. In our study, we investigated commercially available compounds, namely, sinapic acid, catechin, rutin, tannic acid, procyanidin B_2, as well as crude acetonic extracts of almonds, red lentil, buckwheat, and their low-molecular-weight and tannin fractions separated from the crude extracts by Sephadex LH-20 column chromatography. Even as little as 0.5 mg of tannins added per control sample resulted in high absorbance values to the extent of 0.4 for red lentil and almonds, and 1.3 for buckwheat. A strong correlation (r^2 = 0.98) between the content of condensed tannins, as determined by the vanillin reaction, and absorbance of control samples by the TMM assay was found for the plant extracts and their fractions. A more useful colorimetric assay to investigate the Fe(II) chelating ability of tannin-rich preparations may be the method that uses ferrozine.

KEYWORDS: Fe(II) chelation; tetramethylmurexide (TMM) assay; phenolic compounds; tannins; ferrozine assay

INTRODUCTION

Transition metal ions are routinely present in foods and can generate highly reactive free radicals such as the hydroxyl radical (•OH) from peroxides in the presence of O_2 via Fenton's reaction (*1*). Major primary catalysts that initiate oxidation are metal ions with high redox potential such as iron or copper, although pH plays an important role in determining this potential. A class of compounds known as chelating agents can stabilize prooxidative transition metals. A chelating agent is a molecule that is capable of seizing and holding a metal ion. Ligand atoms (usually nitrogen, oxygen, or sulfur) donate two electrons to form the coordinate covalent bond. In most cases, the central atom can be grasped by more than one molecule, so that the ion is held in a set of rings (*2*). The metal chelation ability of organic compounds, such as ethylenediaminetetraacetic acid (EDTA) and its derivatives, nitrilotriacetic acid (NTA), citric acid, and acetic acid, is commonly known and for this reason they are often incorporated in food formulations. Plant phenolic compounds are also excellent chelators of metal ions. The presence of 3′-4′ and/or 7-8 *o*-dihydroxyphenyl (i.e., catechol) groups on the B- and A-rings of flavonoids, respectively, or 5-OH and/or 3-OH moiety with a 4-oxo group in the A/C ring structure or a large number of hydroxyl groups in the makeup of phenolic compounds is important for the binding/chelation of iron ions (*3*−*5*). A double bond between C2 and C3 of flavones also influences the chelation of metal ions (*6*). On the other hand, hydroxyl groups in conjugation with methyl groups or a carbohydrate moiety are not involved in complexing of Fe (*7*, *8*).

Tannins are abundant in the plant kingdom and in foods of plant origin. They are complex polyphenolic compounds with molecular weights greater than 500 Da (*9*). In terms of their chemical structure, they are divided into two groups: hydrolyzable tannins (i.e., gallotannins and ellagitannins), which are gallic acid derivatives, and condensed tannins (i.e., proanthocyanidins) which are predominantly polymers of flavan-3-ols. The presence of numerous catechol and galloyl groups enables tannin molecules to chelate transition metal ions, such as Fe among others. Mila et al. (*10*) reported that ellagitannins and gallotannins, with several chelating groups per molecule, form a blue-black precipitate with ferric ions and also remove Fe(III) from other iron/ligand complexes more efficiently than monofunctional low-molecular-weight phenols. Tannic acid forms complexes with either ferric or ferrous ions (*11*, *12*). Proanthocyanidins also act as a strong chelator of iron ions (*13*). The lowest molecular weight tannin, epigallocatechin gallate, can form a very stable complex at pH 7.0 with Fe(II) at a stoichiometric ratio of 2:1. Tannins can be oxidized only by very strong oxidants, but even when this is the

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case, the corresponding phenoxyl radical formed can still participate in the coordination with metal ions (*14*).

Both proanthocyanidins and hydrolyzable tannins are great free-radical scavengers and are considerably more effective than simple phenolics (*15*). The antioxidant properties of tannins are the result of not only their radical-quenching abilities but also their transition-metal ions chelating activity; this is particularly so for Fe(II) and Cu(II). Lopes et al. (*12*) reported on the antioxidant properties of tannic acid, resulting from the formation of stable complexes with Fe(II), that are unable to participate in Fenton's reaction. Grinberg et al. (*16*) suggest that a portion of the antioxidant activity of the polyphenols from black and green teas act via a mechanism of iron chelation rather than via direct hydroxyl radical scavenging.

The metal chelating ability of tannins has a number of disadvantageous biological consequences. For instance, it is well established that tannins reduce the absorption of nonheme dietary iron (*17*−*20*). The decrease in bioavailability is explained by the formation of stable tannin−iron complexes in the gastrointestinal lumen (*17*). Upon the basis of in vivo studies, Brune et al. (*18*) concluded that the inhibitory effect of phenolic compounds on iron absorption is influenced by the number of galloyl groups in phenolic molecules, whereas the presence of catechol groups seemed to be of minor importance. Other complexing compounds present in food (naturally or as an additive) such as ascorbic acid and EDTA can decrease inhibition of iron absorption evoked by phenolic compounds. EDTA, for example, prevents iron from complexing with tannic acid and can even remove Fe(III) from complexes already formed (*11*). On the other hand, citric acid acts as a ferric ion chelator and as a strong reductant, which reduces ferric ions to the more soluble ferrous ions (*11*, *21*).

One of the methods typically reported in the literature to evaluate the chelating properties/potentials of phenolic compounds is an assay with tetramethylmurexide (TMM), in which this chemical forms complexes with divalent metal ions, such as Fe(II), Cu(II), and Zn(II) (*22*−*27*). The objective of this study was to investigate the utility/limitation of this method for evaluation of Fe(II) chelating efficiencies of selected pure phenolic compounds as well as acetonic plant extracts, and their isolated low-molecular-weight and tannin fractions. A second colorimetric assay, which utilizes ferrozine to form a stable complex with ferrous ions as an estimation of the chelating ability of selected plant extracts, was employed for comparative purposes.

MATERIALS AND METHODS

Chemicals. Sinapic acid, (+)-catechin hydrate, rutin trihydrate, tannic acid, ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4′′-disulfonic acid sodium salt], hexamethylenetetramine (hexamine), Sephadex LH-20, and tetramethylmurexide ammonium salt (TMM) were purchased from Sigma-Aldrich Co. Ltd. (Poznań, Poland). Procyanidin B_2 was acquired from Extrasynthese S.A. (Genay Cedex, France). Ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), ethylenediaminetetraacetic acid (EDTA), and other chemicals, all analytical grade, were acquired from P.O.Ch. Company (Gliwice, Poland).

Materials. The plant materials investigated included almonds (*Prunus dulcis*), red lentil (*Lens culinaris*), and buckwheat (*Fagopyrum esculentum*). Raw almonds with their skins on were purchased from a local market in Olsztyn (Poland), whereas authenticated seeds of red lentil and buckwheat were acquired from the Plant Breading Station in Olsztyn (Poland).

Extraction and Chromatographic Separation of Phenolic Compounds. The almonds and seeds of red lentil and buckwheat were ground in a commercial coffee mill. The ground almonds were defatted with hexanes at 65 °C using a standard Soxhlet apparatus. Phenolic compounds were then extracted from 20 g of each type of plant material using 80% (v/v) acetone (*28*). Briefly, extraction was carried out at 60 °C for 15 min at a solids-to-solvent ratio of 1:8 (w/v). The extraction was repeated 2 more times, supernatants were combined, and acetone was removed using a Büchi rotary evaporator (Rotavapor R-200, Büchi Labortechnik AG, Flawil, Switzerland) at 40 °C. The resultant aqueous residue was lyophilized for ∼48 h at −70 °C and 0.013 mbar (Lyph Lock 6 freeze-dry system, Labconco Co., Kansas City, MO). A 2-g portion of each lyophilized crude extract was dissolved in 20 mL of 95% (v/v) ethanol and applied onto a chromatographic column (5 × 40 cm) filled with Sephadex LH-20. Low-molecular-weight compounds were eluted from the column using 800 mL of 95% (v/v) ethanol, and the tannin fraction was eluted with 600 mL of 50% (v/v) acetone (*29*). Solvents from both fractions were evaporated using the rotary evaporator and water was removed by lyophilization.

Determination of Condensed Tannins Content by the Vanillin Assay. The content of tannins was determined using the modified vanillin assay (*30*). Almond, red lentil, and buckwheat extracts as well as their low-molecular-weight and tannin fractions were tested. To 1 mL of methanolic solution of test material, 5 mL of 0.5% (w/v) vanillin solution in 4% (v/v) concentrated hydrochloric acid were added. Samples and controls (i.e., those without the vanillin reagent) were vortexed vigorously (Vortex Genie 2, Scientific Industries Inc., Bohemia, NY) and subjected to a 20-min quiescent period for maximum color development in darkness. Then, absorbance of the reaction mixture was recorded at 500 nm using a Beckman DU 7500 diode array spectrophotometer (Beckman Instruments Inc., Fullerton, CA). Results were expressed as absorbance units per 1 mg of extract or fraction (A_{500}/mg).

Evaluation of the Fe(II) Chelation Activity by the TMM Assay. One milliliter of a phenolic compounds solution for each extract/fraction in 0.01 M hexamine/HCl buffer containing 0.01 M KCl (pH 5.0) and 1 mL of a 4 mM $FeSO_4$ solution in the same buffer were combined; then, 0.1 mL of a 1 mM aqueous TMM solution was added (*31*). Absorbance was recorded at 480 and 530 nm using the Beckman DU 7500 diode array spectrophotometer and the ratio of A_{480}/A_{530} was calculated. A standard curve of absorbance ratio versus iron concentration in the range of 0−2.5 mM $FeSO_4$ was constructed. The percentage of bound Fe(II) was calculated using the following equation:

$$\text{bound Fe(II) (\%)} = \left(1 - \frac{\text{concentration of free Fe(II)}}{\text{concentration of total Fe(II)}}\right) \times 100$$

Control samples were prepared in a similar fashion: 1 mL of a phenolic compounds solution for each extract/fraction was mixed with 1 mL of 4 mM $FeSO_4$ solution, but 0.1 mL of redistilled water was added instead of the TMM reagent. Change in absorbance readings at 1-min intervals during a 10-min time was recorded. Absorbance measurements of control samples were made for the pure phenolic compounds: sinapic acid, catechin, rutin, tannic acid, procyanidin B_2, and the crude phenolic extracts of almonds, red lentil, and buckwheat as well as their low-molecular-weight and tannin fractions. The final concentration of standards and extracts/fractions was 1 and 0.5 mg/assay, respectively.

Evaluation of Fe(II) Chelation Activity by the Ferrozine Assay. Aqueous solutions of crude phenolic extracts from almond, red lentil, and buckwheat as well as their low-molecular-weight and tannin fractions were prepared at a concentration of 1 mg/mL. Samples (2.5 mL) were mixed with 0.25 mL of 0.4 mM $FeCl_2$ solution and 0.5 mL of 5 mM ferrozine reagent (*32*, *33*). The mixture was left to stand for 10 min at ambient temperature, and then absorbance readings were taken at λ = 562 nm. The percentage of bound Fe(II) was calculated similarly to that described for the TMM assay.

Statistical Analysis. Results of condensed tannins content and Fe(II) bound by extracts and fractions were expressed as means ± standard deviation (n = 3). The correlation analysis between the content of tannins in crude extracts and their fractions determined by the vanillin method, and absorbance of the control samples for the TMM assay was performed using Microsoft Excel software (*34*).

RESULTS AND DISCUSSION

The colorimetric reaction with TMM is commonly used to evaluate divalent metal ions chelation activity of phenolic compounds. "Free" metal ions [i.e., Fe(II)], which are not bound by phenolic compounds, are complexed with TMM. The UV−vis spectrum of a TMM solution shows an absorption maximum at

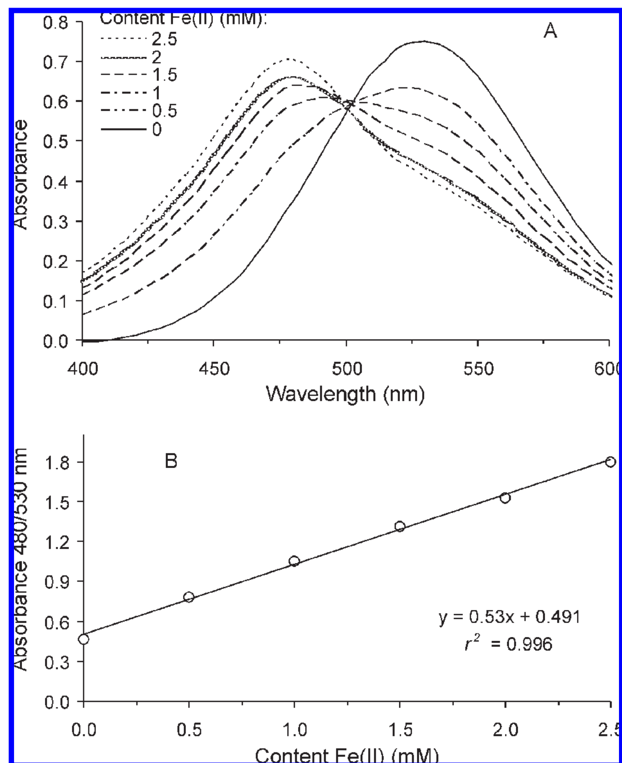

Figure 1. Absorption spectra of TMM in the presence of various concentrations of Fe(II) **(A)** and the standard curve derived from them **(B)**.

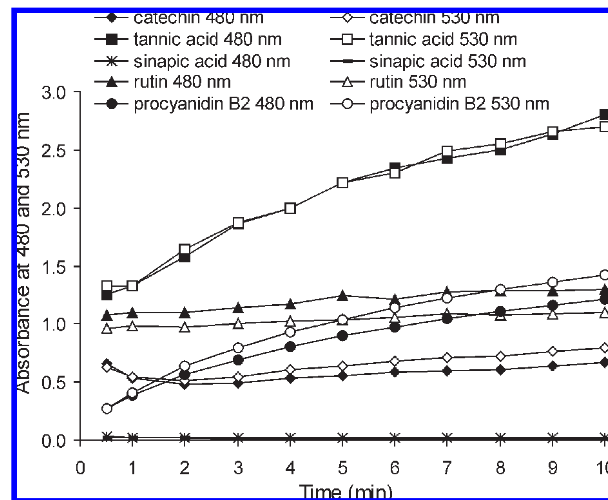

Figure 2. The absorbance values of control samples of some phenolic compounds.

530 nm, whereas spectra of TMM−Fe(II) complexes are characterized by a maximum at 480 nm (**Figure 1A**). Addition of ferrous ions to the sample increases the amount of TMM−Fe(II) complex formed and naturally reduces the quantity of excess TMM in the reaction medium. In other words, the observed 530-nm band diminishes while that at 480 nm rises simultaneously (**Figure 1A**). The ratio of absorbance at 480/530 nm is linearly correlated with the Fe(II) concentration over a certain range; that is, 0−2.5 mM (**Figure 1B**). On the basis of the standard curve, the amount of Fe(II) complexed with TMM can be estimated, and knowing the total quantity of iron ions added to the reaction mixture, the % of Fe(II) bound by the test phenolic compound can be calculated. However, when using the TMM method to study the chelating activity of phenolic compounds, we sometimes encountered analytical problems. Some of the control samples (i.e., phenolic compounds and Fe(II) without added TMM) exhibited a high absorbance value. We will try to investigate which phenolic compounds can form colored reaction products with Fe(II) and how this will affect the TMM assay.

Figure 2 depicts the absorbance values of control samples over the time course of the TMM assay recorded at two wavelengths, 480 and 530 nm, as a means to evaluate the Fe(II) chelation activity of isolated phenolic compounds. The lowest absorbance value was noted for sinapic acid. This value was steady over time and below 0.023 absorbance units. Phenolic molecules with a more complex structure, such as catechin, rutin, and procyanidin B_2, yielded products with a more intensive color after reaction with Fe(II). Absorbances recorded at both aforementioned wavelengths were several times higher than the absorbance of the control sample with sinapic acid added. The highest absorbance value was noted for tannic acid, the compound characterized by the greatest molecular mass (FW = 1701.23 g/mol) and the most complicated structure among the test compounds investigated; after a 10-min incubation, an optical density reading of 2.80 was observed. For catechin and procyanidin B_2, absorbance values recorded at 530 nm were a bit higher than those at 480 nm. This relationship, however, was inverted for rutin. Another significant observation was that over the time course of the experiment, absorbances for catechin and rutin systems hardly rose unlike those for procyanidin B_2 and tannic acid, which continued to increase even after 10 min, thereby indicating that the latter systems were not stable and that complexation might be occurring by a cascade of reactions.

The findings obtained for commercial phenolic compounds of high purity allowed us to hypothesize that absorbance readings of the standards by the TMM assay depend on both the molecular weight and chemical nature of the molecules in question when exposed to Fe(II). Therefore, the next step of our study was to test the response of crude phenolic extracts of almond, red lentil, and buckwheat as well as two fractions separated chromatographically from each on a column of Sephadex LH-20. The two fractions obtained from each plant material included low-molecular-weight compounds eluted by 95% (v/v) ethanol and a high-molecular-weight or tannin fraction eluted by 50% (v/v) acetone.

Figure 3 depicts absorbance values measured at 480 and 530 nm over the time course of the TMM assay for reaction products generated from the interaction of crude phenolic extracts or collected fractions of almond, red lentil, and buckwheat with Fe(II). It should be noted that even a small quantity of the tannin fraction (or the crude phenolic extract in the case of buckwheat), to the extent of 0.5 mg/assay, yielded high absorbance readings for control samples, that is, 0.47, 0.39, and 1.45, for almonds, red lentil, and buckwheat tannins, respectively, and 0.56 for the crude extract of buckwheat. When the absorbance value of a control sample is so high, the proper measurement of a test sample is not feasible. This is where the limitation in the TMM assay lies. An example of the absorption spectra of properly measured samples and controls of the tannin fraction from red lentil is presented in the **Figure 4**. Spectra were recorded at very low addition levels of the tannin fraction (0.2 mg/assay). That addition gave a high absorbance reading of 0.9 at 480 nm for the test sample [tannin fraction + TMM + Fe(II)]. The absorbance of the control sample [tannin fraction + Fe(II)] causes the absorbance of the test sample to be higher than the absorbance of the complex of TMM + Fe(II) over a wide range of wavelengths, not only at λ = 530 nm. It is important to realize that the addition of the tannin fraction to the extent of 0.2 mg/assay was not sufficient to cause significant Fe (II) chelation. The percentage of Fe(II) bound was only 4.8%! In

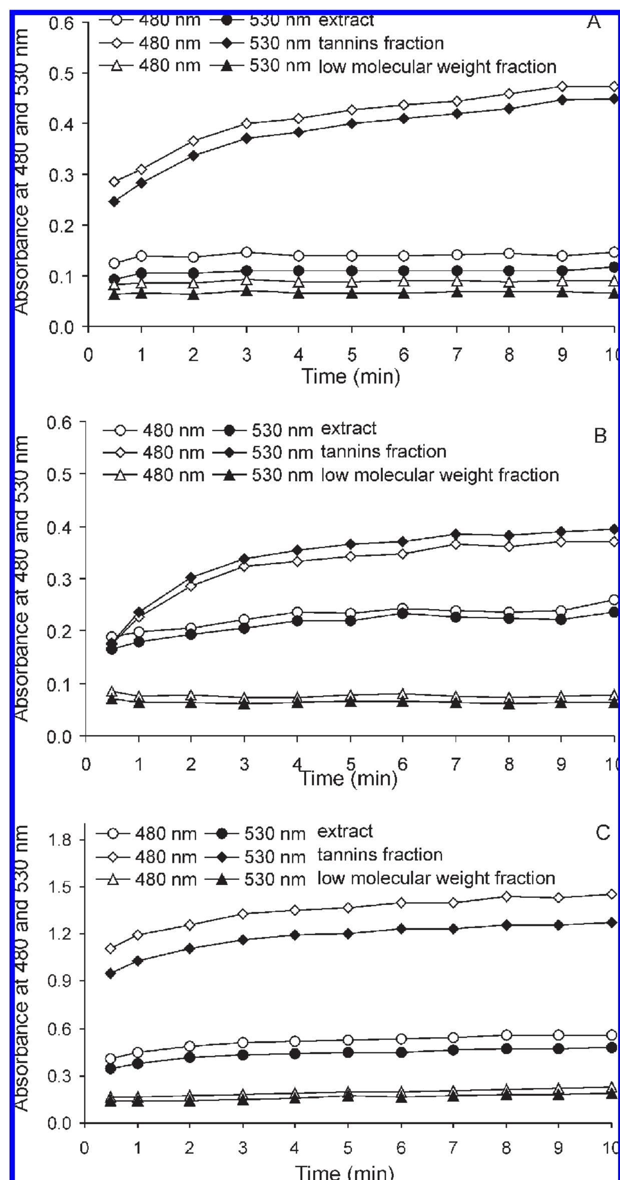

Figure 3. The absorbance values of control samples of extracts and their fractions: **(A)** almonds, **(B)** red lentil, **(C)** buckwheat.

comparison, EDTA chelated Fe(II) to 50% when 0.8 mg/assay was added to the system (**Figure 5B**). The absorbance value of the control sample was low, and that for the proper test sample was below 0.58 (**Figure 5A**).

The content of condensed tannins in the crude phenolic extracts and their fractions was determined by reaction with the vanillin/HCl reagent (**Table 1**). Among the extracts, the highest condensed tannins content was found for buckwheat, A_{500} was 0.420 per mg, while the lowest was for almonds at 0.033 per mg. Condensed tannins were not detected in the low-molecular-weight fractions (i.e., eluted with 95% (v/v) ethanol) of almonds and red lentil, but for the buckwheat low-molecular-weight fraction, an A_{500} reading of 0.061 per mg was found. Fractions eluted with 50% (v/v) acetone were, as expected, abundant in condensed tannins with values ranging from 0.202/mg for red lentil up to 1.036/mg for buckwheat.

Correlations determined between the content of condensed tannins assayed by the vanillin/HCl method and absorbance values of control samples from the TMM assay for crude phenolic extracts and fractions of almonds, red lentil, and buckwheat are shown in **Figure 6**. In accordance with our supposition,

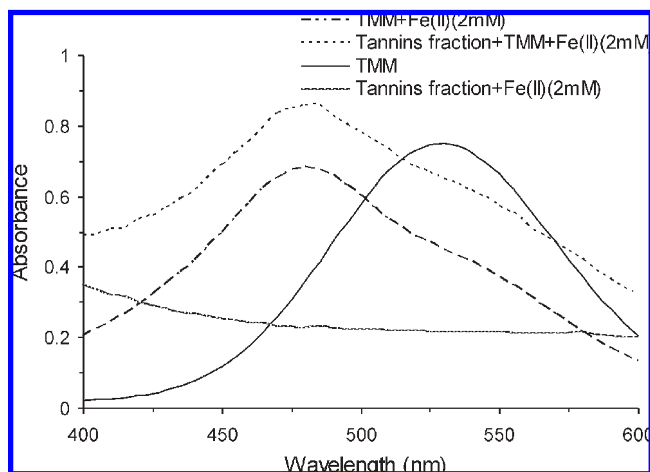

Figure 4. Absorption spectra of sample with 0.2 mg/assay of red lentil tannin fraction and its controls by the TMM assay.

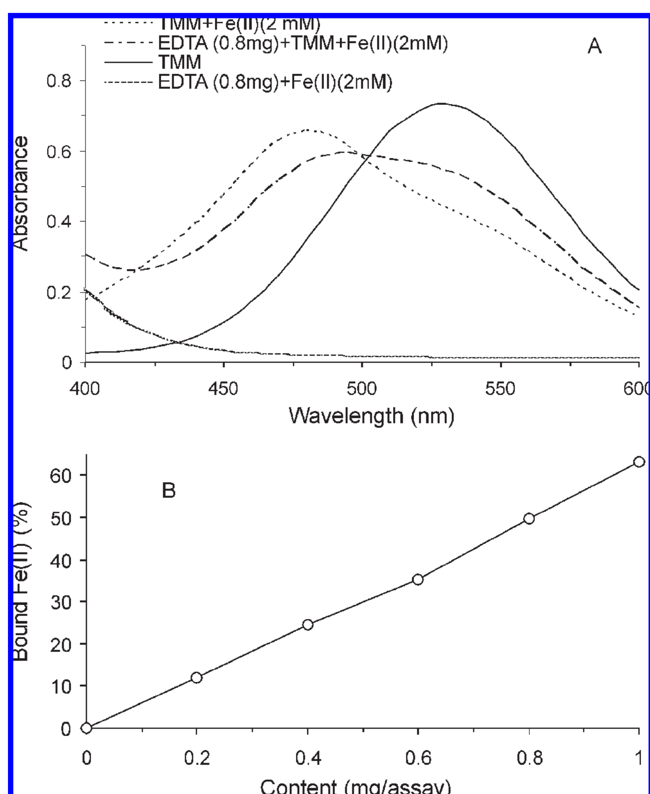

Figure 5. Absorption spectra of sample with 0.8 mg/assay of EDTA and its controls by the TMM assay (A) and % bound Fe(II) by EDTA (B).

absorbance readings for the controls were strictly correlated with the content of tannins in the samples. High correlation coefficients (r^2) were determined for measurements made at both the 480-nm and 530-nm bands, that is, 0.984 and 0.981, respectively.

It is well-known that tannins form blue color complexes with ferric ions. Mila et al. (*10*) asserted that ellagitannins and gallotannins form blue-black precipitates with ferric ions. Tannic acid at acidic pH or gallocatechins at pH 7.0 forms a violet-blue complex in the presence of Fe(III) ions; the complex exhibits an absorption maximum in the visible range of 540−580 nm (*11*, *14*, *35*). Galloyl groups participate in the formation of these complexes. On the other hand, phenolic compounds possessing catechol moieties form green-colored complexes in the presence of Fe(III) ions under acidic conditions

Table 1. Content of Condensed Tannins in Extracts, Low-Molecular-Weight (LMW) Fractions, and Tannin Fractions Expressed as Absorbance Units per 1 mg of Extract/Fraction (A_{500}/mg)[a]

plant	A_{500}/mg		
	crude phenolic extract	LMW fraction	tannin fraction
almonds	0.033 ± 0.006	0.000	0.372 ± 0.004
red lentil	0.078 ± 0.005	0.000	0.207 ± 0.002
buckwheat	0.420 ± 0.005	0.061 ± 0.001	1.036 ± 0.011

[a] Data are expressed as means ± standard deviation (n = 3).

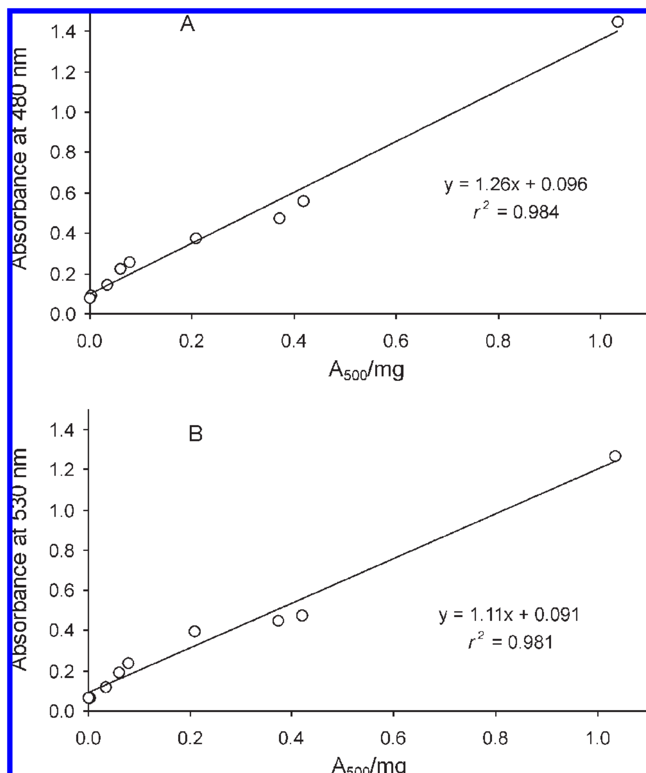

Figure 6. Correlation between results of the vanillin/HCl method for almonds, red lentil, buckwheat extracts/fractions and values of absorbance of control samples measured at **(A)** 480 nm and **(B)** 530 nm.

with an absorption maximum in the visible region of ~680 nm (*35*). Lopes et al. (*12*) noted that spectra for complexes of tannic acid with either ferric or ferrous ions were quite similar, and possessed a maximum between 520 and 500 nm at pH 7.2. Furthermore, UV−vis spectra of complexes of simple phenolic compounds, that is, phenolic acids possessing catechol and galloyl groups with ferrous ions, possess a small band in the range of 450−650 nm with a maximum lying somewhere between 550 and 600 nm (*7*). Jungbluth et al. (*36*) reported that Fe(II) causes no immediate change in the UV−vis spectra of flavonoids. Only after a few hours of mixing, due to oxidation of ferrous to ferric ions via molecular oxygen, did the spectra of Fe(II) and fisetin appear similar to that of the Fe(III)−flavonoid complex. Chvátalová et al. (*37*) also concluded that phenolic acids bearing catechol groups do not form complexes with ferrous ions, only with ferric ones. The lack of differences observed between UV−vis spectra of catecholic acids with added ferrous and ferric ions is explained by the rapid acceleration of ferrous ion oxidation by molecular oxygen in the presence of catecholic acids. In our study, an intensive blue color for control samples containing tannins by the TMM assay was certainly derived from a phenolic−iron ion complex. Yet, it is hard to conclude whether ferrous ions added to the reaction mixture directly take part in complex formation or whether some oxidized via molecular oxygen to the ferric state and then bound to the phenolic residues. The first mechanism was confirmed by studies of Lopes et al. (*12*), which reported that tannic acid formed a stable complex with Fe (II) and even was able to reduce Fe(III) to Fe(II). The second possibility, nonetheless, seems to be more probable because chelators that ligate Fe(II) via oxygen ligands promote the autoxidation of Fe(II) to Fe(III) (*38*). This would also explain the observed increase in absorbance readings of control samples, especially for those complexes formed possessing a large number of catechol or galloyl moieties, over the time course (e.g., tannic acid in **Figure 2**, and the tannin fractions of **Figure 3**).

Phenolic acids possessing monohydroxyl groups are not able to form complexes with iron ions (*7*, *37*, *39*). In our study, the control sample carried out with sinapic acid (i.e., 3,5-dimethoxy-4-hydroxycinnamic acid) did not absorb at the analyzed wavelengths due to its chemical structure (**Figure 2**). The absorbance reading of the Fe−catechin complex after a 10-min incubation was lower than those for complexes of iron with rutin and procyanidin B_2 (**Figure 2**); catechin bears only one catechol group that is able to bind iron, unlike rutin and procyanidin B_2 which possess two binding sites. Apart from the catechol moiety, rutin possesses a 5-hydroxy-4-keto group, which also takes part in complexation with Fe ions. (*3*, *6*, *40*). Owing to its large number of galloyl groups, tannic acid has the ability to chelate more iron ions than any of the other compounds tested in this study; hence, this accounts for the high absorbance values of control samples by the TMM assay (**Figure 2**). Simple phenolic compounds react with iron ions in stoichiometric ratios of 1:1, 2:1, or 3:1 (*14*, *41*). The maximum ratio of iron ions reacting with tannic acid has been reported to be almost 10:1 for the complex at pH 7.2 (*12*). Considering this and the fact that absorbance values of control samples with TMM are developed by Fe ions−tannin complexes formation, the excellent correlation between the tannin content and the absorbance (**Figure 6**) obtained in this study is not surprising.

Some literature data reports using the TMM assay for Fe(II) chelation of phenolic compounds. For example, Wettasinghe and Shahidi (*23*) investigated the Fe(II) chelation activity of borage and evening primrose extracts, Shahidi et al. (*25*) studied extracts of white and black sesame seeds and their hulls, while Wijeratne et al. (*42*) examined extracts of almonds seeds, brown skin, and green shell covers. The authors of these studies never mentioned problems with absorbance readings for their control samples. It should be pointed out that the aforementioned investigations were carried out on extracts obtained using 80% (v/v) aqueous ethanol solutions or on fractions eluted from a Sephadex column with methanol, not 50% (v/v) acetone. The authors' interests did not concern tannins (very strong chelators of iron). From our reading of these works, the extraction and chromatographic conditions employed likely eliminated a portion of the tannins, especially those with the highest degree of polymerization. Apropos standards: the authors used catechin and sinapic acid, which we also used in our study, and these did not create absorbance problems with the TMM assay (**Figure 2**). Complexes formed between TMM and divalent metal ions other than Fe(II) have been investigated for various food products to determine the extent of ion binding. Examples include Zn(II) in coffee brews (*24*, *31*); Cu(II) in alcoholic and nonalcoholic beverages (*43*), an extract of tropical plants (*26*), a ginkgo (*Ginkgo biloba*) extract (*44*); as well as Ca(II) and Ni(II) in pectin studies (*45*, *46*).

Because of the high sensitivity of the ferrozine assay (i.e., the ferrous complex of ferrozine exhibits an $\varepsilon_{562\ nm}$ = 27900 M^{-1} cm^{-1}, (*32*)), it seems that this technique would be quite useful

Table 2. Bound Fe(II) by 2.5 mg of Extracts, Low-Molecular-Weight Fractions (LMW), and Tannin Fractions as Determined Using the Ferrozine Assay[a]

	bound Fe(II), %		
plant	crude phenolic extract	LMW fraction	Tannin fraction
almonds	12.05 ± 0.48	5.23 ± 0.11	90.13 ± 0.90
red lentil	30.03 ± 0.85	14.78 ± 0.50	42.71 ± 0.68
buckwheat	34.05 ± 0.77	18.65 ± 0.36	50.31 ± 0.71

[a] Data are expressed as means ± standard deviation (n = 3).

for the determination of chelation activity for tannin-rich extracts. Complexation resulting from 0.031 mM Fe(II) with ferrozine gave an absorbance reading of 0.812 (data not shown), whereas to obtain an optical density of 0.701 from the TMM assay, a concentration of 2.5 mM Fe(II) is required (**Figure 1**). The ferrozine assay can be employed with much more diluted solutions in comparison to the TMM assay, and resultant complexes formed between phenolic compounds and iron ions (i.e., control samples) demonstrated much lower absorbances. Catechin, rutin, and tannic acid exhibited less than 0.1 absorbance values for control samples under conditions employed by the ferrozine assay (data not shown). Of ions from copper, cobalt, calcium, magnesium, lead, silver, molybdenum, aluminum, nickel, zinc, arsenic, manganese, hexavalent chromium, and trivalent chromium, only Co^{2+} and Cu^{+} were the metal ions other than iron which formed colored species with ferrozine under the test conditions of the assay. Many heavy metals can also react with ferrozine in competition with iron, but the excess reagent used in the assay overcomes such issues (*32*).

Table 2 reports the findings of chelating Fe(II) by almond, red lentil, and buckwheat crude phenolic extracts, their low-molecular-weight and tannin fractions as determined by the ferrozine assay. Iron ions were most efficiently chelated by the tannin fractions (i.e., from 42.7% to 90.13%), and least efficiently by the low-molecular-weight fractions (i.e., less than 19%). Absorbance values of control samples were low, that is, below 0.3 (data not shown). Until now, researchers have employed ferrous ion−ferrozine complex formation to study the chelation characteristics of isolated phenolic compounds, for example, tannic acid (*12*), melatonin, BHT, BHA, α-tocopherol (*47*), and a few plant extracts including those from red bean (*48*), nettle (*49*), seaweeds (*50*), cashew nut skins (*51*), as well as the tannin fractions from buckwheat seeds and groats (*27*). It is difficult to compare results of chelating activity of plant extracts obtained by various researchers, because the reaction of complex formation was carried out at various ratios of Fe(II) and extracts. A comparison is only possible when the researchers use the same material of reference (the most frequent being EDTA). In our study, 0.02 mg of EDTA added per ferrozine assay caused 53% chelation of ferrous ions (data not shown). On the basis of results depicted in **Table 2** it can be calculated that extracts of almonds, red lentil, and buckwheat chelate Fe(II) about 500, 220, and 200 times weaker than EDTA, respectively. Comparable results were obtained by Wang et al. (*50*) for aqueous and 70% (v/v) aqueous acetonic extracts of seaweed. Those extracts complexed about 100 and 300 times less Fe(II) than EDTA, respectively. Moreover, 4.5 mg of an ethanolic extract of cashew nut skins chelated Fe(II) to the same extent as 5 μg of EDTA (*51*).

The results obtained from this study indicate that the TMM method should not be employed to evaluate Fe(II) chelation activity of tannins or of plant extracts rich in tannin constituents, because of the high absorbance values observed in control samples. A valid alternative may be the employment of the ferrozine assay to test for Fe(II) chelating ability of tannin-rich material until such a time when another technique has been proposed and validated.

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