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Solid-Phase Extraction Spectrophotometric Determination of Total Antioxidant Capacity in Antioxidant-poor Samples by Using the Ferric-Ferrozine Method

Kadriye Isil Berker,* Dilek Ozyurt,* Birsen Demirata,* and Resat Apak,***†**

**Department of Chemistry, Faculty of Science and Letters, Istanbul Technical University, 34469 Maslak, Istanbul, Turkey*
***Department of Chemistry, Faculty of Engineering, Istanbul University, 34320 Avcilar, Istanbul, Turkey*
****Turkish Academy of Sciences (TUBA), Piyade Street No: 27, 06690 Cankaya, Ankara, Turkey*

Ferrozine (FZ) preferentially stabilizes Fe(II) over Fe(III) to raise the ferric reduction potential and oxidize antioxidants. The advantages of the ferric-ferrozine method over other iron-based total antioxidant capacity assays were: (i) higher molar absorptivity and enhanced sensitivity, (ii) lower interference from foreign ions, (iii) wide pH tolerance (iv) additivity of the absorbances for mixtures. Solid-phase extraction (SPE) could be combined with spectrophotometry, because the magenta-colored anionic Fe(II)-FZ complex was quantitatively sorbed on Sephadex QAE A-25 resin. The sensitivity enhancement using the resin enabled us to conduct total antioxidant capacity (TAC) measurements of antioxidant-poor samples. The apparent molar absorptivity, linear concentration range and trolox equivalent antioxidant capacities (TEAC) of certain antioxidants were found. The calibration curves (lines) of trolox, rutin, and rosmarinic acid individually and in herbal infusions—by using the method of standard additions—were parallel, confirming that the added antioxidants did not interact with herbal constituents to cause chemical deviations from Beer's law.

Keywords Antioxidant capacity, solid-phase extraction, ferric ferrozine, spectrophotometry, fruit juice

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Introduction

Reactive oxygen and nitrogen species (ROS/RNS) can attack biomacromolecules (*i.e.* lipid, protein, DNA) under "oxidative stress" conditions, causing tissue damage and various diseases. Intrinsic (including enzymatic) and extrinsic antioxidants combat against oxidative stress by counterbalancing ROS/RNS *via* the mechanisms of chemical reduction and free-radical quenching. Extrinsic antioxidants (*e.g.*, antioxidant vitamins, mineral cofactors, flavonoids, carotenoids, and other special antioxidants) cannot be synthesized in an organism, and must be taken through the diet. Thus, total antioxidant capacity (TAC) as a measure of the total antioxidative power of food components needs to be measured with reliable methods. The TAC parameter is generally accepted to be a more realistic measure of the collaborative effect of food antioxidants than their individual determination, which does not take into account possible synergistic interactions among antioxidants.

Fruit juices and herbal teas are the mostly consumed examples of the beverage sector, to which TAC measurements can be directly applied without any sample pretreatment. There are many TAC assay methods in the literature based on Fe(III) oxidation of antioxidative compounds in the presence of iron-binding ligands to regulate the Fe(III)/Fe(II) reduction potential and create strongly absorbing chromophores.[1–9] Mostly cheap and common reagents are used for the measurement of the Fe(III)-based antioxidant capacity, which do not suffer from the common problems of radicalic reagents (*i.e.* ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)), DPPH (2,2-diphenyl-1-picrylhydrazyl)) such as high cost, limited availability and instability.

Fe(III)-L based reducing assays (where L is a iron coordinating ligand, such as cyanide, *ortho*-phenanthroline, *batho*-phenanthroline and tripyridyltriazine, which does not leave the coordination center when Fe(III) is reduced to Fe(II) with an electron donated by an antioxidant) operate in accordance with the following reaction:[10]

$$\text{Fe(III)-L} + \text{AOx} \longrightarrow \text{Fe(II)-L} + \text{oxidized AOx}^{\bullet}$$

The most widely used ferric-reducing antioxidant capacity assays are those utilizing cyanide[11] and tripridyltriazine, also known as the ferric reducing antioxidant power (FRAP) assay.[12]

Ferrozine-type bidentate ligands containing the –N=C(Ar)–C=N– functionality have been known for a long time to chelate the lower valencies of transition-metal ions (such as Cu(I), Co(II) and Fe(II)) to form stable and colored complexes. In 1970, Stookey[13] was the first to use the disodium salt of 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (known as ferrozine: FZ) for the determination of iron(II) in an acetic acid/acetate buffer at pH 5.5. Fe(II)-FZ is magenta-colored, absorbing at 562 nm, and the complex stability is very high for ferrous ion.

† To whom correspondence should be addressed.
E-mail: rapak@istanbul.edu.tr

The formation constant for the ferrous ferrozine ($Fe^{II}(FZ)_3$) chelate was given as $(7.2 \pm 0.3) \times 10^{15}$ M.[14] Using the ferrozine ligand, Fe(II)/Fe(III) speciation analysis was made in natural water samples[15,16] and indirect ascorbic acid (AA) determination was performed based on Fe(III) oxidation of ascorbic acid (AA) in hexamethylenetetramine buffer medium with flow-injection (FI) spectrophotometry.[17] Ferrozine found the highest usage in the assay of Fe(II)-chelating activity of phenolic compounds and herbal extracts, with some examples.[18,19] In the literature, iron binding capacity has been mostly measured in food and serum with the use of ferrozine. Berker *et al*. were the first to modify the Fe(III)-FZ assay and use it in the determination of TAC for various antioxidants (AOx); the basic reaction used in the TAC assay, disregarding charges, was[20]

$$\text{Fe(III)} + \text{AOx} + \text{FZ} \longrightarrow \text{Fe(II)-FZ} + \text{oxidized AOx}^{\bullet}$$

One of the distinct advantages of the Fe(III)-ferrozine method when compared to the FRAP method (using Fe(III)-tripyridyltriazine)—being the first iron-based total antioxidant capacity assay method in literature—is that its working pH is closer to the physiological pH. Because of the very low solubility product of ferric hydroxide as $K_{sp}[Fe(OH)_3] = 6 \times 10^{-38}$, most Fe(III)-based methods (including FRAP) must be carried out in acidic medium to overcome the hydrolysis problem of ferric ion.[9] Unfortunately, in acidic pH values at which these assays are performed, most antioxidants containing $-NH^+$ and phenolic Ar–OH groups are in an undissociated state, meaning that the measured TAC may be lower than the true value. However, when FZ is used along with ferric ion, the Fe(II) state is so stabilized in comparison to Fe(III) that the assay can be carried out at pH 5.5. Another advantage of the proposed method compared to FRAP is that the strong complexation of Fe(II) with FZ prevents the formation of Fenton-type reactions caused by unbound or weakly bound Fe(II), which may give rise to inaccurate TAC determinations through redox cycling of antioxidants. Additionally, the magenta-colored complex has a very high molar absorptivity (ε at the order of 3×10^4 M^{-1} cm^{-1}) enabling sensitive determinations within a wider pH range.

Indirect determinations of ascorbic acid (AA) in drugs were carried out with the use of ferrozine ligand and Sephadex resin with solid phase spectroscopy (SPS).[21] The same authors had published a previous paper, where they introduced a method for the determination of iron with ferrozine using SPS.[22] The main advantage of this technique—introduced in 1976—is high sensitivity due to the preconcentration of the concerned species on a solid support.[23] However, only ascorbic acid assay was carried out in this way without any TAC estimation. In the more recently conducted studies, the use of ferrozine is not common in TAC measurement although it was used for Fe quantitation in hair.[24]

This study aims to measure the TAC of antioxidant-poor food sources and fruit juice industrial waste products by solid-phase extraction–spectrophotometry using the Fe(III)-FZ method with the use of an anion-exchanger resin for preconcentration of the resulting chromophore, *i.e.* the $\{Fe(II)\text{-}FZ\}^{4-}$ anionic chelate. Surprisingly, there is very few information in literature regarding the TAC measurement of "low antioxidant" foods and food industry wastes. Thus, food samples with a poor AOx content, including certain fruit juices, medicinal plant infusions and industrial wastes whose assay is not possible with other TAC methods, can be efficiently measured by preconcentration on a Sephadex QAE A-25 anion exchanger resin and subsequent absorbance measurement directly in the transparent resin phase, opening up new prospects for food science and technology with possible commercial applications (especially for "antioxidant-poor" diets).

Experimental

Materials and methods

Plant materials. Separate infusion solutions of commercially purchased daisy (*Matricaria chamomilla L.*), linden (*Tilia*), bay leaf (*Laurus nobilis*), white pepper (*Piperis albi*), fanugrek (*Trigonella foenum-graecum*), ground cinnamon (*Cinnamomum verum*) and apple pulps (supplied from the fruit juice producer company) were prepared. Each infusion containing pulverized dry plant was agitated for 2 min in freshly boiled water, and then steeped for an additional 3 min without any external heating. At the end of a 5-min total time, the prepared infusions were let to cool, and then filtered from quantitative (equivalent white tape) filter paper. The prepared infusions were freshly used on the day of each experiment.

Chemicals

Ferrozine monosodium salt (FZ), quercetin (QR), ellagic acid (EA), and catechin (CA) were purchased from Fluka; caffeic acid (CF), ferulic acid (FR), trolox (TR) (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), rosmarinic acid (RA), and ascorbic acid (AA) were from Aldrich; glutathione, cysteine, $FeCl_3 \cdot 6H_2O$, CH_3COONa, $NH_4Fe(SO_4)_2 \cdot 12H_2O$, Na_2CO_3, $NaKC_4H_4O_6$, $CuSO_4$, $CuCl_2$ and CH_3COONH_4 were purchased from E. Merck, Germany; rutin and sodium dodecyl sulfate were from Sigma Chemicals, Germany; conc. HCl, conc. NH_3, NaOH, CH_3COOH, and C_2H_5OH (96%, by wt.) were supplied from Riedel de Haen. The ion-exchanger resin used for preconcentration of Fe(II)-FZ colored anionic complex was QAE Sephadex® A-25 chloride form (diethyl-(2-hydroxypropyl)-aminoethyl Sephadex®) with 40 – 125 μm bead size in dry form, has a declared (by manufacturer) ion-exchange capacity of 2.6 – 3.4 meq/g. The anion exchange resin was used in the chloride-form in original dry state as obtained from the supplier, without pretreatment. All other chemicals were of analytical-grade and doubly distilled water was used for the preparation and dilution of solutions.

Apparatus, reagent solution, working conditions, and dilution factor

During preparation of solutions, weighing, agitation, thermostatic incubation, and pH measurements were made with AX200 precision balance, Heidolph swirl mixer, Clifton water bath, and E512 Metrohm Herisau pH-meter were used, respectively. Spectral measurements were conducted in a pair of matched HELMA quartz cuvettes of 10 mm light path, using a Varian Cary 1E Model UV-Vis spectrophotometer. Although the original spectrophotometric method was carried out at ambient temperature, an incubation temperature of 50°C was chosen in the proposed method (of SPE spectrophotometry) because the reference (blank) was not colored and absorption peaks were sharper when incubation was made. In addition, spectral measurements gave different results when the volume of ethanol (used in preparation of standard antioxidant solutions) exceeded 200 μL in the proposed method, which necessitated setting of the maximal EtOH amount at this value.

To prepare a reagent solution composed of Fe(III) and FZ, 0.123 g ferrozine was dissolved with a little amount of water; 0.0246 g $NH_4Fe(SO_4)_2 \cdot 12H_2O$ was dissolved separately in some water, to which 1.0 mL of 1 M HCl solution was added.

These two solutions were mixed and diluted to 25 mL with water. This reagent solution was stored in a dark and cold environment, and prepared daily. All antioxidant solutions were prepared and used daily in absolute ethanol at 1 mM concentration, and diluted with EtOH where necessary.

The working solutions were diluted to different final volumes with 0.4 g resin after the reagent and buffer were added to 150 µL of 1.0×10^{-4} M quercetin solution, and the absorbance values of the resins were measured. The same trials were repeated by adding the reagents after the dilution step, and identical results were obtained.

For all absorbance measurements made with the resin, the reference (blank) was the resin without an antioxidant, *i.e.* a mixture of Fe(III) and ferrozine in pH 5.5 buffer was equilibrated with the resin, as described below.

For a reference (blank) solution equilibrated with the resin, a volume of 1.5 mL ferric-ferrozine reagent and 2.0 mL pH 5.5 buffer solution (containing 0.2 M HAc/NaAc) were added to 0.2 mL of an EtOH (96%) solution, and 0.8 mL distilled water was added to a final dilution of 4.5 mL. This mixture solution was incubated at 50°C for 30 min. After incubation, 5 mL of pH 5.5 buffer was added, and diluted to a final volume of 50 mL with H_2O. Following the addition of 0.4 g of Sephadex resin, the suspension (*i.e.* solution containing resin) was agitated at 450 rpm for 30 min. The separated resin was called the "reference resin".

For antioxidant standard solutions equilibrated with the resin, a volume of $(0.2 - x)$mL EtOH (96%), 1.5 mL of ferric-ferrozine reagent, 2.0 mL of pH 5.5 buffer (containing 0.2 M HAc/NaAc) and H_2O were added to (x)mL antioxidant solution to make the final volume at 4.5 mL. This mixture solution was incubated at 50°C for 30 min. After incubation, 5 mL of a pH 5.5 buffer, the necessary amount of distilled water to make the final volume 50 mL, and 0.4 g of Sephadex resin were added in this order. The final suspension (*i.e.* resin + solution) was agitated at 450 rpm for 30 min. The absorbance against the "reference resin" was measured at 567 nm.

Optimizing agitation time for resin-solution equilibration

Volumes of 150 µL 1.0×10^{-4} M QR, 50 µL ethanol, 1.5 mL Fe(III)-FZ complex reagent, 2.0 mL of 0.2 M acetate buffer at pH 5.5, and finally 0.8 mL water were added into a flask, and incubated in a thermostatic water bath for 30 min at 50°C. Finally, ten more milliliters of a pH 5.5 acetate buffer solution was added, and the mixture was diluted to 100 mL. This solution was added onto 0.4 g resin, and agitated for different time periods of 20, 40 and 60 min at 450 rpm. The optical absorbance of the resin phase was directly read at 567 nm.

Ferric-ferrozine assay of total antioxidant capacity

The amounts of x mL antioxidant, $(0.2 - x)$mL 96% ethanol, 1.5 mL Fe(III)-FZ complex solution,

2.0 mL 0.2 M pH = 5.5 acetate buffer were mixed and diluted to 4.5 mL final volume with H_2O. The mixture solution was incubated for 30 min at 50°C. After the addition of 10.0 mL of 0.2 M acetate buffer at pH 5.5, the final volume was completed to 100 mL with water. The prepared solution was poured onto 0.4 g of Sephadex resin in a quartz cuvette, and agitated batchwise for 30 min at 450 rpm. The colorless supernatant solution was decanted, and the colored resin retaining all Fe(II)-FZ product—formed from the reaction of ferric-ferrozine reagent with antioxidant—remaining at the bottom of the quartz cuvette was transferred to a UV-Vis spectrophotometer for optical absorbance reading at 567 nm. It was observed that when the final solution volume was completed to 100, 250 and 500 mL (as was done to 50 mL), almost the same absorbance was recorded by using the same amount of resin. In this way, the volume enrichment factor could be increased up to 100.

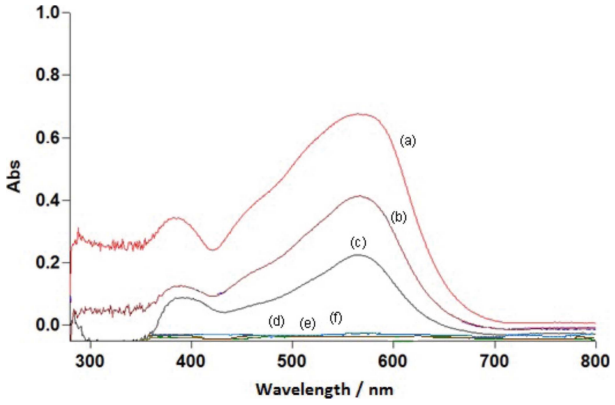


Fig. 1 UV-Vis spectra of Fe(II)-ferrozine complex held on 0.4 g Sephadex resin at pH 5.5 for different initial concentrations of quercetin; (a) 4.0×10^{-7} M QR, (b) 2.0×10^{-7} M QR, (c) 7.5×10^{-8} M QR. The UV-Vis spectra of Fe(II)-ferrozine complex without being held on Sephadex resin at pH 5.5 for different initial concentrations; (d) 4.0×10^{-7} M QR, (e) 2.0×10^{-7} M QR, (f) 7.5×10^{-8} M QR.

Finding the adsorption capacity of Sephadex QAE A-25 resin for the Fe(II)-FZ complex

The standard assay procedure was applied to 100 mg of Sephadex resin in dynamic column mode as: 30 mL Fe(III)-ferrozine reagent, 25 mL of 1.0×10^{-3} M TR, 100 mL 0.2 M acetate buffer at pH 5.5, 840 mL H_2O of dilution; V_{total} = 1000 mL. The weighed resin (0.1065 g) was filled to C18 column and placed in an SPE system, and the prepared colored solution (containing Fe(II)-FZ anionic complex) was passed through the resin. The absorbance of the effluent at each 50 mL solution portion was measured at 567 nm.

Results and Discussion

Determination of maximum absorption wavelength

The UV-Vis spectra of Fe(II)-ferrozine complex held on to Sephadex resin (obtained from different concentrations of quercetin reacted with Fe(III)-FZ reagent) were recorded within the wavelength range of 300 – 800 nm. The absorption spectra of Fe(II)-FZ complex (*i.e.* $Fe(FZ)_3^{4-}$) in solution and adsorbed phases at the same dilutions are simultaneously shown in Fig. 1. The maximal absorption wavelength of the complex in solution is 562 nm,[13] which is slightly different from that in the adsorbed phase, *i.e.* 567 nm. As is apparent from Fig. 1, the solution phase complexes at the same dilution ratios did not yield a noticeable absorption spectrum, showing the advantage of resin preconcentration. The anionic Fe(II)-FZ complex was quantitatively sorbed on Sephadex resin, enabling direct absorbance readings on the transparent resin phase with a significant increase in sensitivity. The enhanced sensitivity is apparent from the very low concentrations of quercetin (Fig. 1) determined with the proposed assay, which is not possible with classical TAC assays not involving SPE preconcentration.

Selection of optimal pH and buffer type

Although the Fe(II)-ferrozine chelate was stable in solution in the pH range of 4 – 9, the colored complex was quantitatively

Table 1 Effect of dilution on the absorbance of 0.4 g resin (150 µL of 1.0×10^{-4} M QR) ($N = 3$)

Total volume/mL	Absorbance
100	0.4350 (± 0.0025)
200	0.4600 (± 0.0046)
300	0.4450 (± 0.0034)
400	0.4480 (± 0.0064)

retainable by the resin between pH 4 and 7, and the optimal pH of retention of this complex on Sephadex resin was pH 5.5 (Table S1, Supporting Information). It is noteworthy that the proposed method has a large pH tolerance which is not encountered in most antioxidant capacity assays. Three different buffers, all at the same buffer concentration (the total concentration of weak acid and conjugate base was 0.2 M in each case) were tested at the predetermined optimal pH. Among the three buffers tested (*i.e.* hexamethylenetetramine hydrochloride, acetic acid/sodium acetate, and sodium dihydrogen phosphate/monohydrogen phosphate combinations), HAc/NaAc buffer gave the most suitable response, as determined from the relative magnitude of the absorbance on the resin phase compared to that remaining in solution.

Dilution factor

The effect of the dilution factor on the absorbance of the resin was studied for different total volumes of solution at a fixed amount of QR and resin. The resin absorbance readings did not significantly differ, showing that about 10-fold dilution did not adversely affect the adsorption of the Fe(II)-FZ anionic chelate on the resin, and that a single-step batchwise contact of adsorbent and solution was sufficient to adsorb all the color from a relatively large volume of solution. Table 1 summarizes the effect of dilution on the absorbance of the resin. Two factor ANOVA without replication data analysis tool gave the result of F-test as $P = 0.05$; $F_{exp.} = 1.00$; $F_{crit.}(1, 3) = 9.27$, $F_{exp.} < F_{crit.}$, *i.e.* the null hypothesis, H_0, stating that there is no significant difference between the resin-phase absorbance values at different dilutions (100 – 400 mL solutions containing the same amount of analyte), is accepted. This finding shows that the used amount of resin is capable of fully extracting the colored complex from solutions at moderate-to-high dilutions. In other words, the distribution coefficient of the complex between the sorbent resin and the solution phase is favorable enough to fully extract the conversion product (*i.e.* Fe(II)-FZ) of the antioxidant analyte in a single run.

Effect of agitation time

The resin was equilibrated for different agitation times (varying between 20 and 60 min) with the Fe(II)-FZ solution resulting from the oxidation of QR with ferric-ferrozine reagent. The absorbance of the resin at 567 nm as a function of agitation time was tabulated in Table S2 (Supporting Information).

The results in Table S2 showed that an agitation time of 20 – 30 min was sufficient for equilibration of the adsorbent resin with the adsorbate (*i.e.* Fe(II)-FZ chelate), and over-agitation may result in a decrease of the net absorbance, possibly due to unexpected coloration of the reagent blank.

Dynamic adsorption capacity of resin for colored Fe(II)-FZ anionic complex

The Fe(II)-FZ solution (of 1 L total volume)—resulting from the redox reaction of 30 mL ferric-ferrozine reagent with 25 mL of 1.0×10^{-3} M TR—was passed through 0.1 g resin column with the help of an SPE manifold, and effluents were collected and A_{567nm}-read at 50 mL-intervals. The breakthrough and saturation of the Sephadex resin for Fe(II)-ferrozine complex were observed when 410 and 830 mL volumes of the effluent, respectively, were collected from the resin outlet. Integrating the upper portion of the curve (recorded as effluent concentration *versus* volume) yielded a dynamic adsorption capacity of (0.145 ± 0.004) mmol TR per gram of resin (Fig. 2).

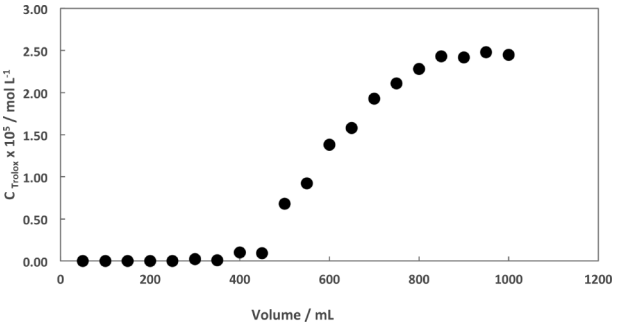


Fig. 2 Saturation of Fe(II)-ferrozine complex on the Sephadex resin, enabling the calculation of dynamic adsorption capacity (30 mL ferric-ferrozine reagent reacted with 25 mL of 1.0×10^{-3} M TR in a total volume of 1 L was passed through 0.1065 g resin column).

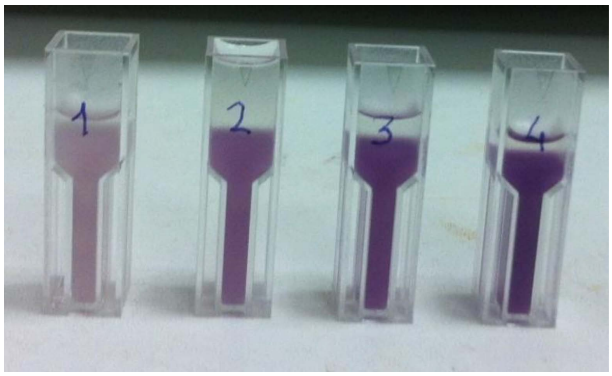


Fig. 3 Photograph of a series of magenta-colored Fe(II)-FZ chelate complexes concentrated on the Sephadex resin using different concentrations of QR.

This is naturally below the declared ion exchange capacity of the resin for ordinary anions, possibly due to the steric hindrance of the ferrozine ligands bound to the Fe(II) coordination center. The total anion exchange capacity of a resin represents the maximum achievable capacity for exchanging anions measured under ideal laboratory conditions. The capacity that will be realized when loading an ionic mineral (or complex ion) onto ion exchange resin will be influenced by such factors as the inherent selectivity of the ion exchange resin for the complex ion having variable size and bulky substituents, the ion's concentration in the loading solution and the concentration of competing ions also present in the loading solution, as well as by kinetic factors that may cause an incomplete achievement of true equilibrium conditions between the solute and sorbent.[25]

Calibration curve

The ferric-ferrozine assay was applied (as described in the proposed procedure) using AOx solution volumes varying in the

Table 2 Calibration equation, apparent molar absorptivity (ε), linear concentration range, linear correlation coefficient, and TEAC values of selected antioxidant compounds

Antioxidant	ε/M^{-1} cm^{-1}	Intercept/$\times 10^2$	Linear range/$10^7 \times$ M	Correlation coefficient	TEAC
Trolox	5.03×10^5	3.54	2.00 – 8.00	0.9954	1.00
Ascorbic acid	4.85×10^5	0.17	2.00 – 8.00	0.9874	0.96
Caffeic acid	5.50×10^5	–2.27	1.00 – 8.00	0.9904	1.09
Ferulic acid	5.05×10^5	2.50	1.00 – 8.00	0.9946	1.00
Gallic acid	9.25×10^5	5.25	0.60 – 4.00	0.9897	1.84
Quercetin	1.58×10^6	5.87	0.50 – 4.00	0.9959	3.14
Rutin	8.85×10^5	6.61	1.00 – 4.00	0.9990	1.75
Catechin	1.50×10^6	3.03	0.50 – 3.00	0.9963	2.98
Rosmarinic acid	1.47×10^6	9.12	0.25 – 2.00	0.9974	2.92

Table 3 Expected and found total antioxidant capacities (TAC) of selected binary and ternary antioxidant mixtures, found with the proposed procedure in mM trolox-equivalents

AOx constituents (each 200 µL of 1.0×10^{-3} M) in admixture	Expected TAC/ mM TR	Found TAC/ mM TR
Gallic acid (GA) Rosmarinic acid (RA)	2.44×10^{-4}	$(2.39 \pm 0.11) \times 10^{-4}$
Gallic acid(GA) Rosmarinic acid (RA) Quercetin (QR)	3.47×10^{-4}	$(3.04 \pm 0.09) \times 10^{-4}$
Ferulic acid (FR) Rutin (RT) Gallic acid (GA)	2.59×10^{-4}	$(2.74 \pm 0.14) \times 10^{-4}$
Rosmarinic acid (RA) Rutin (RT) Quercetin (QR)	3.44×10^{-4}	$(3.56 \pm 0.03) \times 10^{-4}$
Ferulic acid (FR) Rosmarinic acid (RA) Gallic acid (GA)	2.53×10^{-4}	$(2.33 \pm 0.05) \times 10^{-4}$
Ferulic acid (FR) Quercetin (QR) Rutin (RT)	2.56×10^{-4}	$(2.36 \pm 0.18) \times 10^{-4}$

range of $x = 0.025$ – 0.2 mL (Fig. 3). The trolox-equivalent antioxidant capacity (TEAC) of each antioxidant was found as: TEAC = $\varepsilon_{AOx}/\varepsilon_{trolox}$. The calibration equation, apparent molar absorptivity (ε), linear concentration range, linear correlation coefficient (r) and TEAC values of selected antioxidant compounds are listed in Table 2.

The antioxidants selected in constructing the calibration curves were trolox (TR), ascorbic acid (AA), caffeic acid (CF), ferulic acid (FR), gallic acid (GA), quercetin (QR), rutin (RT), catechin (CA) and rosmarinic acid (RA), and the TEAC values found in this study are in accordance with the values found with the original Fe(III)-ferrozine method.[20] The calibration curves of quercetin by using Fe(II)-ferrozine Sephadex QAE 25 and conventional Fe(II)-FZ methods are shown in Fig. S1 (Supporting Information), where the sensitivity enhancement with resin-preconcentration is apparent. The sensitivity enhancement ($\varepsilon_{FeFZ\text{-}resin}/\varepsilon_{FeFZ\text{-}soln.}$) provided by the resin-preconcentration, defined as the ratio of the molar absorptivity of Fe(II)-FZ method with resin to that without resin, is given in Table S3 (Supporting Information). There is an order-of-magnitude sensitivity enhancement, depending on the antioxidant tested.

TAC determination of synthetic mixtures

Theoretically, the expected TAC value of an antioxidant mixture should be equal to the sum of individual TAC values of mixture constituents (Eq. (1)), provided that the selected spectrophotometric method of measurement is free from chemical deviations from Beer's law.

In this equation, $TEAC_i$ is the trolox-equivalent antioxidant capacity coefficient of ith constituent at concentration c_i. The expected and found TAC of selected binary and ternary antioxidant mixtures, found with the proposed procedure in mM trolox-equivalents were depicted in Table 3. In general, the expected and found TAC values agreed well (within spectrophotometrically expected error limits).

$$\text{Total antioxidant capacity } (TAC)_{expected} = (TEAC)_1(concn.)_1 + (TEAC)_2(concn.)_2 + (TEAC)_3(concn.)_3 + ... + (TEAC)_n(concn.)_n \quad (1)$$

A simple way to test the presence of chemical deviations from Beer's law was to construct calibration curves of the selected antioxidant compounds in pure aqueous solution and in a real sample, such as a medicinal plant extract. For this purpose, the calibration curves of RA (alone and in daisy tea infusion) and of RT (alone and in linden tea infusion) were drawn (Figs. 4 and 5). Since the calibration lines for a given AOx in pure water and in a medicinal plant extract were almost parallel in each case, it could be deduced that the real sample constituents did not chemically interact with the tested AOx in response to the ferric-ferrozine spectrophotometric method combined with SPE, and that any chemical deviations from Beer's law were basically absent (Figs. 4 and 5).

Application to real samples

The proposed ferric-ferrozine spectrophotometric method combined with SPE enrichment was applied to a number of antioxidant-poor plant infusions and industrial waste products (Table 4). The TAC of the tested dry plants in the units of mmol TR equivalents per gram of dry plant was calculated with the use of Eq. (2).

$$TAC\ (mmolTR/g) = (A/\varepsilon_{TR}) \times (V_f/V_s) \times r(V_{cup}/m) \quad (2)$$

where A, measurement absorbance; V_f, final volume; V_s, sample volume; r, dilution factor; V_{cup}, initial volume (extraction or infusion prepared from (m) grams of dried sample).

It is apparent from the data in Table 4 that some antioxidant-poor plants, fruit juices and industrial wastes can be assayed for their TAC values using the proposed enrichment method, which cannot be carried out with the use of other widely-used spectrophotometric procedures lacking that much sensitivity.

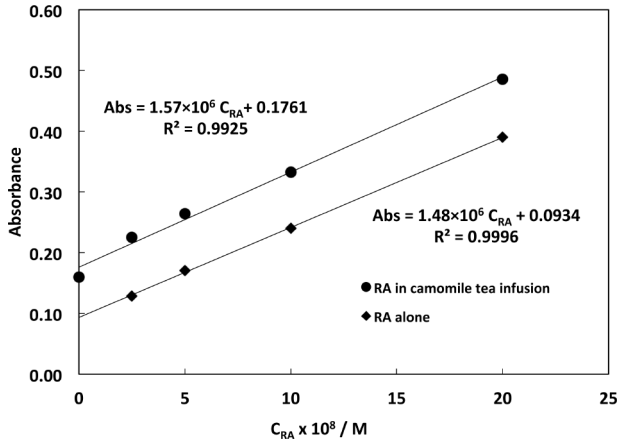


Fig. 4 Calibration curves for rosmarinic acid in pure water and in camomile tea infusion (measured by concentrating the resulting Fe(II)-FZ complex on Sephadex resin). RA alone: Abs. = $1.48 \times 10^6 C_{RA} + 9.34 \times 10^{-2}$, $r = 0.9996$. RA in camomile tea infusion: Abs. = $1.57 \times 10^6 C_{RA} + 0.1761$, $r = 0.9925$.

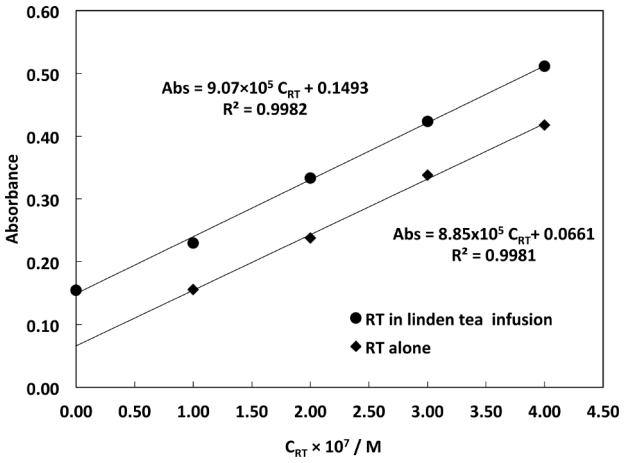


Fig. 5 Calibration curves for rutin in pure water and in linden tea infusion (measured by concentrating the resulting Fe(II)-FZ complex on Sephadex resin). RT alone: Abs = $8.85 \times 10^5 C_{RA} + 0.0661$, $r = 0.9981$. RT in linden tea infusion: Abs = $9.07 \times 10^5 C_{RA} + 0.1493$, $r = 0.9982$.

In this regard, very few results from the literature can be mentioned, without any improvement in sensitivity. For example, Karakaya *et al.*[26] and Pellegrini *et al.*[8] could not detect the TAC value Cola drinks with the use of ABTS/TEAC and TRAP assays, respectively. Furthermore, as opposed to the results for vegetables and fruits, the TAC values of soft beverages found with different assays could not be correlated well.[8] The proposed resin-preconcentration Fe(II)-FZ method was applied to three plant infusions and wastes, namely white pepper (piperis albi) and apple pomace I, II (industrial wastes), and the found TAC values are reported in Table S4 (Supporting Information), whereas the classical solution-phase Fe(II)-FZ method could not detect antioxidants in these samples.

Conclusions

In this study, spectrophotometry was used in combination with a solid phase on which the sample conversion product was adsorbed, and the analytical signal was obtained by direct measurement of the absorbance on a solid support. This work combined the use of a very sensitive TAC assay (of very high apparent molar absorptivity) using a stable reagent applicable within a wide pH range with a solid-phase extraction enrichment of antioxidants, where AOx compounds chemically reduced ferric-ferrozine to the ferrous-ferrozine anionic chelate concentrated on an anionic Sephadex QAE resin. Since common antioxidants lie within the redox potential range of 0.2 – 0.8 V, ferric-ferrozine is capable of oxidizing most antioxidants. Moreover, since the resin used for concentrating the Fe(II)-FZ chelate was transparent, the absorbance reading could be made directly in the solid resin phase. The ferrous iron was highly stabilized in the resulting ferrozine chelate, preventing possible redox cycling of antioxidants (proceeding through unbound or weakly-bound Fe(II)) and thereby preventing incorrect TAC measurement. A significant increase in sensitivity was observed, as a result of the SPE combination with the proposed assay. Thus, antioxidant-poor samples and industrial effluents could be easily assayed for their TAC values with the proposed method, because very high dilution and concentration factors were attainable without losing from precision and sensitivity.

Another important advantage of the proposed methodology of TAC determination is the "antioxidant sensing" capability on a solid substrate, because the response on a resin surface can be potentially used for *in situ*/on site sensing and for automated flow analysis. As opposed to similar other ferric-based TAC assays like FRAP, the proposed assay does not necessitate the presence of an acidic medium, and can be carried out at pH 5.5 which is closer to physiological pH. The TEAC values of selected antioxidant compounds observed with the proposed assay were close to those found by the original Fe(III)-ferrozine assay without preconcentration. The proposed method also bears the property of the additivity of TAC values (in accordance with the principle of additivity of absorbances as a consequence of Beer's law), which is rarely found in certain assays. Since the method was effectively adapted to the determination of the total antioxidant capacity in samples whose antioxidant content is low, it has potential for further applications to other complex problems of antioxidant analysis (such as beverages,

Table 4 Total antioxidant capacities (TAC) of some AOx-poor plants and industrial wastes found with the proposed ferric-ferrozine SPE method, in the units of mmol TR/g and mmol TR/mL equivalents

Plant infusions, juices and wastes	TAC
Daphne leaf (*Laurus nobilis*)	$(1.14 \pm 0.03) \times 10^{-1}$ mmol TR/g
White pepper (*Piperis albi*)	$(7.15 \pm 0.01) \times 10^{-3}$ mmol TR/g
Fenugrek (*Trigonella foenum-graecum*)	$(2.56 \pm 0.05) \times 10^{-2}$ mmol TR/g
Ground cinnamon (*Cinnamomum verum*)	$(2.33 \pm 0.07) \times 10^{-1}$ mmol TR/g
Commercial apple juice (100%)	$(3.12 \pm 0.04) \times 10^{-3}$ mmol TR/mL
Apple juice (Granny Smith)	$(2.19 \pm 0.03) \times 10^{-3}$ mmol TR/mL
Apple pomace I (industrial waste)	$(2.38 \pm 0.15) \times 10^{-2}$ mmol TR/g
Apple pomace II (industrial waste)	$(7.80 \pm 0.09) \times 10^{-3}$ mmol TR/g
Pomegranate peel (industrial waste)	$(1.20 \pm 0.11) \times 10^{-1}$ mmol TR/g
Pomegranate seed (industrial waste)	$(6.60 \pm 0.08) \times 10^{-2}$ mmol TR/g

pharmaceuticals, antioxidant-poor diet foods and fruit-processing effluents, *etc.*).

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Supporting Information

This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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