http://dx.doi.org/10.21577/0103-5053.20190025

J. Braz. Chem. Soc., Vol. 30, No. 6, 1293-1301, 2019
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Article

A Procedure for Assessment of the Reducing Capacity of Plants-Derived Beverages Based on the Formation of the Fe^{II}/2,2'-Bipyridine Complex

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An alternative spectrophotometric ferric reducing activity power (FRAP) method for quantification of total reducing capacity (TRC) was developed. The method is based on the reduction of Fe^{III} to Fe^{II} by antioxidant compounds containing 2,2'-bipyridine (bipy) in aqueous solution. Absorbance values recorded at 521 nm, characteristic of the $Fe(bipy)_3^{2+}$ complex formed, were used to determine the TRC of some plants-derived beverages. For the teas samples, the TRC values obtained with the proposed method and cupric reducing antioxidant capacity (CUPRAC) reagent had an excellent agreement (adjusted correlation coefficient (r^2) = 0.951). Concerning herbs samples, the TRC values obtained with the proposed FRAP method correlated very well with values obtained using the 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$) method (adjusted r^2 = 0.975). It can be inferred from these results that other beverages derived from plants (e.g., beers, wines, and fruits juices) could also be analyzed with this new FRAP assay. In addition, the reducing capacity of 21 phenolic derivatives was determined with the proposed method in order to elucidate their structure-reactivity relationship. As expected, the phenolic derivative structure changes greatly the TRC values obtained with this proposed FRAP assay.

Keywords: total reducing capacity, teas, medicinal herbs, 2,2'-bipyridine, Fe^{III}

Introduction

Originally, the acronym for ferric reducing activity power (FRAP) was employed to designate the ferric reducing ability of plasma, an assay designed to measure the antioxidant power of this biological sample. This spectrophotometric test was developed based on the reduction reaction of Fe^{III} to Fe^{II} in aqueous solution (pH 3.6; acetate buffer) containing the 2,4,6-tripyridyl-*s*-triazine (TPTZ) ligand, being the absorbance measurements (at 593 nm) of the Fe^{II}/TPTZ complex formed related to the reducing capacity of these biological samples.[1]

Over the last twenty years or so most of the researchers have used this acronym to also designate the ferric (ion) reducing antioxidant power assay.[2-5] Based on this more comprehensive definition, other methods using the reduction reaction of Fe^{III} in solution containing different complexing agents for Fe^{II} have also been developed.

Phenanthroline and batho-phenanthroline, both chelating agents that form stable and colored complexes with Fe^{II} at pH 4.6 (acetate buffer), were utilized in a thorough study dealing with the quantification of reducing capacity of many mixtures of standard polyphenols.[6] However, these iron complexes, as far as we know, have not yet been applied to determine the reducing capacity in any sample of plant origin.

On the other hand, the reduction of Fe^{III} in acid solution (1.0 mol L^{-1} HCl) containing 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine) was utilized to quantify the total reducing capacity (TRC) of teas leading to sound results.[7]

Recently, a comprehensive study based on the reduction of Fe^{III} in aqueous solution (pH 8.0, tris(hydroxymethyl) aminomethane (tris) buffer) containing the 3-hydroxy-4-nitroso-2,7-naphthalenedisulfonic acid was carried out. Several antioxidant agents were evaluated with this alternative FRAP assay before it was effectively used to determine the reducing capacity of aqueous extracts of many medicinal plants.[8]

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All these FRAP assays have in common the use of an electron-transfer reaction between the antioxidants (present in the samples) and the oxidant agent (Fe^{III}/complexes), both in the same solution, but whose order of added reagents may be quite different.

It is well known that Fe^{II} forms with 3-fold excess of the organic bidentate ligand 2,2'-bipyridine (bipy; Figure 1) a very stable chelate $Fe(bipy)_3^{2+}$ ($\log \beta_3 = 17.2$ at 25 °C).[9] This aqueous orange-red complex shows a maximum absorption at 521 nm ($\varepsilon_{521\ nm} = 7.5 \times 10^3$ L cm^{-1} mol^{-1})[10] and has been commonly used for direct determination of total iron content in different type of samples after reduction of Fe^{III} by addition of a suitable reducing agent.[11] Consequently, if Fe^{III} and bipy (1:3 ratio) are in excess when compared to the reducing agent, it is possible to determine indirectly this own reducing agent based on the formation of the $Fe(bipy)_3^{2+}$ complex.

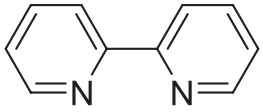


Figure 1. Structural formula of 2,2'-bipyridine.

In fact, a recent study employed the reduction reaction of Fe^{III} to Fe^{II} in presence of 3-fold excess bipy (pH 4.6; acetate buffer) to quantify the total polyphenolic content in nineteen medicinal plants expressing the results in pyrogallic acid (PA).[12] In that work it was also described that other antioxidants compounds (AOs), particularly tannic acid, 1,2,4-benzenetriol, 1,2-dihydroxybenzene, phenol and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), can also reduce Fe^{III} in presence of bipy.

Based on those findings, the reduction reaction of $Fe(bipy)_3^{3+}$ to $Fe(bipy)_3^{2+}$ complex was used in this present work to determine the reducing capacity of several standard AOs (mostly phenolic acids and flavonoids). This detailed study elucidates which is the oxidation ability of $Fe(bipy)_3^{3+}$ complex towards these AOs under the same experimental conditions used in the aforementioned study developed to the quantification of polyphenol content (pH 4.6; acetate buffer).[12]

Additionally, this same redox reaction was used to develop a spectrophotometric FRAP method to quantify the reducing capacity of aqueous extracts of twelve Brazilian medicinal plants. The plants analyzed have been used as a food source, for their healing properties (utilized in folk medicine) and in religious rituals. Besides, this reaction was also employed to quantify the TRC of twelve teas found in the local market and largely consumed by the population.

For comparison purposes, TRC values obtained with the suggested method were compared with two well-established methods. Regarding teas samples the TRC values obtained were compared with the cupric reducing antioxidant capacity (CUPRAC) method which is based on reduction of Cu^{II} to Cu^{I} in the presence of neocuproine.[13] For medicinal plants the TRC values were checked out with the method based on the extinction of the free radical derived from the 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS).[14]

Finally, the TRC values of the two groups of samples (teas and medicinal plants) obtained with the proposed method were also compared with the total polyphenolic content values obtained with the Folin-Ciocalteu reagent (FCR), as recommended by Brazilian Pharmacopoeia.[15]

Experimental

Apparatus

All spectrophotometric measurements were made in an HPUV 8453 (Agilent, USA) spectrophotometer using a 1.00 cm glass cell.

Materials

Reverse osmosis water (Quimis Q842-210, Brazil) was used to prepare the analytical-grade chemicals and in all sample dilutions (except when another solvent is indicated).

Reagents used for the total polyphenolic content quantification

The FCR was prepared as described elsewhere.[15,16]

A 10% (m/v) sodium carbonate (Na_2CO_3, formula mass (FM) 105.99 g mol^{-1}, 99%; Vetec, Brazil) solution was prepared in water.

A 1.89 mg mL^{-1} PA ($C_6H_6O_3$, FM 126.11 g mol^{-1}, 99%; Synth, Brazil) stock solution was prepared by dissolving 0.189 g in 100.0 mL of water. Diluted 0.0189 mg mL^{-1} working solutions were prepared accurately.

A 0.188 mg mL^{-1} gallic acid (GA, $C_7H_6O_5.H_2O$, FM 188.13 g mol^{-1}, 98%; Carlo Erba, Brazil) solution was prepared by dissolving 0.0188 g in 100.0 mL of water. A 0.0188 mg mL^{-1} working solution was obtained by dilution.

Reagents used for TRC quantification (proposed method)

A 4.90 mg mL^{-1} iron(III) sulfate ($Fe_2(SO_4)_3.5H_2O$, FM 489.95 g mol^{-1}, 97%; Fluka, Brazil) solution was prepared by dissolving 0.490 g in 100.0 mL of water.

Acetate buffer solution (pH 4.6) was prepared by dissolving 14.3 mL of glacial acetic acid (HAc, CH_3COOH, FM 60.05 g mol^{-1}, 99.8%; Merck, Brazil) and 20 g of potassium acetate (KAc, CH_3COOK, FM 98.15 g mol^{-1}, 99%; Merck, Brazil) in water in a 1.0 L volumetric flask.

A 2.58 mg mL^{-1} 2,2'-bipyridine (bipy, $C_{10}H_8N_2$, FM 156.19 g mol^{-1}, 99%; Fluka, Brazil) solution was prepared by dissolving 0.644 g in 10.0 mL ethanol (CH_3CH_2OH, FM 46.06 g mol^{-1}, 99.5%; Synth, Brazil) and then diluted with water in a 250.0 mL volumetric flask.

A 1.76 mg mL^{-1} ascorbic acid (AA, $C_6H_8O_6$, 99.7%, FM 176.13 g mol^{-1}; Merck, Germany) solution was freshly prepared by dissolving 0.176 g in a 100.0 mL volumetric flask containing water. A 0.0352 mg mL^{-1} solution was obtained by accurate dilution.

Tannic acid ($C_{76}H_{52}O_{46}$, FM 1701.20 g mol^{-1}, 99%; J. T. Baker, USA); GA ($C_7H_6O_5.H_2O$, FM 188.13 g mol^{-1}, 99%; Synth, Brazil); 2,3,4-trihydroxybenzoic acid (2,3,4-THB, $C_7H_6O_5$, FM 170.12 g mol^{-1}, 97%; Sigma-Aldrich, USA), PA, phloroglucinol and 1,2,4-benzenetriol ($C_6H_6O_3$, FM 126.11 g mol^{-1}, 99%; Sigma-Aldrich, USA); hydroquinone, resorcinol and *o*-pyrocatechol ($C_6H_6O_2$, FM 110.11 g mol^{-1}, 99%; Synth, Brazil); caffeic acid ($C_9H_8O_4$, FM 180.16 g mol^{-1}, 98%; Sigma-Aldrich, USA); *p*-coumaric acid ($C_9H_8O_3$, FM 164.16 g mol^{-1}, ≥ 98%; Sigma-Aldrich, USA); ferulic acid ($C_{10}H_{10}O_4$, FM 194.18 g mol^{-1}, 99%; Sigma-Aldrich, USA); sinapic acid ($C_{11}H_{12}O_5$, FM 224.21 g mol^{-1}, 98%; Sigma-Aldrich, USA); vanillic acid ($C_8H_8O_4$, FM 168.15 g mol^{-1}, > 97%; Merck, Germany); vanillin ($C_8H_8O_3$, FM 152.15 g mol^{-1}, 99%; Sigma-Aldrich, USA); quercetin ($C_{15}H_{10}O_7$, FM 302.24 g mol^{-1}, 98%; Sigma-Aldrich, USA); rutin ($C_{27}H_{30}O_{16}$, FM 610.52 g mol^{-1}, 95%; Sigma-Aldrich, USA); (–)-epigallocatechin gallate ($C_{22}H_{18}O_{11}$, FM 458.37 g mol^{-1}, 80%; Sigma-Aldrich, USA); phenol (C_6H_6O, FM 94.11 g mol^{-1}, 99%; Synth, Brazil) and Trolox ($C_{14}H_{18}O_4$, FM 250.29 g mol^{-1}, > 97%; Sigma-Aldrich, USA) solutions of 1.0×10^{-2} or 1.0×10^{-3} mol L^{-1} (except 0.1 mol L^{-1} phenol) were prepared by dissolving in water. Dilute solutions (1.0×10^{-4} to 5.0×10^{-5} mol L^{-1}) were also obtained by dilution with water. These antioxidant solutions need to be maintained in this unit of concentration (mol L^{-1}) for proper calculation of the reducing capacity of each.

Reagents used for TRC quantification (reference methods)

A 3.84 mg mL^{-1} 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, 548.68 g mol^{-1}, 99%; Sigma, Brazil) solution was prepared dissolving 192 mg in water in a 50.0 mL volumetric flask.

A 37.9 mg mL^{-1} potassium persulfate ($K_2S_2O_8$, FM 270.32 g mol^{-1}, 99%; Sigma, Brazil) solution was prepared dissolving 379 mg in water in a 10.0 mL volumetric flask.

A 604 mg mL^{-1} copper(II) perchlorate ($Cu(ClO_4)_2$, FM 262.45 g mol^{-1}) solution was prepared by reaction of copper(II) basic carbonate ($CuCO_3.Cu(OH)_2$, FM 221.12 g mol^{-1}, > 95%; Sigma, Brazil) with a 5% excess of perchloric acid ($HClO_4$, FM 100.46 g mol^{-1}, 70%; Merck, Brazil) and standardized by complexometric titration with ethylenediaminetetraacetic acid (EDTA) as described elsewhere.[17-19] A 24.4 mg mL^{-1} diluted solution used was prepared by dilution in water.

A 1.54×10^2 mg L^{-1} ammonium acetate ($C_2H_3O_2NH_4$, FM 77.08 g mol^{-1}, 97%; Merck, Brazil) solution was prepared by dissolution in water and used as buffer solution (pH 7.0).

A 3.21 mg mL^{-1} monohydrated neocuproine hydrochloride (NC, $C_{14}H_{12}N_2.HCl.H_2O$, FM 262.73 g mol^{-1}, 99.5%; Synth, Brazil) solution was prepared by dissolution of 0.320 g in 100 mL of 99% ethanol.

CUPRAC reagent was prepared by mixing 0.75 mL of 24.4 mg mL^{-1} copper(II) perchlorate solution, 3.0 mL of 1.54×10^2 mg L^{-1} ammonium acetate, and 15.0 mL of 3.21 mg mL^{-1} neocuproine hydrochloride monohydrated in a 50.0 mL volumetric flask completed with 99.5% ethanol.

Methods

Preparation of tea samples

A procedure previously described was used for the preparation of tea samples.[19] Briefly, 300 mg of commercial tea were transferred to a 100.0 mL beaker containing 50 mL of water and kept in water bath (65 °C, 30 min). After cooling, this solution was transferred to a 100.0 mL volumetric flask, completed with water and then filtered. When necessary a 5-fold dilution was used, transferring 5.0 mL of this solution to a 25.0 mL volumetric flask.

These aqueous samples were used for the total polyphenolic content (TPC) determination, with FCR, and for the TRC quantification with CUPRAC and the proposed method.

Preparation of herbal extracts

Aqueous samples

The extraction procedure described in previous studies[8,19] was used to prepare the aqueous extracts of medicinal herbs. These aqueous extracts were used for two quantifications: the TPC, with FCR, and the TRC, with the proposed method.

Samples in organic solvents

The extracts in methanol/acetone mixture were obtained as described elsewhere and used to quantify the antioxidant capacity with the $ABTS^{\bullet+}$ method.[14,20]

TPC determination with the FCR

The multiple standard addition method used for the TPC quantification was already described.[8,12,16] For teas, a

0.0188 mg mL^{-1} GA standard solution was used to express the TPC (mg GA g^{-1} dry material) as used in another study.[19] For the aqueous extracts of medicinal herbs, a 0.0189 mg mL^{-1} PA standard solution was used to express the TPC (g PA 100 g^{-1} dry material) as recommended by Brazilian Pharmacopoeia.[15]

Reducing capacity quantification with proposed method

Calibration graph with a standard antioxidant (AA)

In eight 5.0 mL volumetric flasks the following reactants were added: 0.50 mL of 4.90 mg mL^{-1} $Fe_2(SO_4)_3$ solution, different volumes (0.2 to 0.9 mL) of a 0.0352 mg mL^{-1} AA standard solution, 0.50 mL of acetate buffer (HAc/KAc; pH 4.6) solution and 1.0 mL of 2.58 mg mL^{-1} bipy solution. The AA final concentration (C_{AA}) was (1.41-6.34) × 10^{-3} mg mL^{-1}. Absorbance measurements at 521 nm ($A_{521\ nm}$) were recorded using a mixture containing 0.490 mg mL^{-1} $Fe_2(SO_4)_3$ and 0.515 mg mL^{-1} bipy in the same acetate buffer solution as reference solution (blank reagent). A calibration graph ($A_{521\ nm}$ *vs.* C_{AA}, where C_{AA} is in mg mL^{-1}) obtained is described by the equation $A_{521\ nm} = a + b\ C_{AA}$.

Calibration graphs with some phenolic compounds (PC)

Calibration graphs with standard PC were performed like the one made with AA standard solution. For each PC analyzed, at least three calibration graphs ($A_{521\ nm}$ *vs.* [PC], where [PC] is the concentration of PC in mol L^{-1}) were obtained. In these studies, only straight lines were considered, originating from the calibration graphs that showed very good linearity (adjusted correlation coefficient (r^2) ≥ 0.99) described by the equation $A_{521\ nm} = a + b$ [PC]. These calibration graphs need to be obtained in mol L^{-1} in order to comply with the definition of the reducing capacity of standard AOs.[7,8,21]

Calibration graphs with samples (teas and aqueous extracts of medicinal herbs)

In five 5.0 mL volumetric flasks were added: 0.50 mL of 4.90 mg mL^{-1} $Fe_2(SO_4)_3$ solution, 100 to 1000 µL (depending on the kind of sample) of 3.0 mg mL^{-1} aqueous extracts of teas or herbs (both obtained with dry material), 0.50 mL of acetate buffer solution (pH 4.6) and 1.0 mL of 2.58 mg mL^{-1} bipy solution. $A_{521\ nm}$ were recorded using the same blank reagent above described. A calibration graph ($A_{521\ nm}$ *vs.* C_{DM}, where C_{DM} is the dry material (DM) concentration in mg mL^{-1}) obtained is described by the equation $A_{521\ nm} = a' + b'\ C_{DM}$.

Calculation of reducing capacity of standard PC

The reducing capacity of each PC investigated was expressed as ascorbic acid equivalent capacity (AA_{EC}), defined as the concentration in 10^{-3} mol L^{-1} of AA standard solution which presented a reducing capacity value equivalent to a 1.0 × 10^{-3} mol L^{-1} of PC solution under the same experimental conditions.[7,8,21]

Calculation of reducing capacity in samples (aqueous extracts of teas and medicinal herbs)

The equation $A_{521\ nm} = a + b\ C_{AA}$ is applied to calculate the $A_{521\ nm}$ value corresponding to a 1.0 mg mL^{-1} AA standard solution. This $A_{521\ nm}$ value is replaced in the equation $A_{521\ nm} = a' + b'\ C_{DM}$ providing the concentration (mg mL^{-1}) of the solution analyzed (and the corresponding DM mass), which is equivalent to the TRC of a 1.0 mg mL^{-1} AA. The TRC values obtained (corrected to 5-fold dilution when necessary) were expressed as g DM g^{-1} AA and can be more easily calculated using the equation 1:

$$\mathrm{TRC}\left(\mathrm{g\ DM\ g^{-1}AA}\right) = \frac{\left(1000 \times m_{DM}\right)}{\left\{\frac{\left[\left(a+b\right) - a'\right] \times fd}{b'}\right\}} \qquad (1)$$

where a, b, a' and b' are the coefficients of the straight line equations above described, fd is the dilution factor and m_{DM} is the mass (in grams) of dry material.

Determination of the reducing capacity of tea with CUPRAC reagent

This method, based on the reduction of Cu^{II} to Cu^{I} in solution containing neocuproine (pH 7.0), was performed as described elsewhere.[13,19]

Determination of the TRC of herbal extracts using the $ABTS^{\bullet+}$

The preparation of $ABTS^{\bullet+}$ solution and the procedure used here were carried out as previously described.[14] The antioxidant capacity values were expressed in µM Trolox g^{-1} dry material.

Results and Discussion

Bipy is partially protonated in aqueous solutions in pH < 4.0 (pK_{a1} = –0.2; pK_{a2} = 4.3),[9-11] and ferric hydroxo complexes (e.g., $FeOH^{2+}$ and $Fe(OH)_2^{+}$) may be present in unbuffered solutions in pH > 3.5.[22] Thus, in the present study, the pH was maintained at 4.6 with acetate buffer solution, which has also been used with the same reduction reaction of Fe^{III} to Fe^{II} in a solution containing bipy, in a recently proposed method for the quantification of the polyphenolic content in medicinal plants.[12]

The above considerations support the experimental

conditions adopted in this proposed method (0.490 mg mL^{-1} $Fe_2(SO_4)_3$, 0.515 mg mL^{-1} bipy as final concentration at pH 4.6 kept with acetate buffer). The procedure described here can be performed in few minutes and it might be adapted for flow injection analysis, though it is not the purpose of the present study.

AA was chosen and used as the standard antioxidant to express the reducing capacity due to its fast reaction (ca. 10 min), low cost and being biologically active. A typical calibration graph ($A_{521\ nm}$ *vs.* C_{AA}) obtained from the absorption spectra (Figure 2) leads to a straight line described by the equation $y = -0.0217 + 115x$ (n = 8; adjusted $r^2 = 0.997$) for a linear range from (1.41 to 6.34) × 10^{-3} mg mL^{-1} AA (Figure 2, inset). The angular coefficient, defined as apparent absorptivity (at 521 nm) for AA, was 115 ± 4 mL cm^{-1} mg^{-1} for 20 calibration curves (relative standard deviation (RSD) = 3.3%).

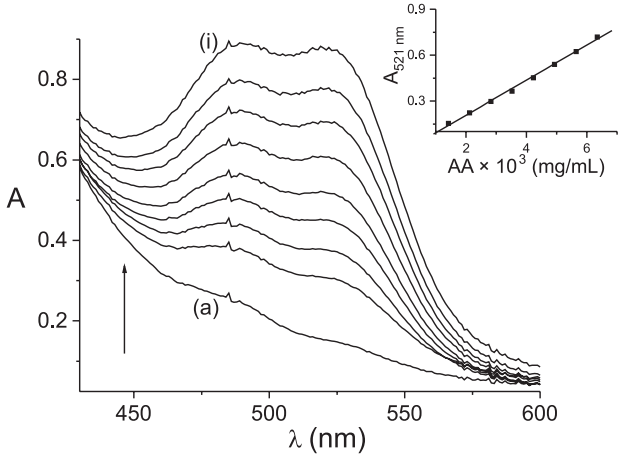


Figure 2. Absorption spectra of (a) 0.490 mg mL^{-1} $Fe_2(SO_4)_3$, 0.515 mg mL^{-1} bipy at pH 4.6 with acetate buffer solution; (b) to (i) (1.41, 2.11, 2.82, 3.52, 4.23, 4.93, 5.64 and 6.34) × 10^{-3} mg mL^{-1} ascorbic acid (AA) + (a), respectively, using water as reference solution. Inset: calibration curve for AA using the $A_{521\ nm}$ of the $Fe(bipy)_3^{2+}$ complex (b = 1.0 cm).

Trolox (a water-soluble compound analogous to vitamin E) also reduces Fe^{III} to Fe^{II} in solution containing bipy, but has a current cost about 35 times greater (considering a pack of 25 g) and almost half of the capacity reduction value of AA (AA_{EC} value of 0.79, Table 1).

The reducing capacity of polyphenolic compounds

In Table 1, there is a basic structure of phenol that helps in the interpretation of AA_{EC} obtained for the antioxidant compounds investigated.

Tannic acid has the highest AA_{EC} value (7.07), which is due to the highest number of hydroxyl groups (HG).

GA (pK_{a1} = 4.4) and its isomer 2,3,4-THB (pK_{a1} = 3.0) have the –COOH group partially deprotonated under these experimental conditions (pH 4.6).[9] In 2,3,4-THB the –COOH group is in vicinal position to the three HG, but in GA the –COOH group is symmetrically opposed to the three HG. This seems to be the reason that GA (AA_{EC} 2.76) has a reducing capacity value 2 times higher than 2,3,4-THB (AA_{EC} 1.39). In fact, it has been pointed out that the less acidic the phenol the easier its oxidation.[5] In addition, (–)-epigallocatechin gallate (an ester of GA with epigallocatechin) has an AA_{EC} value (2.44) about 10% lower than the GA, despite having eight HG. This shows that it is not only the number of HG that influences the reducing capacity of polyphenols, but also their acidity and the position of HG in the benzene ring.[6,23]

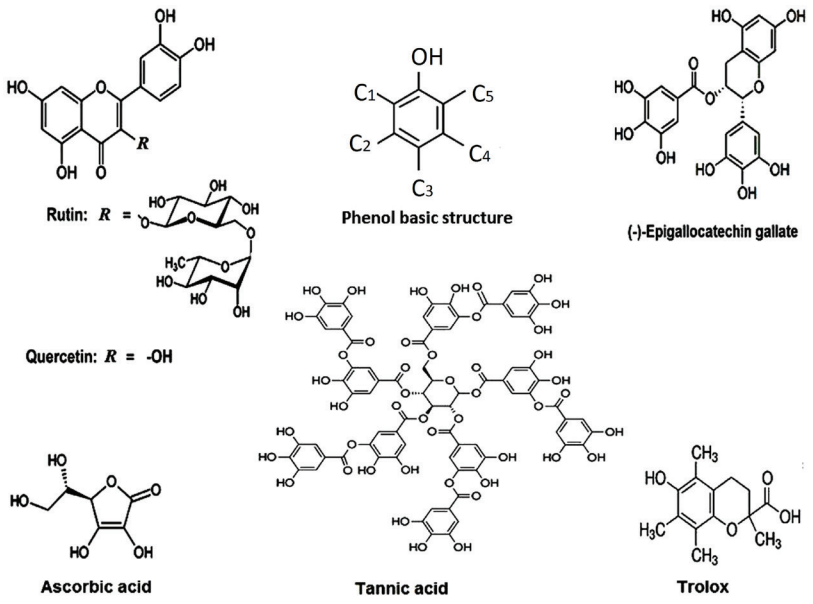
Among benzenotriols isomers the 1,2,4-benzenetriol (AA_{EC} 2.74) is a stronger reducing agent than PA (AA_{EC} 2.14). It is well known that the HGs in *ortho* position increase the reducing capacity, but the presence of an HG in the C2 position of PA makes it a weaker reducing agent than 1,2,4-benzenetriol (HG in C1 and C3 positions). In phloroglucinol the 3 HGs are proportionally distributed in the benzene, which strongly decreases the AA_{EC} value to 0.07. All these observations are in agreement with theoretical information.[24,25]

Regarding benzenediols, the AA_{EC} values follow the order: *o*-pyrocatechol (1.03) > hydroquinone (0.82) > resorcinol (0.01). It shows that reducing capacity is higher with second HG in *ortho* position and that the *para* position provides an electron donation more easily than *meta* position.[25] These results are in accordance with previous experimental findings[8,21] and also with theoretical information, which points out that oxidation of phenols to quinones seems to be easier if two HG are in *ortho* or *para* positions in the benzene ring.[24,25]

Phenol (pK_a = 9.8),[9] with only one HG, is the weakest reducing agent evaluated with the lowest AA_{EC} value (0.001), confirming that HG participates actively in the reduction reaction of Fe^{III} to Fe^{II} in solution containing bipy.

The introduction of radicals (other than –OH) in the aromatic ring modifies significantly the AA_{EC} value when compared to phenol. For instance, addition of –CH=CH–COOH group (which happens to be an electron-releasing radical) into C3 position originates *p*-coumaric acid (pK_{a1} = 4.64),[9] which has an AA_{EC} value (0.10) 100 times higher than phenol. Adding a donating group $–OCH_3$ in C1 position of *p*-coumaric acid forms ferulic acid (pK_{a1} = 3.60)[9] that presents an AA_{EC} value (0.66) about 600 times higher than phenol. Another $–OCH_3$ group added at C5 position of ferulic acid gives the sinapic acid (pK_{a1} = 4.58)[9] that presents an AA_{EC} value (0.99) about 1000 times higher than phenol. These three

Table 1. Parameters of the linear regression of the calibration graphs ($A_{521\ nm}$ = a + b [PC]) and reducing capacity values (AA_{EC}) of some polyphenolic compounds obtained with the proposed method



Polyphenolic compound (PC)	FM / (g mol^{-1})	C_1	C_2	C_3	C_4	C_5	HG	HGP	LR / (μmol L^{-1})	n	a	b / 10^3	r^2	AA_{EC}
Tannic acid	1701.23	–	–	–	–	–	25	–	1.0-8.0	7	–0.0980	145	0.996	7.07
Gallic acid	170.12	OH	H	COOH	H	OH	3	–	0.2-28	8	–0.0459	56.6	0.998	2.76
2,3,4-THB	170.12	OH	OH	COOH	H	H	3	2,3,4	4.0-28	7	–0.0551	28.4	0.998	1.39
Pyrogallic acid	126.11	OH	OH	H	H	OH	3	1,2,3	3.0-24	8	0.0386	43.8	0.996	2.14
1,2,4-Benzenetriol	126.11	OH	H	OH	H	H	3	1,2,4	4.0-16	7	–0.0253	56.1	0.999	2.74
Phloroglucinol	126.11	H	OH	H	OH	H	3	1,3,5	100-450	6	–0.0108	1.43	0.988	0.07
o-Pyrocatechol	110.11	OH	H	H	H	H	2	1,2	10-70	7	–0.0156	21.2	0.994	1.03
Resorcinol	110.11	H	OH	H	H	H	2	1,3	200-1400	6	–0.0249	0.186	0.980	0.01
Hydroquinone	110.11	H	H	OH	H	H	2	1,4	1.0-20	7	0.0071	16.9	0.990	0.82
Phenol	94.11	H	H	H	H	H	1	–	2000-50000	6	–0.0708	0.03	0.990	0.001
p-Coumaric acid	164.16	H	H	CH=CH–COOH	H	H	1	3	200-480	7	–0.1871	2.03	0.984	0.10
Ferulic acid	194.18	OCH_3	H	CH=CH–COOH	H	H	1	3	10-80	8	0.0314	13.6	0.996	0.66
Sinapic acid	224.21	OCH_3	H	CH=CH–COOH	H	OCH_3	1	3	4.0-32	8	0.0385	20.3	0.990	0.99
Caffeic acid	180.16	OH	H	CH=CH–COOH	H	H	2	3,5	20-48	8	–0.0366	21.9	0.992	1.07
Vanillin	152.15	OCH_3	H	CHO	H	H	1	3	200-1300	7	–0.0664	0.565	0.982	0.03
Vanillic acid	168.15	OCH_3	H	COOH	H	H	1	3	10-100	7	0.0455	5.14	0.992	0.25
Quercetin	302.24	–	–	–	–	–	5	–	1.0-20	7	–0.0785	66.4	0.988	3.24
Rutin	610.52	–	–	–	–	–	10	–	2.0-30	8	–0.0266	26.5	0.998	1.29
(–)-Epigallocatechin gallate	458.37	–	–	–	–	–	8	–	2.0-16	8	0.0250	50.1	0.998	2.44
Trolox[a]	250.29	–	–	–	–	–	–	–	2.0-30	8	0.0180	16.2	0.999	0.79
Ascorbic acid[a]	176.12	–	–	–	–	–	–	–	8.0-36	8	–0.0724	20.5	0.996	1.00

[a]Trolox and ascorbic acid were included in this table for comparison purposes. FM: formula mass; HG: hydroxyl group in benzene ring; HGP: position of the hydroxyl group on the benzene ring; LR: linear range; n: number of points of the calibration graphs; a, b and r^2: linear, angular and correlation coefficients of the calibration graphs, respectively; AA_{EC}: reducing capacity expressed as ascorbic acid equivalent; 2,3,4-THB: 2,3,4-trihydroxybenzoic acid.

monohydroxylated phenols have a –COOH group and the AA_{EC} values do not seem to be strictly connected to the acidity conditions of proposed method (pH 4.6). In fact, in sinapic acid the presence of the two $–OCH_3$ adjacent to the HG favors significantly its reduction capacity, which is in agreement with theory.[24,25]

Caffeic acid ($pK_{a1} = 3.0$)[9] is a dihydroxylated cinnamic acid derivative, has one more –OH group than *p*-coumaric acid, being both –OH in opposite position to the –CH=CH–COOH group. The presence of this second –OH group (in *ortho* position) increases ten times the reducing capacity value (AA_{EC} 1.07) with respect to *p*-coumaric acid. In this case, the number of HG contributes more strongly to the AA_{EC} value than the acidity of the phenol derivative.[8,23]

Vanillin (a phenolic aldehyde with an $–OCH_3$ in C1 position) is the main component of the vanilla seed extract. Vanillic acid ($pK_a = 4.45$)[9] is an oxidized form of vanillin. Although partially dissociated in these experimental conditions, vanillic acid has an AA_{EC} value (0.25) about 9-fold higher than vanillin (AA_{EC} 0.03), probably due to the proton dissociation.

The number of –OH group also plays an important role in the AA_{EC} values of flavonoids, another class of antioxidant compounds that exhibit great reducing capacity.[26,27] Two flavonoids with the same framework (quercetin and rutin) were analyzed using the proposed method. Quercetin has an AA_{EC} value (3.24) 2.5 times greater than rutin (AA_{EC} 1.29), which can be attributed to the replacement of an –OH group in quercetin by a disaccharide group in rutin, in agreement with the theory.[6]

Even though the reaction of a polyphenol standard solution may not reproduce the analytical response of complex matrices (like extracts of medicinal herbs or teas), the results present in Table 1 are useful for assessment of the reactivity of single polyphenol. As expected, these data revealed that type, number, and position of a given chemical radical (mainly –OH groups) attached to the benzene ring change the reducing capacity values obtained with the proposed method.

Eventually, as the linear range of most of the phenolic acid derivatives analyzed (Table 1) is between $(1\text{-}500) \times 10^{-6}$ mol L^{-1} (with exception of resorcinol, phenol and vanillin), the procedure presented here can be used in more diluted samples. The results might be expressed in another standard compound instead of AA. In this context, a polyphenol with a high AA_{EC} value, but with a more affordable cost (e.g., GA or quercetin), could be used.

The reducing capacity of teas samples

Table 2 shows the AA_{EC} results for twelve teas. The TRC values obtained with the proposed method ($Fe(bipy)_3^{2+}$ complex) and CUPRAC reagent had an excellent agreement (adjusted $r^2 = 0.951$). This shows that despite the different values of the conditional reduction potential of the Fe^{III}/Fe^{II} couple in solution containing bipy (1.08 V *vs.* normal hydrogen electrode (NHE))[11,12] and Cu^{II}/Cu^{I} in neocuproine medium (0.635 V *vs.* NHE),[18,19,28] both seem to oxidize (at least proportionally) the compounds present in the tea samples.

Table 2. Reducing capacity values and polyphenolic content of some teas

Tea	Part used[a]	Total polyphenol	Reducing capacity	
		Folin-Ciocalteu / (mg gallic acid g^{-1} dry material)	$Cu(NC)_2^{+}$ / 10^{-2} (g dry material mg^{-1} ascorbic acid)	$Fe(bipy)_3^{2+}$ / (g dry material g^{-1} ascorbic acid)
Peumus boldus Molina	leaves	63.5 ± 1.8	3.5 ± 0.4	2.37 ± 0.04
Matricaria recutita L. (sample 1)	receptacle scale	16.9 ± 1.2	0.5 ± 0.1	0.026 ± 0.001
Matricaria recutita L. (sample 2)	receptacle scale	9.49 ± 0.8	0.5 ± 0.2	0.035 ± 0.002
Matricaria recutita L. (sample 3)	receptacle scale	14.1 ± 0.6	0.5 ± 0.1	0.017 ± 0.001
Baccharis genistelloides (Lam.) Pers.	leaves	9.47 ± 0.7	0.8 ± 0.1	0.040 ± 0.001
Camellia sinensis (L.) Kuntze	leaves	92.7 ± 1.9	6.9 ± 1.2	6.36 ± 0.42
Ilex paraguariensis A. St.-Hil.	aerial parts	60.3 ± 2.9	5.5 ± 0.3	3.39 ± 0.21
Camellia sinensis (L.) Kuntze (sample 1)	aerial parts	58.6 ± 1.6	4.5 ± 0.4	3.38 ± 0.15
Camellia sinensis (L.) Kuntze (sample 2)	aerial parts	83.9 ± 3.1	5.5 ± 0.5	4.74 ± 0.17
Cymbopogon citratus (DC.) Stapf	leaves and receptacle scale	8.33 ± 0.1	0.8 ± 0.2	0.027 ± 0.002
Erythroxylum coca Lam.	leaves	3.49 ± 0.2	1.9 ± 0.1	1.67 ± 0.140
Mentha piperita L.	leaves and branches	61.9 ± 1.4	2.3 ± 0.3	2.00 ± 0.01

[a]As informed by suppliers. NC: neocuproine; bipy: 2,2'-bipyridine.

Since it was observed a good correlation between the TPC quantified with the Folin-Ciocalteu reagent and the TRC values obtained with CUPRAC reagent (adjusted $r^2 = 0.811$) and with the proposed method (adjusted $r^2 = 0.816$), it can be assumed that in these samples the agents responsible for the reducing capacity are polyphenols.

The reducing capacity of aqueous extracts of medicinal herbs

TRC values obtained with both assays (proposed and $ABTS^{\bullet+}$ methods) showed a very good agreement (adjusted $r^2 = 0.975$), indicating that both methods can be used to quantify the reducing capacity of herbs. The proposed method has the advantage of being conducted in aqueous solution, unlike $ABTS^{\bullet+}$ method, which uses organic solvents such as acetone and methanol. These two procedures do not present much difference in the completion time of the reaction, although the $ABTS^{\bullet+}$ solution requires at least 16 h of previous preparation.[14,29] Regarding the price of reagents (ABTS is currently about 14 times more expensive than bipy) the proposed method is more attractive from an economic point of view. Besides, the suggested method is conducted in aqueous medium and the ligand used (bipy) can be recycled, making it environmentally attractive.[30]

In addition, good correlations between the TPC quantified with the Folin-Ciocalteu reagent and the reducing capacity values obtained with the $ABTS^{\bullet+}$ method (adjusted $r^2 = 0.792$) and the proposed method (adjusted $r^2 = 0.835$) were found, showing that the polyphenols present in these herbs should be responsible for this reducing capacity (Table 3).

Additionally, the results of the TRC obtained with the $Fe(bipy)_3^{2+}$ complex for teas and herbs suggested that the proposed method can also be used to quantify the reducing capacity of other samples derived from plants that are rich in polyphenolic compounds (e.g., fruits and fruit juices).

As other methods based on the reduction of metal ion M^{n+} to $M^{(n-1)+}$ developed to quantify the TRC (in a solution containing a complexing agent for $M^{(n-1)+}$), the assay suggested here does not require a lag phase type of measurement. In this context, the values of TRC can also be used to express the total antioxidant capacity.

Table 3. Reducing capacity values and polyphenolic content of aqueous extracts of some Brazilian medicinal herbs

Plant	Brazilian typical name	Intake preparation	Use in folk medicine	Total polyphenol	Reducing capacity	
				Folin-Ciocalteu / (g pyrogallic acid 100 g^{-1} dry material)	ABTS / (μM Trolox g^{-1} dry material)	$Fe(bipy)_3^{2+}$ / (g dry material g^{-1} ascorbic acid)
Bauhinia splendens Kunth (bark)	"escada de jabuti"	infusion	syphilis; rheumatism; hemorrhoids	1.36 ± 0.09	25585 ± 699	1.85 ± 0.13
Brosimum gaudichaudii Trécul (bark)	"mamica de cadela"	infusion	bronchitis; blood circulation	1.72 ± 0.03	11009 ± 540	0.05 ± 0.01
Carapa guianensis Aubl. (bark)	andiroba	infusion	bacterial infection; psoriasis	2.44 ± 0.05	42687 ± 2389	3.88 ± 0.26
Cordia ecalyculata Vell. (leaves)	"porangaba"	infusion or decoction	diuretic; fatigue; edema	1.86 ± 0.05	12022 ± 182	0.09 ± 0.01
Dipteryx odorata (Aubl.) Willd. (seeds)	cumaru	infusion	antispasmodic; ulcer; cardiotonic	1.55 ± 0.10	16895 ± 2176	0.12 ± 0.01
Geissospermum laeve (Vell.) Mier (bark)	"pau pereira"	decoction	inappetence; indigestion	1.09 ± 0.02	10971 ± 558	0.08 ± 0.01
Hymenaea courbaril L. (bark)	"casca de jatobá"	decoction	bronchitis; rhinitis; diuretic	1.21 ± 0.07	24373 ± 1854	0.10 ± 0.01
Plantago major L. (leaves)	"tanchagem"	infusion or decoction	skin diseases; diarrhea; gastritis	2.02 ± 0.10	15401 ± 518	0.12 ± 0.01
Vismia japurensis Reichardt (leaves)	"lacre"	infusion or decoction	rheumatism; dermatosis	0.40 ± 0.06	12604 ± 554	0.04 ± 0.01
Inga alba (Sw.) Willd. (leaves)	"ingá vermelha"	infusion	rheumatism; diarrhea; headache	8.03 ± 0.42	95594 ± 1627	10.3 ± 0.51
Piranhea trifoliata Baill. (leaves)	"piranheira do Xingu"	infusion	uterine inflammation	4.05 ± 0.56	83654 ± 2643	7.69 ± 0.40
Minquartia guianensis Aubl. (leaves)	"acariquara"	decoction	viruses and inflammations	1.49 ± 0.18	17380 ± 1097	0.13 ± 0.01

ABTS: 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid diammonium salt; bipy: 2,2'-bipyridine.

Conclusions

The method suggested here for quantifying the total reduction capacity of teas and herbs is simple, fast, reliable and easy to perform. The good results obtained allow us to infer that the proposed procedure can also be used to quantify the reducing capacity of other samples of plant origin (e.g., fruit juices, beers and wines). This study also revealed that the reducing capacity of polyphenolic compounds with the proposed method depends on their chemical structure (mainly the presence and position of hydroxyl groups).

Both the equipment (spectrophotometer) and the reagents (iron(III) sulfate, 2,2'-bipyridine and acetate buffer) used in the proposed method are not expensive, so they can be adopted by laboratories performing routine analyses. Furthermore, as this method is conducted in aqueous medium and the ligand (2,2'-bipyridine) can be recycled, it becomes environmentally attractive.

Acknowledgments

The authors acknowledge the financial support from Brazilian agency CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for the scholarship granted to R. L. S. and by funding projects and research grants for C. V. N.

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Submitted: September 5, 2018
Published online: February 12, 2019