A Procedure for Assessment of the Reducing Capacity of Plants-Derived Beverages Based on the Formation of the FeII/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 FeIII to FeII by antioxidant compounds containing 2,2’-bipyridine (bipy) in aqueous solution. Absorbance values recorded at 521 nm, characteristic of the Fe(bipy)32+ 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 (r2) = 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•+) method (adjusted r2 = 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, FeIII

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 FeIII to FeII by antioxidant compounds containing 2,2’-bipyridine (bipy) in aqueous solution. Absorbance values recorded at 521 nm, characteristic of the Fe(bipy)32+ 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 (r2) = 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•+) method (adjusted r2 = 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.

Phenanthroline and batho-phenanthroline, both chelating agents that form stable and colored complexes with FeII 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. 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 FeIII 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. Recently, a comprehensive study based on the reduction of FeIII 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.
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). This aqueous orange-red complex shows a maximum absorption at 521 nm ($\varepsilon_{521\text{ nm}} = 7.5 \times 10^3$ L cm$^{-1}$ mol$^{-1}$) 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. 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.

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). 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). 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 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. 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).

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.

**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. A 10% (m/v) sodium carbonate (Na$_2$CO$_3$, formula mass (FM) 105.99 g mol$^{-1}$, 99%; Vetec, Brazil) solution was prepared in water.

A 0.188 mg mL$^{-1}$ gallic acid (GA, C$_7$H$_6$O$_5$·H$_2$O, 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**

A 0.0188 mg mL$^{-1}$ gallic acid (GA, C$_7$H$_6$O$_5$·H$_2$O, 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 0.490 mg mL$^{-1}$ iron(III) sulfate (Fe$_2$(SO$_4$)$_3$·5H$_2$O, FM 489.95 g mol$^{-1}$, 97%; Fluka, Brazil) solution was prepared by dissolving 0.490 g in 1.00 mL of water.

Acetate buffer solution (pH 4.6) was prepared by dissolving 14.3 mL of glacial acetic acid (HAc, CH$_3$COOH, FM 60.05 g mol$^{-1}$, 99.8%; Merck, Brazil) and 20 g of potassium acetate (KAc, CH$_3$COOK, FM 98.15 g mol$^{-1}$, 99%; Merck, Brazil) in water in a 1.0 L volumetric flask.

**Figure 1.** Structural formula of 2,2’-bipyridine.
A 2.58 mg mL⁻¹ 2,2'-bipyridine (bipy, C₁₀H₈N₂, FM 156.19 g mol⁻¹, 99%; Fluka, Brazil) solution was prepared by dissolving 0.644 g in 10.0 mL ethanol (CH₃CH₂OH, 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, C₆H₈O₆, 99.7%, FM 176.13 g mol⁻¹; Merck, Germany) solution was freshly prepared by dissolving 0.176 g in 100.0 mL volumetric flask containing water. A 0.0352 mg mL⁻¹ solution was obtained by accurate dilution.

Tannic acid (C₇₆H₅₂O₄₆, FM 1701.20 g mol⁻¹, 99%; J. T. Baker, USA); GA (C₁₀H₂₀O₂₆, FM 188.13 g mol⁻¹, 99%; Synth, Brazil); 2,3,4-trihydroxybenzoic acid (2,3,4-THB, C₇H₆O₅, FM 170.12 g mol⁻¹, 97%; Sigma-Aldrich, USA), PA, phloroglucinol and 1,2,4-benzenetriol (C₆H₆O₃, FM 126.11 g mol⁻¹, 99%; Sigma-Aldrich, USA); p-hydroquinone, resorcinol and o-pyrocatechol (C₆H₆O₂, FM 110.11 g mol⁻¹, 99%; Synth, Brazil); caffeic acid (C₉H₆O₄, FM 180.16 g mol⁻¹, 98%; Sigma-Aldrich, USA); p-coumaric acid (C₈H₇O₃, FM 164.16 g mol⁻¹; ≥98%; Sigma-Aldrich, USA); ferulic acid (C₁₀H₁₀O₄, FM 194.18 g mol⁻¹, 99%; Sigma-Aldrich, USA); sinapic acid (C₁₁H₁₀O₅, FM 224.21 g mol⁻¹, 98%; Sigma-Aldrich, USA); vanillic acid (C₇H₆O₃, FM 168.15 g mol⁻¹, >97%; Merck, Germany); vanillin (C₇H₆O₃, FM 152.15 g mol⁻¹, 99%; Sigma-Aldrich, USA); quercetin (C₁₅H₁₀O₇, FM 302.24 g mol⁻¹, 98%; Sigma-Aldrich, USA); rutin (C₂₀H₁₆O₁₁, FM 610.52 g mol⁻¹, 95%; Sigma-Aldrich, USA); (–)-epigallocatechin gallate (C₂₂H₁₈O₁₁, FM 458.37 g mol⁻¹, 80%; Sigma-Aldrich, USA); phenol (C₆H₅O, FM 94.11 g mol⁻¹, 99%; Synth, Brazil) and Trolox (C₁₄H₁₈O₄, FM 250.29 g mol⁻¹, >97%; Sigma-Aldrich, USA) solutions of 1.0 × 10⁻⁴ or 1.0 × 10⁻⁵ mol L⁻¹ (except 0.1 mol L⁻¹ phenol) were prepared by dissolving in water. Dilute solutions (1.0 × 10⁻² to 5.0 × 10⁻³ 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 (K₂S₂O₈, 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(ClO₄)₂, FM 262.45 g mol⁻¹) solution was prepared by reaction of copper(II) basic carbonate (CuCO₃,Cu(OH)₂, FM 221.12 g mol⁻¹, > 95%; Sigma, Brazil) with a 5% excess of perchloric acid (HClO₄, FM 100.46 g mol⁻¹, 70%; Merck, Brazil) and standardized by complexometric titration with ethylenediaminetetraacetic acid (EDTA) as described elsewhere. A 2.44 mg mL⁻¹ diluted solution used was prepared by dilution in water.

A 1.54 × 10⁻⁴ mg L⁻¹ ammonium acetate (C₂H₃O₂NH₄, 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, C₁₄H₁₂N₂.HCl.H₂O, 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. 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 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⁺ method.

TPC determination with the FCR

The multiple standard addition method used for the TPC quantification was already described. 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.\(^\text{19}\) 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.\(^\text{15}\)

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\(_3\)(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) \(\times\) 10\(^{-3}\) mg mL\(^{-1}\). Absorbance measurements at 521 nm (A\(_{521}\)) were recorded using a mixture containing 0.490 mg mL\(^{-1}\) Fe\(_3\)(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\(_{521}\) vs. C\(_{AA}\), where C\(_{AA}\) is in mg mL\(^{-1}\)) obtained is described by the equation A\(_{521}\) = 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\(_{521}\) 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\(^2\)) \(\geq\) 0.99) described by the equation A\(_{521}\) = 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.\(^\text{7,8,21}\)

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\(_3\)(SO\(_4\))\(_3\) solution, 100 to 1000 \(\mu\)L (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\(_{521}\) were recorded using the same blank reagent above described. A calibration graph (A\(_{521}\) vs. C\(_{DM}\), where C\(_{DM}\) is the dry material (DM) concentration in mg mL\(^{-1}\)) obtained is described by the equation A\(_{521}\) = 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 \(\times\) 10\(^{-3}\) mol L\(^{-1}\) of PC solution under the same experimental conditions.\(^\text{7,8,21}\)

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

The equation A\(_{521}\) = a + b C\(_{AA}\) is applied to calculate the A\(_{521}\) value corresponding to a 1.0 mg mL\(^{-1}\) AA standard solution. This A\(_{521}\) value is replaced in the equation A\(_{521}\) = 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:

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

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.\(^\text{13,19}\)

Determination of the TRC of herbal extracts using the ABTS\(^{•+}\)

The preparation of ABTS\(^{•+}\) solution and the procedure used here were carried out as previously described.\(^\text{14}\) The antioxidant capacity values were expressed in \(\mu\)M 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),\(^\text{9-11}\) and ferric hydroxo complexes (e.g., FeOH\(^{2+}\) and Fe(OH)\(_2\)^+) may be present in unbuffered solutions in pH > 3.5.\(^\text{22}\) 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.\(^\text{12}\)

The above considerations support the experimental
conditions adopted in this proposed method (0.490 mg mL⁻¹ 
Fe₃(SO₄)₂, 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 (A₅₂₁ nm vs. Cₐₐ) 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](image-url) Absorption spectra of (a) 0.490 mg mL⁻¹ Fe₃(SO₄)₂, 
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 A₅₂₁ nm of the Fe(bipy)₃²⁺ complex 
(b = 1.0 cm).

Trolox (a water-soluble compound analogous to 
vitamin E) also reduces Fe³⁺ to Fe²⁺ 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 (AAEC 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 AAEC obtained for the antioxidant 
compounds investigated.

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

GA (pKₐ = 4.4) and its isomer 2,3,4-THB (pKₐ = 3.0) 
have the –COOH group partially deprotonated under 
these experimental conditions (pH 4.6). 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 
(AAEC 2.76) has a reducing capacity value 2 times higher 
than 2,3,4-THB (AAEC 1.39). In fact, it has been pointed 
out that the less acidic the phenol the easier its oxidation. 
In addition, (–)-epigallocatechin gallate (an ester of GA 
with epigallocatechin) has an AAEC 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.

Among benzenetriols isomers the 1,2,4-benzenetriol 
(AAEC 2.74) is a stronger reducing agent than PA 
(AAEC 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 AAEC value 
to 0.07. All these observations are in agreement with 
theoretical information.

Regarding benzenediols, the AAEC 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. These results are in accordance with 
previous experimental findings 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.

Phenol (pKₐ = 9.8), with only one HG, is the weakest 
reducing agent evaluated with the lowest AAEC value 
(0.001), confirming that HG participates actively in the 
reduction reaction of Fe³⁺ to Fe²⁺ in solution containing bipy.

The introduction of radicals (other than –OH) in 
the aromatic ring modifies significantly the AAEC 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 (pKₐ = 4.64), which has an AAEC value 
(0.10) 100 times higher than phenol. Adding a donating 
group –OCH₃ in C1 position of p-coumaric acid forms 
ferulic acid (pKₐ = 3.60) that presents an AAEC value 
(0.66) about 600 times higher than phenol. Another 
–OCH₃ group added at C5 position of ferulic acid gives 
the sinapic acid (pKₐ = 4.58) that presents an AAEC value 
(0.99) about 1000 times higher than phenol. These three
Table 1. Parameters of the linear regression of the calibration graphs ($A_{521\text{ nm}} = a + b \text{ [PC]}$) and reducing capacity values (AAEC) of some polyphenolic compounds obtained with the proposed method.

<table>
<thead>
<tr>
<th>Polyphenolic compound (PC)</th>
<th>FM / (g mol$^{-1}$)</th>
<th>C$_1$</th>
<th>C$_2$</th>
<th>C$_3$</th>
<th>C$_4$</th>
<th>C$_5$</th>
<th>HG</th>
<th>HGP</th>
<th>LR / (µmol L$^{-1}$)</th>
<th>n</th>
<th>a</th>
<th>b / 10$^3$</th>
<th>$r^2$</th>
<th>AAEC</th>
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<tbody>
<tr>
<td>Tannic acid</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>25</td>
<td>–</td>
<td>1.0-8.0</td>
<td>7</td>
<td>–0.0980</td>
<td>145</td>
<td>0.996</td>
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<td>H</td>
<td>COOH</td>
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<td>OH</td>
<td>3</td>
<td>2,3,4</td>
<td>0.2-28</td>
<td>8</td>
<td>–0.0459</td>
<td>56.6</td>
<td>0.998</td>
<td>2.76</td>
</tr>
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<td>OH</td>
<td>COOH</td>
<td>H</td>
<td>H</td>
<td>3</td>
<td>2,3,4</td>
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<td>7</td>
<td>–0.0551</td>
<td>28.4</td>
<td>0.998</td>
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<td>3</td>
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<td>OH</td>
<td>H</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>3</td>
<td>1,2,4</td>
<td>4.0-16</td>
<td>7</td>
<td>–0.0253</td>
<td>56.1</td>
<td>0.999</td>
<td>2.74</td>
</tr>
<tr>
<td>Chlorogallicin</td>
<td>126.11</td>
<td>H</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
<td>H</td>
<td>3</td>
<td>1,3,5</td>
<td>100-450</td>
<td>6</td>
<td>–0.0108</td>
<td>1.43</td>
<td>0.988</td>
<td>0.07</td>
</tr>
<tr>
<td>o-Pyrocatechol</td>
<td>110.11</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>2</td>
<td>1,2</td>
<td>10-70</td>
<td>7</td>
<td>–0.0156</td>
<td>21.2</td>
<td>0.994</td>
<td>1.03</td>
</tr>
<tr>
<td>Resorcinol</td>
<td>110.11</td>
<td>H</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>2</td>
<td>1,3</td>
<td>200-1400</td>
<td>6</td>
<td>–0.0249</td>
<td>0.186</td>
<td>0.980</td>
<td>0.01</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>110.11</td>
<td>H</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>2</td>
<td>1,4</td>
<td>1.0-20</td>
<td>7</td>
<td>0.0071</td>
<td>16.9</td>
<td>0.990</td>
<td>0.82</td>
</tr>
<tr>
<td>Phenol</td>
<td>94.11</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>1</td>
<td>–</td>
<td>2000-50000</td>
<td>6</td>
<td>–0.0708</td>
<td>0.03</td>
<td>0.990</td>
<td>0.001</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>164.16</td>
<td>H</td>
<td>H</td>
<td>CH=CH-COOH</td>
<td>H</td>
<td>H</td>
<td>1</td>
<td>3</td>
<td>200-480</td>
<td>7</td>
<td>–0.1871</td>
<td>2.03</td>
<td>0.984</td>
<td>0.10</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>194.18</td>
<td>OCH$_3$</td>
<td>H</td>
<td>CH=CH-COOH</td>
<td>H</td>
<td>H</td>
<td>1</td>
<td>3</td>
<td>10-80</td>
<td>8</td>
<td>0.0314</td>
<td>13.6</td>
<td>0.996</td>
<td>0.66</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>224.21</td>
<td>OCH$_3$</td>
<td>H</td>
<td>CH=CH-COOH</td>
<td>H</td>
<td>OCH$_3$</td>
<td>1</td>
<td>3</td>
<td>4.0-32</td>
<td>8</td>
<td>0.0385</td>
<td>20.3</td>
<td>0.999</td>
<td>0.99</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>180.16</td>
<td>OH</td>
<td>H</td>
<td>CH=CH-COOH</td>
<td>H</td>
<td>H</td>
<td>2</td>
<td>3,5</td>
<td>20-48</td>
<td>8</td>
<td>–0.0366</td>
<td>21.9</td>
<td>0.992</td>
<td>1.07</td>
</tr>
<tr>
<td>Vanillin</td>
<td>152.15</td>
<td>OCH$_3$</td>
<td>H</td>
<td>CHO</td>
<td>H</td>
<td>H</td>
<td>1</td>
<td>3</td>
<td>200-1300</td>
<td>7</td>
<td>–0.0664</td>
<td>0.565</td>
<td>0.982</td>
<td>0.03</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>168.15</td>
<td>OCH$_3$</td>
<td>H</td>
<td>COOH</td>
<td>H</td>
<td>H</td>
<td>1</td>
<td>3</td>
<td>10-100</td>
<td>7</td>
<td>0.0455</td>
<td>5.14</td>
<td>0.992</td>
<td>0.25</td>
</tr>
<tr>
<td>Quercetin</td>
<td>302.24</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>5</td>
<td>–</td>
<td>1.0-20</td>
<td>7</td>
<td>–0.0785</td>
<td>66.4</td>
<td>0.988</td>
<td>3.24</td>
</tr>
<tr>
<td>Rutin</td>
<td>610.52</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>10</td>
<td>–</td>
<td>2.0-30</td>
<td>8</td>
<td>–0.0266</td>
<td>26.5</td>
<td>0.998</td>
<td>1.29</td>
</tr>
<tr>
<td>(−)-Epigallocatechin gallate</td>
<td>458.37</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>8</td>
<td>–</td>
<td>2.0-16</td>
<td>8</td>
<td>0.0250</td>
<td>50.1</td>
<td>0.998</td>
<td>2.44</td>
</tr>
<tr>
<td>Trolox$^a$</td>
<td>250.29</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>2.0-30</td>
<td>8</td>
<td>0.0180</td>
<td>16.2</td>
<td>0.999</td>
<td>0.79</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>176.12</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>8.0-36</td>
<td>8</td>
<td>–0.0724</td>
<td>20.5</td>
<td>0.996</td>
<td>1.00</td>
</tr>
</tbody>
</table>

$^a$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; AAEC: reducing capacity expressed as ascorbic acid equivalent; 2,3,4-THB: 2,3,4-trihydroxybenzoic acid.
monohydroxylated phenols have a –COOH group and the AAEC 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 –OCH3 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 (AAEC 1.07) with respect to p-coumaric acid. In this case, the number of HG contributes more strongly to the AAEC value than the acidity of the phenol derivative.8,23

Vanillin (a phenolic aldehyde with an –OCH3 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 AAEC value (0.25) about 9-fold higher than vanillin (AAEC 0.03), probably due to the proton dissociation.

The number of –OH group also plays an important role in the AAEC 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 AAEC value (3.24) 2.5 times greater than rutin (AAEC 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 (1-500) x 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 AAEC 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 AAEC results for twelve teas. The TRC values obtained with the proposed method (Fe(bipy)32+ 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 FeIII/FeII couple in solution containing bipy (1.08 V vs. normal hydrogen electrode (NHE))11,12 and CuII/CuI 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>
<thead>
<tr>
<th>Tea</th>
<th>Part used</th>
<th>Total polyphenol</th>
<th>Reducing capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Folin-Ciocalteu/</td>
<td>Cu(NC)2+/10^{-2}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(mg gallic acid g^{-1}</td>
<td>(g dry material mg^{-1}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dry material)</td>
<td>ascorbic acid)</td>
</tr>
<tr>
<td>Peumus boldus Molina</td>
<td>leaves</td>
<td>63.5 ± 1.8</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>Matricaria recutita L. (sample 1)</td>
<td>receptacle scale</td>
<td>16.9 ± 1.2</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Matricaria recutita L. (sample 2)</td>
<td>receptacle scale</td>
<td>9.49 ± 0.8</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Matricaria recutita L. (sample 3)</td>
<td>receptacle scale</td>
<td>14.1 ± 0.6</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Baccharis genistelloides (Lam.) Pers.</td>
<td>leaves</td>
<td>9.47 ± 0.7</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Camellia sinensis (L.) Kuntze</td>
<td>leaves</td>
<td>92.7 ± 1.9</td>
<td>6.9 ± 1.2</td>
</tr>
<tr>
<td>Ilex paraguariensis A. St.-Hil.</td>
<td>aerial parts</td>
<td>60.3 ± 2.9</td>
<td>5.5 ± 0.3</td>
</tr>
<tr>
<td>Camellia sinensis (L.) Kuntze (sample 1)</td>
<td>aerial parts</td>
<td>58.6 ± 1.6</td>
<td>4.5 ± 0.4</td>
</tr>
<tr>
<td>Camellia sinensis (L.) Kuntze (sample 2)</td>
<td>aerial parts</td>
<td>83.9 ± 3.1</td>
<td>5.5 ± 0.5</td>
</tr>
<tr>
<td>Cymbopogon citratus (DC.) Stapf</td>
<td>leaves and receptacle scale</td>
<td>8.53 ± 0.1</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>Erythroxylum coca Lam.</td>
<td>leaves</td>
<td>3.49 ± 0.2</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>Mentha piperita L.</td>
<td>leaves and branches</td>
<td>61.9 ± 1.4</td>
<td>2.3 ± 0.3</td>
</tr>
</tbody>
</table>

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$^+$ 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$^+$ 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$^+$ 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$^+$ 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

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

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