



Analytical Methods

High-throughput assay comparison and standardization for metal chelating capacity screening: A proposal and application

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ABSTRACT

Aiming to standardize the experimental protocols to assess the ability to chelate Fe²⁺ and Cu²⁺ using 96-well microplates, we analyzed Brazilian coffees (n = 20) as a study-case in relation to their antioxidant activity using conventional methods (DPPH and FRAP assays) and correlated the results with the total phenolic content (TPC) using bivariate and multivariate statistical approaches. Complementarily, we assessed the repeatability, reproducibility, recovery, and linearity of both methods. Data showed that the proposed assays presented a good repeatability and reproducibility (<7% RSD) and mean recovery values of 96.66% and 98.91% for the iron and copper assays, respectively. Both methods were linear in the range of 0–100 mg EDTA equivalents/L. Cu²⁺-chelating ability was significantly correlated to FRAP, DPPH, and TPC, while sparse (p < 0.05) correlations were obtained with Fe²⁺-chelating ability. Overall, both micro assays can be used to assess the ability of plant-based extracts to chelate Fe²⁺ and Cu²⁺ *in vitro*.

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1. Introduction

According to Halliwell and Gutteridge (2007), an antioxidant is a substance that, when present at a low concentration compared with that of an oxidizable substrate in the medium, inhibits oxidation of the substrate. Phenolic compounds, by nature, are able to protect cells from oxidizing agents and, therefore, can be considered antioxidants. In this regard, an antioxidant compound may be classified according to their function, i.e., free-radical scavenger (such as vitamin E), compounds that induce the *in vivo* production of antioxidants (i.e., isothiocyanates), scavengers of oxidants that are not free radicals (i.e., catalase), and chemical compounds that hinders and inhibits the generation of free radicals via redox reactions (i.e., phenolic compounds that chelate transition metal ions). Antioxidants may also be classified according to the source: *endogenous* (i.e., glutathione reductase, uric acid, L-carnitine and glutathione peroxidase) or *exogenous* (quercetin, isoflavones, and stilbenes). The chemical polarity is also one factor that classifies antioxidants in water-soluble (i.e., anthocyanins and some phenolic acids) or lipophilic antioxidants (i.e., vitamin E and carotenoids). In addition to these classifications, the mechanism of action of antioxidants is another classification criterion: antioxidants may be clustered in three different mechanisms, that is electron transfer

(ET), hydrogen atom transfer (HAT), and chelation of transition metals, such as Fe²⁺, Cu²⁺, and Zn²⁺ (Prior, Wu, & Schaich, 2005). Following this criterion, antioxidants (AH/PheOH) that are able to quench free radicals by hydrogen donation (ROO• + AH/PheOH → ROOH + Phe-O•; ROO• + A• → ROOA) are considered HAT antioxidants. Oxygen radical absorbance capacity (ORAC) and chemiluminescence using luminol as probe are the main assays used to assess the HAT of extracts and chemical compounds. In the same sense, if the antioxidant is able to transfer one electron to reduce free radicals (ROO• + AH/PheOH → ROO⁻ + AH⁺/PheOH⁺ → A⁺/Phe-O• + H₃O⁺; ROO⁻ + H₃O⁺ → ROOH + H₂O) pro-oxidant metals [Fe(III) + AH/PheOH → Fe(II) + AH⁺/PheOH⁺] and carbonyls, they are considered ET antioxidants (Shahidi & Zhong, 2015). Among the assays, the scavenging of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) and ferric reducing antioxidant power (FRAP) as well as the cupric-ion reducing antioxidant capacity (CUPRAC) are the most frequently used in routine screening studies (Apak, Güçlü, Özyürek, & Celik, 2008; Apak et al., 2007, 2013). In this aspect, the ability to chelate transition metals is considered a secondary mechanism to estimate the antioxidant activity of an extract/compound and recent studies have repeatedly demonstrated that some transition metals, especially Fe²⁺ and Cu²⁺, are involved in the offset of some non-communicable degenerative diseases, such as Alzheimer's, Parkinson's and Wilson's as well as cardiovascular diseases and oxidative stress (Bandmann, Weiss, & Kaler, 2015; Dusek et al., 2015; Loeff & Walach, 2012; Salonen et al., 1992).

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The metal chelation of iron and copper ions is important approach as these metals are involved in the formation of reactive oxygen species (ROS) *in vivo* via Fenton and Haber-Weiss reactions ($\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^\bullet$), such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^\bullet), which are very strong oxidant agents (Kehrer, 2000). Herein, the *in vitro* and *in vivo* assessment of a product's ability to chelate these transition metal ions are important means to understand the role of food extracts on the chelating properties of transition metals involved in oxidative damage *in vivo* (Bandmann et al., 2015).

UV/VIS spectrophotometric assays are inexpensive, widely used and require low-cost equipment for routine analysis, presenting a suitable precision (Granato, Santos, Maciel, & Nunes, 2016). The UV/VIS spectrophotometric assays used to assess the ability to chelate Fe^{2+} and Cu^{2+} are based on the methods proposed by Carter (1971) and Saiga, Tanabe, and Nishimura (2003), respectively. These methods use ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid monosodium salt hydrate) and pyrocatechol violet (3,3',4-trihydroxyfuchson-2''-sulfonic acid), respectively, as chromophores and have been used to assess the antioxidant activity of a wide variety of food extracts, beverages and phytochemicals (Carrasco-Castilla et al., 2012; Félix-Silva et al., 2014; Granato, Grevink, Zielinski, Nunes, & van Ruth, 2014; Granato, Margraf, Brotzakakis, Capuano, & Ruth, 2015). Although the use of those methods has increased, there is no standardization on the experimental protocols (*i.e.*, concentration of reagents, pH of the reactional medium, reaction time) and how the results should be expressed: as a metallic complex is formed between Fe^{2+} -ferrozine or Cu^{2+} -pyrocatechol violet, results can be expressed as % of inhibition of complex formation, % of formed complex, or as the IC_{50} concentration, that is, the concentration that chelates 50% of the free metal ion. Alternatively, an analytical curve with a known and stable metal chelator can be plotted and the results compared with this chemical standard using linear regression analysis. In addition, the procedures can be carried out using test-tubes (>2.5 mL) (Sánchez-Vioque et al., 2013) or by using 96-well microplates (Canabady-Rochelle et al., 2015; Jia et al., 2013), which makes a standardization of the experimental protocol and the understanding of the chemical reactions in the system highly necessary (Granato, Santos et al., 2016).

High-throughput chemical methods that can be used for first-level screening of antioxidant activity of food extracts are a suitable experimental approach as they are low-cost, robust and sensitive (Herald, Gadgil, & Tilley, 2012). Based on this fact and trying not to generate a large amount of chemical reagents, this work was aimed to standardize the experimental protocols to assess the metal chelating ability (Fe^{2+} and Cu^{2+}) of food extracts using clear, flat-bottom 96-well polystyrene microplates (300 μL /well). For this purpose, we also analyzed Brazilian commercial coffee samples (*Coffea arabica*) as a study-case in relation to their antioxidant activity using conventional methods (DPPH and FRAP assays) and correlated the results with the total phenolic content using bivariate and multivariate statistical approaches. Complementarily, we assessed the repeatability, reproducibility, recovery, and linearity of the metal chelating methods.

2. Material and methods

2.1. Chemical reagents

Gallic acid, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), ethyl alcohol, cupric sulphate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), ferrozine (3-(2-pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5',5''-disulfonic acid disodium salt), pyrocatechol violet (3,3',4-trihydroxyfuchson-2''-sulfonic acid), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), ferric chloride

hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), and ascorbic acid were obtained from Sigma-Aldrich (St Louis, USA) while potassium hexacyanoferrate (III), $\text{K}_3[\text{Fe}(\text{CN})_6]$, was obtained from Merck (Germany). Ultra-pure water was used in all experiments. All other reagents were of analytical grade.

2.2. Coffee samples and extraction procedure

A total of $n = 20$ coffee samples (*Coffea arabica*) produced in different locations of Brazil were analyzed in this study. Coffee powders (60 Tyler mesh particle size) were extracted using ultrapure water at a proportion of 1:10 (m/v) at 85 °C for 10 min under magnetic stirring and covered with a lid. After extraction, coffee brews were filtered using qualitative filter paper and centrifuged for 5 min at 6200g. Samples were stored at -80°C until analysis.

2.3. Total phenolic content

The total phenolic content (TPC) was quantified using the Prussian Blue assay as described by Margraf, Karnopp, Rosso, and Granato (2015). Briefly, samples were diluted with ultrapure water at a proportion of 1:150 (v/v) and an aliquot (100 μL) was added in a flat-bottom 96-well polystyrene microplate with 100 μL of a 0.50 mmol/L $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution diluted in HCl 0.01 mol/L. After 5 min rest, the reaction was initiated by adding 100 μL of a 0.50 mmol/L $\text{K}_3[\text{Fe}(\text{CN})_6]$ solution. The increase of absorbance was monitored at $\lambda = 725$ nm after 15 min reaction using a microplate reader (Epoch, BioteK, USA). For quantification purposes, the TPC was estimated using an analytical curve of gallic acid (linearity: 0–40 mg/L; $R^2 = 0.979$) and results were expressed as mg of gallic acid equivalents/L of coffee brew (mg GAE/L).

2.4. Determination of metal chelation ability

2.4.1. Fe^{2+} chelation ability

The ability of extracts to chelate Fe^{2+} was assessed using the colorimetric method proposed by Carter (1971), with modifications. In slightly acidic medium (pH 6), phenolic compounds bind a certain amount of Fe^{2+} , but the remaining Fe^{2+} reacts with ferrozine, forming a blue-colored complex that can be monitored spectrophotometrically (Fig. 1). However, in the presence of phenolic compounds, there is disruption of the formation of the metallic complex (due to the binding of Fe^{2+} in the phenolic structure) which leads to a decrease in the absorbance at $\lambda = 562$ nm (Perron et al., 2010). Herein, measurement of color reduction is an estimation of the binding ability of the extract. Therefore, the higher the absorbance at $\lambda = 562$ nm (which is due to the ferrous ion-ferrozine complex), the weaker the ferrous iron binding ability (antioxidant activity) of the chemical compounds present in the test sample.

Initially, samples were diluted with ultrapure water (1:30 v/v) and aliquots of coffee (50 μL) were placed in 96-well microplates in triplicates. Then, 160 μL of ultrapure water and 20 μL of a 0.30 mmol/L FeSO_4 solution were added in each well followed by the addition of 30 μL of a 0.80 mmol/L ferrozine solution after 5 min reaction, totaling 260 μL in each well. Distilled water (50 μL) replaced the ferrozine solution to be used as a control, which was used to correct for unequal color of the sample solutions. The reactional medium had a mean pH value of 6.00 and the absorbance at $\lambda = 562$ nm of test samples was recorded after 15 min. A decrease in absorbance corresponds to an increase in iron chelating ability. The percentage of Fe^{2+} -ferrozine complex formation was calculated as: Fe^{2+} chelating ability (%) = $[(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{solution without ferrozine}}) / \text{Abs}_{\text{control}}] \times 100$. Six evenly spaced concentrations of disodium ethylenediamine tetra-acetic acid (EDTA-Na_2) were used to plot an analytical curve, where the

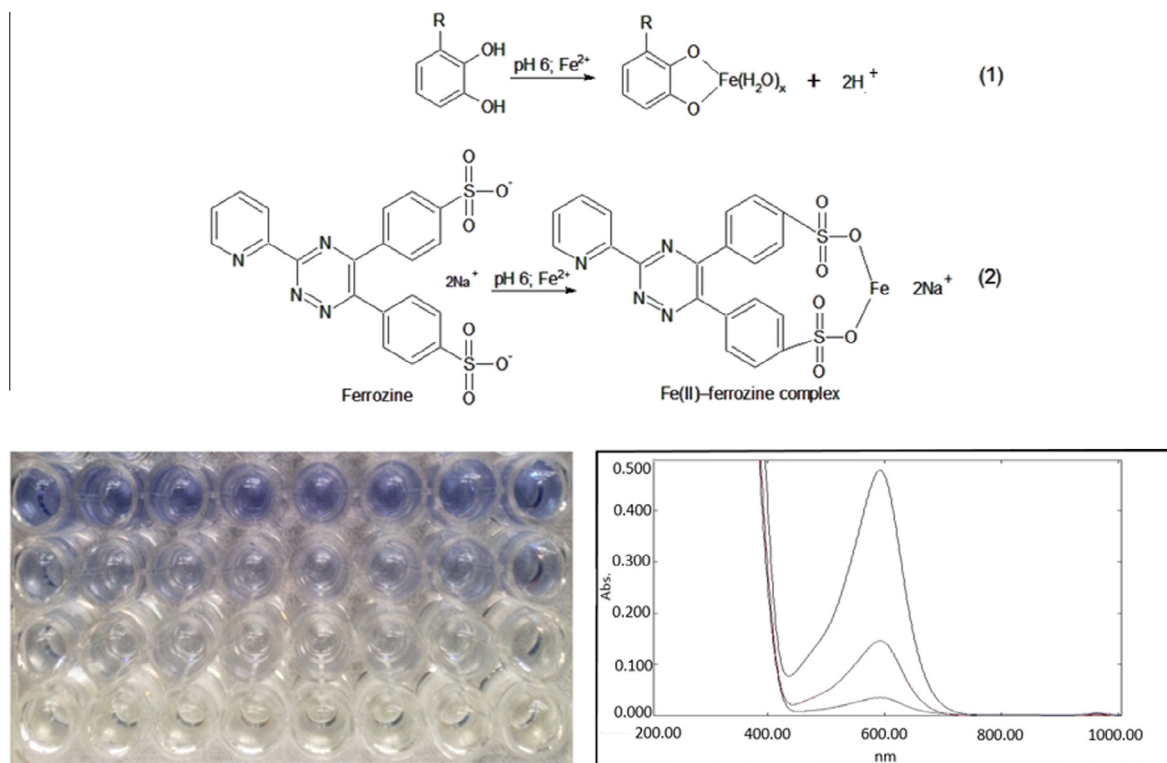


Fig. 1. Reaction between a phenolic compound and iron (II) in the iron-binding assay using ferrozine as chromogen agent together with the UV/VIS spectrum of the Fe²⁺-ferrozine complex and a microplate containing different concentrations of EDTA.

EDTA-Na₂ concentrations were plotted in the Y-axis and the respective data of the Fe²⁺-ferrozine complex formation were inserted in the X-axis. Results were expressed as mg EDTA equivalents/L of coffee brew (mg EDTAE/L).

2.4.2. Cu²⁺ chelation ability

The ability of coffee extracts to chelate Cu²⁺ was assessed using the method that employs pyrocatechol violet (PV) as the chromogen agent (Saiga et al., 2003). In aqueous medium buffered at slightly acidic medium (pH 6.0), phenolic compounds may bind Cu²⁺, but depending on the chemical structure, the reactivity of formation of the complex phenol-Cu²⁺ is increased, but there is usually a leftover of Cu²⁺ in the reactional medium because not all the copper is bound by chemical compounds present in a test sample. Thereafter, the remaining Cu²⁺ reacts with pyrocatechol violet at a proportion of 2:1, forming a dark-colored complex that can be monitored at $\lambda = 632$ nm (Fig. 2) (Su, Sun, Huo, Yang, & Yin, 2010). However, the dark color turns into yellow in the presence of chelating agents that dissociate the complex, and the chelating activity can thus be estimated by the measurement of the rate of color reduction.

Briefly, in each well, 30 μ L of diluted beverage (1:30 v/v) or water (control) were mixed with 200 μ L of sodium acetate buffer (50 mmol/L, pH 6.0). Then, 30 μ L of a 100 mg/L CuSO₄·5H₂O solution was added in each well and let react for 2 min, when 8.5 μ L of a 2 mmol/L PV solution were added to initiate the reaction. The microplate was shaken for 10 min and allowed to react for more 10 min at 25 °C, when the absorbance was read at $\lambda = 632$ nm. The percentage of formation of Cu²⁺-PV complex was calculated as: Cu²⁺ chelating ability (%) = [(Abs_{sample})/Abs_{control}] × 100. Seven evenly spaced concentrations of EDTA-Na₂ were used to plot an analytical curve, where the EDTA-Na₂ concentrations were plotted in the Y-axis and the respective data of the Cu²⁺-PV complex formation were inserted in the X-axis. Results were expressed as mg EDTA equivalents/L of coffee brew (mg EDTAE/L).

2.4.3. Repeatability, reproducibility, linearity, and recovery

A stock solution of EDTA was prepared at a concentration of 100 mg/L and diluted to 50, 35, 25, 12.5, and 6.25 mg/L, each one in triplicate. These solutions were used to perform the analyses of linearity, repeatability, reproducibility, and recovery. Linearity was assessed by constructing an analytical curve using the formation of the metallic complex versus nominal concentrations of EDTA-Na₂ from 0 to 100 mg/L. The ordinary least-squares regression was applied and the linear model was proposed and the residuals were checked for normality using the Shapiro-Wilk's test. The regression equation ($y = ax \pm b$), coefficient of determination (R²), adjusted R², and correlation coefficient (r) were also calculated for each method. P-values below 5% were regarded as significant (Granato, de Araújo Calado, & Jarvis, 2014).

Precision of both assays was assessed by the repeatability (intra-day) and reproducibility (inter-day) and reported as relative standard deviation (% RSD). Reproducibility was analyzed by measuring of n = 18 readings divided in three consecutive days (n = 6 readings/day) of a stock solution of EDTA. Repeatability was evaluated by means of n = 12 successive measurements of the same sample of EDTA solution (25 mg/L and 60 mg/L for the iron and copper assays, respectively) that was analyzed under the same conditions in a short time (analyst, spectrophotometer, calibration, and place) (Thompson, Ellison, & Wood, 2002). The recovery was measured by reading n = 12 replicates and the $\pm 95\%$ confidence interval was calculated using Statistica v. 7 software (Statsoft, USA).

2.5. Antioxidant activity

2.5.1. Scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH)

The free-radical scavenging activity toward DPPH radical (DPPH[•] + PheOH → DPPH(H) + PheO[•]) was assessed in a system buffered at pH 6.0 using a 50 mmol/L sodium phosphate solution as described by Zheng, Lin, Su, Zhao, and Zhao (2015) with the

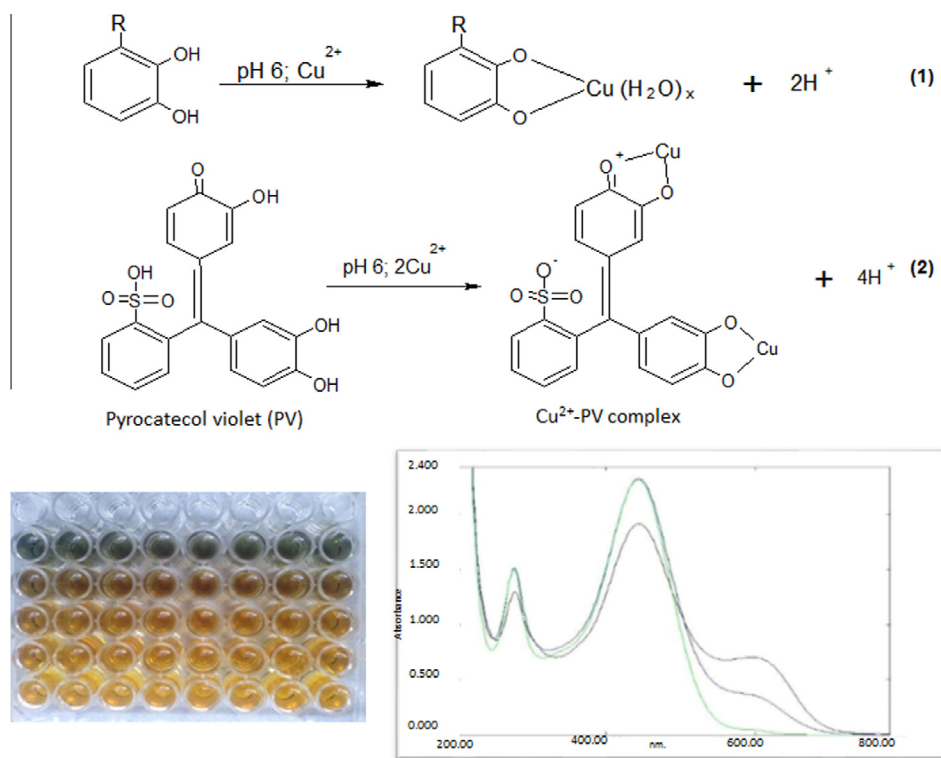


Fig. 2. Reaction between a phenolic compound and copper (II) in the copper-binding assay using pyrocatechol-violet as chromogen agent together with the UV/VIS spectrum of the Cu^{2+} -pyrocatechol-violet complex and a microplate containing different concentrations of EDTA.

experimental conditions proposed by Granato, Grevink et al. (2014). Pure ethyl alcohol and a 50 mmol/L sodium phosphate solution buffered at pH 6 were used as the solvent (1:1 volume ratio) of the DPPH radical. For this purpose, 40 μL of diluted sample (1:150 v/v) were mixed with 260 μL of a 0.10 mmol/L DPPH radical solution in a 96-well microplate and let react for 30 min at 25 $^{\circ}\text{C}$. The decrease of absorbance was monitored at $\lambda = 525$ nm against a blank (sodium phosphate buffer and ethyl alcohol at 1:1 v/v) and the antioxidant effect was calculated using an analytical curve plotted with different evenly spaced concentrations of ascorbic acid (linearity: 0–15 mg/L; $R^2 = 0.995$). Results were expressed as mg ascorbic acid equivalents per liter of coffee (mg AAE/L).

2.5.2. Ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power (FRAP) of beverages was quantified using the method proposed by Benzie and Strain (1996), with slight modifications. An aliquot of 280 μL of the freshly prepared FRAP reagent and 20 μL of diluted test sample (1:150 v/v) were added to each well, and after 30 min reaction the absorbance was read at $\lambda = 593$ nm. The FRAP reagent was prepared by mixing sodium acetate buffer (300 mmol/L, pH 3.6), a solution of TPTZ (10 mmol/L) in 40 mmol/L HCl, and 20 mmol/L $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ using the proportion 10:1:1 (v/v/v). An analytical curve with different concentrations of ascorbic acid (linearity: 15–120 mg/L; $R^2 = 0.998$) was plotted to quantify the ferric reducing antioxidant power of the selected coffees. The results were expressed in mg ascorbic acid equivalents per liter (mg AAE/L).

2.6. Statistical analysis

Results are expressed as the means followed by the standard deviation of three readings. Grubb's test ($\alpha = 0.05$) was used to check for possible outliers. Correlation analysis based on the Pearson's correlation coefficient (r -value) was performed to

measure the degree of association between pairwise variables. For that purpose, the adherence to the normal distribution was checked by the Normal P-Plot and by Shapiro-Wilk test, taking on $p < 0.05$ (Granato, Calado et al., 2014; Nunes, Alvarenga, de Souza Sant'Ana, Santos, & Granato, 2015). Principal component analysis based on linear correlation was used to analyze the data using a multivariate projection of all coffee samples and antioxidant properties in a two-dimensional graph (Granato, Margraf et al., 2015). Mean values of each response variable were standardized using the auto-scaling procedure prior to PCA. Statistica v. 7 (Statsoft, USA) and Action v. 2.9 (Statcamp, Brazil) were used for the statistical analyses.

3. Results and discussion

In foods, divalent transition metals, such as Fe^{2+} and Cu^{2+} , depending on the pH and substrates, may start the lipid peroxidation and generate reactive species that lead to the deterioration of odor and taste (Benedet & Shibamoto, 2008). In humans, an overload of copper and iron ions in blood is also associated with a higher risk of some non-communicable diseases, DNA damage (Miura, 2015), and neurodegenerative diseases (Bettencourt et al., 2016). Thus, the ability of a food extracts or chemical compound found in herbal extracts in chelating transition metals is of paramount importance.

Before analyzing the coffee samples, the high-throughput methods to screen the ability to chelate Fe^{2+} and Cu^{2+} were tested and standardized. For that purpose, EDTA- Na_2 was used as the chemical standard to generate the analytical curve (linearity) and to assess the repeatability, reproducibility, and recovery of both assays (Table 1). Data showed that the Cu^{2+} -chelating ability assay presented a good repeatability and reproducibility ($<5\%$ RSD) and recovery of $98.91 \pm 4.51\%$. Additionally, the method was linear in the range of 0–100 mg EDTA equivalents/L, presenting

Table 1

Experiments to test the assays to chelate transition metal ions.

Validation parameter	Fe ²⁺ -chelating ability	Cu ²⁺ -chelating ability
Recovery (%)	96.66 ± 6.66 (CI _{95%} = 22.97–25.36 mg/L)	98.91 ± 4.51 (CI _{95%} = 57.53–61.17 mg/L)
Repeatability (% RSD)	6.90	4.56
Reproducibility (% RSD)	6.75	3.57
Linearity	Range: 6.25–100 mg/L Curve: $y = -1.1888 \cdot x + 108.374$ Statistical parameters: $R^2 = 0.9942$; $R^2_{adj} = 0.9927$ $p < 0.0001$	Range: 0–100 mg/L Curve: $y = -1.6172 \cdot x + 161.292$ Statistical parameters: $R^2 = 0.9993$; $R^2_{adj} = 0.9991$ $p < 0.0001$

$R^2 = 0.9993$ and $R^2_{adj} = 0.9991$ and no trend was observed in the residual analysis (Shapiro Wilk's statistics = 0.832, $p = 0.084$), proving that the generated analytical curve is appropriate to be used in the range tested. Regarding the Fe²⁺-chelating ability assay, a good repeatability and reproducibility were also obtained (<7% RSD) and the method was linear (Shapiro Wilk's statistics = 0.927, $p = 0.556$) in the range of 6.25–100 mg EDTA equivalents/L, presenting $R^2 = 0.9942$, $R^2_{adj} = 0.9927$.

While some authors (Sánchez-Vioque et al., 2013) state their results as the amount of Fe²⁺-ferrozine complex or amount of Cu²⁺-pyrocatechol violet complex development $[(Abs_{sample})/Abs_{control}] \times 100$, others express the results as the % of inhibition of the complex formation, $[(Abs_{blank} - Abs_{sample})/Abs_{blank}] \times 100$ (Alemán et al., 2011; Liu, Cao, & Jiang, 2015; Torres-Fuentes, Alaiz, & Vioque, 2012; Wang, Jonsdottir, & Ólafsdóttir, 2009). As well outlined by Canabady-Rochelle et al. (2015), in order to compare data reported in various studies, there is an urgent need

to standardize the experimental conditions (i.e., pH of the medium, concentration of reagents, reaction time, type of solvent used, among others). For instance, some authors do not consider the color provided by ferrozine in the formula (Carrasco-Castilla et al., 2012; Farhan et al., 2012; Liu et al., 2015); however, the ferrozine solution is slightly yellow and it is recommended to correct the final absorbance of the test sample (or EDTA when preparing the analytical curve) for unequal color of the sample solutions. In some tests (data not shown), when the color of the ferrozine solution was not taken into consideration, results showed inconsistencies (i.e., formation of the Fe²⁺-ferrozine complex higher than 100% for some diluted – lower than 15 mg/L – EDTA solutions). We also verified that for the ferrous-ion chelating assay, when the analytical curve is constructed using EDTA as chemical standard, at least six repetitions for each concentration should be prepared to assure repeatability and reproducibility of data.

Table 2

Antioxidant activity, metal chelating ability and total phenolic content of different Brazilian coffee samples.

Coffee samples	Cu ²⁺ – chelating ability (mg EDTAE/L)	Fe ²⁺ – chelating ability (mg EDTAE/L)	Total phenolic content (mg GAE/L)	DPPH (mg AAE/L)	FRAP (mg AAE/L)
1	1242 ± 129	1307 ± 136	2043 ± 81	1490 ± 49	4179 ± 196
2	1502 ± 9	1689 ± 128	2214 ± 77	1865 ± 87	5614 ± 146
3	1162 ± 13	833 ± 90	2166 ± 28	1675 ± 73	4503 ± 154
4	1710 ± 49	1817 ± 102	2298 ± 29	2293 ± 64	7579 ± 142
5	1549 ± 44	1642 ± 118	2196 ± 24	1757 ± 34	5643 ± 50
6	1434 ± 53	1055 ± 89	2184 ± 29	1788 ± 111	5356 ± 99
7	1294 ± 26	1595 ± 73	2250 ± 40	1806 ± 278	5151 ± 84
8	1660 ± 98	1023 ± 69	2331 ± 4	2120 ± 125	6225 ± 163
9	1707 ± 64	1707 ± 44	2260 ± 36	1973 ± 256	5606 ± 199
10	1632 ± 63	1298 ± 205	2243 ± 40	1871 ± 89	5631 ± 353
11	1509 ± 64	1772 ± 47	2290 ± 28	1894 ± 214	5614 ± 191
12	1349 ± 212	1784 ± 31	2253 ± 17	1941 ± 181	4995 ± 308
13	1269 ± 37	1322 ± 137	2152 ± 47	1753 ± 114	4597 ± 191
14	1552 ± 101	1310 ± 90	2237 ± 52	1773 ± 108	5274 ± 292
15	1499 ± 72	1002 ± 144	2351 ± 9	1816 ± 91	5389 ± 109
16	1422 ± 232	1698 ± 67	2220 ± 26	1806 ± 62	4806 ± 31
17	1549 ± 185	1615 ± 106	2198 ± 63	1835 ± 227	5241 ± 169
18	1692 ± 71	1758 ± 13	2306 ± 50	1728 ± 126	5487 ± 189
19	1559 ± 81	1589 ± 77	2306 ± 44	1757 ± 89	5430 ± 223
20	1682 ± 101	1778 ± 79	2298 ± 19	1743 ± 163	4843 ± 195

Table 3

Correlation analysis (r-value) between antioxidant activity, metal chelating ability and total phenolic content of coffees (n = 20).

Variables	Total phenolic content	DPPH	FRAP	Cu ²⁺ chelating ability	Fe ²⁺ chelating ability
Total phenolic content	1.0000				
DPPH	$p = -0.5855$	1.0000			
FRAP	$p = 0.007$	$p = -0.5825$	1.0000		
Cu ²⁺ chelating ability	$p = 0.007$	$p < 0.001$	$p = -0.6684$	1.0000	
Fe ²⁺ chelating ability	$p = 0.001$	$p = 0.013$	$p = 0.001$	$p = -0.3956$	1.0000
	$p = 0.376$	$p = 0.311$	$p = 0.293$	$p = 0.084$	$p = -$

Taking all these initial experimental data analysis, in the current work we propose the expression of results by comparing the metallic complex formation with a known concentration of a cheap and widely employed metal chelator, EDTA. Using the reagent as a standard, comparison inter and intra research groups can be made easily.

The total phenolic content and antioxidant activity of coffee samples measured by the FRAP and DPPH assays together with

the sample's ability to chelate Fe^{2+} and Cu^{2+} are presented in Table 2. For both metal chelating methods, no outliers were found using the Grubb's test ($n = 20$ samples, $\alpha = 0.05$). Using linear correlation analysis (Table 3), it is possible to observe that the TPC was highly ($p < 0.05$) correlated to DPPH, FRAP, and Cu^{2+} -chelating ability. As shown in Fig. 3A, higher values of TPC provides higher mean values of Cu^{2+} -chelating ability, which is in-line with the results obtained by Melo et al. (2013) and Granato, Margraf et al. (2015). The Cu^{2+} -chelating ability assay was also significantly correlated to DPPH ($r = 0.5466$, $p = 0.013$) and FRAP ($r = 0.6931$, $p = 0.001$) (Fig. 3B; Table 3), showing a suitable association with widely used antioxidant assays.

According to Guo et al. (2007), phenolic compounds present some functional sites that are recognized to bind transition metals in their structure, such as the 4-keto group with a 3-and/or 5-hydroxyl group, or the presence of a 3'-4' and/or 7-8 o-dihydroxyphenyl (catechol) structure in the B benzene ring of flavonoids or type of substituents (Khokhar & Apenten, 2003). Additionally, flavonoids also display a high binding of divalent transition metals (Cu^{2+} , Fe^{2+} , Zn^{2+}), and more specifically, Říha et al. (2014) verified that the most efficient copper chelation sites in flavonoids were the 3-hydroxy-4-keto group in flavonols and the 5,6,7-trihydroxyl group in flavones. Phenolic compounds bearing only one hydroxyl group in the structure do not present transition metal chelating ability (Zhou, Yin, & Yu, 2006). Andjelković et al. (2006) showed that some phenolic compounds not bearing catechol or galloyl moiety (3',4',5'-OH trihydroxybenzene), such as ferulic, vanillic, and syringic acids do not present any Fe^{2+} -chelating ability, while chlorogenic, caffeic, and gallic acids have a strong ability to bind Fe^{2+} . Indeed, the presence of methoxyl and hydroxyl groups in the *ortho* position of phenolic acids increases Cu^{2+} chelation ability (Zhou et al., 2006). Therefore, the ability to chelate $\text{Fe}^{2+}/\text{Cu}^{2+}$ is dependent on the phenolic structure, location and number of hydroxyl groups.

As observed in Table 3 and Fig. 3C, Fe^{2+} -chelating ability showed sparse and non-significant ($p > 0.05$) correlations with TPC, FRAP, DPPH, and Cu^{2+} -chelating ability. These results corroborate the data obtained in other works with different food and herbal matrices (Granato, Koot, Schnitzler, & van Ruth, 2015; Wang et al., 2009) in which phenolic compounds seem to play a minor role in binding Fe^{2+} in some extracts. On the other hand, Granato, Grevink et al. (2014) found significant correlations between total phenolic content ($r = 0.724$), total flavanols ($r = 0.747$) and epigallocatechin gallate ($r = 0.676$) with iron-chelating ability of $n = 10$ *Camellia sinensis* teas. As coffee is a rich source of phenolic acids and some flavonoids (Felberg et al., 2015; Oliveira-Neto et al., 2016; Regazzoni et al., 2016), a high Cu^{2+} and Fe^{2+} chelating effect was obtained. These results altogether clearly indicate that not only the chemical components present in the extracts but also the matrix effects (i.e., pH and redox potential) are also responsible for the binding ability of transition metals (Sunda & Huntsman, 2003).

In order to gain an overview of all data structure, including the coffee samples, TPC, metal chelating effect and antioxidant activity, PCA was applied to investigate the relationship between all variables simultaneously (Fig. 1 Supplementary Material). Two PCs were extracted and explained up to 80% of data variability, in which the 1st PC correlated the TPC, DPPH, FRAP, and copper-chelating ability, indicating that these antioxidant assays are highly associated. The 2nd PC was only correlated to the iron-chelating ability, corroborating the linear correlation analysis data that the iron chelating ability of coffees is not significantly associated with FRAP, DPPH, and TPC. A very similar projection of variables (PC1 versus PC2) was obtained by Wang et al. (2009) analyzing ten species of Icelandic seaweeds.

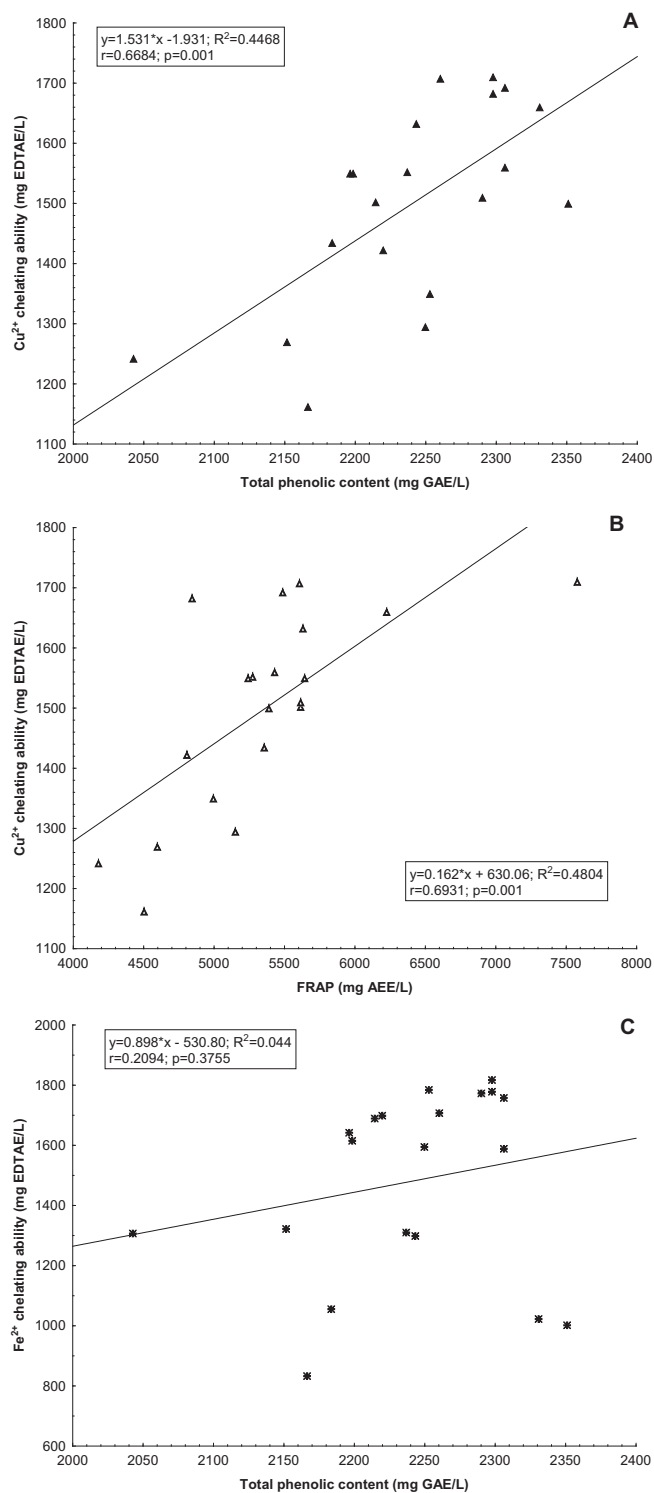


Fig. 3. Correlation analysis between total phenolic content and the Cu^{2+} chelating ability (A) and Fe^{2+} chelating ability (B) as well as Cu^{2+} chelating ability versus FRAP.

4. Conclusions

In the current work, two spectrophotometric high-throughput assays were tested and standardized to assess the Fe^{2+} and Cu^{2+} chelating ability of coffee samples and a good repeatability and reproducibility were obtained. Correlation analysis showed that the Cu^{2+} chelating ability is significantly and positively correlated to DPPH, FRAP and total phenolic content, while Fe^{2+} chelating ability was not ($p > 0.05$). We strongly suggest that the expression of results should be made by using the complex formation (Cu^{2+} -pyrocatechol violet and Fe^{2+} -ferrozine) comparing these data with an analytical curve prepared with EDTA or other metal chelators. This is the most suitable way to obtain standardization and comparable results for different food matrices (extracts and even isolated compounds) inter and intra research groups.

Conflict of interest statement

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2016.07.091>.

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