Assessment of the reducing capacity of processed fruit juices with the copper(I)/4,4'-dicarboxy-2,2'-biquinoline complexes

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Abstract An alternative method for quantification of the total reducing capacity (TRC) of processed ready-to-drink fruit juices (orange, grape, peach, mango, cashew, strawberry, apple and guava) is suggested. The spectrophotometric procedure is based on the reduction of Cu(II) to Cu(I) by antioxidants (present in the samples) in aqueous buffered solution (pH 7.0), containing 4,4'-dicarboxy-2,2'-biquinoline acid (H_2BCA), yielding the $Cu(BCA)_2^{3-}$ complexes. The absorbance values at 558 nm ($A_{558\text{ nm}}$) of the $Cu(BCA)_2^{3-}$ complexes obtained with juice samples were compared with $A_{558\text{ nm}}$ values of the same complexes obtained with a standard ascorbic acid solution and used to quantify and express the reducing capacity of each sample. Regarding orange juices a positive relationship between the TRC values using the $Cu(BCA)_2^{3-}$ complexes and the labelled ascorbic acid (AA) content along with the total polyphenol content (TPC) was measured. Grape juices showed the best positive correlation was verified between the TRC (with the $Cu(BCA)_2^{3-}$ complexes) and the TPC. While other fruit juices showed good agreement of TRC values with $Cu(BCA)_2^{3-}$ complexes and DPPH reagent. The proposed method may be applied to measure the TRC of beers and wines and also for biological samples like serum and follicular fluid.

Keywords Reducing capacity · Processed food · Fruit juices · Copper(I) · 4,4'-dicarboxy-2,2'-biquinoline acid

Introduction

A regular intake of vegetables, fruits and fruit juices is associated with a lower risk of several illnesses such as inflammatory diseases (e.g. arthritis), cardiovascular disorders, stroke, cancer and also prevention of premature aging (Knekt et al. 2002; Halliwell 2007). One reasonable justification for this, is the presence of various phytochemical compounds that have antioxidant properties (Kris-Etherton et al. 2002).

The main antioxidant compounds present in citrus fruits (orange, lemon, mandarin and lime etc.) are ascorbic acid and flavonoids (Xu et al. 2008). Grape and berries juices contain mainly polyphenols, flavonoids (anthocyanins and flavanols), stilbenes (resveratrol), phenolic acids (derivatives of cinnamic and benzoic acids) and a wide variety of tannins (Perales et al. 2008).

The antioxidant capacity of processed juices, is owed to the presence of phenolic compounds and vitamins (Klimczak et al. 2007; Toaldo et al. 2013).

According to a survey conducted in Brazil (Nielsen 2013), the ready-to-drink fruit juices consumption increased by 12.5% in 2013 when compared with the previous year, which was attributed to the convenience in daily consumption along with a greater tendency to maintain healthier lifestyles.

Given that fruit juices are a very complex mixture, separating, identifying and quantifying each antioxidant compound individually emerges as a laborious, tedious and often expensive process. Considering this, it is always desirable to have available a simpler, low cost, and

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environmentally friendlier analytical method to estimate the antioxidant capacity of such a food commodity.

The 4,4'-dicarboxy-2,2'-biquinoline acid (H_2BCA , $C_{20}H_{12}N_2O_4$, Fig. 1) is an organic diacid derivative of quinoline commercially found in its disodium salt form (Na_2BCA), and is a specific ligand for Cu(I). When Cu(II) ions are reduced in buffered aqueous solution ($pH = 7$) containing Na_2BCA the red–violet Cu(I)/BCA complexes are formed, which exhibit absorption peaks at 357 nm ($\epsilon_{357\text{ nm}} = 4.2 \times 10^4 \text{ L cm}^{-1} \text{ mol}^{-1}$) and 558 nm ($\epsilon_{558\text{ nm}} = 7.7 \times 10^3 \text{ L cm}^{-1} \text{ mol}^{-1}$) (Manoel and Moya 2015).

Our group has been involved in using Cu(I)/BCA complexes in the development of analytical methods for indirect determination of calcium channel blocker (Sabino et al. 2010) and some nonsteroidal anti-inflammatory (Braga et al. 2011) drugs and for the total polyphenol content in wine (Moya et al. 2008) and in plant extracts (Marino et al. 2009).

Recently, the reduction capability of 25 standard antioxidants (phenolic derivatives, flavonoids, stilbenoids, vitamins, etc.) was evaluated in order to obtain an individual structure–activity relationship with the proposed reaction. From this comprehensive investigation, a method for the determination of total reducing capacity (TRC) in herbal extracts was proposed (Manoel and Moya 2015).

In this present work an alternative methodology for the TRC quantification of processed fruit juices based on the formation of the Cu(I)/BCA complexes has been suggested. Since these food products contain natural (or added) antioxidants the addition of aliquots of these samples in a solution containing Cu(II) promotes reduction to Cu(I) which, in the presence of Na_2BCA ($pH = 7$), forms the red-violet Cu(I)/BCA complexes. The absorbance values at 558 nm ($A_{558\text{ nm}}$) of these Cu(I)/BCA complexes obtained with juice samples were compared with $A_{558\text{ nm}}$ values of the same complexes formed after the addition of an ascorbic acid solution (a standard antioxidant compound) and used to quantify and express the reducing capacity of each juice. As far as we know this is the first study to assess the TRC of processed fruit juices using Cu(BCA) $_2^{3-}$ complexes.

Fig. 1 Structure of the 4,4'-dicarboxy-2,2'-biquinoline acid (H_2BCA)

The TRC values of the same samples were compared with the antioxidant capacity values obtained with the DPPH method (Sanchez-Moreno et al. 1998; Santo et al. 2013) and the total polyphenol content using the Folin–Ciocalteu reagent (The Brazilian Pharmacopoeia 2010).

Materials and methods

Apparatus and reagents

All absorbance measurements were made in a HPUV 8453 (Agilent) spectrophotometer using a 1.0 cm optical path length using glass or quartz cell.

Reverse osmosis water (Quimis, Q842-210, Brazil) was used to prepare all solutions (except when another solvent is indicated).

Copper(II) perchlorate, $Cu(ClO_4)_2$, 2.328 mol/L stock solution was synthesized and standardized as described in previous studies (Marino et al. 2009; Sabino et al. 2010; Braga et al. 2011; Manoel and Moya 2015). A 1.0×10^{-2} mol/L working solution was prepared by accurate dilution in water.

Dissodium salt of 4,4'-dicarboxy-2,2'-biquinoline, Na_2BCA , 3.0×10^{-2} mol/L stock solution was prepared by dissolution of the 1.165 g ($Na_2C_{20}H_{10}N_2O_4$, > 98%, Sigma-Aldrich) in 100.0 mL volumetric flask and completed with water (Marino et al. 2009; Sabino et al. 2010; Braga et al. 2011; Manoel and Moya 2015).

Ammonium acetate ($NH_4(H_3C-COO)$, 98%, Merck) 2.0 mol/L was prepared by dissolving 77.08 g in 500.0 mL volumetric flask and used as buffer solution (pH 7.0).

Ascorbic acid ($C_6H_8O_6$, 99.7%, Merck) 1.0×10^{-2} mol/L (1.76 mg/mL) was freshly prepared by dissolution of 0.176 g in a 100.0 mL volumetric flask. A 1.0×10^{-3} mol/L (0.176 g/mL) working solution was prepared by accurate dilution in water.

D(+) lactose ($C_{12}H_{22}O_{11} \geq 98\%$), d(+) glucose ($C_6H_{12}O_6$, $\geq 96\%$), d(−) fructose ($C_6H_{12}O_6 \geq 99\%$), d(+) sucrose ($C_{12}H_{22}O_{11} \geq 99.8\%$), citric acid ($C_6H_8O_7$, $\geq 99.5\%$) and sodium erythorbate monohydrate ($C_6H_7NaO_6 \cdot H_2O$, 97%) 1.0×10^{-2} mol/L stock solutions (all from Sigma-Aldrich) were prepared by dissolution in water. A 1.0×10^{-3} mol/L working solutions were prepared by accurate dilution in water.

Riboflavin ($C_{17}H_{20}N_4O_6$, 99.5%, Calbiochem, South Korea) 1.0×10^{-2} mol/L stock solution was dissolved in water adding 2.0 mL 1.0 mol/L NaOH drop by drop until total dissolution. A 1.0×10^{-3} mol/L working solution was obtained by accurate dilution in water.

O-acetyl–tocopherol ($C_{31}H_{52}O_3$, 96%, Sigma-Aldrich) 1.0×10^{-2} mol/L stock solution was prepared by dissolution in ethanol.

BHA (a mixture of 2-*tert*-Butyl-4-hydroxyanisole and 3-*tert*-butyl-4-hydroxyanisole, $C_{11}H_{16}O_2$, > 98%, Sigma Aldrich) 1.0×10^{-3} mol/L stock solutions were prepared by dissolution in 50:50 v/v water:ethanol solution.

Trolox[®] ($C_{14}H_{18}O_4$, 97%, Sigma-Aldrich) 4.0×10^{-4} mol/L stock solution was prepared in an 8% (v/v) ethanol:water solution by adding 1.0 mol/L NaOH solution drop by drop until total dissolution in a 100.0 volumetric flask.

The Folin–Ciocalteu (FC) reagent was prepared as recommended by Brazilian Pharmacopeia (2010) and it is described elsewhere (Santo et al. 2013; Silva et al. 2013).

Sodium carbonate (Na_2CO_3 , 99%, Vetec) 10% (w/v) solution was prepared in water.

Gallic acid ($C_7H_6O_5$, 99%, Sigma) 1.0×10^{-3} mol/L standard solution was prepared by dissolving 0.0170 g in 100 mL volumetric flask and the volume completed with water. A 1.0×10^{-4} mol/L working solution was prepared by accurate dilution.

Methyl alcohol (CH_3COH , 99.8%), ethyl alcohol (C_2H_5OH , $\geq 99.8\%$) and acetone (CH_3OCH_3 , $\geq 99.5\%$) were from Merck. A 50% (v/v) methyl alcohol and a 70% (v/v) acetone solutions were prepared in water.

DPPH (2,2-diphenyl-1-picrylhydrazyl, $C_{18}H_{12}N_5O_6$, Sigma) 23.7 mg/L solution was prepared just before use by dissolving 2.4 mg in pure methyl alcohol in a 100 mL volumetric flask.

General procedures

Sample preparation

The juice samples (all cartons packed) were purchased from local supermarkets in the city of São Paulo, Brazil, and kept in the laboratory at room temperature. The types of juice were selected based on their availability on supermarket shelves and that is the reason for having more grape and orange samples. All samples were within the expiration date established by the manufacturer. The density of each sample was determined using a 25.0 mL calibrated pycnometer immediately after opening the package.

For determination of both the total reducing capacity (with the proposed method) and of the total polyphenol content (with FC reagent), 1.0 mL of sample was weighed and transferred to a 10.0 mL volumetric flask.

For quantification of antioxidant capacity with the DPPH method 1.0 mL of each sample was weighed and transferred to 10.0; 25.0; 50.0 and 100.0 mL volumetric flasks and the volume was completed with water.

Determination of the total polyphenol content (TPC) in fruit juices with the FC reagent

A calibration graph was obtained by mixing aliquots (300–1000 μ L) of a 1.0×10^{-4} mol/L (0.0170 mg/mL) gallic acid (GA) standard solution with 200 μ L of FC reagent in a 5.0 mL volumetric flask. The volume was completed with a 10% Na_2CO_3 solution.

The multiple standard addition method (Marino et al. 2009; Santo et al. 2013; Silva et al. 2013) was used in the analysis of all samples as follows: 500 μ L of diluted fruit juices were transferred to five volumetric flasks of 5.0 mL followed by the addition of 200 μ L of FC reagent. In four out of five volumetric flasks, aliquots of 200–800 μ L from a 1.0×10^{-4} mol/L GA solution were added and the volume was completed with the same Na_2CO_3 solution.

In both graphs (calibration and multiple standard additions with the samples) the absorbance values were recorded at 715 nm ($A_{715 \text{ nm}}$) after 30 min using water as reference solution. All analyses were performed in triplicate and the TPC was expressed as g GA per g juice fruit.

Quantification of the antioxidant capacity based on the DPPH radical scavenging

The DPPH assay was based on the procedure described previously (Sánchez-Moreno et al. 1998; Souza and Moya 2015) with minor modifications. Absorbance values were recorded at 515 nm ($A_{515 \text{ nm}}$) after 30 min using methanol as reference solution. All analyses were performed in triplicate.

First, a calibration curve with five DPPH dilute solutions (2.37–18.9 mg/L) was obtained (Eq. 1). Then a calibration curve with fruit juice was obtained using four different dilute solutions: 10; 20; 40 and 100 mg/mL (see “Sample Preparation” section). Aliquots of 0.125–0.500 mL of each dilution were transferred to a 5.0 mL volumetric flask which was completed with a 23.7 mg/L DPPH solution. These $A_{515 \text{ nm}}$ values originated the Eq. 2 (C_{JF} is the concentration in mg/mL of fruit juice).

A control solution was prepared transferring 0.125 mL of a solvent solution (40 mL 50% (v/v) methanol + 40 mL 70% (v/v) acetone + 20 mL water) to a 5.0 mL volumetric flask and the volume was completed with the same 23.7 mg/L DPPH solution. $A_{515 \text{ nm}}$ half value of the control solution is then substituted in Eqs. (1) and (2) to obtain the mass (in g) of DPPH and fruit juice, respectively. The EC_{50} values are calculated dividing the mass of fruit juice by mass of the DPPH (both in grams) (expressed as g juice fruit/g DPPH). These EC_{50} values mean the mass of fruit juice which reduces 50% of the initial DPPH concentration (Tables 1, 2 and 3).

$$A_{515\text{ nm}} = a + b \times \text{DPPH} \quad (1)$$

$$A_{515\text{ nm}} = a - b \times C_{\text{JF}} \quad (2)$$

Proposed procedure

Calibration graph with ascorbic acid (AA) standard solution

In eight volumetric flasks of 5.0 mL were added: 250 μL 1.0×10^{-2} mol/L Cu(II), 1.0 mL 2.0 mol/L ammonium acetate (pH 7.0) and 200–900 μL aliquots 1.0×10^{-3} mol/L (0.176 mg/mL) AA standard solution. After homogenization 250 μL 3.0×10^{-2} mol/L Na_2BCA were transferred to the volumetric flasks and the volume completed with the same ammonium acetate solution. AA final concentration ranged from 4.0×10^{-5} mol/L (3.52×10^{-3} mg/mL) to 1.8×10^{-4} mol/L (2.82×10^{-2} mg/mL). Absorbance values at 558 nm ($A_{558\text{ nm}}$) were recorded using the above mixture freshly prepared (without AA) as a reference solution. A calibration graph ($A_{558\text{ nm}}$ versus C_{AA} , in mg/mL) obtained is described by the Eq. (3):

$$A_{558\text{ nm}} = a + b \times C_{\text{AA}} \quad (3)$$

Quantification of the total reducing capacity (TRC) in juice fruits

In five volumetric flasks of 5.0 mL were added: 250 μL 1.0×10^{-2} mol/L Cu(II), 1.0 mL 2.0 mol/L ammonium

acetate and aliquots (100; 200; 300 and 400 μL) of the dilute juice sample (see “Sample Preparation” section). After homogenization 250 μL 3.0×10^{-2} mol/L Na_2BCA were transferred to each 5.0 mL volumetric flasks and the volume was completed with the same ammonium acetate solution. Taking into account the density of each juice sample, results were expressed as g/L. $A_{558\text{ nm}}$ values were obtained after 10 min using the same reference solution described in calibration graph with AA (5.0×10^{-4} mol/L Cu(II), 1.5×10^{-3} mol/L Na_2BCA and mol/L ammonium acetate). A graph ($A_{558\text{ nm}}$ versus C_{JF} , juice fruit concentration in mg/mL) obtained is described by the Eq. (4):

$$A_{558\text{ nm}} = a + b \times C_{\text{JF}} \quad (4)$$

From Eq. 1 is calculated the $A_{558\text{ nm}}$ value corresponding to a 1.0 mg/mL AA standard solution. This $A_{558\text{ nm}}$ value is then substituted to Eq. 2, giving the concentration of juice fruit (in mg/mL) which corresponds to the reducing capacity of a 1.0 mg/mL AA standard solution. This value is corrected considering the sample dilution (ten-fold). Using the density value (g/mL) is possible to calculate the mass of juice in the 25.0 mL volumetric flask (see “Sample Preparation” section). All analyses were performed in replicates ($n = 3$). The TRC values obtained for all samples were expressed as g AA/g juice as shown in Tables 1, 2 and 3.

Table 1 Quantification of the total reducing capacity of orange juices using the $\text{Cu}(\text{BCA})_2^{3-}$ complexes

Orange juice	Antioxidant capacity		TPC	AA	Additives
	DPPH EC_{50}	$\text{Cu}(\text{BCA})_2^{3-}$			
1	ND	0.015 ± 0.001	0.13 ± 0.01	6.7	E316; E300; vitamins A, B3, B6, B12, D and E
2	ND	1.07 ± 0.16	0.43 ± 0.10	70	E316; E300
3	0.87 ± 0.04	1.11 ± 0.01	0.25 ± 0.03	65	E300; proteín
4	15.6 ± 0.6	ND	0.21 ± 0.01	34	E316; E300; dietary fiber; iron; sugar; calcium; omega 3
5	1.58 ± 0.02	0.014 ± 0.003	0.22 ± 0.01	NI	E316, nominated as light.
6	0.35 ± 0.01	0.39 ± 0.01	0.34 ± 0.07	42	E300; E316; dietary fiber
7	1.97 ± 0.10	0.56 ± 0.02	0.19 ± 0.01	70	E300; E316
8	1.49 ± 0.18	0.52 ± 0.05	0.34 ± 0.01	20	E300; dietary fiber
9	5.17 ± 0.27	0.017 ± 0.004	0.23 ± 0.03	NI	E300; dietary fiber; proteín
10	0.76 ± 0.03	0.023 ± 0.003	0.17 ± 0.01	NI	E316
11	2.24 ± 0.27	0.016 ± 0.002	0.20 ± 0.01	30	E300

TPC = total polyphenolic content expressed as g GA/g juice fruit; AA = ascorbic acid content labelled as mg AA/200 mL juice; Additives as reported in the package (all samples contain carbohydrates); DPPH EC_{50} = expressed as g juice fruit/g DPPH; $\text{Cu}(\text{BCA})_2^{3-}$ (proposed method) = expressed as g AA/g juice; E316 = sodium erythorbate; E300 = ascorbic acid; NI = not informed

All numeric values are mean and relative standard deviation obtained with the three replicates ($n = 3$)

Table 2 Quantification of the total reducing capacity of grape juices using the $\text{Cu}(\text{BCA})_2^{3-}$ complexes

Grape juice	Antioxidant capacity		TPC	AA	Additives
	DPPH EC_{50}	$\text{Cu}(\text{BCA})_2^{3-}$			
1	0.65 ± 0.05	0.66 ± 0.02	0.59 ± 0.01	14	E316; E300.
2	0.72 ± 0.02	0.63 ± 0.03	0.92 ± 0.03	NI	E316.
3	0.55 ± 0.05	0.57 ± 0.10	0.46 ± 0.03	32	E316; E300.
4	0.29 ± 0.01	0.37 ± 0.03	1.22 ± 0.10	NI	E316; E300.
5	0.48 ± 0.02	0.23 ± 0.01	0.28 ± 0.16	NI	NI
6	1.39 ± 0.04	0.29 ± 0.04	0.027 ± 0.006	NI	NI
7	0.32 ± 0.01	0.21 ± 0.01	0.17 ± 0.01	NI	NI
8	0.34 ± 0.01	0.38 ± 0.05	0.19 ± 0.01	NI	NI
9	0.94 ± 0.07	0.90 ± 0.05	0.80 ± 0.09	26	E300; E316; nominated as light
10	1.9 ± 0.6	0.29 ± 0.01	0.63 ± 0.05	30	E316; E300; nominated as light
11	1.21 ± 0.31	0.36 ± 0.02	0.87 ± 0.1	5.0	E316; E300; iron, nominated as light
12	1.95 ± 0.02	0.56 ± 0.11	0.41 ± 0.08	NI	E316; nominated as light

TPC = total polyphenolic content expressed as g GA/g juice fruit; AA = ascorbic acid content labelled as mg AA/200 mL juice; Additives as reported in the package (all samples contain carbohydrates); DPPH EC_{50} = expressed as g juice fruit/g DPPH; $\text{Cu}(\text{BCA})_2^{3-}$ (proposed method) = expressed as g AA/g juice; E316 = sodium erythorbate; E300 = ascorbic acid; NI = not informed

All numeric values are mean and relative standard deviation obtained with the three replicates (n = 3)

Table 3 Quantification of the reducing capacity of others fruit juices using the $\text{Cu}(\text{BCA})_2^{3-}$ complexes

Sample juice	Antioxidant capacity		TPC	AA	Additives
	DPPH EC_{50}	$\text{Cu}(\text{BCA})_2^{3-}$			
Peach 1	0.79 ± 0.01	1.87 ± 0.03	0.27 ± 0.04	19	E300
Peach 2	0.68 ± 0.03	1.11 ± 0.12	0.19 ± 0.01	23	E316; E300.
Mango 1	0.35 ± 0.02	1.47 ± 0.16	0.32 ± 0.03	34	E300; E316; dietary fiber
Mango 2	0.71 ± 0.04	1.45 ± 0.23	0.30 ± 0.02	NI	E316; dietary fiber
Cashew 1	0.28 ± 0.01	0.93 ± 0.08	0.33 ± 0.03	40	E316; E300; dietary fiber
Cashew 2	0.04 ± 0.01	0.31 ± 0.01	0.45 ± 0.01	NI	E316
Strawberry 1	0.86 ± 0.04	3.17 ± 0.17	0.27 ± 0.01	NI	
Strawberry 2	0.51 ± 0.01	1.79 ± 0.12	0.33 ± 0.06	28	E300; E316; iron
Apple 1	0.18 ± 0.01	0.87 ± 0.03	0.19 ± 0.02	NI	
Apple 2	0.12 ± 0.01	0.003 ± 0.001	0.24 ± 0.02	32	E300; E316; dietary fiber
Guava 1	0.10 ± 0.01	0.81 ± 0.04	0.14 ± 0.02	40	E300; E316; iron; dietary fiber
Guava 2	0.09 ± 0.01	0.31 ± 0.04	0.15 ± 0.05	NI	E316; dietary fiber

TPC = total polyphenolic content expressed as g GA/g juice fruit; AA = ascorbic acid content labelled as mg AA/200 mL juice; Additives as reported in the package (all samples contain carbohydrates); DPPH EC_{50} = expressed as g juice fruit/g DPPH; $\text{Cu}(\text{BCA})_2^{3-}$ (proposed method) = expressed as g AA/g juice; E316 = sodium erythorbate; E300 = ascorbic acid; NI = not informed

All numeric values are mean and relative standard deviation obtained with the three replicates (n = 3)

Results and discussion

Some chemical aspects of the proposed reaction

In the present method, Cu(I) was generated by reduction of Cu(II) by the antioxidant compounds (AOs) present in the fruit juices containing BCA solution ($\text{pH} \geq 7$). According to literature two molecules of BCA coordinate to one Cu(I)

ion forming the $\text{Cu}(\text{BCA})_2^{3-}$ (Manoel and Moya 2015), which is described by Eq. (5). Thus, BCA should be maintained in excess at least 2.5 times over Cu(II) so that the colour of the solution remains constant (Manoel and Moya 2015) to get the highest sensitivity and reproducibility in the analyses.

As mentioned before (Moya et al. 2008; Marino et al. 2009, Sabino et al. 2010; Braga et al. 2011; Manoel and Moya 2015), the best experimental conditions for analytical purposes were achieved in a solution (Cu(II):BCA in a 1:3 ratio) containing 0.5×10^{-3} mol/L Cu(II), 1.5×10^{-3} mol/L BCA and 0.8 mol/L ammonium acetate (pH = 7). At higher concentrations, a light green Cu(II)/BCA compound precipitate was formed and at lower concentrations a decrease in the absorbance values was noted.

Ascorbic acid (AA) was used as standard compound to express the antioxidant capacity of fruit juice since is not expensive as Trolox[®] and promptly reduces Cu(II) in solution containing BCA. Figure 2 shows the absorption spectra of aqueous solution containing Cu(II) 1.0×10^{-4} mol/L and BCA 3.0×10^{-4} mol/L before and after the addition of AA 5.0×10^{-5} mol/L with formation of the Cu(BCA)_2^{3-} complexes.

The Cu(BCA)_2^{3-} complexes formed, after reduction of Cu(II) by reducing agents in medium containing BCA^{2-} , had two peaks of maximum absorption: 357 nm ($\epsilon_{357 \text{ nm}} = 4.2 \times 10^4$ L/cm mol) and 558 nm ($\epsilon_{558 \text{ nm}} = 7.7 \times 10^3$ L/cm mol) being the former 5-fold more sensitive than the latter. In spite of that, the solution containing only Cu(II) and BCA^{2-} (used as a reference solution) also absorbed at 357 nm. Therefore, the absorbance measurements in the proposed assay were performed at 558 nm, where only the Cu(BCA)_2^{3-} complexes absorb.

Fig. 2 Absorption spectra of aqueous solutions containing: (I) = Cu(II) 1.0×10^{-4} mol/L + BCA 3.0×10^{-4} mol/L + ammonium acetate 0.8 mol/L; (II) = (I) + AA 5.0×10^{-5} mol/L. (Absorbance measurements after 5 min; pathlength = 1.0 cm; water as reference solution)

Data treatment

In the present study three experimental parameters obtained from the juices samples were evaluated: the antioxidant capacity with DPPH method, the TRC with the Cu(BCA)_2^{3-} complexes and the TPC with FC reagent. AA content informed by suppliers was also included in this comparative study but since this parameter was not quantified any conclusion should be taken carefully. An attempt to compare these values considering all fruit juice samples as a single group was not successful. Thus, they were divided into three groups: orange juices (8), grape juices (7), and miscellaneous fruits (2 peaches, 2 mangos, 2 cashews, 2 strawberries, 2 apples and 2 guavas), which are shown in Tables 1, 2 and 3, respectively. In order to evaluate the parameters described above, Pearson's correlation coefficients (*r*) were obtained after linear regression using Origin[®] 7.0.

Considering orange juice samples, there was observed no positive correlation between antioxidant capacity values (obtained with the DPPH method) and those of TRC with Cu(BCA)_2^{3-} complexes or even with the TPC (FC reagent). It could be speculated that the compounds responsible for reductive capacity in all these industrialized samples analysed are less detected by DPPH. In samples 1 and 2, for instance, it was possible to detect the TRC with Cu(BCA)_2^{3-} complexes and the TPC (FC reagent) but not with DPPH.

Instead, there was a positive relationship between the values obtained with the Cu(BCA)_2^{3-} complexes and the TPC (*r* = 0.646) and with the labelled AA content (*r* = 0.769). TPC showed a slight positive correlation with labelled AA content (*r* = 0.381). Since both the Cu(BCA)_2^{3-} complexes and the FC reagent react indistinctly with AA and phenols (Manoel and Moya 2015), it is not possible to state that the TCR values obtained with the proposed method in the orange juices samples analysed are due to the presence of AA (natural or added) or polyphenols. Additionally, the discrepancies between the TRC values obtained with Cu(BCA)_2^{3-} complexes and the antioxidant capacity achieved with DPPH method can be also ascribed to the different experimental conditions of the analytical methods.

Despite this, the good agreement of the TRC values obtained with Cu(BCA)_2^{3-} complexes with TPC and labelled AA suggest that the proposed method could be used in samples of industrialized orange juice.

Regarding the grape juices (Table 2) a slight positive correlation (*r* = 0.220) between the DPPH reagent and Cu(BCA)_2^{3-} complexes was verified but no correlation between DPPH and the TPC was observed. A better

positive relationship between the TRC values obtained with the Cu(BCA)_2^{3-} complexes and the TPC ($r = 0.390$) was observed. In most of the grape juice samples the AA content was not labelled. Therefore establishing any correlation with the investigated parameters was not possible. Based on this and knowing that the TPC values of grape juices, (0.17–0.88) g GA/g juice, were higher than those of orange juices, (0.13–0.43) g GA/g juice, it may be concluded that these differences are owed to polyphenols. This is also in agreement with a study carried out with different types of honey (natural products such as fruit juices), which indicates that the antioxidant activity of these samples is owed also to polyphenols (Karabagias et al. 2016).

For miscellaneous fruit juices (Table 3) a very good agreement between the TRC values measured with the Cu(BCA)_2^{3-} complexes and DPPH ($r = 0.852$) reagent was found. TPC values presented in Table 3, (0.14–0.33) in g GA/g juice, also showed a good positive correlation with TRC values measured with Cu(BCA)_2^{3-} complexes ($r = 0.702$) and with DPPH ($r = 0.654$) reagent. Since only negative correlation between these three parameters (Cu(BCA)_2^{3-} , DPPH and TPC) and the AA content was found it can be speculated that other antioxidant compounds, that not only AA (Wang et al. 1996), should be responsible for the reducing activity of the juice samples presented in Table 3. It is necessary to mention that of all these juice samples (strawberry exception) contained some amount of pulp (dietary fiber) in their composition but due to the sample dilution no turbidity was observed.

Advantages and disadvantages of the proposed method in the quantification of total reducing capacity of fruits juice

1. The formation of the Cu(BCA)_2^{3-} complexes is fast as in FRAP (ferric reducing activity power) and CUPRAC (cupric reducing antioxidant capacity) methods (Prior et al. 2005) and can be performed in few minutes. Even though it was not the main purpose of the present study, it might be adapted for flow injection systems.
2. The aim of the present study was to determine the TRC of fruit juices but not the individual contribution of antioxidants. Knowing that AA is widely used as a vitamin supplement it should be mentioned that the proposed method cannot distinguish the AA present naturally in fruits or added.
3. Considering the possible interfering compounds in the proposed method it was verified that reducing sugars (such as fructose, glucose and lactose), riboflavin and citric acid do not react even when the concentration is about 10^{-4} mol/L. It could be assigned to the

conditional potential value of the Cu(II)/Cu(I) couple in the BCA^{2-} medium, ($E_x^{\text{Cu(II)/Cu(I)/BCA}} = 0.630$ V vs. NHE) (Braga et al. 2011), which prevented any positive interference of these compounds in the TRC quantification of these fruit juices samples.

4. Sodium erythorbate (E316) and BHA (E320) generally used as preservatives in soft drinks and packed food, respectively, reacted positively with the proposed method. A typical calibration curve for erythorbate aqueous solution was linear, $(20\text{--}180) \times 10^{-6}$ mol/L, with an apparent molar absorptivity at 558 nm, $(7.7 \pm 0.4) \times 10^3$ L/cm mol). For BHA the calibration curve was linear, $(2\text{--}7) \times 10^{-6}$ mol/L, with $\epsilon_{558 \text{ nm}} = (1.8 \pm 0.2) \times 10^4$ L/cm mol. Thus, BHA is 2.3 more sensitive than erythorbate, but since BHA is prepared in hydroethanolic solution (50%), these values cannot be strictly compared. In addition, they cannot be differentiated by the proposed method and if are present in the sample, may both contribute to the overall reducing capacity.
5. Similarly, both *o*-acetyl- α -tocopherol and Trolox® (an analogous water soluble compound of vitamin E) gave a positive reaction. Nevertheless, addition of *o*-acetyl- α -tocopherol in aqueous solution containing Cu(II) , ammonium acetate and BCA results to a turbid solution, probably because *o*-acetyl- α -tocopherol stock solution was prepared in pure ethanol. This may indicate that under these experimental conditions less hydrophilic antioxidants (like vitamin E) if present, do not contribute to the overall reducing capacity of processed juices. On the other hand, using Trolox® (which was prepared in an 8% (v/v) ethanol:water) is possible to obtain linear calibration curves from $(10\text{--}80) \times 10^{-6}$ mol/L with $\epsilon_{558 \text{ nm}} = (1.9 \pm 0.2) \times 10^4$ L/cm mol. Despite this good sensitivity, it is almost 100 times more expensive than AA.
6. The most commonly methods used to estimate the antioxidant activity of fruits juice was based on the DPPH (Klimczak et al. 2007) and ABTS (Cilla et al. 2009; Gülçin 2012) free radical scavenging procedures, which required organic solvents (acetone and methanol) that need to be recycled or properly disposed. Like other methods based on metallic redox couple (e.g. FRAP and CUPRAC) (Prior et al. 2005), the proposed method is performed in aqueous solution and without needing the use of organic solvents. On the other hand, the experimental procedure described in the proposed method is not suitable to measure the TRC of non-aqueous samples (e.g. olive oil).
7. No extraction process is required in the proposed method, which making it less tedious. Also it was not necessary to centrifuge or filter juice samples.

8. One limitation of spectrophotometric methods is the possible interference of coloured and turbid samples. In the proposed method, the absorbance values were taken at 558 nm ($A_{558\text{ nm}}$) and the coloration of grape or strawberry juices could interfere. However, since it is required a 25-fold dilution the sample itself does not contribute to the final $A_{558\text{ nm}}$. For the same reason, it was noted that the pulp of peach, apple, mango and cashew juices do not cause any turbidity.
9. The order of added reagents must be followed to obtain reproducibility and to prevent also precipitation of the light green precipitate of Cu(II)/BCA complexes.
10. Finally, the possibility of recovering H_2BCA , as described in our previous work (Braga et al. 2011), makes this method more friendly from an environmental point of view.

Conclusion

The reductive reaction of Cu(II) to Cu(I) in an aqueous solution containing 4,4'-dicarboxy-2,2'-biquinoline was suitably utilized in an alternative method to measure the total reducing capacity in processed fruit juices. As expected, samples with higher polyphenol content (e.g. grape juice) showed the higher total reducing capacity. For orange juice a correlation between labelled AA content and the total reducing capacity obtained with the proposed method seemed more consistent.

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Compliance with ethical standard

Conflict of interest The authors declare that they have no conflicts of interest.

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