Current Topics in Analytical Chemistry
Vol. 9, 2012

Review

Estimation of antioxidant properties of teas using DPPH assay

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ABSTRACT

Several studies indicate that the active dietary constituents of fruits, vegetables and beverages prevent free radical-induced diseases and protect against foodstuff oxidative deterioration. Antioxidant capacity is widely used as a parameter to characterize different plant materials (fruits, vegetables, wines, teas, oils). It can also be used to control variation within or between products as well as different geographical origin. There are several methods for the evaluation of the efficiency of antioxidants. 2,2-dipheny-1-picrylhydrazyl radical (DPPH) assay is one of the most popular and frequently employed methods to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of foods. This paper presents an overview of the publications regarding the use of DPPH method for *in vitro* evaluation of antioxidant properties of different kind of teas which are consumed worldwide as a desirable beverage.

KEYWORDS: tea, antioxidant properties, free radical scavenging, DPPH assay

1. INTRODUCTION

Tea is consumed worldwide as a desirable beverage and traditionally is the “cup that cheers”. It is appreciated because of its attractive aroma and taste characteristics as well as beneficial health effects. Tea is obtained from the young, tender leaves of *Camellia sinensis*, which undergo different manufacturing procedures to give various types of teas: black, green, white or oolong, they differ in appearance, organoleptic taste, chemical content as well as flavour due to their respective enzymatic oxidation process. In recent years, tea is extensively investigated mainly regarding its influence on human health [1]. Tea is an important source of polyphenols, plant derived antioxidants that are believed to explain some of the health benefits. The antioxidant activity of polyphenolic compounds is mainly due to their properties, which allow them to act as reducing agents, hydrogen donors and metal chelators.

In foods, antioxidants have been defined as a substance that in small quantities is able to prevent or greatly retard the oxidation of easily oxidizable materials such as fats. However, in biological systems the definition for antioxidants has been extended to any substance that when present at low concentrations compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substrate [2, 3]. The bioactivity of an antioxidant is dependent on several factors like its structure, physico-chemical characteristics and *in vivo* radical generating conditions. Antioxidant capacity (or activity, parameter, potential, power) is widely used as a parameter (together with others) to characterize different plant materials (fruits, vegetables, wines, teas, oils). It can also be used to control variation within or between products and allows for provision of quality standards for regulatory issues and health claim.

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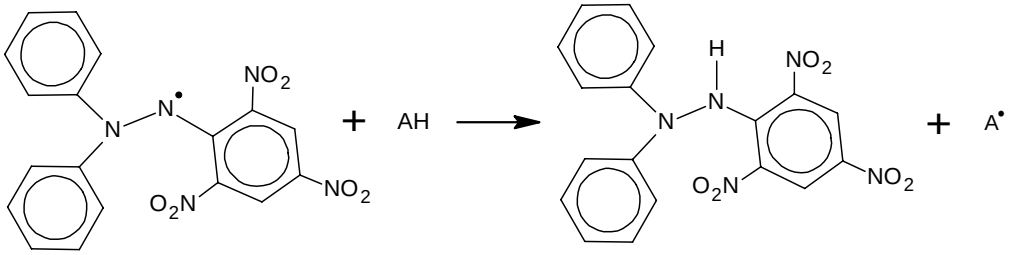
There are several methods for the evaluation of the efficiency of antioxidants or the extracts of plant materials [3-8]. 2,2-dipheny-1-picrylhydrazyl radical (DPPH) assay is one of the most popular and frequently employed methods to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant properties of foods. When a solution of radical DPPH$^{\bullet}$ is mixed with antioxidant/ reducing compound, its color turns from purple to yellow of the corresponding hydrazine (Fig. 1). The reducing ability of antioxidants towards DPPH$^{\bullet}$ can be evaluated by monitoring the absorbance decrease at about 515-528 nm until the absorbance remains stable and the resulting decolorization is stoichiometric with respect to the number of electrons taken up [4].

However, from the analytical point of view it is very difficult to compare the results of different laboratories due to different ways for their quantitation. Various research groups have used different concentration of DPPH, incubation time and pH value. This review shows to what extent the mentioned parameters have influence on the presented results.

2. Preparation of tea infusion

The content of antioxidants, mainly polyphenols, in tea infusion as simultaneously tea brew antioxidant properties may depend on many factors including type of tea (loose tea leaf, standard tea bag), amount of tea used or present in the tea bag, the size of tea leaves, how long the tea is left to infuse in water, temperature of water used for brewing as well as whether the tea bag is squeezed. A lot of cups of tea, which are drunk everyday around the world, are brewed using tea bags due to their convenience in handling and disposal. The shapes of tea bags used by manufacturers are aimed at improving the rate of tea infusion and customer convenience. Majchrzak *et al*. [9] (2004) did not observe any statistically significant differences between leaf and bagged black teas of the same type. The crucial factor is that the bag must have enough space for the free movement of tea leaves to take place. On the other hand, Rusak *et al*. [10] reported that the extraction of catechins from green tea was significantly affected by the form (bagged or loose) of the tea, whereas this effect was shown not to be statistically significant for white tea.

Teas are usually consumed as 1% w/v solutions (1 g in 100 mL) or one teabag (about 2 g) per tea cup (~220 mL). However, different ratios of dry tea leaves to volume of water have been also used for evaluation of antioxidant properties of tea infusions [11-14]. The effect of different extraction conditions (water temperature, extraction time and multiple extractions) and storage time of prepared infusions on the content of phenolic compounds have been studied [10, 15-17]. Komes *et al*. [16] reported that maximum extraction efficiency from green tea was achieved during aqueous brewing at 80^{o}C for 5 min from powder, 15 min (bagged) and 30 min (loose leaves). At 20 mg/mL concentration level, scavenging abilities of cold (4^{o}C for 24 h) and hot (90^{o}C for 20 min) water extracts from steaming green tea on DPPH radicals were 31.7-36.3% and 29.1-34.0%, respectively [11]. Thus, it seems that tea infusion after cold water brewing possesses higher antioxidant properties. Apparently, tea prepared using cold water contains lower



2,2-diphenyl-1-picrylhydrazyl 2,2-diphenyl-1-picrylhydrazine

Fig. 1. DPPH$^{\bullet}$ chemical structure and its reaction with a scavenger indicated by AH.

amount of caffeine, reduced bitterness ad higher aroma [17]. For its preparation, tea leaves are steeped in water at 25°C for at least 2 h before consumption. During 24 h storage at room temperature, fluctuations in the antioxidant capacities of green tea infusions were observed [16]. The strong tendency of polyphenols to undergo polymerization reactions increase molecular complexity and steric hindrance reduce the availability of hydroxyl groups in reactions with radicals.

The quality of a cup of tea can depend also on the kind of water used for brewing. In some countries, where the quality of tap water is poor, bottled spring, mineral or well water is used for preparation of tea infusion. Fig. 2 shows the influence of kind of water used for preparation of infusion (deionized, spring and tap water) on the results in DPPH assay for very popular *Yellow Label* black tea (Lipton brand). This tea exhibits the highest activity when deionized water was used. Danrong *et al*. [18] reported that green tea infusion prepared with deionized water contained more polyphenols in comparison with tap and activated carbon adsorbed water.

3. Chemical conditions for DPPH assay

The excess of DPPH$^{\bullet}$ reagent should be used in order to exhaust the H-donating capacity of polyphenols. However, in accordance with Beer's law and the normal practice in spectrophotometry, the initial DPPH$^{\bullet}$ concentration in a cuvette should be chosen to give absorbance values less than 1.0 (which corresponds to the light intensity being reduced no more than ten-fold in passing through the sample). This implies the final concentration for the DPPH$^{\bullet}$ solution in the cuvette in the range of 25-70 μM [19]. The experiments in the published papers have been performed using much higher DPPH$^{\bullet}$ concentration [12, 13, 20-23].

For most of the compounds that exhibited antioxidant properties, their reaction with DPPH$^{\bullet}$ is biphasic, with a fast decay in absorbance in the first minutes, followed by a slower step [24]. As can be seen from Fig. 3, black tea infusions exhibit high capacity to scavenging the DPPH$^{\bullet}$ radicals already in 5 min [25]. This fast step essentially refers to the electron transfer process from B ring (3'-OH and 4'-OH) of flavonoid molecules to DPPH$^{\bullet}$ and latter kinetic reflects the remaining activity of the oxidation-degradation products. For fruit teas, constant slow decay of DPPH$^{\bullet}$ absorbance was observed. The higher antioxidant properties of black teas could be due to the content and composition of major polyphenols. As the rate of reaction varies widely

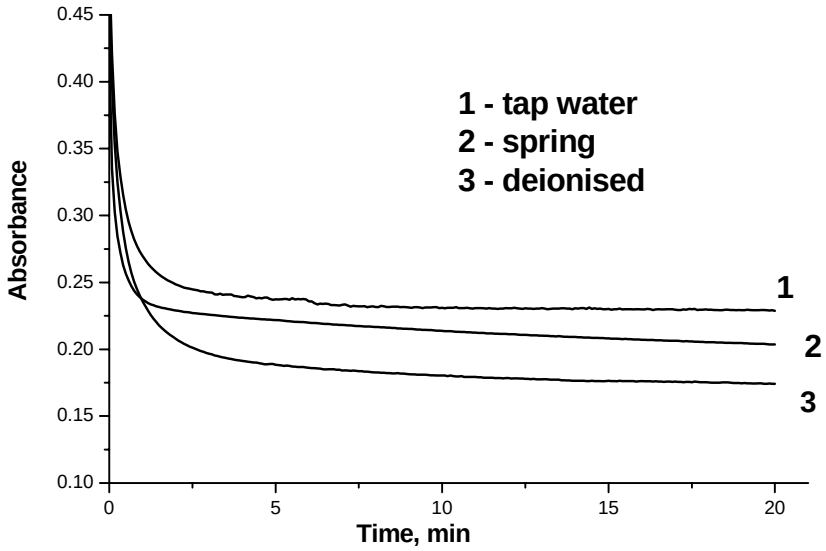


Fig. 2. The reaction rate of tea infusion (Lipton *Yellow label*) with DPPH$^{\bullet}$ radical when different waters were used for brewing.

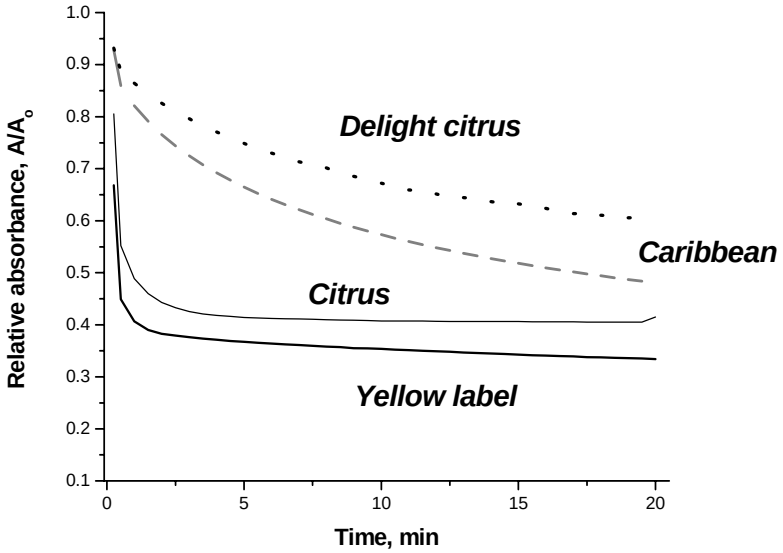


Fig. 3. The kinetic curves of scavenged DPPH$^{\bullet}$ by tea infusions. Solid lines-black teas, dotted lines - fruit teas [25].

among different antioxidants, the best practice is to follow the reaction till completion, *e.g.* when the absorbance of DPPH$^{\bullet}$ reaches a steady state. The DPPH assay is most frequently simplified by measuring DPPH$^{\bullet}$ concentration at the beginning of the reaction and after a certain incubation time, but not less than 20 min.

The results of DPPH assay have been presented in many ways. The majority of studies express the results in terms of the reduction percentage of the DPPH$^{\bullet}$ solution, referred also as percent of inhibition or quenching, and calculated as: I% = $[(A_0 - A_t)/A_0]$ x 100, where A_0 and A_t are the absorbance in the absence and presence of antioxidant. The results are also presented in the form of percentage of residual DPPH$^{\bullet}$ calculated in the following way: $DPPH^{\bullet}_{res} = [(DPPH^{\bullet})_t/(DPPH^{\bullet})_0]$ x 100, where $DPPH^{\bullet}_0$ and DPPH$^{\bullet}$ are the concentrations at initial and steady state, respectively, obtained from a calibration curve. The antioxidant concentration necessary to decrease the initial DPPH$^{\bullet}$ concentration by 50% inhibition (named as efficiency concentration EC_{50} or inhibition concentration IC_{50}) is often used for the comparison of antioxidant capacity of different compounds or extracts of natural samples. EC_{50} value is calculated using the graph by plotting inhibition percentage against extract or compound concentration and its lower value indicates higher antioxidant activity. However, this parameter highly depends on the initial DPPH$^{\bullet}$ concentration as it was shown in Fig. 4 for black tea infusion. Probably for this reason different values of EC_{50} could be found in the literature for the same compounds. One from the several examples is ascorbic acid, for which EC_{50} equals to 2.7 μM [26], 50 μM [27], 56 μM [28] and 629 μM [29] were reported. It should be noted that EC_{50} becomes unsuitable as a parameter characterizing the antioxidant properties of a given sample when we deal with the inhibition of chain processes [30].

The change of DPPH$^{\bullet}$ absorbance could be compared to the change induced by a reference compound. Several compounds have been used as a standard antioxidant in the performed experiments for tea infusions, it includes ascorbic acid [13, 31, 32], α-tocopherol [33], quercetin [34] and trolox (water soluble analogue of vitamin E) [16, 24, 35]. Selection of suitable reference compounds for *in vitro* antioxidant capacity is not an easy task to achieve [36]. The choice has to remain at the convenience of the researchers, with regard to the aim of the study. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) does not have any physiological significance and its choice as the standard for antioxidant activity is arbitrary. However, the expression of the results

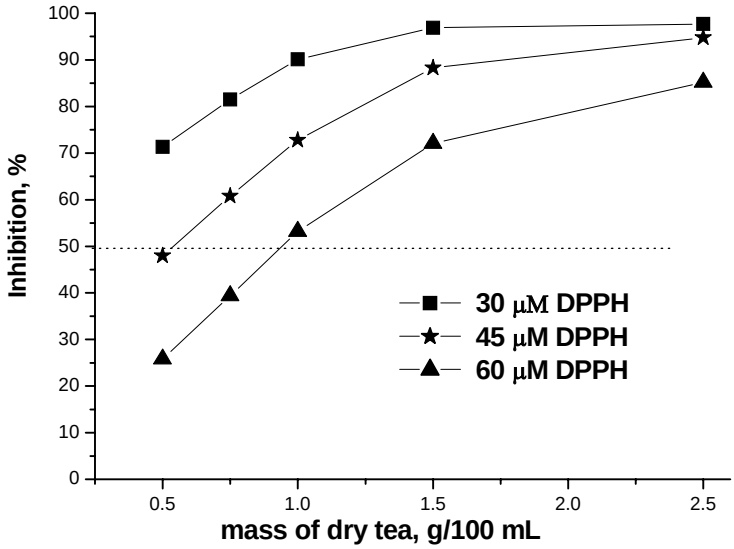


Fig. 4. The scavenging ability of black tea extracts with different concentration of DPPH•.

as Trolox equivalent (μmol of trolox necessary to provide the same antioxidant activity as a gram of the sample) helps to compare the published data.

In the original paper introducing DPPH assay [37], it was suggested that the system should be maintained at pH in the range 5.0-6.5 by using acetate buffers. However, these conditions were abandoned in the latter current practice as there is great uncertainty in the meaning of pH values for methanol or ethanol used mainly as reaction media. Generally under acidic conditions the reducing capacity may be suppressed, whereas under basic conditions proton dissociation of polyphenolics would enhance the reducing capacity of compounds [38]. Effect of pH on DPPH assay was only studied for model solutions [39, 40]. Sharma and Bhat [40] examined the DPPH• radical scavenging activity of ascorbic acid, butylhydroxytoluene (BHT) and propyl gallate using the reagent prepared in methanol and in "buffered" methanol, which was prepared by mixing 0.1 M acetate buffer (pH 5.5) with methanol (2:3, v/v). They found that the radical scavenging profiles of ascorbic acid and propyl gallate were comparable with both regent media, while for BHT it was markedly higher in buffer-methanol solution (EC_{50} = 10 μmol) in comparison to that in methanol alone (EC_{50} = 60 μmol). Similar dependence can be observed in Fig. 5. Catechin exhibits similar antioxidant activity in methanol and "buffered" methanol, while for quercetin higher value was obtained when DPPH• was prepared in 0.1 M acetate buffer at pH 5.5. The influence of pH of tea infusion on their scavenging ability is also presented in Fig. 5. *Yellow label* black tea infusion has pH equals to 4.97 and for this sample very small difference between the results were obtained. For both fruit teas - *Rosehip* (pH 2.92) and *Delight Citrus* (pH 3.37) the influence of using "buffered" DPPH• in comparison with its methanolic solution is much higher.

Metal ions are natural components of plants and their content is influenced by the type of plant, the soil composition and local environment. Their concentration in the obtained extracts of food samples depends on metal, plant type as well as extraction conditions. As the transition metal ions play a vital role in the initiation of free radical processes (*via* the Fenton reaction), metal chelation by polyphenolic compounds is widely considered as another mechanism of their antioxidant activity [41]. In addition, some flavonoids exhibit the ability to reduce Fe(III) and Cu(II) ions [42, 43]. Dawidowicz *et al.* [39] studied the difference between BHT/DPPH• reaction rates in the systems with the presence of Fe(III) and Cu(II) and without metal ions. The increase of metal concentration (up to 1 μg/mL and 20 μg/mL for Cu and Fe, respectively) caused

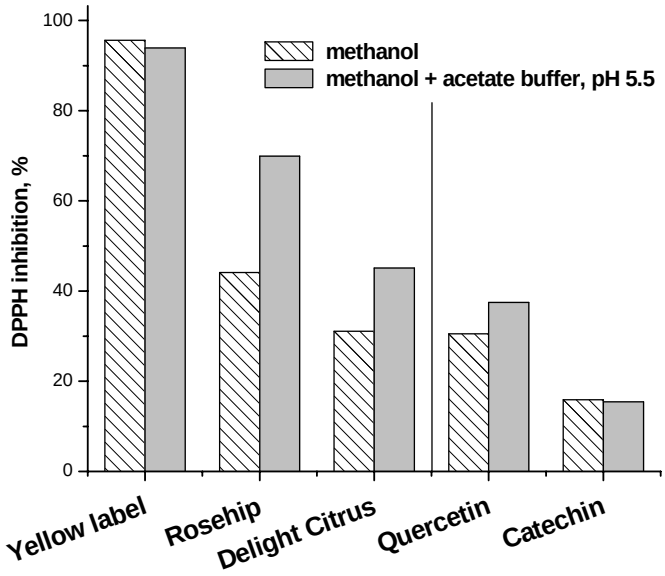


Fig. 5. Antioxidant capacity of different tea infusions as well as quercetin (100 µM) and catechin (100 µM) using DPPH$^{\bullet}$ prepared in methanol and the mixture of methanol and acetate buffer, pH 5.5.

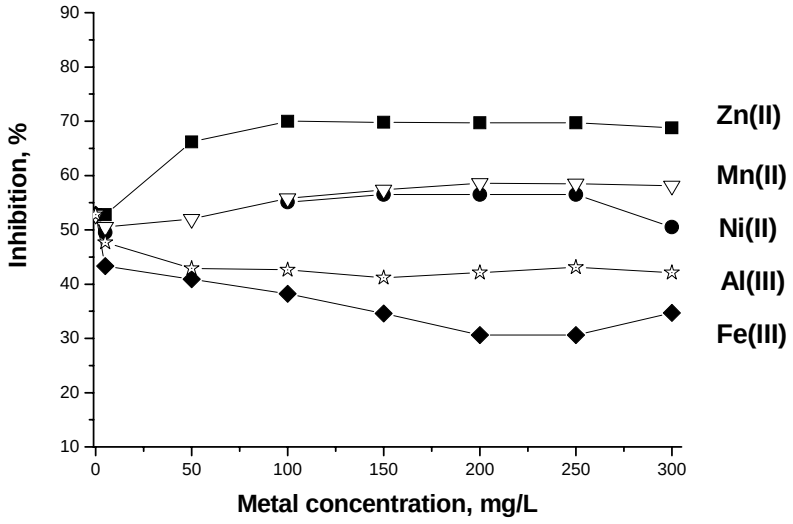


Fig. 6. The effect of the presence of metal ions on the results in DPPH assay for quercetin (100 µM).

an almost linear deceleration of the reaction kinetics. Cu(II) suppressed BHT/DPPH$^{\bullet}$ reaction kinetics more than Fe(III). The influence of some metal ions on the inhibition of quercetin in DPPH assay is presented in Fig. 6. The presence of Zn(II) gave higher results, while Al(III) and Fe(III) gave much lower.

The reported differences in the antioxidant properties of the extracts obtained from the same plant material (growing for example under different conditions) can be caused not only by their different composition of polyphenolic compounds but also by differences in the types of metal ion and their concentration in the final extract. The results in DPPH assay for samples polluted with metal ion can be different in relation to the samples free of these metal ions. Thus, it can be a source of erroneous conclusions in comparison of different samples.

4. Comparison with other assays

There are several different methods to assess antioxidant capacity as well many measures used to express the relative antioxidant capacity of different substances as well as biological samples. An antioxidant is first a reducing agent and actually most assays of antioxidant activity *in vitro* are measures of reducing power. ORAC (oxygen radical absorbance capacity) method utilizes fluorescence detection, while CUPRAC (cupric reducing antioxidant capacity), Folin-Ciocalteu (FC) assay, FRAP (ferric ion reducing antioxidant power) stable $DPPH^{\bullet}$ radical and the chemically generated $ABTS^{+}$ radical cation (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) are applied with spectrophotometric measurements. Electrochemical detection has been also used for the analysis of the anodic current waveform in cyclic voltammetry and for a potentiometric measurement of redox potential. Spectrophotometric methods are still the most widely used because the reagents are easy to get, results are given relatively quickly and the experiments are convenient.

However, these assays differ from each other in terms of substrates, reaction conditions and quantitation methods. Folin-Ciocalteu method, very popular and widely used for the determination of so-called total polyphenols content, is based on a non specific phenol oxidation reaction by the two strong inorganic oxidants (phosphotungstic and phosphomolibdic acids). This assay is conducted in alkaline medium and gives different responses to different phenolic compounds, depending on their chemical structures [7]. Moreover, the FC reagent could simultaneously oxidize several nonphenolic organic compounds as well some inorganic substances to give elevated apparent phenolic content, thus it can be used for the measurement of total reducing capacity of samples. CUPRAC method is based on reduction of Cu(II) to Cu(I) at neutral pH by antioxidants present in a sample utilizing the copper(II)-neocuproine reagent as the chromogenic oxidant. Slow reacting antioxidants needed increased temperature incubation to complete their oxidation [44]. FRAP method utilizes the reduction of ferric tripyridyltriazine (Fe^{III} -TPTZ) complex at low pH to ferrous form which has an intense blue colour and can be monitored at 593 nm. Radical cation $ABTS^{\bullet+}$ during its reaction with an antioxidant at pH 7.4 is converted to its colorless neutral form and the decrease in absorbance at 734 nm is monitored. This assay is often referred to as the Trolox Equivalent antioxidant capacity (TEAC) assay. However, there is no perfect system available to establish the “true” antioxidant capacity of a single component or a complex mixture of antioxidants. Determination of antioxidants depends strongly on the conditions selected for a test system and the obtained antioxidant values are rather relative. These methods have different reduction potentials as well as different experimental conditions, and do not yield the same values for the antioxidant capacity. Thus, there is no single, widely-acceptable assay applicable to a reasonable variety of compounds in food matrices. Therefore, to ensure that a sample is indeed the one of higher antioxidant activity, several methods should be used and their results compared.

Usually linear correlations between the results obtained by different assays have been checked. The DPPH assay showed similar trends for studied tea infusions to FC and CUPRAC method [25]. No good correlation was found between total phenolic content determined by Folin-Ciocalteu and DPPH radical scavenging capacity for green tea preparation [34, 42]. Apak *et al*. [45] obtained very high correlation coefficient (0.966) between CUPRAC antioxidant capacity and Folin-Ciocalteu total phenolic content of herbal teas. To the contrary, the same authors [46] reported that in CUPRAC method trolox-equivalent capacities of a wide range of polyphenolic compounds were linearly correlated (0.8) to those found by ABTS, but not to those by FC assay. The total quantities of polyphenols in tea samples as well as the percentage of the individual compounds are varied with the varieties and the change of tea plant living conditions. Besides, every individual component has different extent of reactions with analytical reagents, particularly in the presence of certain food components. The facts that the obtained results did not show good correlations could be the result of the synergies or antagonisms of tea infusion components in a given assay and

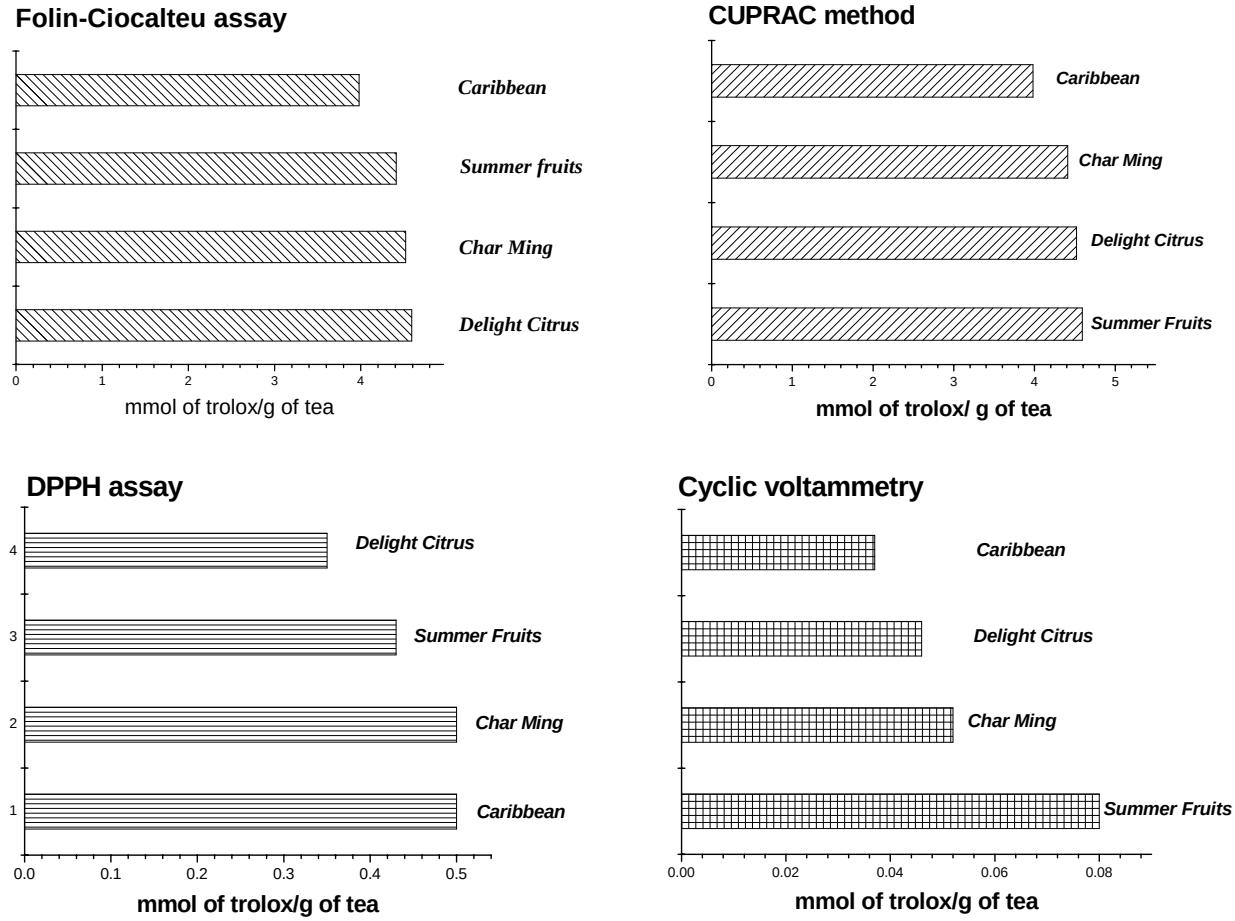


Fig. 7. Comparison of antioxidant capacity of four black flavored tea infusions obtained using different assays. All results are expressed in Trolox equivalent (mM of trolox/g of dry tea).

these processes are under investigations [47, 48]. Fig. 7 shows the comparison of antioxidant capacity of five popular black favored tea infusions obtained using DPPH, FC and CUPRAC assays as well as cyclic voltammetry. As can be seen, the order of these teas is different for each method, although, all the results are expressed in Trolox equivalent.

It should be emphasized that the employed assays are strictly based on chemical reactions *in vitro* and they bear no similarity to biological systems. It is of great importance to study whether there is a correlation between the intake of high potent antioxidants and the level of oxidative stress. Recently, the biological model systems to screen in vivo natural antioxidants have been proposed [50, 51]. They are more relevant methods than the popular chemistry assays because they account for some aspects of uptake, metabolism, and location of antioxidant compounds within cells. However, they are more complicated. Evaluation of the antioxidant status of different kind of commercially available teas will promote research on the identification and quantification of active components of these teas that may help protect consumers against free radical damage and oxidative stress-related diseases.

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