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# A new parameter to simultaneously assess antioxidant activity for multiple phenolic compounds present in food products

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ABSTRACT

In this work, we established a new methodology to simultaneously assess the relative reaction rates of multiple antioxidant compounds in one experimental set-up. This new methodology hypothesizes that the competition among antioxidant compounds towards limiting amount of free radical (in this article, DPPH) would reflect their relative reaction rates. In contrast with the conventional detection of DPPH decrease at 515 nm on a spectrophotometer, depletion of antioxidant compounds treated by a series of DPPH concentrations was monitored instead using liquid chromatography coupled with quadrupole time-of-flight (LC-QTOF). A new parameter, namely relative antioxidant activity (RAA), has been proposed to rank these antioxidants according to their reaction rate constants. We have investigated the applicability of RAA using pre-mixed standard phenolic compounds, and also extended this application to two food products, *i.e.* red wine and green tea. It has been found that RAA correlates well with the reported $k$ values. This new parameter, RAA, provides a new perspective in evaluating antioxidant compounds present in food and herbal matrices. It not only realistically reflects the antioxidant activity of compounds when co-existing with competitive constituents; and it could also quicken up the discovery process in the search for potent yet rare antioxidants from many herbs of food/medicinal origins.



## 1. Introduction

Antioxidant activity usually refers to the ability to scavenge free radicals (Brewer, 2011; Halliwell, 2011). On the basis of anti-free radical reactions, antioxidant compounds have been characterized primarily by two parameters, total antioxidant capacity (total stoichiometry, $n$) and reaction rate constant ($k$). The former is a static parameter that evaluates how much free radical one mole of the compound (or a standardized amount of mixture) can consume given sufficient reaction time. The latter is a kinetic parameter that focuses on the rate of the anti-free radical reaction (Macdonald-wicks, Wood, & Garg, 2006). Total antioxidant capacity can be used to evaluate both pure compounds and food/herbal matrices (Cai, Luo, Sun, & Corke, 2004; Deng, Cheng, & Yang, 2011). For pure compounds, the total antioxidant capacity is equivalent to the number of labile hydrogen present in one compound that can be abstracted by free radical, or expressed as stoichiometric factor ($n$), ranging from 1 to 5 for common antioxidant compounds (Goupy, Dufour, Loonis, & Dangles, 2003). As for food/herbal matrices, total antioxidant capacity has great variation which is attributed by the different abundances of responsible compounds found in food and herbs. Total antioxidant capacity provides a basis for quantitative comparison between food and herbs of different origins (Balasundram, Sundram, & Samman, 2006; Cai et al., 2004; Kasote, Katyare, Hegde, & Bae, 2015). For example, among the commonly consumed fruits, red grapes were reported to be at least 15-fold of watermelon in terms of total antioxidant capacity (Floegel, Kim, Chung, Koo, & Chun, 2011). In contrast, kinetic parameter, *i.e.* reaction rate constant ($k$) generally evaluates only pure compounds, and it categorizes antioxidants into fast, intermediate and slow kinetics according to the obtained value of $k$ in unit of $M^{-1}\,s^{-1}$ (Mishra, Ojha, & Chaudhury, 2012; Xie & Schaich, 2014). Epigallocatechin was reported to be a fast free radical scavenger giving $k$ of 3900–5500 $M^{-1}\,s^{-1}$ (Goupy et al., 2003); while $k$ of trans-ferulic acid was reported to be 110–130 $M^{-1}\,s^{-1}$ (Foti, Daquino, & Geraci, 2004).

Among a great variety of assays that are used to evaluate total antioxidant capacity and kinetic behavior, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical method is one of the most common assays due to its high stability and the ease of conducting the experiment (Huang, Ou, & Prior, 2005; Stasko, Brezová, Biskupic, & Misík, 2007; Tang, Li, Chen, Guo, & Li, 2008). DPPH is a stable nitrogen-bearing free radical that gives a maximal absorbance at 515 nm, which has been utilized to assess antioxidant capacity in that antioxidant candidates scavenge this free radical resulting in

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the decrease in absorbance. In all these assays, DPPH was given in excess, 3–10 times of the test sample; after co-incubating with antioxidant candidates for a certain period of time, ranging from 10 min to 120 min, the residual DPPH was quantified on the basis of proportionality between its concentration and the absorbance at 515 nm (Goupy et al., 2003; Huang et al., 2005; Mishra et al., 2012). To study the kinetics of antioxidant compounds, generally, following the addition of potential antioxidant compounds into DPPH, the decrease of DPPH absorbance at 515 nm was monitored over a certain period of time, from 1 to 10 min. In such an experimental setting, DPPH was also provided in much excess, 3–10 times the test compounds. A few assumptions were also made regarding the reaction mechanisms, the reaction order and reversibility of the reaction (Bondet, Brand-Williams, & Berset, 1997), in order to propose the mathematical equations that can best fit the observed curve of DPPH decrease over time; subsequently the *k* values can be calculated for each test antioxidant compound. The experimental procedure is tedious and the compounds with different mechanisms require different assumptions made to ensure the best curve-fitting. Besides, different kinetic models proposed in different laboratories can give rise to very different *k* values for the same compound (Butković, Klasinc, & Bors, 2004; Foti et al., 2004; Goupy et al., 2003; Villaño, Fernández-Pachón, Moyá, Troncoso, & García-Parrilla, 2007).

In reality, antioxidant compounds would not be prescribed or given individually, except some vitamins. All antioxidant foods or herbal supplements rich in antioxidant ingredients would be taken as what they are, *i.e.* a complex mixture with antioxidants and non-antioxidants co-existing. Although the overall antioxidant capacity demonstrated by crude extracts allows relative comparison, it would never answer the questions which compounds are responsible and how they contribute to the overall antioxidant activity. When a mixture of compounds exerts antioxidant protection, as a rule of thumb, the compounds of faster kinetics (larger *k*) would be more effective free-radical scavengers as opposed to those of slower kinetics (Goupy et al., 2003). Therefore, it is of prime importance to determine rate constants instead of static parameters such as half-effective concentration or stoichiometry.

In this paper, we intend to propose a new methodology to evaluate reaction rates of different antioxidant compounds. In contrast with the conventional method using spectrophotometer to monitor the de-colorization of DPPH at 515 nm, we use an advanced analytical instrument, *i.e.* liquid chromatography coupled with quadrupole time-of-flight (LC-QTOF) to monitor the decrease of antioxidant compounds treated by limiting amount of DPPH. Instead of assessing the compounds individually, the new methodology is able to evaluate multiple antioxidant compounds simultaneously in one experimental setting. This new methodology hypothesizes that the competition between antioxidants towards limited amount of DPPH should reflect their relative kinetic behavior. To quantitatively measure this competitiveness, we have come up with a new parameter, relative antioxidant activity (RAA), using this new analytical platform DPPH-LC-QTOF and Chou's Median-Effect equation (Chou, 2007, 2010). In this paper, we intend to show that RAA is able to give a clear ranking for compounds with different reaction rate constants, which correlates well with the reported *k* values. We also show that RAA, as a new parameter, is primarily affected by rate constants though the concentration of compounds present in the mixture also plays a role.

## 2. Experimental

### 2.1. Reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH), (±)-catechin hydrate, gallic acid, quercetin, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), protocatechuic acid, caffeic acid, epicatechin, kaempferol, kaempferol 3-O-rutinoside, myricetin, naringenin, p-coumaric acid, resveratrol, 2,4-dihydroxybenzoic acid and trans-ferulic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). LC–MS grade acetonitrile and formic acid were also purchased from Sigma-Aldrich. MilliQ water (<18.2 mΩ) was used in LC–MS analysis.

### 2.2. Preparation of mixture of reference antioxidant compound, red wine samples and tea infusions

All reference standards were dissolved in ethanol to prepare 1000 mg/L stock solutions. Further dilution using ethanol/water (50/50) was done to prepare the test solutions containing 2 mg/L of trolox and 14 phenolic compounds, respectively. This was labeled as mixture **A**. Mixture **B** and **C** were prepared similar to mixture **A** except that the concentration of gallic acid was reduced to 0.4 mg/L in mixture **B** while increased to 10 mg/L in mixture **C**.

25 μL of each red wine sample, namely Jacob's Creek shiraz cabernet (JC) and French cellars Merlot (FM), and 20 μL of trolox at 100 mg/L were added to 955 μL of ethanol/water.

One green tea bag with brand name as CheZai ® was soaked in 250 mL boiling water for 5 min. The infusion 50 μL and 20 μL of trolox at 100 mg/L were added to 930 μL of ethanol/water.

### 2.3. Experimental conditions

#### 2.3.1. DPPH assay

DPPH stock solution 1000 mg/L was freshly prepared in ethanol. Further dilution using ethanol/water (50/50) was performed to prepare 9 concentrations, *i.e.* 5, 10, 20, 50, 100, 200, 250, 400 and 500 mg/L. Samples included three mixtures (**A**, **B** and **C**), two red wine samples (JC and FM), and one green tea infusion (CheZai®). Assay was carried out by mixing 100 μL of sample and 100 μL of DPPH solutions, and subsequently kept in the dark for 120 min. At the end of reaction, 2 μL of reaction mixture was injected to LC-QTOF for analysis.

#### 2.3.2. LC-QTOF-MS conditions

LC-QTOF analysis was performed on an Agilent series 1290 Infinity HPLC instrument (Agilent, Waldbronn, Germany) coupled with an Agilent 6550 iFunnel QTOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with an electrospray (ESI) interface. The HPLC instrument includes a binary pump, an online degasser, an auto-sampler and a thermostatically controlled column compartment. Chromatographic separation was carried out at 35 °C on an Agilent Poroshell® EC $C_{18}$ column (150 mm × 3 mm, 2.7 μm). Chromatographic conditions were as follows: flow rate at 0.4 mL/min, sample injection volume of 2 μL. A binary gradient elution system consisted of ultra-pure water with 0.1% formic acid (A, v/v) and acetonitrile containing 0.1% formic acid (B, v/v). The gradient elution was programmed as follow: 0–3 min, 5% B; 3–12 min, 5–35% B; 12–18 min, 35–95% B; 18–22 min, 95% B; 22–23 min, 95–5% B. For mass detection, the operating parameters were as follows: drying gas temperature, 170 °C; drying gas flow rate, 16 L/min; nebulizer, 35 psi; sheath gas temperature, 320 °C; sheath gas flow, 11 L/min. All the acquisition and analysis of data were controlled by MassHunter® software (Agilent Technologies). Each sample was analyzed in negative mode to provide abundant information for structural identification. Mass spectra were recorded across the m/z range of 100–1100 with accurate mass measurement of all mass peaks. Accurate mass measurements of each peak from the total ion chromatogram (TIC) were obtained by means of an automated calibrate delivery system using a dual-nebulizer ESI source that introduces a low flow (40 μL/min) of a calibrating solution (calibration solution A,