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# The relative antioxidant activity and steric structure of green tea catechins – A kinetic approach

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ABSTRACT

There are eight most abundant green tea catechins (GTCs) existing in four pairs of eipimers, and carbon-3 configuration represents the only steric difference within each pair. This study aimed to use a new kinetic approach to elucidate the effect of stereochemical changes on the antioxidant activity. A mixture of eight GTCs was treated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) prepared in a series of concentrations, their relative reaction rates towards scavenging DPPH were revealed by the recently introduced parameter, *i.e.* $D_m$. The 3-*R* configuration in (−)-epicatechin, (−)-epigallocatechin and (−)-epigallocatechin gallate gave lower $D_m$ values demonstrating faster kinetics as opposed to their 3-*S* counterparts, with the only exception of the pair of (−)-catechin gallate and (−)-epicatechin gallate in which 3-*S* configuration was faster. These results suggested that the kinetic approach adopted in this study could reflect the different antioxidant activity of GTCs attributed by minor steric changes.

## 1. Introduction

Green tea is rich in polyphenolic compounds that contribute greatly to its anti-aging, anti-carcinogenetic and anti-atherosclerotic properties (Brown et al., 2011; Wu et al., 2004; Yan, Zhao, Suo, Liu, & Zhao, 2012). The majority of polyphenolic compounds present in green tea belongs to flavan-3-ol (Graham, 1992), which is structurally unique among the other flavonoids in that two chiral carbons exist at carbon-2 and carbon-3 imparting the stereoisomerism to this sub-class of flavonoids. The most abundant flavan-3-ols are referred to as the green tea catechins (GTCs). Primarily, there are four pairs of epimers, namely, (+)-catechin (C) and (−)-epicatechin (EC), (−)-gallocatechin (GC) and (−)-epigallocatechin (EGC), (−)-catechin gallate (CG) and (−)-epicatechin gallate (ECG), (-)-gallocatechin gallate (GCG) and (−)-epigallocatechin gallate (EGCG). In terms of stereochemistry, these eight compounds can be grouped as the form with (2*R,* 3*S*) configuration, including C, GC, CG and GCG, and its *epi* form with (2*R*, 3*R*) configuration, including EC, EGC, ECG and EGCG.

The eight GTCs are reported to contribute to the antioxidant properties of the green tea infusions (Šilarová, Meloun & Lenka, 2017; Peluso & Sserafini, 2017). Numerous reports have been published in order to elucidate the antioxidant mechanisms of GTCs, and their antioxidant stoichiometry and kinetics are usually evaluated by their free radical scavenging activity (Sang et al., 2002; Sawai & Sakata, 1998; Zhu et al., 2001; Zhu et al., 2000). C and EC are the most-studied epimer pair in terms of their radical scavenging activity (Muzolf, Szymusiak, Gliszczyn-Swiglo, Rietjens, & Tyrakowska, 2008; Tsimogiannis & Oreopoulou, 2006). C and EC have been found not significantly different in terms of antioxidant stoichiometry, expressed as similar half-effective concentrations ($EC_{50}$) towards quenching 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Tsimogiannis & Oreopoulou, 2006) or peroxyl radical (Kang et al., 2002). By contrast, the kinetic behavior of C and EC towards free radicals has been found very different, with EC being invariably faster than C (Butković, Klasinc & Bors, 2004; Villaño, Fernández-Pachón, Moyá, Troncoso, & García-Parrilla, 2007). This might suggest that kinetic study could be able to differentiate these epimers.

As for the rest of GTCs, their radical scavenging activities have also been studied extensively over the years (Meng et al., 2018; Kalai & Nagrarajan, 2018; Ong & Annuar, 2017; Toyo'oka, Kashiwazaki & Kato, 2003; Unno, Yayabe, Hayakawa, & Tsuge, 2002; Guo et al., 1999; Nanjo, Mori, Goto, & Hara, 1999). The majority of these studies focused on antioxidant stoichiometry. The study done by Guo et al. (1999) found that at low concentrations GCG, GC and C were stronger than EGCG, EGC and EC, respectively; while some other studies (Nanjo et al., 1999; Unno et al., 2002; Toyo'oka, Kashiwazaki & Kato, 2003) found

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that the scavenging activity was independent of their steric differences. The inconsistency in these findings could suggest that the antioxidant stoichiometry could not reflect the steric difference within each epimer pair of GTCs. Much fewer reports approached the radical scavenging activity of GTCs from the kinetic perspective. In one article that studied the antioxidant kinetics of GTCs in the DPPH-scavenging reactions (Ong & Annuar, 2017), EGCG, EGC, ECG and EC were studied for their reaction rate constants. However, due to the inclusion of only *epi* form (2*R*, 3*R*), this study was not designed to explore the steric impact on the scavenging activity. As suggested by these studies, the kinetic study that usually yields the reaction constants of a wide numeral range could be able to differentiate epimer pairs. Although C and EC have been well studied, the rest of these epimers have yet to be studied from the kinetic perspective in order to elucidate the steric impact on the antioxidant activity.

The aim of this study was to clarify if the steric change within each epimer pair of GTCs could affect the antioxidant kinetics in scavenging DPPH. When these eight GTCs as a whole were exposed to limiting amount of DPPH and their competition towards quenching DPPH could reflect their intrinsic kinetic difference. As a result, antioxidants themselves were consumed. Faster antioxidants would be consumed more while slower ones would be consumed less. A quantitative way to compare the intrinsic kinetic difference was represented by a newly coined parameter as $D_m$ value. The lower was the $D_m$ value, the faster was the antioxidant towards free radicals. Hence, the specific aim of this study was to study all the abundant GTCs using $D_m$ values in order to discover if there was significant difference in the $D_m$ within each epimer pair of GTCs.

## 2. Materials and methods

### 2.1. Reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH), C, EC, GC, EGC, CG, ECG, GCG and EGCG were purchased from Sigma-Aldrich (St. Louis, MO, USA). LC-MS grade acetonitrile and formic acid were also purchased from Sigma-Aldrich (St. Louis, MO, USA). MilliQ water (< 18.2 mΩ) was used in LC-MS analysis (Merck Millipore, Germany).

### 2.2. Preparation of the mixture of GTCs standards

All GTCs standards were individually dissolved in ethanol to prepare 400 mg/L stock solutions that were stored at −80 °C. Further dilution using ethanol/water (50/50) was done to prepare the solution containing 0.75 mg/L of eight GTCs, respectively, which was labeled as mixture-**A**. Stock solutions kept at -80 °C within 3 months were considered suitable for experiments.

### 2.3. Preparation of green tea infusions

Lipton®, Goldkili® and Chezai® green tea bags bought in local supermarkets (Singapore) were used in this experiment. Specifically, one tea bag was soaked in 200 mL boiling water for a duration of 5 min, and Lipton®, Goldkili® and Chezai® were further diluted 140-, 80- and 160-fold, respectively, by ethanol/water (50/50) prior to DPPH treatment. The dilution factor was based on the pre-test results. Briefly, 100 μL of different dilutions of tea infusion was mixed with 100 μL of 50 mg/L DPPH prepared in ethanol/water (50/50), and the absorbance at 517 nm taken by the microplate reader of Infinite® 200 Pro (Tecan Group Ltd., Switzerland) should be approximately 0.1 after incubation for 2 h at ambient temperature.

### 2.4. DPPH assay of mixture-A and green tea infusions

DPPH assay was conducted according to the previously reported with minor modification (Yang et al., 2017). DPPH stock solution 500 mg/L was freshly prepared in ethanol. Further dilution using ethanol/water (50/50) was performed to prepare eight concentrations, *i.e.* 2, 5, 10, 15, 20, 30, 40 and 50 mg/L. Assay was carried out by mixing 100 μL of sample and 100 μL of DPPH solutions, and subsequently kept in the dark for 2 h. A control was the mixture of ethanol/water 100 μL and 100 μL of sample. Samples referred to the mixture-**A** and the diluted tea infusions. At the end of reaction, 2 μL of reaction mixture was injected to LC-QTOF for analysis. For each sample, three independent experiments were conducted. Tea infusions were prepared freshly each time according to Section 2.3, and mixture-**A** was freshly prepared from the stock solutions that were stored at −80 °C.

### 2.5. Instruments and conditions

#### 2.5.1. LC-QTOF-MS conditions

The instrumental analysis was similar to the previously published except the gradient elution was adjusted to ensure the baseline separation of all epimer pairs (Li et al., 2016; Yang et al., 2017). Briefly, the analysis was performed on an Agilent series 1290 Infinity HPLC instrument coupled with Agilent 6550 ESI-Q-TOF mass spectrometer. Chromatographic separation was carried out at 35 °C on an Agilent Poroshell® EC $C_{18}$ column (150 mm × 3 mm, 2.7 μm). Mobile phase was delivered at 0.4 mL/min consisting of MilliQ water with 0.1% formic acid (**A**, v/v) and acetonitrile containing 0.1% formic acid (**B**, v/v), and they were delivered in the programmed gradient elution as follows, 0–12 min, 10–22% **B**; 12–15 min, 22–95% **B**; 15–16 min, 95% **B**; 16–17 min, 95–10% **B**; 17–19 min, 10% **B**. The key settings in acquiring mass information under negative mode were as follows, drying gas temperature at 170 °C, drying gas flow rate at 16 L/min, nebulizer at 35 psi, sheath gas temperature at 320 °C and sheath gas flow at 11 L/min. Agilent MassHunter® software was used for data acquisition and processing. Mass spectra were recorded in the range of *m/z* 100–1000. An in-line calibration was performed by an automated calibrate delivery system that introduced a low flow of a calibrant solution which gave ions at *m/z* 112.9855 and 966.0007 under negative mode.

#### 2.5.2. LC-QTOF-MS data process

Background noise and unrelated ions were removed from LC-MS raw data by the molecular feature extraction algorithm (MFE, MassHunter® Qualitative Analysis Software; Agilent). Target compounds finding was based on the accurate mass of eight reference compounds purchased commercially, with the mass tolerance of ± 10 ppm and retention time tolerance of ± 0.12 min. The MS/MS spectra obtained from tea infusions were also manually compared with that of GTCs standards to ensure unequivocal compounds identification. Results were exported as .csv file, in which peak area (PA) of each compound would be generated. Further processing of PA was done on Microsoft® Excel (2016) to calculate $D_m$ values according to the description in Section 2.6.

### 2.6. $D_m$ calculation

$D_m$ calculation was based on our previous publication (Yang et al., 2017). Briefly, Chou's median-effect equation (Chou, 2007) was employed to find $D_m$ which is defined as the median effective concentration of DPPH that consumes half of the antioxidant compound in a particular system, expressed in mg/L. $f_a$ is the fraction of an antioxidant compound that is affected by DPPH at a specific concentration and $f_u$ is the fraction of this compound that is not affected by DPPH at the same concentration. The sum of $f_a$ and $f_u$ gives 100%.

$$F_a = (PA_{control} - PA_D)/PA_{control} \times 100\% \quad (1)$$

$PA_{control}$: peak area of control;$PA_D$: peak area at concentration $D$

A linear regression was plotted where $y$ is logarithm of division of $f_a$ over $f_u$, and $x$ is logarithmic conversion of DPPH concentration ($D$)

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