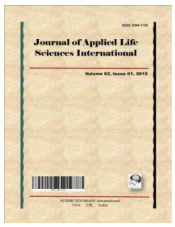

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Effect of pH on the Radical Quenching Capacity of Tea Infusions Using the ABTS$^{\bullet+}$ Assay

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Authors' contributions

This work was carried out in collaboration between all authors. Author ROA designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors YMC and NKC managed the literature searches, performed the spectroscopy analysis. Authors ROA and PSN managed the experimental process. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The aims of this study were to assess the impact of pH on the free radical quenching activity of tea infusions using a modified 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay and three antioxidant compounds as reference.
Study Design: *In-vitro* method.
Place and Duration of Study: Faculty of Life and Health Science, School of Biomedical Sciences, Ulster University, UK. From Sept 2014 and May 2016.
Methodology: Free radical quenching capacity of tea (Earl grey, black tea, Ceylon tea, & green tea) infusions were investigated using persulfate activated ABTS with acetate buffer (pH 4.5) or phosphate buffer saline (pH 7.0) as solvent. Tests were performed using 96-well microplates, 20 µl of sample and 280 µl of ABTS reagent, and calibrated using ascorbic acid, trolox or gallic acid as reference antioxidants.
Results: Gallic acid free radical quenching was pH dependent and unsuitable as reference. The free radical quenching capacity of trolox and ascorbic acid was not significantly different at pH 4.5

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and pH 7.0. The radical quenching capacity of tea infusions expressed as Trolox Equivalent Antioxidant Capacity (TEAC) or Ascorbic Acid Equivalent Antioxidant Capacity (AAEAC) was greater by 50-300% at pH 7 compared to pH 4.5.
Conclusion: The modified ABTS assay is suitable for examining the influence of pH on free radical quenching ability of tea samples. Gallic acid was not a suitable reference compound. The radical quenching capacity of tea infusions increases with rising pH.

Keywords: Radical quenching; ABTS; tea; gallic acid; ascorbic acid; TEAC value.

1. INTRODUCTION

The role of different reference compounds for standardizing antioxidant assays has not been explored in detail [1]. The first international congress on antioxidant methods recommended the "TEAC assay" for collaborative study [2]. The radical quenching assay, which uses 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and persulfate radical initiator [3], is sensitive for lipophilic and hydrophobic antioxidants over a wide pH-range [4-10]. Recent investigations highlighted the effect of pH on ATBS assay kinetics [11,12].

Consumption of tea increases plasma antioxidant capacity [13-15]. There is increasing interest also in the possible health benefits of tea extracts [16]. Currently, the evaluation of tea antioxidant capacity remains problematic due to chemical ionizations and the varying pH levels observed for the human digestive tract and also plasma [17,18]. The extraction efficiency and stability of antioxidants are also influenced by pH [19].

Free radical quenching capacity is frequently expressed as the Trolox Equivalent Antioxidant Capacity (TEAC) as measured using the ABTS assay. In a recent publication, trolox was demonstrated to be a more suitable reference compared to gallic acid for the FRAP assay of tea infusions at different pH values [20]. The aims of the present study were to examine the effect of pH on the free radical quenching activity of tea infusions using the ABTS assay. Four tea varieties were examined with trolox, ascorbic acid or gallic acid as reference antioxidants. Radical quenching capacity was evaluated at pH 7.0 and pH 4.5 and described in terms of TEAC, Ascorbic Acid Equivalent Antioxidant Capacity (AAEAC) or Gallic Acid Equivalent Antioxidant Capacity (GAEAC). The results indicate that the radical quenching capacity was increased for tea infusions at pH 7.0 compared to pH 4.5. The size of the pH effect on free radical quenching capacity varied with the antioxidant reference adopted. Such findings were confirmed using values for the IC_{50}, which is the concentration of tea required for 50% inhibition of ABTS radicals, independent of any particular reference antioxidant.

2. MATERIALS AND METHODS

2.1 Materials

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), methanol, trolox, gallic acid, ascorbic acid and most reagents were purchased from Sigma Aldrich Ltd. Four of tea bag brands were purchased from local supermarkets as follows: green tea (ASDA), Ceylon tea (Marks & Spencer), English black tea (Knightsbridge), Earl grey (Twinning's).

2.2 ABTS Reagent Formulations at pH 7.0 and pH 4.5

Free radical quenching was evaluated using the ABTS persulfate assay described previously with modification [21]. Briefly, ABTS stock solution (0.53mM) was prepared with phosphate buffer saline (pH 7) or sodium acetate (0.1M, pH 4.5) buffer. Sodium persulfate (7.4 mM) was prepared in the corresponding buffer and an ABTS working solution was prepared by adding 10ml persulfate solution and 90 ml ABTS stock solution and incubating in the dark for 16 hrs. The resulting solution was diluted with buffer so that the initial absorbance at 734 nm ($A_{734\perp}$) was 0.85 (~55 µM) as measured in a standard colorimeter (Ultrospec 2000 UV/VIS Spectrophotometer; Pharmacia Biotech).

2.3 Tea Infusions and Antioxidant Standard

Tea infusions were prepared by soaking pre-weighed tea bags with boiling water (100 ml) for 10 minutes and allowing cooling to room temperature. Samples were centrifuged (x11, 000 RPM for 5 min) using a microcentrifuge and analyzed as described below.

2.4 Microplate ABTS Assay for Tea Infusions

Exactly 20 µl of reference compounds (trolox, ascorbic acid or gallic acid 0-1 mM) was added to 96-well microplates followed with 280 µl of ABTS solution. The mixtures were agitated briefly, stored in the dark for 30 minutes and absorbance was read at 734 nm using a microplate reader. Tea infusions were pre-diluted with deionized water (250-200 fold) and analyzed similarly.

2.5 Data Analysis and Statistical Analysis

2.5.1 Calibration parameters for the ABTS assay

The ABTS colorimetric analysis conforms to Beer's Law, (eq. 1), where ΔA equal $A_{734\perp}$ minus the absorbance of the ABTS solution with added test sample ($\Delta A_{734\ (S)}$); C = concentration of test compound, and ε (M^{-1} cm^{-1}) is the molar absorptivity when the *test compound* reacts with excess ABTS.

$$\Delta A = \varepsilon\ l\ C \quad (1)$$

Calibrations graphs (ΔA versus C) were fitted with linear trend-lines (Y= x. GRAD) and values for GRAD and standard errors (GRAD ± SEM) were determined using 16 data points collected from triplicate experiments.

Calibration parameters such as the minimum detectable concentration for antioxidant (MDC) were determined; MDC = $3SD_0$/GRAD where SD_0 = standard deviation for colorimetric analysis for a reagent blank. Values for GRAD equal to the apparent molar absorptivity ($\varepsilon\ \iota$) for microplate analysis were subjected to pathlength correction as described previously [22]. In brief, ε (M^{-1} cm^{-1}) = GRAD/ microplate pathlength (0.73 cm). The stoichiometry (n) showing the number of ABTS radicals transformed per molecule of antioxidant, was determined using the molar absorptivity for $ABTS^{\bullet+}$ (1.5×10^4 M^{-1} cm^{-1}) [3] and the relation, n = ε (M^{-1} cm^{-1})/1.5×10^4 M^{-1} cm^{-1}. All routine data manipulation was conducted in Microsoft excel.

2.5.2 ABTS radical quenching referenced to trolox and other standards

The radical quenching capacity (RQC) of tea infusions was calculated from eq. (2) below;

$$RQC = \frac{\Delta A}{GRAD} * Av * \frac{Vex}{Sv} * DF * \frac{FW}{W} * 10^5 \quad (2)$$

where, Av = microplate assay volume (300 x 10^{-6}l), Vex = tea extract volume (1x 10^{-2}l), Sv = tea infusion sip volume assayed (20 x10^{-6}l), DF = dilution factor for sample (1 if undiluted), FW = formula weight of the reference antioxidant (g/mole), W = dry weight of tea sample. With trolox as reference, the units for RQC are mg-Trolox Equivalent Antioxidant Capacity per 100 g dry weight of sample (mg-TEAC/100 g). By replacing the terms for GRAD and FW in eq. (2) using the corresponding calibration parameters for ascorbic acid and gallic acid the radical quenching capacity was expressed as Ascorbic Acid Equivalent Antioxidant Capacity (AAEAC) or Gallic Acid Equivalent Antioxidant Capacity (GAEAC), respectively.

2.5.3 Radical quenching referenced to ABTS inhibitory concentration (IC_{50})

Values for the IC_{50}, which is concentration of test compound that inhibits 50% of ATBS, were determined based on the percent ABTS inhibition (%INH) defined in (eq. 3).

$$\%INH = (\Delta A / A_{734\perp}) * 100 \quad (3)$$

Combining eq. (3) and eq. (1) gives eq. (4) and eq. (5).

$$\%INH = (\varepsilon\ \iota\ C / A_{734\perp}) * 100 \quad (4)$$

$$\%INH = m.\ C \quad (5)$$

It may be expected from eq. (5) that a graph %INH versus concentration (C) produces a straight-line graph with the gradient (m) and IC_{50} was calculated from eq. (6).

$$IC_{50} = 50/\ m \quad (6)$$

3. RESULTS

3.1 ABTS Assay Calibration Using Three Reference Compounds

Table 1 show calibration parameters for the ABTS assay using trolox, gallic acid or ascorbic acid as reference antioxidants. The graphs of antioxidant concentration (mol/l) plotted versus ΔA produced straight-lines with regression coefficients (R^2) of 0.96-0.99. Tables 1 also lists the "true" molar absorptivity values obtained by

adjusting microplate values to match output from a 1-cm spectrophotometer, allowing for a pathlength of 0.73 cm [22]. The reaction stoichiometry (n) indicated that one molecule of antioxidant could quench 1-10 moles of ABTS radical (Table 1). Moreover, the response with gallic acid was sensitive to pH whilst trolox and ascorbic acid were less affected. All assays had 30-minute duration and so kinetic effects were not considered.

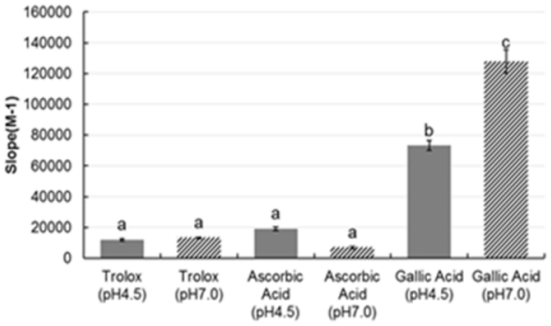

Fig. 1. ABTS calibration slopes using trolox, ascorbic acid, or gallic acid as standards at pH 4.5 and pH 7.0

Analyses were performed using 96-microplates, with 20 µl of sample and 280 µl of ABTS solution and absorbance at 734 nm. Solvents were acetate buffer (pH 4.5) or phosphate buffer saline (pH 7.0)

From Fig. 1 and Table 1, rising solvent pH increased the reaction stoichiometry for gallic acid from ~6 to 11 molecules of ABTS. In addition, the molar absorptivity change arising from gallic acid reaction with ABTS radical increased from 9.1 x 10^4 (M^{-1} cm^{-1}) to 16.0 x 10^4 (M^{-1} cm^{-1}). By contrast, the molar absorptivity with ascorbic acid or trolox were not significantly different (P= 0.05) as a function of pH. On the basis of molar absorptivity value, the ranking of free radical quenching capacity was, gallic acid (pH 7.0) > gallic acid (pH 4.5) > trolox = ascorbic acid (pH 4.5 & pH 7.0).

3.2 Radical Quenching by Tea Infusions Referenced to Standard Compounds

Fig. 2 shows the ABTS radical quenching activity for Earl Grey, black tea, green tea, and Ceylon tea infusions expressed as mg-GAEAC/100 g (Fig. 2a), mg-AAEAC/100 g (Fig. 2b) or mg-TEAC/100 g (Fig. 2c) of dried sample. Based on values for GAEAC the free radical quenching capacity for three tea infusions (green tea, Ceylon tea, and black tea) were slightly greater at pH4.5 compared to pH7 or unaffected by pH (Earl grey). By contrast values TEAC or AAEAC for tea infusions were higher at pH7.0 compared to pH4.5 (Figs. 2b and 2c).

3.3 Radical Quenching Referenced to IC_{50} Value for Tea Infusions

Fig. 3 shows values for IC_{50} ranged from 8(±0.6) µg/ml for the green tea (pH 7) rising to 24 (±1.2) µg/ml for Earl gray tea infusion (pH 4.5). All tea infusions showed *decreased* IC_{50} (increasing radical quenching) at pH 7 compared to pH 4.5. These results are not dependent on any particular reference antioxidant.

4. DISCUSSION

4.1 Effect of Reference Compounds and pH for Calibration

Trolox and ascorbic acid produced pH-stable radical quenching activity and their calibration characteristics were not statistically different over pH 4.5 to pH 7.0. In contrast, gallic acid showed pH-sensitive radical quenching capacity with $ABTS^{\bullet+}$. Such results are consistent with past reports showing that rising pH increases polyphenol hydroxyl group ionization, facilitates electron transfer and increases antioxidant capacity [20]. The current results agree also with suggestions for adopting ascorbic acid as a reference standard for the ABTS assay under some circumstance [1]. ABTS radical quenching was reportedly unchanged from pH 3-pH 6 with each molecule of ascorbic acid scavenging two ABTS molecules [7]; the current results showed an average ABTS: ascorbic acid stoichiometry of 1:1 in agreement with other results [21].

4.2 Effect of pH on the Free Radical Quenching Capacity of Tea Samples

The radical quenching capacity for tea samples showed clear differences at pH 7.0 and pH 4.5 where trolox or ascorbic acid were used as the reference antioxidant. Since, free radical quenching is ultimately a reflection of electron-transfer ability, the tendency for tea components to quench ABTS radical should decrease at low pH with increasing H^+ ion concentration. Interestingly, antioxidant capacity was sometimes found to be elevated with decreasing pH due to enhanced extractability and stability of plant components [23]. Other investigators employed trolox as a reference compound in order to compare results for ABTS, DPPH and FRAP assays [24].

Tea antioxidant capacity was demonstrably higher at pH 7.0 compared to pH 4.5, using values for IC_{50} which were not dependent on using trolox, ascorbic acid or gallic acid as a reference. However, it can be anticipated that the IC_{50} depend on the initial concentration of ABTS free radical. From eq. 4- eq. 6, it may be noted that, $m = 100\varepsilon\iota / A_{734\perp}$ and consequently the IC_{50} increases if one uses higher initial concentrations of ABTS (e.g. $A_{734\perp}/\varepsilon\iota$). This observation highlights that need to adopt well-defined ABTS concentrations during free radical quenching analysis.

Table 1. Calibration parameters for the ABTS assay at pH 4.5 and pH 7.0

pH 4.5	R^2	MCD	ε (M^{-1} cm^{-1})	CV (%)	n
Gallic acid	0.9956	5.5E-07	91080	3.3	6.1
Ascorbic acid	0.9676	6.2E-07	24170	3.3	1.6
Trolox	0.9993	7.3E-07	17500	0.5	1.2
pH 7.0	**R^2**	**MCD**	**ε (M^{-1} cm^{-1}•)**	**CV (%)**	**n**
Gallic acid	0.9914	2.9E-07	160428	6	10.7
Ascorbic acid	0.9898	1.6E-05	10903	5.8	0.7
Trolox	0.9971	4.5E-06	17166	3.9	1.1

Notes: Analysis were performed in 96-well microplates, ε (M^{-1} cm^{-1}) = molar absorptivity normalized for 1-cm pathlength instrument with a microplate pathlength = 0.76 cm, reaction stoichiometry (n) = moles of ABTS quenched per mole of reference antioxidant, MDC = minimum detectable concentration of test compound, CV (%) is the within-assay coefficient of variation (n = 16 data points)

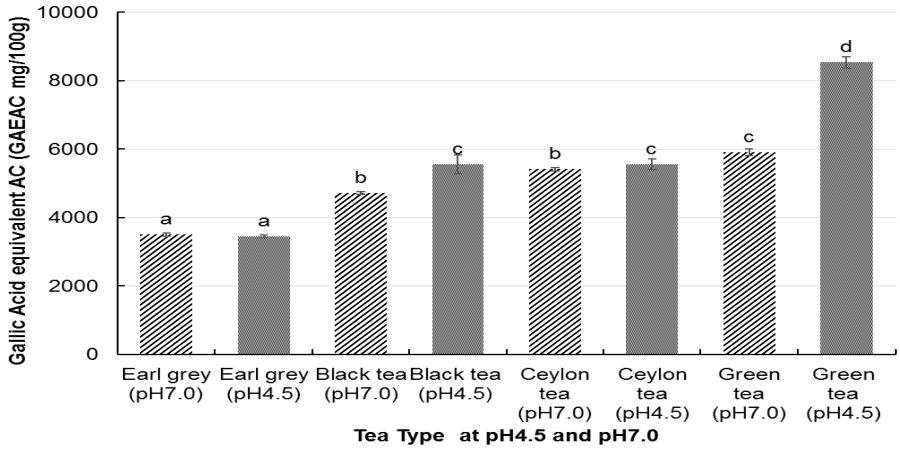

Fig. 2a. Free radical quenching activity of tea infusions at pH 4.5 and pH7.0
Data is referenced to gallic acid as standard (see text for details)

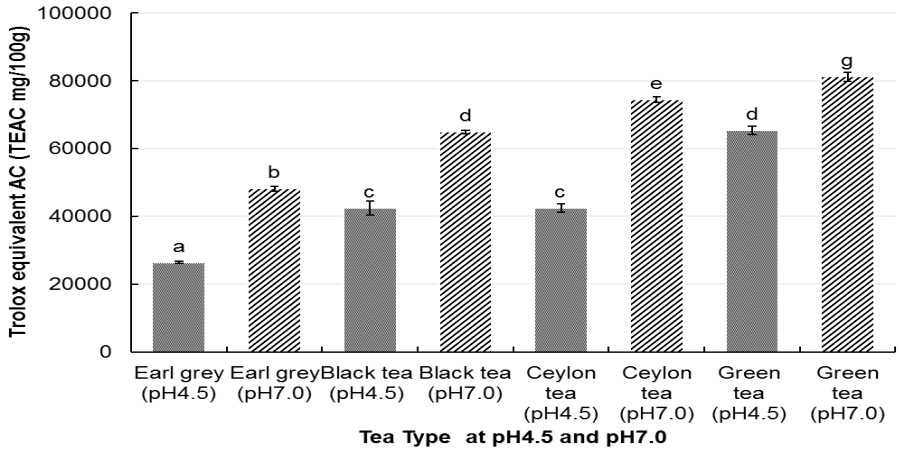

Fig. 2b. Free radical quenching activity of tea infusions at pH 4.5 and pH7.0
Data is referenced to trolox as standard (see text for details)

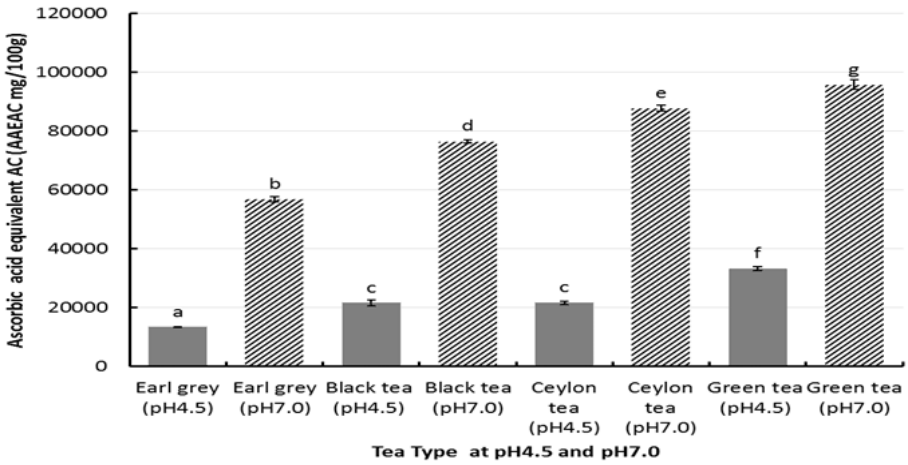

Fig. 2c. Free radical quenching activity of tea infusions at pH 4.5 and pH7.0
Data is referenced to ascorbic acid as standard (see text for details)

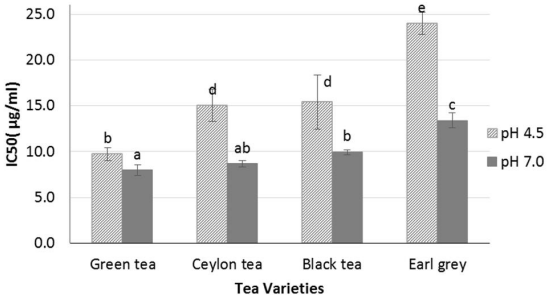

Fig. 3. Free radical inhibitory activity of tea infusions at pH 4.5 and pH7.0
Values are expressed as the 50% inhibitory concentration of tea infusion (IC50 µg/ml). Bars with different letters are significantly different (P = 0.05)

In agreement with current results, previous studies showed that the radical quenching activity of hydroxyflavones were increased due to the dissociation of hydroxyl groups at high pH [4]. The antioxidant capacity of anthocyanins were increased at neutral pH compared to acidic pH [5,6]. Caffeic acid showed increased radical quenching capacity with increases of pH but the kinetics slowed at high pH [8].

5. CONCLUSION

The free radical quenching capacity for tea infusions increases with pH but the magnitude observed depends on choice of antioxidant reference compound. Trolox or ascorbic acid are more suitable as reference compound for the ABTS assay as compared to gallic acid, which is itself pH-sensitive. As a future recommendation, assays for total antioxidant capacity should be checked for potential confounding responses which may arise from adopting a single internal reference compound.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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