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A FRAP Assay at pH 7 unveils Extra Antioxidant Activity from Green, Black, White and Rooibos Tea but not Apple Tea

Wong CW, Cheung WSM, Lau YY, Bolanos de la Torre AAS, and Owusu-Apenten R*

Faculty of Life and health sciences, School of Biomedical Sciences, University of Ulster, Cromore Road, Coleraine BT52 1SA, UK

***Corresponding Author:** Dr. Richard Owusu-Apenten, Faculty of Life and health sciences, School of Biomedical Sciences, University of Ulster, Cromore Road, Coleraine BT52 1SA, UK, Tel: (0)44-028-701-24314; Email: r.owusu-apenten@ulster.ac.uk

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Abstract

Aim: Realization of a ferric reducing antioxidant power (FRAP) assay at neutral pH and re-evaluation of tea antioxidant activity for comparisons with the standard FRAP assay.

Method: A FRAP assay at neutral pH utilized ferrozine (7.3-(2-Pyridyl)-5, 6-diphenyl-1, 2, 4-triazine-4', 4"-disulfonic acid; ferrozine) dye in conjunction with Tris-HCl buffer (0.1M. pH 7.0) with 280 µl of regent addition to 20 µl of tea infusions and absorbance measurements at 562 nm with a microplate reader.

Results: The microplate ferrozine FRAP assay (mFzFRAP) gave linear calibrations for ascorbic acid, gallic acid, ammonium ferrous sulphate, (AFS), trolox, cysteine and glutathione (R^2 = 0.998 -1.000) with molar absorptivity (measures of sensitivity) similar to literature values. The analytical precision was 5-7% and the minimum detectable concentrations (MDC) were 1.4-2.8 µM (0.4-0.8 nanomoles).

Discussion: Values for FRAP were higher at pH 7.0 compared to pH 4.0 for gallic acid, ascorbic acid, glutathione, and cysteine possibly due to their ionization at high pH. The assay sensitivity for AFS and trolox were unchanged at pH 4.0 and pH 7.0. When assayed at pH 7 the water infusions from green tea, black tea, white tea, and rooibos tea had 200-360% antioxidant activity normally observable at low pH.

Conclusion: A FRAP assay at pH 7 unveils extra antioxidant activity for green, black, white and Rooibos teas compared to values from the standard TptzFRAP (pH 3.6) method. As a recommendation, the antioxidant activity of teas and other herbal preparations should be re-evaluated over a wide pH range.

Keywords: Tea, Antioxidant activity, microplate, FRAP, Ferrozine, pathlength calibration

Highlights

A FRAP assay at pH 7.0 reveals the antioxidant activity for tea infusions maybe 200-360% of the values reported previously

Introduction

There is an ongoing need for developing new assays for antioxidant activity and for optimizing existing methods [1-3]. The ferric reducing antioxidant power (FRAP) assay [4] is a convenient method for assessing antioxidant activity by reduction of Fe (III) to Fe(II) and complexation with 2,4,6-Tripyridyl-s-Triazine (TPTZ) as a chromogenic ligand [5]. A low pH medium is thought to be essential for the TptzFRAP (pH 3.6) assay to ensure reagent longevity and antioxidant stability [4]. However, the standard TptzFRAP (pH 3.6) method has limited sensitivity to albumins which may constitute up to 28% of serum antioxidant activity [6-9]. A particular limitation relevant to foods is that polyphenols from fruits and vegetables show decreasing antioxidant activity at low pH [10-15] which might affect results obtained with the TptzFRAP (pH3.6) method. Our laboratory recently highlighted the benefits of calibrating the microplate TptzFRAP (pH 3.6) assay, using a manual pathlength correction, to achieve results consistent with a 1-cm spectrophotometer but retaining the high throughput characteristics so important for basic research [16].

Ferrozine (3-(2-Pyridyl)-5, 6-diphenyl-1, 2, 4-triazine-4', 4"-disulfonic acid) is an alternative to TPTZ for total iron determination. Compared to TPTZ, ferrozine has a higher water solubility, and wider pH (pH4-11) range [17]. The difference in physical characteristics is due to the presence of two charged sulfonate groups in the ferrozine molecule (Figure 1).

Three ferrozine molecules bind with Fe(II) species strongly with a low dissociation constant ($4x10^{15}$ M^{-3}) which may shift the

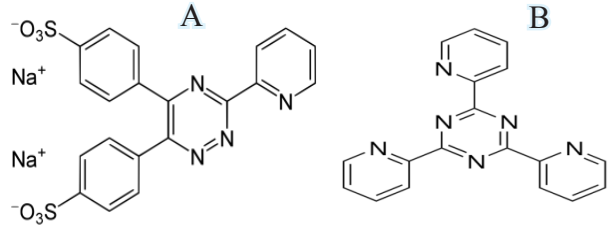

Figure 1: Structures for (A) ferrozine (3-(2-Pyridyl)-5, 6-diphenyl-1, 2, 4-triazine-4', 4''-disulfonic acid sodium salt.) and (b) TPTZ (2, 4, 6-Tris (2-pyridyl)-s-triazine; 2, 4, 6-Tripyridyl-s-triazine) used for the FRAP assays at pH 4.0 / pH 7.0 (FzFRAP) or the standard TptzFRAP assay at pH 3.6.

redox potential for Fe(III) /Fe(II) half reaction by (= 0.059 log ($4x10^{15}$) about +1.2 Volt. Ferrozine is widely applied for iron determination [18, 19], for ascorbate determination and for monitoring iron-chelating reactions [20-26]. The reductions of Fe (III) by a variety of agents were monitored using ferrozine at pH 5-8.5 [27]. Berker et al employed ferrozine as dye for FRAP analysis pH 5.5 and compared the performance for tea samples using a standard TptzFRAP (pH 3.6) assay and CUPRAC assay [28].

Tea (*Camellia sinensis*) drink is consumed worldwide and is considered an important source of polyphenol antioxidants [29]. Tea antioxidant activity was evaluated using the standard TptzFRAP (pH 3.6) assay [30-33]. Consuming tea increased plasma antioxidant activity in healthy subjects as measured using the TptzFRAP (pH 3.6) assay [31, 34-36]. The tea catechins showed enhanced antioxidant activity with increasing pH when studied individually [15]. The antioxidant activity of tea infusions analysed using the 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) method apparently depended on the intrinsic pH of the original tea [37]. The preceding data suggests a need to reassess the antioxidant activity of tea.

The overall aims of this study were, to evaluate the antioxidant activity of tea infusions at pH 7.0 using a ferrozine based FRAP (FzFRAP(pH 7)) assay adapted to the 96-well format and to compare the results with values obtained using the calibrated microplate TptzFRAP(pH 3.6) assay reported recently and which we applied to honey samples not tea [16]. In this investigation the antioxidant activity for several pure antioxidant compounds were also tested as a function of pH. Overall, this study found that the antioxidant activity of tea infusions was 200-360% times higher compared to values reported previously. The results are discussed in relation to general pH-effects on antioxidants and some design requirements for FRAP assays at pH 7.0.

Materials and methods

Materials and reagents

Sodium acetate, glacial acetic acid, Trizma base, ferrozine ((3-(2-pyridyl)-5,6-bis(4-phenylsulfonicacid)-1,2,4-triazine)), ferric chloride hexahydrate, ascorbic acid, gallic acid (GA), ammonium ferrous sulphate (AFS), trolox, glutathione, cysteine and all other reagents were purchased from Sigma-Aldrich Ltd unless otherwise stated and used as received. Tea bag samples were purchased from Twining's online, including "Pure White Tea", "Pure Green Tea", "Apple Crunch", with expiry date at least one year from the date of purchase. Redbush rooibos tea bags were bought in a local supermarket.

Reference antioxidants

Reference antioxidant compounds used as calibration standards were prepared from 1000 μM solutions. The standard antioxidants (ascorbic acid, gallic Acid, AFS, trolox, cysteine or glutathione) were diluted to 500 μM, 250 μM and 125 μM, before use. All standards were prepared daily before use.

Ferrozine FRAP reagents

Ferrozine reagent was prepared essentially as described previously [21, 38] with modifications. A stock ferrozine solution (2.2 mM) was produced by adding ferrozine (55mg) to 50 ml sodium acetate buffer (0.73M, pH 4.0), phosphate buffer saline (0.1M, pH 7.0) or Tris-HCl buffer (0.1M, pH 7.) as solvent. Ferric chloride; (10mM) was prepared by dissolving 54mg with 10 ml deionized water. A ferrozine working reagent was prepared by daily combining ferrozine stock solution with the ferric chloride solution in a volume ratio of 9:1.

Preparation of TptzFRAP regents

The TptzFRAP (pH 3.6) regent was produced as described by Benzie and Strain [4] and adapted for microplate format described recently [16].

Microplate FRAP analysis using ferrozine or TPTZ

Microplate analysis were performed by adding 20 μl of sample to flat-bottomed 96-well microplates (NUNC, Sigma Aldrich, UK) followed by 280ul of FzFRAP (pH 7) reagent or TptzFRAP (pH 3.6) reagent. The reaction mixtures were incubated in the dark at 37°C for 30 minutes and absorbance readings were recorded at 562 nm or 593 nm, respectively, using a microplate reader (VERSAmax model; Molecular devices, Sunnydale, California, USA).

Pathlength corrections for microplate data

Pathlength correction was performed as described recently to adjust microplate results to values corresponding to a 1-cm spectrophotometer [16]. Briefly, calibrations were run using microplate reader and the same solutions were evaluated using a 1-cm pathlength UV/Visible spectrophotometer (Ultrospec 2000, Pharmacia Biotech, Uppsala Sweden) in conjunction with 1-cm polystyrene cuvettes (Sarsted Ltd, Leicester, UK). The pathlength for mFzFRAP (pH 7) assay (L') was calculated from the slope (GRAD) of the respective calibration graphs; L' (cm) = GRAD (microplate)/ GRAD (1cm-spec).

Analysis of tea infusions

Individual tea bags (2-3g) were weighed intact and extracted with freshly boiled distilled water (90°C) for 15 minutes. The infused bags were removed and tea samples were allowed to cool and then centrifuged x11, 000 RMP using a Centaur microcentrifuge. Tea infusions were prepared daily and analysed for antioxidant activity as described in Microplate FRAP analysis Section).

Determination of total phenols by the Folin-Ciocalteu method

The total phenol content of tea infusions were analysed using the Folin-Denis reagent [39] with modification. Pre-diluted tea infusions (100μl) were added to microcentrifuge tubes followed by 100μl Folin reagent and 800μl sodium carbonate solution (7.5% w/v). The samples were vortexed briefly, incubated at 37°C for 20 minutes and then centrifuged at 11,000rpm for 5 minutes. After transferring 200ul of supernatants to 96-well plates samples were read using a microplate reader (VERSAmax model reader; Molecular devices, Sunnydale, California, USA) at 750nm (A750). The total phenols assay was calibrated using gallic acid (0-3mM).

Data and statistical analysis

Microplate data were exported to excel for graphing and routine calculations. FRAP calibration graphs were constructed by plotting absorbances (minus reagent blanks) versus the concentration of standard in the assay system (mol/l) on the x-axis. Data points were fitted with a linear regression trend line (y= x.GRAD) and apparent molar absorptivity (GRAD) was determined from the

graph slope by eliminating the high X-points sequentially until the squared regression coefficient (R^2) was maximum [40]. Other routine calibration parameters were determined as described previously [41]. Significant differences were tested by 1-way ANOVA with Turkey post-hoc testing for separation of means using SPSS version 22 (IBM Corporation).

Results

Calibration of the mFzFRAP assay

Table 1 summarizes calibration results the mFzFRAP assay at pH 4 and pH 7 using several pure antioxidant compounds. Ammonium ferrous sulphate (AFS) was included as non-reducing calibration standard. All calibration responses fitted to straight line equations with squared regression coefficient (R2) >0.99. The gradient (GRAD) from calibration graphs yielded apparent molar absorptivity for microplate analysis (Table 1). The detection efficiencies for different antioxidants were expressed as FRAP-values determined by dividing the GRAD for different antioxidants with the GRAD value for AFS. The mFzFRAP (pH 4) method showed FRAP-values for trolox, ascorbic acid and gallic acid were 2:2:3 respectively. In contrast, the FRAP values using the FzFRAP (pH7) for trolox, ascorbic acid and gallic acid were 2:3.4:7.2 (Table 1). For the mFzFRAP (pH 7) method we found the average precision was 5.0% with a minimum detectable concentration (MDC) of antioxidant was 2.0 μM. For antioxidant analysis using the FzFRAP (pH 4) method the precision was 6.9% and MDC was 2.8 μM (Table 1). An attempted FzFRAP (pH7) regent formulation using phosphate buffer as medium led to the formation of precipitates.

Pathlength corrected molar absorptivity

Values for GRAD (Table 1) are apparent molar absorptivity (εL', M-1) values obtained from microplate analysis and subjected to pathlength correction to make these directly comparable to data from a 1cm spectrophotometer. For example, the actual molar absorptivity for ascorbic acid was determined using the FzFRAP (pH 7) reagent and a standard 1-cm spectrophotometer as 89757 M^{-1} cm^{-1}. The light pathlength corresponding to a filling volume of 300 μL was then estimated as 0.8 cm (=71960/ 89757). The corresponding analysis at pH 4.0 yields L'= 0.73 cm and the average pathlength value for 0.76 cm was adopted for all other assays. Estimates of the "true" molar absorptivity values using the mFzFRAP (pH 7) assay for antioxidants are shown in Tables 1, column 5.

Microplate TptzFRAP (pH 3.6) assay of Benzie and Strain

The standard mTptz.FRAP (pH 3.6) assay produced linear responses for all reference antioxidants with zero intercepts and correlation coefficients (R^2) values >0.99. After pathlength correction, the molar absorptivity using the mTptzFRAP (pH 3.6) assay was 51383, 172284, 41266 and 20951 M^{-1} cm^{-1} for ascorbic acid, gallic acid, trolox and AFS respectively, with an average MDC equal to 0.43μM for antioxidants similar to values reported previously [16].

Tea antioxidant activity at pH 3.6 and pH 7.0

The antioxidant activity of tea infusions was determined using the mTptzFRAP (pH 3.6) assay or the mFzFRAP (pH 7) assay and the results were expressed, in terms of ferric ions reduced (μmol Fe) per gram tea dry-weight basis using the relation,

Table 1: Ferrozine microplate assays at pH 4.0 and pH 7.0

	GRAD ε.L' (M^{-1}) /10^4	R^2	MDC (10^{-7} M)	Absorptivity *ε.($M^{-1}cm^{-1}$) /10^4	Error %	FRAP value
mFzFRAP(pH 4)						
Ascorbic Acid	4.120±0.0283 (b)	0.998	29.5	5.386	6.2	1.8
Gallic Acid	6.988±0.1317 (c)	0.992	7.56	9.136	2.8	3.0
Trolox	4.4067±0.3705 (b)	1.000	1.50	5.768	3.6	1.9
AFS	22992±0.0175 (a)	1.000	64.1	3.006	10.2	1.0
mFzFRAP(pH 7)						
Ascorbic Acid	7.196±0.4962 (d)	0.999	9.2	9.407	5.9	3.4
Gallic Acid	15.22±0.1394(e)	0.997	3.9	1.990	1.4	7.2
Trolox	45.81±0.2870 (b)	1.000	4.7	3.152	7.0	1.9
AFS	2.118±0.949 (a)	1.000	38.4	2.769	6.8	1.0
Cysteine	1.544±0.1553 (f)	1.000	51.9	2.018	5.5	0.7
Glutathione	2.664±0.1389(g)	0.999	8.3	3.483	3.1	1.3

GRAD = gradient of calibration graphs for microplate ferrozine FRAP assay, R2 = correlation coefficient, MDC = minimum detectable concentration, * = molar absorptivity corresponding to a 1-cm spectrophotometer, Error (%) is a measure of assay precision, Effective pathlength (L') = 0.76 cm. In column 2 values with different letters are significantly different ($p<0.05$)

$$FeEAC = \frac{\Delta A}{GRAD} * \frac{A_v}{Sp_v} * D * \frac{1}{C_{ext}} * 10^5 \quad (1)$$

In the above relation, FeEAC is the ferric ion equivalent antioxidant activity (µmol /g), ΔA = absorbance change corrected for the reagent blank, GRAD (M-1) is gradient from the AFS calibration graph, A_v = total assay volume (300µl), Sp_v = sip volume (20µl) of tea infusion analysed, C_{ext} = concentration of tea extract that is, weight of tea bags (grams) divided by volume (litres) of hot water during infusions (g /l), D = dilution for tea infusions prior to analysis (D = 1 for undiluted infusion). To express tea antioxidant activity in terms of trolox equivalent antioxidant activity (TEAC; µmole/g) then GRAD from the trolox calibration graph was inserted into equation (1).

Table 2 summarizes values for total antioxidant activity for apple tea, black tea, green tea, Rooibos tea, and white tea expressed in terms of FeEAC, gallic acid equivalent antioxidant activity (GAEAC), ascorbic acid equivalent antioxidant activity (AAEAC) or TEAC. Table 2 also summarizes estimates for the *average* FRAP-value based on tea infusion data. The order of antioxidant activity at pH 3.6 was, green tea > white tea > black tea > apple tea > rooibos tea. At pH 7 the order of antioxidant activity was green tea > white tea = black tea > rooibos tea > apple tea.

Discussion

The study was concerned not with improving the efficiency of antioxidant *extraction* from tea. Therefore only hot water infusions were studied. Teas were prepared under identical conditions and analysed using the mTptzFRAP (pH 3.6) method or the mFzFRAP (pH 7) method; in addition the total phenols content was determined. All analyses were performed contemporaneously. Previous research noted the low sensitivity of the TptzFRAP (pH 3.6) for thiol antioxidants including, serum albumin, glutathione, and cysteine and so two thiol antioxidants were included in the analyses [6]. The main concern was to evaluate possible systematic differences between FRAP assay results at low pH and results performed at pH 7.0.

PH and antioxidant activity

Redox potential is a measure of antioxidant activity and is related to pH by the Nernst equation, Eh = $E^o \pm (0.059/n)$ pH, where E^o is standard redox potential at a nominal hydrogen ion activity of IM (pH =0), and Eh is the observed redox potential at any other pH. For a one-electron reduction (n=1) then each unit pH change produces a 59 mV change in the redox potential provided that the redox reaction involves an ionisable reductant or oxidant and one of these compounds has an appropriate pKa value near the pH range of interest [42]. Tea polyphenols, under consideration in this study, show rising antioxidant activity with increasing pH due to ionization of phenol groups [12-15].Thiol groups for which there is low FRAP at low pH are relatively basic (pKa 8-9) and protonation at low pH is expected to increase the value for Eh and thereby lower their antioxidant activity; similarly the nucleophilic character of thiol compounds decreases with decreasing pH [43] The Nernst equation predicts that a FRAP assay at pH 7 could unveil extra antioxidant activity compared to values at pH 3.6 assuming tea contains moderately acidic or basic antioxidants with pKa near about pH 7.

Table 2: Antioxidant activity of tea infusions determined using a FRAP assay at pH 3.6 and pH 7.0

mTptzFRAP (pH 3.6)	**GAEAC (µmol/g)**	**AAEAC (µmol/g)**	**TEAC (µmol/g)**	**FeEAC (µmol/g)**	**TP** (µmol/g)**
Apple Tea	90.3	255.0	324.0	503.0	104.0
Black Tea	153.1	432.0	549.0	853.0	540.0
Green Tea	318.0	898.0	1141.0	1771.0	666.0
Rooibos Tea	54.0	154.0	195.0	303.0	166.0
White Tea	274.0	774.0	982.0	1525.0	501.0
FRAP value*	6	2	2	1	
mFzFRAP (pH 7)	**GAEAC (µmol/g)**	**AAEAC (µmol/g)**	**TEAC (µmol/g)**	**FeEAC (µmol/g)**	**TP** (µmol/g)**
Apple Tea	90.0	190.0	297.0	510.0	104.0
Black Tea	350.0	750.0	1174.0	3090.0	540.0
Green Tea	580.0	1230.0	1925.0	4180.0	666.0
Rooibos Tea	100.0	220.0	348.0	750.0	166.0
White tea	420.0	890.0	1394.0	3190.0	501.0
FRAP value*	7	3	2	1	

Data for tea 1.3-2% infusions prepared at 90°C. Antioxidant activity is expressed as µmole (reference antioxidant) per gram dry weight (tea); labels are gallic acid equivalent antioxidant activity (GAEAC), ascorbic acid equivalent antioxidant activity (AAEAC), ferric iron equivalent antioxidant activity (FeEAC) or trolox equivalent antioxidant activity (TEAC). *Average FRAP values using FeEAC as reference. **Folin total phenols results (µmol GAE/ g tea)

Effect of pH on pure antioxidants

The molar absorptivity for AFS using the mFzFRAP (pH 7) assay was not statistically different from results at pH 4.0 (Table 1). The average molar absorptivity from the current study for AFS was 28871 (M^{-1} cm^{-1}) in agreement with literature values of 27900

to 28600 M^{-1} cm^{-1} at pH 4-pH 10 [17, 44]. By contrast results for ascorbic acid showed the molar absorptivity at pH 4 was statistically different from the value at pH 7 (Table 1). Published data shows the molar absorptivity for ascorbic acid using ferrozine at pH 3.0 was 55800 M^{-1} cm^{-1} [20] and the value rises to 60700 M^{-1} cm^{-1} at pH 5.5 [28]. For the mFzFRAP assay for gallic acid we found the molar absorptivity at pH 4 (this study) increasing at pH 7.0 (Table 1). Previous research also reported molar absorptivity of 158000 M^{-1} cm^{-1} for gallic acid at pH 5.5 [28].

A synthesis of pH effects on antioxidant activity combining current results and published data is shown in Figure 2. Clearly, the antioxidant activity for trolox, and AFS were not pH sensitive and there is some justification for presenting results in terms of FRAP-values and or trolox values (not shown). However, the available data suggests that the antioxidant activity for ascorbic acid increases at pH > 5.5. For gallic acid there was a linear increase in antioxidant activity with increasing pH (Figure 2).

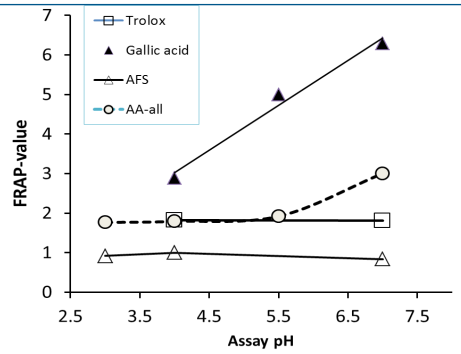

Figure 2: Effect of pH on the antioxidant activity for gallic acid, ascorbic acid and trolox expressed as the FRAP-value. Data synthesis of results from present study at pH 4 & 7 and literature data at pH 3.0 and pH 5.5 For gallic acid the equation of the straight line is, FRAP-value =1.14 pH – 1.54. For ascorbic acid at pH 5.5-7.0 the straight-line equation is, FRAP-value = 0.7882pH – 2.24. (See text for details).

Past research confirms that the redox potential for ascorbic acid and glutathione decreases by 60mV for each unit-pH rise. Ascorbic acid showed increasing reducing power with rising pH in line with ionizable groups with pKa_1= 4.2 & pKa_2 = 11.6. The pH effect on glutathione could be explained by thiol group ionizations with pKa = 8.92 [42]. The rises in FRAP-value for gallic acid (Figure 2) are also in agreement with recent reports which showed the TEAC value gallic acid increase from pH 2 – pH 9 due to the ionization of phenyl hydroxyl groups with pKa = 7.68-7.73. The other tea polyphenols (catechin, epecatechin, epigallocatechin gallate) also showed increasing antioxidant activity with increasing pH in line with phenyl hydroxyl group ionizations with pKa = 8.64-8.97[15].

Trolox is a water-soluble analogue for vitamin E and is used frequently as the reference antioxidant for the ABTS antioxidant assay [45]. Past research showed the antioxidant activity trolox was unaffected by pH values below 10 which is consistent with results in Figure 2. However, the antioxidant activity of trolox increased above pH 10 due to phenyl hydroxyl group ionization with pKa = 11.9-12.1 [46]. As further support, the 4-tocopherol isomers also showed a constant reducing activity at pH 4-10 and rising antioxidant activity at high pH corresponding to ionization transition(s) with pKa = pH 13.0 [14].

In short, the synthesis of evidence from this paper and past investigations (Figure 2) suggest the antioxidant activity of trolox is unaffected at pH 4-pH 10. In contrast, gallic acid and ascorbic acid show rising antioxidant activity with increasing pH (Figure 2). Such pH-dependant changes should raise concerns for using ascorbic acid and gallic acid as reference compounds for in-vitro antioxidant assays (Table1).

Tea antioxidant activity, total phenols and effect of pH

Not surprisingly some green tea brands will show higher antioxidant activity detected by TptzFRAP (pH 3.6) method when compared to the brands examined in this work. Differences in extraction conditions [47] and agronomic conditions [48] will also produce variations in results. Distilled water tea infusions showed higher antioxidant activity compared to tea infusions prepared using tap water [49]. Hot and cold water infusions had broadly similar antioxidant activity [50]. Soultani et al. reported 2839 µmol FeEAC/g (green tea) and 1768 µmol FeEAC/g (black) for samples with total phenols content of 1111.0 µmol GAE/g and 723 µmol GAE/g(black tea) [33]. Other tea brands gave TptzFRAP(pH 3.6) results similar to values in this report (Table 2). For instance, Sato and Benzie [30] reported the antioxidant activity for tea infusions on a dry basis as 272-1144 FeEAC µmol/g (green tea), and 132-654 µmol FeEAC/g (black teas). For 26 different tea samples the overall range of values for FRAP was 132-1144 µmol FeEAC/g whereas the total phenols content for teas ranged from 387-624 µmol (gallic acid)/g. The FRAP assays results for tea could vary by 2-3 fold within each class of (green, white, black etc.) due to possible differences in terms of agronomic/ production factors, and differences in brands [51]. Irrespective of any brand differences in tea antioxidant activity, evaluating antioxidant activity at low pH may underestimate results by 200-360% (Figure 3).

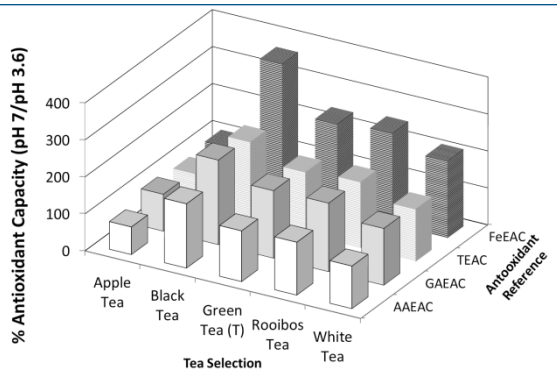

Figure 3: Relative antioxidant activity of tea infusions measured using standard TptzFRAP method (pH 3.6) FzFRAP assay. Antioxidant activity cited as Gallic acid equivalent (GAEAC), ascorbic acid equivalents (AAEAC), trolox equivalents (TEAC) or Ferric ion equivalent (FeEAC) values

We found literature support for the FzFRAP (pH 7.0) results in the current study which showed that the FRAP of tea infusions were underestimated at low pH (Figure 3). The antioxidant activity for a variety of seven tea types were consistently higher using the FzFRAP(pH5.5), compared to results from standard TptzFRAP(pH 3.6) assay, but lower that than CUPRAC (pH 7) results. For green tea samples the FeEAC was ~684 µmol/g and 1264 µmol/g evaluated using the TptzFRAP(pH 3.6) or FzFRAP (pH5.5) methods, respectively [28]. Of the major flavonoids in

tea about 86% of the FRAP characteristics were accounted for by (–)-epicatechin and (+)-catechin. [52]. In agreement with our data, changing pH4 to pH 7 increased the TEAC value for all the major tea catechins by about 2-3 fold [15]. Finally, the antioxidant activity of wine [12] and beer [53] polyphenols were also demonstrated to increase with increasing pH indicating that this phenomenon (Figure 3) may be general and not unique to tea.

General issues for FRAP assay formulations at pH 7.0

The original TptzFRAP (pH 3.6) assay was designed for centrifugal analysers where reagent stability and a high rate of reduction of Fe(III) ion at low pH were important [4]. A range of factors could militate against using neutral pH media in conjunction with the TptzFRAP assay; (a) TPTZ has a large bulky structure with zero charge (Figure 1) and low water-solubility under neutral conditions, (b) alkaline conditions are thought to promote Fe(II)/ Fe(III) hydrolysis forming insoluble hydroxides with negative consequence on FRAP reagent stability., (c) ascorbic acid and other plasma antioxidants initially evaluated using the FRAP assay are stabilized by low pH, (d) high concentrations of acetate (the default buffer for the TptzFRAP method) may neutralize interferences with ascorbic acid reduction of Fe(III) caused by other organic acids, (e) excessively high pH (>pH 7.0) or low pH (pH<3.0) were found to decrease the rate of Fe(III) reduction leading to an incomplete reaction [21, 54]. Reduction of Fe(III) by ascorbic acid decreased at pH <3.0 due to protonation of ferrozine. High pH values led to declining ascorbic acid response due to iron (III)-OH formation and a declining rate of reduction for the Fe(III) hydroxyl complex [20]. The reaction time for ascorbic acid determination in fruit juice were sluggish but improved after the addition of aluminium chloride and an assay time of 6 minutes was deemed sufficient for fruit juice antioxidants though longer times might be suited for other plant foods [20], (f) low pH conditions were convenient for the analysis of plasma extracts produced by TCA precipitation of proteins, and (g) solvents other than acetate buffer led to concerns regarding metal-ion buffer interactions, e.g. phosphate binds Fe(III) leading to precipitate formation; (h) oxygen-rich buffers produce a tendency for Fe(II) autoxidation via the Fenton reaction but nitrogen-based buffers were less auto-oxidizing [55].

In support for the FzFRAP (pH 7) assay, prior research showed that ferrozine could be used for Fe (II) detection in conjunction with a variety of buffers and pH values. After reacting Fe(III) with a variety of reductants at pH 5.5 to 8.0 the Fe(II) species was monitored using excess of ferrozine [27] partly because Fe(II) hydroxide formation was inhibited by the presence of the high affinity ferrozine ligand. Ferrozine was also used for iron determination at pH 9.5 (ammonium acetate-ammonium hydroxide buffer) in the presence of strong reducing agents, such as thioglycolic acid [56]. Ferrozine dissolved in HEPES buffer (pH 7.0) was adopted as the medium for ascorbic acid analysis [57]. The upper pH limit for the ferrozine-Fe(II) reaction was not fixed but reliant on the specific buffer choice; pH 6.5 (acetate buffer), pH 7 (phosphate), pH 7.5 (bicarbonate buffer), pH 8.0 (triethanolamine), pH 8.5 (tris-HCl) or pH 9,0 for borate buffer [44]. Past research demonstrated that ferrozine-Fe (II) complex was stable at pH 4-pH11 [27, 44, 54]. Finally, the ferrozine reagent used for this study was identical to (1.85mM Ferrozine plus 0.93mM iron (III)) chloride) that was adopted for autoanalyzer determination of plasma ascorbic acid in acetate buffer at pH 4.0 [21, 38]. There was also a 15-fold sample dilution rate (20 µl of test compound with 280 µl FRAP reagent) in order to ensure sufficient buffering activity.

Conclusions and recommendations

The FRAP assay at pH 7.0 using a ferrozine dye and a microplate format to improve throughput shows that the antioxidant activity of tea infusions is higher than previously estimated at low pH. This paper suggests there is some merit in expanding the scope of existing antioxidant assays to cover a wide range of pH conditions. As a recommendation, current antioxidant activity estimates determined under pH conditions removed from physiological pH values need re-evaluating.

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References

1. Gulcin I. Antioxidant activity of food constituents: an overview. Arch Toxicol. 2012; 86(3):345-91. doi: 10.1007/s00204-011-0774-2.
2. Apak R, Shela Gorinstein, Volker Böhm, Karen M Schaich, Mustafa Özyürek, Kubilay Güçlü. Methods of measurement and evaluation of natural antioxidant capacity/activity (IUPAC Technical Report). Pure and Applied Chemistry. 2013; 85(5):957-998.
3. Fraga CG, Oteiza PI, Galleano M. In vitro measurements and interpretation of total antioxidant capacity. Biochim Biophys Acta. 2014; 1840(2):931-4. doi: 10.1016/j.bbagen.2013.06.030.
4. Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem. 1996; 239(1):70-6.
5. Collins P, Diehl H, Smith GF. 2,4,6-Tripyridyl-s-triazine as reagent for iron. Determination of iron in limestone, silicates, and refractories. Analytical Chemistry. 1959; 31(11):1862–1867. doi: 10.1021/ac60155a056
6. Cao G, Prior RL. Comparison of different analytical methods for assessing total antioxidant capacity of human serum. Clin Chem. 1998; 44(6 Pt 1):1309-15.
7. Prior RL, Wu XL, Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agric Food Chem. 2005; 53(10):4290-4302.
8. Apak R, Güçlü K, Ozyürek M, Karademir SE, Altun M. Total antioxidant capacity assay of human serum using copper(II)-neocuproine as chromogenic oxidant: The CUPRAC method. Free Radic Res. 2005; 39(9):949-961.
9. Benzie IF, Choi SW. Antioxidants in food: content, measurement, significance, action, cautions, caveats, and research needs. Adv Food Nutr Res. 2014; 71:1-53. doi: 10.1016/B978-0-12-800270-4.00001-8.
10. Tyrakowska B, Soffers AEMF, Szymusiak H, Boeren S, Boersma MG, Lemańska K. TEAC antioxidant activity of 4-hydroxybenzoates. Free Radic Biol Med. 1999; 27(11-12):1427-1436.
11. Paiva Martins F, Gordon MH. Effects of pH and ferric ions on the antioxidant activity of olive polyphenols in oil-in-water emulsions. Journal of the American Oil Chemists Society. 2002; 79(6):571-576. doi: 10.1007/s11746-002-0524-4.
12. Borkowski T, Szymusiak H, Gliszczyńska-Rwigło A, Rietjens IM, Tyrakowska B. Radical scavenging capacity of wine anthocyanins is strongly pH-dependent. J Agric Food Chem. 2005; 53(14):5526-34.

13. Amorati R, Pedulli GF, Cabrini L, Zambonin L, Landi L. Solvent and pH effects on the antioxidant activity of caffeic and other phenolic acids. J Agric Food Chem. 2006; 54(8):2932-7.

14. Mukai K, Tokunaga A, Itoh S, Kanesaki Y, Ohara K, Nagaoka S, Abe K. Structure-activity relationship of the free-radical-scavenging reaction by vitamin E (alpha-, beta-, gamma-, delta-Tocopherols) and ubiquinol-10: pH dependence of the reaction rates. J Phys Chem B. 2007; 111(3):652-62.

15. Muzolf M, Szymusiak H, Gliszczyńska-Swigło A, Rietjens IM, Tyrakowska B. pH-Dependent radical scavenging capacity of green tea catechins. J Agric Food Chem. 2008; 56(3):816-23. doi: 10.1021/jf0712189.

16. Bolanos de la Torre AA, Henderson T, Nigam PS, Owusu-Apenten RK. A universally calibrated microplate ferric reducing antioxidant power (FRAP) assay for foods and applications to Manuka honey. Food Chem. 2015; 174:119-23. doi: 10.1016/j.foodchem.2014.11.009.

17. Stookey LL. Ferrozine-a new spectrophotometric reagent for iron. Analytical Chemistry. 1970; 42(7):779-781. doi: 10.1021/ac60289a016.

18. Guo ML, Carlos Perez, Yibin Wei, Elise Rapoza, Gregory Su, Fadi Bou-Abdallah. Iron-binding properties of plant phenolics and cranberry's bio-effects. Dalton Transactions. 2007; 43:4951-4961. doi: 10.1039/b705136k.

19. Dairam A, Fogel R, Daya S, Limson JL. Antioxidant and iron-binding properties of curcumin, capsaicin, and S-Allylcysteine reduce oxidative stress in rat brain homogenate. J Agric Food Chem. 2008; 56(9):3350-3356. doi: 10.1021/jf0734931.

20. Jaselskis B, Nelapaty J. Spectrophotometric determination of microamounts of ascorbic acid in citrus fruits. Anal. Chem. 1972; 44(4):379–381. doi: 10.1021/ac60310a001.

21. Butts WC, Mulvihill HJ. Centrifugal analyzer determination of ascorbate in serum or urine with Fe3+/Ferrozine. Clin Chem. 1975; 21(10):1493-7.

22. Adeniyi W, Jaselskis B. Spectrophotometric determination of microamounts of tocopheryl acetate (vitamin E) in multi-vitamin capsules. Talanta. 1980; 27(11 Pt 2):993-6. doi:10.1016/0039-9140(80)80133-0.

23. McGown EL, Rusnak MG, Lewis CM, Tillotson JA. Tissue ascorbic acid analysis using ferrozine compared with the dinitrophenylhydrazine method. Anal Biochem. 1982; 119(1):55-61.

24. Miller NJ, Diplock AT, Riceevans CA. Evaluation of the total antioxidant activity as a marker of the deterioration of apple juice oil storage. J. Agric. Food Chem. 1995; 43(7):1794-1801. doi: 10.1021/jf00055a009.

25. Molina Diaz A, Ortega-Carmona I, Pascual-Reguera MI. Indirect spectrophotometric determination of ascorbic acid with ferrozine by flow-injection analysis. Talanta. 1998; 47(3):531-536.

26. Chvatalova K, Iva Slaninováb, Lenka Březinováa, Jiří Slaninaa. Influence of dietary phenolic acids on redox status of iron: Ferrous iron autoxidation and ferric iron reduction. Food Chemistry. 2008; 106(2):650-660. doi:10.1016/j.foodchem.2007.06.028.

27. Cowart RE, Singleton FL, Hind JS. A comparison of bathophenanthrolinedisulfonic acid and ferrozine as chelators of iron(ii) in reduction reactions. Anal Biochem. 1993; 211(1):151-155.

28. Berker KI, Kubilay Güçlü, Birsen Demirataa, Reşat Apak. A novel antioxidant assay of ferric reducing capacity measurement using ferrozine as the colour forming complexation reagent. Anal. Methods. 2010; 2(11):1770-1778. doi: 10.1039/C0AY00245C.

29. Prior RL, Cao G. Antioxidant capacity and polyphenolic components of teas: implications for altering in vivo antioxidant status. Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.). 1999; 220(4):255-61. doi: 10.1046/j.1525-1373.1999.d01-44.x.

30. Benzie IFF, Szeto YT. Total antioxidant capacity of teas by the ferric reducing/antioxidant power assay. J Agric Food Chem. 1999; 47(2):633-636.

31. Benzie IFF, Szeto YT, Strain JJ, Tomlinson B. Consumption of green tea causes rapid increase in plasma antioxidant power in humans. Nutr Cancer. 1999; 34(1):83-87.

32. Langley Evans SC. Antioxidant potential of green and black tea determined using the ferric reducing power (FRAP) assay. Int J Food Sci Nutr. 2000; 51(3):181-188.

33. Soultani G, Antonios E Koutelidakis, Maria Kapsokefalou, Michael Komaitis. The effect of pectin and other constituents on the antioxidant activity of tea. Food Hydrocolloids. 2014; 35:727-732. doi:10.1016/j.foodhyd.2013.08.005.

34. Langley Evans SC. Consumption of black tea elicits an increase in plasma antioxidant potential in humans. Int J Food Sci Nutr. 2000; 51(5):309-315.

35. Leenen R, Roodenburg AJ, Tijburg LB, Wiseman SA. A single dose of tea with or without milk increases plasma antioxidant activity in humans. Eur J Clin Nutr. 2000; 54(1):87-92.

36. Rechner AR, Wagner E, Van Buren L, Van De Put F, Wiseman S, Rice-Evans CA. Black tea represents a major source of dietary phenolics among regular tea drinkers. Free Radic Res. 2002; 36(10):1127-1135.

37. Pekal A, Pyrzynska K. Effect of pH and metal ions on DPPH radical scavenging activity of tea. Int J Food Sci Nutr. 2015; 66(1):58-62. doi: 10.3109/09637486.2014.959899.

38. Moore S, Calder KA, Miller NJ, Rice-Evans CA. Antioxidant activity of saliva and periodontal-disease. Free Radic Res. 1994; 21(6):417-425.

39. Ainsworth EA, Gillespie KM. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. Nat Protoc. 2007; 2(4):875-7.

40. Stone D, Ellis. Stats tutorial - Instrumental analysis and calibration. 2011 [cited 2014 June]. Available from: http://www.chem.utoronto.ca/coursenotes/analsci/stats/index.html.

41. Owusu-Apenten R. Food protein analysis: quantitative effects on processing. Boca Raton: CRC Press; 2002.

42. Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic Biol Med. 2001; 30(11):1191-212.

43. Owusu-Apenten R. Colorimetric analysis of protein sulfhydryl groups in milk: applications and processing effects. Crit Rev Food Sci Nutr. 2005; 45(1):1-23.

44. Gibbs CR. Characterization and application of ferrozine iron reagent as a ferrous iron indicator. Anal. Chem. 1976; 48(8):1197-1201. doi: 10.1021/ac50002a034.

45. Walker RB, Everette JD. Comparative reaction rates of various antioxidants with ABTS radical cation. J Agric Food Chem. 2009; 57(4):1156-61. doi: 10.1021/jf8026765.

46. Davies MJ, Forni LG, Willson RL. Vitamin E analogue Trolox C. E.s.r. and pulse-radiolysis studies of free-radical reactions. Biochem J. 1988; 255(2):513-22.

47. Molan AL, De S, Meagher L. Antioxidant activity and polyphenol content of green tea flavan-3-ols and oligomeric proanthocyanidins. Int J Food Sci Nutr. 2009; 60(6):497-506. doi: 10.1080/09637480701781490.

48. Jayasekera S, Molanb AL, Gargc M, Moughan PJ. Variation in antioxidant potential and total polyphenol content of fresh and fully-fermented Sri Lankan tea. Food Chemistry. 2011; 125(2):536-541. doi:10.1016/j.foodchem.2010.09.045.

49. Huang WY, Yu-Ru Lin, Ruei-Fen Ho, Ho-Yen Liu, Yung-Sheng Lin. Effects of water solutions on extracting green tea leaves. ScientificWorldJournal. 2013; 2013:368350.

50. Venditti E, Tiziana Bacchettia, Luca Tianoa, Patricia Carlonib, Lucedio Grecib, Elisabetta Damiani. Hot vs. cold water steeping of different teas: Do they affect antioxidant activity? Food Chemistry. 2010; 119(4):1597-1604. doi:10.1016/j.foodchem.2009.09.049.

51. Yashin A, Yashin Y, Boris N. Determination of antioxidant activity in tea extracts, and their total antioxidant content. American Journal of Biomedical Sciences 2011; 3(4):322-335. doi: 10.5099/aj110400322 2011 [cited 2015 Jan]; Available from: http://nwpii.com/ajbms/papers/AJBMS_2011_4_09.pdf.

52. Veljkovic JN, PAVLOVIĆ ALEKSANDRA N, MITIĆ SNEŽANA S, TOŠIĆ SNEŽANA B, STOJANOVIĆ GORDANA S, KALIČANIN BILJANA M, et al. Evaluation of individual phenolic compounds and antioxidant properties of black, green, herbal and fruit tea infusions consumed in Serbia: spectrophotometrical and electrochemical approaches. Journal of Food and Nutrition Research. 2013; 52(1):12-24.

53. Singh N, Sharma R, Balapure AK. pH regulated scavenging activity of beer antioxidants through modified DPPH assay. Toxicol Ind Health. 2007; 23(2):75-81.

54. Thompsen JC, Mottola HA. Kinetics of the complexation of iron(II) with ferrozine. Anal. Chem. 1984; 56(4):755-757. doi: 10.1021/ac00268a037.

55. Welch KD, Davis TZ, Aust SD. Iron Autoxidation and Free Radical Generation: Effects of Buffers, Ligands, and Chelators. Arch Biochem Biophys. 2002; 397(2):360-369.

56. Viollier E, Inglett PW, Hunter K, Roychoudhury AN, Van Cappellen P. The ferrozine method revisited: Fe(II)/Fe(III) determination in natural waters. Applied Geochemistry. 2000; 15(6):785-790. doi:10.1016/S0883-2927(99)00097-9.

57. Hynes MJ, Coinceanainn MO. Investigation of the release of iron from ferritin by naturally occurring antioxidants. J Inorg Biochem. 2002; 90(1-2):18-21.