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Antioxidant Capacity of Teas and Herbal Infusions: Polarographic Assessment

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ABSTRACT: Hydrogen peroxide scavenging (HPS) activity of unfermented (green, yellow, and white), partially fermented (oolong), and completely fermented (black) tea (*Camellia sinensis*), maté (*Ilex paraguariensis*), and various herbal infusions, as well as individual compounds (flavan-3-ols, flavonols, cinnamic and benzoic acids, and methylxanthines), was assessed by recently developed direct current (DC) polarographic assay. Correlations of tea and herbal infusion HPS activity with total phenolic content determined using the Folin−Ciocalteu assay (FC-GAE) (0.81 and 0.93), ferric reducing/antioxidant power (FRAP) (0.97 and 0.92), 1,1-diphenyl-2-picrylhydrazyl (DPPH) (0.77 and 0.80), and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) scavenging (0.86 and 0.86) were statistically significant. Correlations between relative antioxidant capacity index (RACI), calculated by assigning all applied assays equal weight, and HPS (0.98), FRAP (0.97), ABTS (0.89), and DPPH (0.89) confirmed DC polarographic assay reliability when applied individually. Correlation analysis, ANOVA, and Levene and Tukey's HSD tests unequivocally confirmed this reliable, rapid, and low-cost assay validity, clearly demonstrating its advantages over spectrophotometric assays applied.

KEYWORDS: *tea, herbal infusions, maté, antioxidant activity, hydrogen peroxide, DC polarography*

INTRODUCTION

The measurement of antioxidant (AO) activity is well documented and carried out to compare different foods or commercial products and provide quality standards for regulatory issues and health claims.[1] Simple, direct, and high-throughput assays for the determination of AO capacity find wide applications in research, food industry, and drug discovery. The number of methods proposed to measure antioxidants (AOs) in botanicals has increased considerably. Despite the recent efforts of the scientific community to come to an agreement regarding standardization of one method for determination of AO capacity,[1] a widely accepted standardized method has not yet been established. Limitations of AO activity assays suggest that more than one analytical method should be used to elucidate the capacity of food samples.[2] All of these assays differ in terms of the assay principles and experimental conditions as they include certain essential features, such as suitable substrate, an oxidation initiator, and an appropriate measure of the end point.[3]

Antioxidant capacity assays may be broadly classified as electron transfer (ET)- and hydrogen atom transfer (HAT)-based assays.[1,4] HAT-based assays measure the capability of an AO to quench free radicals (generally peroxyl radicals) by H-atom donation (ORAC assay), whereas in most ET-based assays, the AO action is simulated with a suitable redox-potential probe; that is, the AOs react with a fluorescent or colored probe (oxidizing agent) instead of the peroxyl radical. Spectrophotometric ET-based assays measure the capacity of an AO in the reduction of an oxidant, which changes color when reduced, and these assays generally set a fixed time for the concerned redox reaction and measure thermodynamic conversion (oxidation) during that period. ET-based assays include ABTS/TEAC, DPPH, Folin−Ciocalteu, FRAP, and CUPRAC using different chromogenic redox reagents with different standard potentials. All of these assays are well established and have been used for the determination of the AO capacity of teas, herbal infusions, cocoa products, and coffees.

In comparison with the standard spectrophotometric techniques, an electrochemical approach for the determination of AO activity and quantification and characterization of AOs, as well as for high-resolution antioxidant screening, has certain advantages, such as the possibility of direct measurement, as well as rapidity and sensitivity.[5] Various electrochemical techniques rely upon direct or indirect oxidation of phenolic compounds. Phenolic OH groups act as hydrogen- or electron-donating agents enabling direct determination of the total AO activity, that is, evaluation of the overall reducing power, without the use of reactive species. The analytical potency of electrochemistry in the direct determination of antioxidant capacity has been evaluated.[6] Besides flow injection amperometric detection (FIA-ED), voltammetric techniques are employed the most frequently, above all, cyclic voltammetry (CV).

Teas and herbal infusions are among a wide range of complex samples investigated using voltammetric techniques. Cyclic

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voltammetry at a glassy carbon electrode can provide a rapid measurement of easily oxidizable polyphenols in teas.[7,8] Determination of AO capacity of fruit tea infusions based on cyclic voltammograms was reported.[9] Differential pulse voltammetry at a graphite electrode was applied to determine the polyphenolic content of green tea extract.[10] Abrasive stripping square-wave voltammetry (SWV) using a paraffin-impregnated graphite electrode was employed to access AO properties of tea leaves.[11] Catechins in green and black teas were characterized using SWV as well.[12] Also, several types of commercially available teas from various manufacturers were tested for antioxidant content using the amperometric method.[13]

As an important aspect of AO activity, hydrogen peroxide scavenging (HPS) activity has been determined using various assays. Limitations in the peroxidase-based system, such as the presence of interfering compounds, have been surpassed using enzyme-free methodologies. Highly sensitive chemiluminescent assays developed during the past decade represent a significant advancement over enzyme-based HPS assays, owing to elimination of the effects caused by radical reaction.[14−17] Recently, novel HPS assays have been developed based on (i) the formation of gold nano shells;[18] (ii) cupric reducing AO capacity (CUPRAC) methodology;[19] (iii) chronoamperometry;[20] and (iv) direct current (DC) polarography.[21] An enzyme-free polarographic assay, introduced by our research group, relies on the sensitivity of an anodic current originating from hydrogen peroxide complex formation in alkaline solutions, at potential of mercury dissolution, to the presence of AOs.[21] Briefly, a decrease of the anodic current upon the addition of individual AOs or complex samples has been followed using DC polarography on dropping mercury electrode (DME). Until now, the assay has been successfully applied on various alcoholic beverages including beer,[22] red and white wines,[23] and various strong alcoholics.[24]

In this paper the intention was to explore the feasibility of DC polarography in the determination of HPS activity of worldwide popular types of tea (*Camellia sinesis*) (white, yellow, green, oolong, and black), a tea-like beverage called maté (*Ilex paraguariensis*) that is widely consumed in Argentina, Brazil, Uruguay, and Paraguay and becoming increasingly popular in the United States and Europe, and herbal infusions used globally as traditional medicine and to prove the versatility of the assay in the analysis of various beverages, both alcoholic and nonalcoholic. This is the first report on DC polarographic assay application on nonalcoholic beverages. Whereas the HPS activity of alcoholic beverages was correlated only with total phenolic content estimated by Folin−Ciocalteu assay (FC) and/or scavenging activity against artificial radical species DPPH, the HPS activity of teas and herbal infusions has been correlated with two additional AO assays, total reducing power (FRAP) and scavenging activity against ABTS. The effectiveness of the phenolics present has been estimated on the basis of phenolic antioxidant coefficient (PAC_{HPS}) calculated as the ratio between HPS activity and total phenolics content. The HPS activity of individual compounds, both phenolics (flavan-3-ols, flavonols, cinnamic and benzoic acids) and methylxanthines, has been determined to gain better insight into the significance of particular compounds' contribution to the overall activity of complex samples. To enable more comprehensive comparison between investigated samples, the relative antioxidant activity index (RACI), based on calculation assigning equal weight to all AO tests applied, has been introduced. The polarographic assay validity was confirmed, on the basis of correlations with widely used spectrophotometric assays. ANOVA and *F* tests, as well as the Levene test, were applied to show high accuracy of HPS, compared to other AO assays. Post hoc Tukey's HSD tests at 95% confidence limit were calculated to show any significant differences between teas and herbal infusions samples. Low coefficient of variation of the HPS assay also confirmed the finding of higher accuracy over assays applied in parallel.

MATERIALS AND METHODS

Chemicals. Folin−Ciocalteu reagent, ammonium peroxodisulfate, sodium carbonate, sodium acetate trihydrate, acetic acid, hydrochloric acid, ferric chloride hexahydrate, and ferric sulfate heptahydrate were of analytical grade and supplied by Kemika (Zagreb, Croatia). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was supplied by Fluka (Buchs, Switzerland), and methanol (HPLC grade) was purchased from J. T. Baker (Deventer, The Netherlands). 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,4,6-tripyridyl-*S*-triazine (TPTZ), and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid diammonium salt (ABTS) as well as gallic acid (GA) were obtained from Aldrich (Sigma-Aldrich Chemie, Steinheim, Germany). Hydrogen peroxide was obtained from Merck (Darmstadt, Germany).

Sample Preparation. Six teas [green (Sencha), black (Lingia), white (Silver Needle), yellow (Yin Zhen), oolong (Formosa Fine), and maté (roasted maté) tea] as well as 13 herbal species [lemon balm (*Melissae folium*), thyme (*Serpylli herba*), peppermint (*Menthae piperitae folium*), sage (*Salviae folium*), horsetail (*Equiseti herba*), linden (*Tiliae flos*), raspberry leaves (*Rubi ideae folium*), ground ivy (*Herba glechomae*), yarrow (*Millefolii herba*), hawthorn (*Folium crataegi cum flores*), woodruff (*Asperulae herba*), blackberry leaves (*Rubi fruticosi folium*), and olive leaf (*Oleae folium*)] were purchased from a specialized tea store. To simulate beverage brewing, teas were prepared using an aqueous extraction procedure. Extraction was carried out by pouring 200.0 mL of boiled distilled water into a 250 mL beaker containing plant samples (2.0 g) at room temperature. After extraction (10 min), the infusions were filtered through a tea strainer.

Determination of Total Phenols. Total phenol content (TPC) of teas and herbal infusions was determined spectrophotometrically according to a modified method of Lachman et al.[25] with Folin−Ciocalteu's reagent. Briefly, 0.5 mL of the sample was added into a 50 mL volumetric flask containing 2.5 mL of Folin−Ciocalteu's reagent, 30 mL of distilled water, and 7.5 mL of 20% Na_2CO_3 and filled to the mark with distilled water. After 2 h, the absorbance of blue coloration was measured at 765 nm against a blank sample. Gallic acid was used as the standard, and the results are expressed as milligrams per liter of gallic acid equivalents (GAE). All measurements were performed in triplicate.

Determination of AO Activity Using DC Polarography. The current−potential (*i*−*E*) curves were recorded using the polarographic analyzer PAR (Princeton Applied Research), model 174A, equipped with an X−Y recorder (Houston Omnigraphic 2000). A dropping mercury electrode (DME) with a programmed dropping time of 1 s as working electrode, a saturated calomel electrode (SCE) as a reference, and a Pt-foil as auxiliary electrode were used. Clark Lubb's (CL) buffer (pH 9.8) was prepared by mixing 25 mL of 0.4 M H_3BO_3, 25 mL of 0.4 M KCl, and 40.8 mL of 0.2 M NaOH. Starting 5 mM H_2O_2 concentration was obtained by addition of 100 μL of 1.00 M H_2O_2 into 20 mL of buffer in electrolytic cell. Samples were gradually added into the electrolytic cell with buffered H_2O_2 solution. Before each *i*−*E* curve recording, a stream of pure nitrogen was passed through the cell solution, during 5 min before the first recording and during 30 s after the addition of each aliquot. The inert atmosphere was kept by passing nitrogen above the cell solution. The initial potentials were 0.10 or 0.15 V, and the potential scan rate was 10 mV s^{-1}. The DME current oscillations were filtered with the low-pass filter of the instrument positioned at 3 s. Decrease of the anodic current of H_2O_2, that is,

initial i_l value (i_{l0}), obtained by recording 5 mM H_2O_2 solution, upon addition of investigated samples has been recorded. All experiments were done in triplicate, at room temperature.

Determination of Free Radical Scavenging Ability by the Use of DPPH Radical. The antioxidant capacity of the teas and herbal infusions was determined using the DPPH radical scavenging assay,[26] with some modifications. Briefly, 100 μL of extract was added to 1.9 mL of 0.094 mM DPPH in methanol up to 2 mL. The free radical scavenging capacity of the sample was evaluated by measuring the absorbance after 30 min at 517 nm. The antioxidant capacity was expressed as millimoles per liter Trolox equivalents, using the calibration curve of Trolox (0−1000 μM), a water-soluble vitamin E analogue. All determinations were performed in triplicate.

Determination of Free Radical Scavenging Ability by Use of the ABTS Radical Cation. The Trolox equivalent antioxidant capacity (TEAC) was also estimated by the ABTS radical cation decolorization assay.[27] Stock solutions of ABTS (7 mM) and potassium peroxodisulfate (140 mM) in water were prepared and mixed together to a final concentration of 2.45 mM potassium peroxodisulfate. The mixture was left to react overnight (12−16 h) in the dark, at room temperature. On the day of analysis, the ABTS radical solution was diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm. All measurements were performed as follows: 20 μL of extract was added to 2.0 mL of the ABTS radical solution, and the absorbance readings were taken after exactly 6 min against the appropriate reagent blank of 20 μL of ethanol instead of the sample. The results obtained from triplicate analyses were expressed as Trolox equivalents and derived from a calibration curve determined for this standard (100−1000 μM).

Determination of Ferric Reducing/Antioxidant Power (FRAP Assay). The ferric reducing/antioxidant power (FRAP) assay was carried out according to a standard procedure.[28] FRAP reagent was prepared by mixing acetic buffer, TPTZ, and $FeCl_3 \cdot 6H_2O$ (20 mM water solution) at a ratio of 10:1:1. Briefly, to a volume of 950 μL of FRAP reagent was added 50 μL of tea extract. After 4 min, the absorbance of blue coloration was measured against a blank sample. All measurements were performed in triplicate. Aqueous solutions of $FeSO_4 \cdot 7H_2O$ (100−1000 μM) were used for the calibration, and the results are expressed as millimoles per liter Fe(II).

Determination of Relative Antioxidant Activity Index (RACI). Central tendency is the most widely used to compare the AO activity of complex food samples determined using multiple assays,[29] where samples are ranked according to the mean value and standard deviation of the assays used. Because the units and the scale of the data from various chemical methods are different, the data in each data set should be transformed into standard scores, a dimensionless quantity derived by subtracting the mean from the raw data divided by the standard deviation, according to the equation

$$\text{standard score} = (x - \mu)/\sigma \quad (1)$$

where x represents the raw data, μ the mean, and σ the standard deviation. The standard scores of a sample for different assays when averaged give a single unitless value termed as RACI, which is a specific combination of data from different chemical methods with no unit limitation and no variance among methods.

Statistical Analysis. Descriptive statistical analyses for calculating the means and standard errors of the mean were performed using Microsoft Excel software (Microsoft Office 2007). All obtained results were expressed as the mean ± standard deviation (SD). The evaluation of one-way analysis of variance (ANOVA), Levene test of homogeneity of variances, and F test of obtained results was performed for comparison of means, and significant differences between specific teas and herbal infusions samples are determined according to post hoc Tukey's HSD test at the 95% confidence limit, using StatSoft Statistica 10 software.

RESULTS AND DISCUSSION

The polarographic behavior of anodic current appearing in alkaline-buffered solutions of hydrogen peroxide, at the potential of mercury dissolution, was studied. Confirmation that the anodic current originates from a mixed complex, $[Hg(O_2H)(OH)]$, formed at the electrode surface was provided. The influence of different experimental conditions (pH of medium, H_2O_2 concentration, electrolyte solution temperature) on that current was followed. A rapid, simple, and reliable enzyme-free AO assay based on the anodic current decrease in the presence of AOs was developed and optimized.[21]

The objective of this study was to determine the AO activity of true teas, maté, and herbal infusions, using the DC polarographic assay. Caffeine-containing beverages, true teas and maté, have been considered together as in the previous study by Komes et al.,[30] whereas data related to herbal infusion have been reported in parallel. Such a concept was found to be convenient to emphasize the novel assay applicability on both herbal infusions (various plant species) and teas (one plant subjected to different growing and manufacturing conditions).

All investigated samples (5 true teas, maté, and 13 herbal infusions) have been gradually added into initial buffered peroxide solution. A prominent difference between polarograms of 5 mmol L^{-1} solution of H_2O_2 in CL buffer (pH 9.8), recorded before and after the addition of infusions, has been observed (Figure 1). Comparison of polarograms indicates that

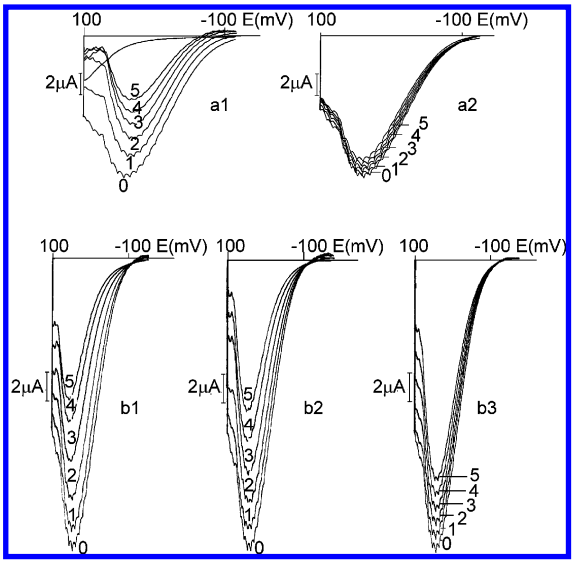

Figure 1. Anodic polarographic curves of 5 mmol L^{-1} solution of H_2O_2 in CL buffer (pH 9.8) before (0) and after addition of five equal aliquots of green tea and maté [(1−5) 100−500 μL (a1, a2)] and lemon balm, peppermint, and hawthorn [(1−5) 200−1000 μL (b1, b2, b3)].

tea infusions exhibit a more potent effect on the anodic current than herbal infusions. Optimal volume of tea aliquots has been estimated to be 100 μL. The effect of the addition of green tea and maté (five equal aliquots of 100 μL) on the anodic current decrease is shown in Figure 1 (a1, a2). Green tea exhibits a very potent effect (surpassed only by yellow tea), whereas the effect of maté addition is the lowest among caffeine-containing samples. Two times higher volumes of herbal infusion aliquots were added into the initial solution of peroxide to obtain an effect comparable to that of the teas. Polarograms of lemon balm, peppermint, and hawthorn (five equal aliquots of 200 μL) are shown in Figure 1(b1, b2, b3). Remarkable differences between the effects of these three herbal infusions are

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observable; lemon balm exhibits the superior effect followed by peppermint, whereas the hawthorn effect is low.

The remaining anodic limiting current (i_{lr}) obtained upon gradual addition of tested samples has been compared with the height of initial limiting current (i_{l0}). The percentage of i_l decrease has been calculated upon each addition of tested infusion according to the equation

$$\%\ \text{scavenged}\ [H_2O_2] = \left(1 - \frac{i_{lr}}{i_{l0}}\right) \times 100 \quad (2)$$

All investigated samples exhibit AO activity in a dose-dependent manner. Plots of percentage of i_l decrease calculated upon each addition versus added volume of tea are shown in Figure 2a. For the sake of clarity, limiting current decrease–

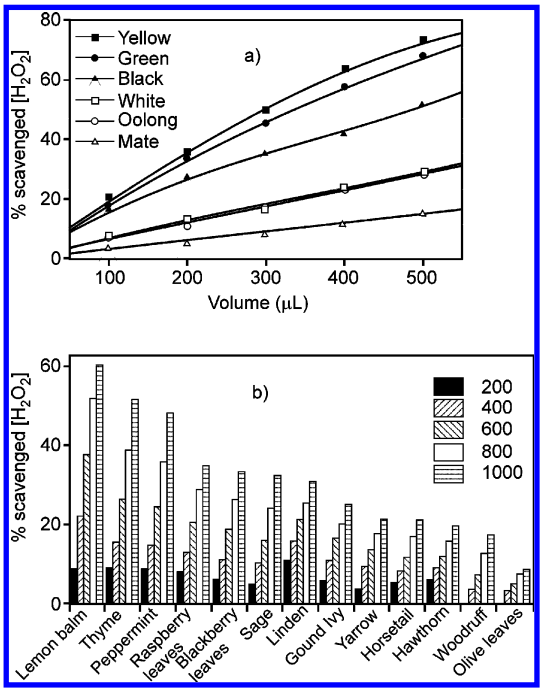

Figure 2. Teas and herbal infusions effects on anodic limiting current of 5 mmol L^{-1} solution of H_2O_2 in CL buffer (pH 9.8) [(% of scavenged H_2O_2 vs volume of added samples) – dose volume curves for teas (100–500 μL)] (a) and dose volume histograms for herbal infusions (200–1000 μL) (b).

volume histograms have been given instead of the curves to illustrate the effect of all herbal infusions in the same time (Figure 2b).

Except for the most active samples among both teas and herbal infusions, in the entire investigated volume range a linear relationship exists. Saturation phenomena at the highest volumes added, 500 μL for yellow tea and 1000 μL for lemon balm, have been noted. Green and black tea infusions deviate from linearity only at volumes >500 μL. The slope of the linear part of the dose–response curves has been used as criterion of AO activity. In our previous papers AO activity was expressed as volume required for 50% decrease of anodic oxidation current (AO_{50}), its reciprocal value (AO_{50}^{-1}),[22,23] or percentage of anodic oxidation current decrease obtained upon addition of 500 μL of samples (HPS500).[24] The slope has been introduced[21] as a more adequate way to express HPS activity, particularly when the activities of the analyzed samples vary significantly.[21] The results obtained by applying the novel electrochemical assay and commonly used spectrophotometric ones have been summarized in Table 1. On the basis of results of all assays employed, the unitless relative antioxidant activity index (RACI) has been calculated by assigning each assay equal weight. Previously, RACI was efficiently used to compare the AO activities of numerous samples obtained from multiple assays.[29] Here, it was introduced to enable more comprehensive comparison between investigated samples, as well as comparison between applied assays.

Finding that teas generally exhibit higher HPS activity and possess higher phenolic content than herbal infusions corroborates well with previous studies.[31−36] The following rank of order of HPS activity has been found: yellow > green > black > white > oolong > maté. Total reducing power measured by FRAP differs only in opposite position of white and oolong tea, whereas ABTS radical scavenging only in the positions of green and yellow tea. According to both HPS and FRAP yellow tea activity surpasses that of green tea, whereas the highest DPPH and ABTS scavenging activities have been ascribed to green tea, followed by yellow and black teas, respectively. Scavenging of DPPH radical decreases in the following order: green > black > yellow > oolong > white > maté. Maté exhibits the lowest activity among caffeine-containing infusions according to all assays applied. Rank of total reducing power (FRAP) as well as free radicals scavenging (ABTS, DPPH) corroborates with previous results.[30,36] On the basis of RACI the following rank of order has been found: green > yellow > black > white > oolong > maté.

The obtained results confirm the well-established structure–antioxidant capacity relationship of polyphenols, indicating that the highest content of total phenolics does not necessarily account for the highest AO capacity. Great variations in total AO activity can be caused by the presence of various polyphenolic compounds, that is, different phenolic profile, as well as different responses of individual polyphenolic compounds in a particular assay. Variations in phenolic contribution to total AO activity determined using different assays can be explained by the differences in the assay principle and experimental conditions. Introduction of phenolic antioxidant coefficient (PAC_{HPS}), calculated here as the ratio between HPS activity and total phenolic content (expressed in g GAE/L), enables the comparison of AO effectiveness of phenolics. Taking into account both AO activity and total content of phenolics, PAC enables more comprehensive insight into the sample behavior. The observed superiority of unfermented over fermented teas might be explained by the prevalent presence of highly active flavan-3-ols such as epicatechin (EC), epigallocatechin-3-gallate (EGCG), epicatechin-3-gallate (ECG), and epigallocatechin (EGC), the very high HPS activities of which were assessed using CUPRAC methodology.[19] The activity of individual compounds commonly present in teas and herbal infusions,[31] including flavan-3-ols, has been determined using DC polarographic assay (Table 2). High HPS activity of flavan-3-ols, found in high amount in unfermented, intermediate amount in semifermented, and in low amount in fully fermented teas,[37] has been followed by flavonols and cinnamic and benzoic acids. The superior HPS activity of EGCG surpasses by far the activities of EC and catechin. A positive correlation between EGCG content and both ABTS and DPPH scavenging, as well as the importance of a gallate group at the 3-position for radical scavenging ability of flavan-3-ols, was confirmed.[36] A significant reduction of catechins noted in black tea is probably only partially

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Table 1. Teas and Herbal Infusions Total Phenolic Content (FC GAE), Antioxidant Activity Determined by DC Polarographic Assay, Expressed as HPS (%/mL), and Phenol Antioxidant Coefficient (PAC_{HPS}), Standard Spectrophotometric Assays (FRAP, ABTS and DPPH), and Relative Antioxidant Capacity Index (RACI)

	HPS[a] %/mL	FC GAE[a] mgGAE/L	PAC_{HPS}[b]	FRAP[a] mM Fe(II)	ABTS[a] mM Trolox	DPPH[a] mM Trolox	RACI[c]
Tea Infusions							
yellow tea	156 ± 8^{k}	824 ± 50^{j}	189	18.44 ± 1.58^{j}	4.99 ± 0.21^{j}	4.37 ± 0.64^{k}	1.96
green tea	134 ± 7^{j}	819 ± 8^{j}	164	17.79 ± 0.73^{j}	7.68 ± 0.15^{k}	4.80 ± 0.40^{l}	2.04
black tea	97 ± 8^{i}	973 ± 29^{k}	100	13.56 ± 0.03^{i}	2.90 ± 0.83^{hi}	4.45 ± 0.57^{kl}	1.04
white tea	59 ± 2^{gh}	383 ± 17^{fg}	154	4.96 ± 0.31^{de}	2.61 ± 0.42^{gh}	3.66 ± 0.26^{i}	0.35
oolong tea	56 ± 3^{fg}	558 ± 33^{i}	101	6.25 ± 0.26^{efg}	0.74 ± 0.23^{b}	3.88 ± 0.06^{ij}	0.09
maté tea	30 ± 2^{cde}	307 ± 8de	99	4.36 ± 0.46^{cd}	0.64 ± 0.59^{b}	1.46 ± 0.21^{de}	−0.55
Herbal Infusions							
lemon balm	66 ± 3^{h}	962 ± 6^{k}	69	10.56 ± 0.48^{i}	3.09 ± 0.08^{i}	2.51 ± 0.34^{h}	0.44
thyme	53 ± 3^{fg}	531 ± 1^{i}	100	6.49 ± 0.15^{gh}	2.65 ± 0.21^{h}	3.63 ± 0.11^{i}	0.22
peppermint	47 ± 3^{f}	591 ± 25^{i}	80	7.18 ± 0.23^{gh}	2.60 ± 0.08^{gh}	4.15 ± 0.04^{jk}	0.22
raspberry leaf	35 ± 1^{e}	467 ± 4^{h}	75	6.13 ± 0.95^{fgh}	2.26 ± 0.01^{fg}	2.02 ± 0.01^{fg}	−0.26
blackberry leaf	34 ± 1^{e}	559 ± 12^{i}	60	7.23 ± 0.52^{h}	2.80 ± 0.17^{hi}	2.37 ± 0.23^{gh}	−0.14
sage	32 ± 2de	340 ± 10^{ef}	95	5.88 ± 1.15^{fg}	1.47 ± 0.25^{cd}	2.44 ± 0.14^{h}	−0.29
linden	29 ± 3^{cde}	409 ± 1^{gh}	71	3.32 ± 0.14^{cd}	2.01 ± 0.04^{ef}	1.46 ± 1.96^{de}	−0.52
ground ivy	24 ± 1^{bcd}	275 ± 8^{cd}	89	3.24 ± 0.22^{cd}	1.73 ± 0.09^{de}	1.74 ± 0.02^{ef}	−0.54
yarrow	22 ± 1^{bc}	259 ± 10^{cd}	85	2.58 ± 0.09^{c}	1.50 ± 0.04^{cd}	1.28 ± 0.17^{cd}	−0.68
horsetail	21 ± 1^{bc}	219 ± 26^{bc}	95	2.03 ± 0.14^{bc}	1.29 ± 0.17^{c}	0.94 ± 0.01^{bc}	−0.74
hawthorn	19 ± 1^{b}	381 ± 14^{fg}	49	4.16 ± 071de	1.47 ± 0.02^{cd}	1.73 ± 0.06^{ef}	−0.68
woodruff	18 ± 2^{b}	159 ± 45^{ab}	110	1.17 ± 0.11^{b}	0.17 ± 0.14^{a}	0.88 ± 0.33^{b}	−0.86
olive leaves	9 ± 1^{a}	105 ± 4^{a}	88	0.81 ± 0.07^{a}	0.51 ± 0.03^{b}	0.30 ± 0.02^{a}	−1.09

[a]Values with the same letter are not statistically different at the $p < 0.05$ level, 95% confidence limit, according to Tukey's HSD test. [b]PAC_{HPS}, phenol antioxidant coefficient, calculated as ratio of HPS (%/mL) and total content of phenolics expressed in g GAE/L. [c]RACI, relative antioxidant capacity index, calculated by assigning each applied assay equal weight (unitless).

Table 2. Hydrogen Peroxide Scavenging Activity of Flavan-3-ols, Flavonols, Cinnamic and Benzoic Acids, and Methylxanthines Present in Teas and Herbal Infusions, Expressed as the Slope of the Dose−Response Curves [Percentage of i_1 Decrease vs Amount of Compound (μmol)]

	intercept (%)	slope (%/μmol^{-1})	R
flavan-3-ols			
epigallocatechin gallate	0.00	72.00 ± 1.55	0.9995
epicatehin	0.36	47.84 ± 2.09	0.9981
catechin	1.46	47.57 ± 3.28	0.9878
flavonols			
myrcetin	0.40	46.84 ± 3.35	0.9924
kaempferol	−1.21	35.83 ± 4.47	0.9774
cinnamic acids			
ferulic	−0.95	13.86 ± 0.52	0.9958
caffeic	−1.30	12.65 ± 0.75	0.9811
benzoic acids			
vanillic	−0.61	10.29 ± 0.89	0.9852
p-hydroxybenzoic	−0.20	3.51 ± 0.28	0.9876
methylxanthines			
theophylline	0.66	22.30 ± 0.95	0.9939
theobromine	−2.19	15.49 ± 0.68	0.9868
caffeine	0.12	4.35 ± 0.23	0.9959

compensated by larger polyphenolic molecules, such as dimeric theaflavins or polymeric thearubigins. The HPS activity of some phenolic acids present in small amount in fresh and processed tea leaves[36] is shown in Table 2. Previously, the activity of a wide range of phenolic acids was reported, and the finding that benzoic acids possesses considerably lower HPS activity than corresponding cinnamate analogues was discussed.[21] Besides phenolics, methylxanthines such as theophylline, theobromine, and caffeine are biologically active constituents of tea samples. The order of DPPH scavenging (EGCG > ECG > EGC > gallic acid > EC > caffeine) indicated a low contribution of caffeine to the overall AO activity of tea.[38] A negligible contribution of caffeine to the overall antioxidant activity of both true teas and maté reported by Komes et al.[30] has been confirmed by DC polarographic measurements. The activity of caffeine has been found the lowest among all individual compounds considered here except *p*-hydroxybenzoic acid (Table 2). Approximately 5- and 3-fold higher activities have been ascribed to theophylline and theobromine, respectively, whereas flavan-3-ols have an order of magnitude higher HPS activity. Caffeoyl derivatives, methylxanthines, and flavonoids are the major bioactive constituents of maté.[39] A positive and significant correlation was observed between the AO activity and the content of caffeoyl derivatives.[40]

Among the herbal infusions, lemon balm exhibits the highest content of phenolics, followed by peppermint, blackberry leaves, and linden. Finding that the TPC of herbal infusions varies largely is consistent with previous results.[32,34] The phenolic profile of herbal infusions showed the presence of a wide variety of flavonoids, phenolic acids, and their derivatives.[31] The HPS activity of common flavonoids and phenolics acids identified in herbal infusions is shown in Table 2. Such diversity can explain different responses in AO activity assays. Considering the HPS assay, lemon balm exhibits the highest activity followed by thyme, peppermint, raspberry and blackberry leaves, and sage. According to the results of the FRAP assay, lemon balm exhibited again the highest reducing power, followed by blackberry leaf, peppermint, thyme, raspberry leaf, and sage. Of 70 medicinal plants screened for

Table 3. Correlation Coefficients between FC GAE, HPS, FRAP, ABTS, and DPPH for Teas and Herbal Infusions

	HPS	FRAP	ABTS	DPPH
		Tea Infusions		
FC GAE	0.806** (0.053)	0.870* (0.024)	0.618^{ns} (0.191)	0.811** (0.050)
DPPH	0.772** (0.072)	0.733** (0.097)	0.690^{ns} (0.129)	
ABTS	0.861* (0.028)	0.860* (0.028)		
FRAP	0.974^{+} (0.001)			
		Herbal Infusions		
FC GAE	0.928^{+} (0.000)	0.955^{+} (0.000)	0.893^{+} (0.000)	0.694^{+} (0.008)
DPPH	0.801^{+} (0.001)	0.782^{+} (0.002)	0.772^{+} (0.000)	
ABTS	0.856^{+} (0.000)	0.892^{+} (0.000)		
FRAP	0.915^{+} (0.000)			

$^{+}$, significant at 0.01 level; *, significant at 0.05 level; **, significant at 0.10 level; ^{ns}, not significant; p values are in parentheses.

AO activity using the FRAP assay, lemon balm was considered to be the "antioxidant queen".[32] Lemon balm exhibits the highest scavenging activity toward ABTS also, followed by blackberry leaves, thyme and peppermint, raspberry leaf, and linden, whereas peppermint exhibits the highest scavenging activity toward the DPPH radical, followed by thyme, lemon balm, sage, and blackberry and raspberry leaves. As in our study, Moraes de Souza et al.[34] reported that according to the DPPH assay, lemon balm was not the most potent radical scavenger, although it exhibited the highest content of total phenols, whereas the DPPH scavenging activity detected with electron paramagnetic resonance (EPR) confirmed the superiority of lemon balm over 29 popular and easily available medicinal plants.[41] Lemon balm exhibits the highest values of RACI among tested infusions, followed by thyme and peppermint, but it is still >5 times lower in comparison with green tea RACI. Variations in the AO activity of one herbal species, among different studies, can be explained by different growing and extraction conditions (climate and soil, harvest time, stage of development, part of plant used, particle size, and extraction method).

Among various herbal infusions included in this study only thyme, sage, horsetail, and woodruff PAC_{HPS} values are high enough to be compared to semifermented and fully fermented teas. Obviously, PAC cannot be considered independently to estimate sample as potential source of powerful AOs. Only infusions with high AO activity can be taken into account as rich source of AOs, which is consistent with the study reported by Katalinić et al.[32]

The novel assay has been validated through correlations with spectrophotometric assays employed in this study. Correlation coefficients obtained for teas and herbal infusions are given in Table 3. Significant positive correlations between FC GAE and HPS for both teas (0.80, significant at $p < 0.05$ level, 95% confidence limit) and herbal infusions (0.93, significant at $p < 0.01$ level, 95% confidence limit) are obtained. Lower correlation coefficients for tea infusions might be explained by changes of tea phenolics content and profile during the manufacturing process. Higher correlations obtained for beer (0.93),[22] red and white wines (0.99),[23] and strong alcoholics (0.97)[24] indicate higher uniformity of these beverages, obtained from the same kinds of raw materials by similar technological process. Because samples included in this study are diverse in terms of their polyphenolic profile, correlation coefficients obtained are considered to be satisfactorily high to confirm the assay validity.

Stronger correlations between HPS and FRAP than between HPS and both DPPH and ABTS scavenging have been obtained for teas (0.98, significant at $p < 0.01$ level, 95% confidence limit) and herbal infusions (0.91, significant at $p < 0.01$ level, 95% confidence limit) (Table 2). Both FRAP and HPS measure the overall reducing power; that is, they reflect activities of all reducing substances present. Higher correlation of HPS with ABTS (0.86, significant at $p < 0.05$ level for teas, and 0.85, significant at $p < 0.01$ level, for herbal infusions, at 95% confidence limit), than with DPPH scavenging (0.77, significant at $p < 0.10$ level, for teas, and 0.80, significant at $p < 0.01$ level, for herbal infusions, 95% confidence limit) is observed (Table 2). Correlations between HPS and DPPH were high for wine samples (0.99)[23] and strong alcoholics (0.92),[24] whereas HPS has been correlated here with ABTS and FRAP for the first time. Finding that HPS correlates better with FC GAE than DPPH supports a previous conclusion that HPS reflects the sum of a wider range of AOs present than DPPH.[23,24] As weak oxidant DPPH reacts slowly with very weak reducing substances. Reaction of phenolics with ABTS radical is rapid, whereas that with DPPH varies depending on the reducing potential. The lower correlation observed between HPS and DPPH, in comparison to HPS and ABTS, may also be due to the fact that the DPPH radical reacts only with lipophilic antioxidants, whereas the ABTS radical reacts with both hydrophilic and lipophilic antioxidants,[1] thus covering a larger share of antioxidants in a sample and resulting in a higher correlation between these two assays. The major drawback of the most widely applied AO assays is the model oxidative system used for evaluation of AO activity; the oxidizing compounds used are not present in the human body. Hydrogen peroxid, as a naturally present, stable reactive oxygen species able to reproduce in vivo state conditions, has a great advantage over nonphysiological, artificial radical species usually used in common spectrophotometric assays. The recent development of four HPS assays[18−21] confirms their increasing popularity.

The one-way ANOVA test has been calculated, accompanied by the F test. There is no significant difference between HPS and FC GAE, FRAP, ABTS, and DPPH assays. Results of spectrophotometric assays are similar to electrochemical ones at the 95% confidence level, at $p < 0.01$ significance level ($F_{FC\ GAE} = 42.54$, $F_{FRAP} = 61.05$, $F_{ABTS} = 526.98$, and $F_{DPPH} = 819.16$, with $F_{crit} = 5.05$). ANOVA also shows the effects of all assays. According to the sum of squares comparison between effects, FC GAE has been found to be the most influential variable for final AO result, whereas HPS has been found to be more influential than FRAP, ABTS, and DPPH assays. The effect of HPS can also be observed from Table 1, where HPS data span a much larger range of values compared to FRAP, ABTS, and DPPH assays, but still, the accuracy of HPS remains

much higher compared to other AO assays. Coefficients of variability for HPS, FRAP, ABTS, and DPPH have been evaluated to be 5.99, 7.51, 17.05 and 15.25, respectively. According to the Levene test of homogeneity of variances, the DC polarographic assay has been found to be more influential compared to other AO assays. In contrast to ANOVA, the Levene test shows that HPS is more influential then FC GAE, which has been found to be statistically significant, but only at the $p < 0.10$ level. Post hoc Tukey's HSD tests have been evaluated for comparison between the teas and herbal infusions samples within each of the used experimental methods, and statistically significant difference have been found in almost all samples, at significance level, $p < 0.05$, 95% confidence limit (Table 1).

Stepwise regression applied between RACI and AO assays reveals that each of them matches RACI considerably. Correlation coefficients between RACI and HPS, FC GAE, FRAP, ABTS, and DPPH assays are 0.98, 0.84, 0.97, 0.89, and 0.89, respectively, with $p < 0.001$ significant level, 95% confidence limit. The highest level of correlation between RACI and HPS (0.98) assay indicates that the DC polarographic assay is reliable enough to be applied as a single assay. FRAP also has a high correlation coefficient with RACI (0.97), but a slightly lower percentage of significant correlation with the other assays used, in comparison with HPS.

In conclusion, rapid and simple polarographic evaluation of the overall AO activity of tea and herbal infusions, as well as their constituents, without the use of reactive radical species has been demonstrated for the first time. The possibility to make a distinction between the AO activity of herbal infusions prepared from various plant species, as well as tea infusions obtained from the same plant subjected to different processing procedures, according to the decrease of polarographic anodic current originating from mixed hydrogen peroxide complex with mercury has been clearly shown. Introduction of a phenolic antioxidant coefficient provides an insight into the effectiveness of the phenolics present. The HPS activity of individual compounds shows that phenolics are major but not unique contributors to total AO activity. By far superior HPS activity has been ascribed to EGCG, whereas the lowest has been ascribed to *p*-hydroxybenzoic acid. Methylxanthines contribute total HPS activity of analyzed complex samples as well. Whereas caffeine HPS activity is similar to that of *p*-hydroxybenzoic acids, both theophylline and theobromine have higher HPS activity than cinnamic acids. Rank of order of considered compounds activity enables better understanding of variations in total activity of complex samples. The high correlations between the overall AO activity determined using DC polarographic assay and well-established spectrophotometric techniques applied have been obtained for both caffeine-containing samples and herbal infusions. The introduction of RACI enables a more comprehensive comparison of both analyzed samples and applied assays. A statistically high significant linear relationship between AO activities measured by all applied AO assays and RACI has been observed. The highest correlation between RACI and HPS activity proves the reliability of the polarographic assay, indicating that the DC polarographic assay can be applied even individually. Comprehensive statistical evaluation of all obtained data unequivocally confirms the validity of this easy-to-handle and low-cost assay. The high accuracy of the assay, compared to spectrophotometric assays applied in parallel, is clearly shown by correlation analysis, ANOVA, and *F* test, as well as Levene test. The accuracy of the assay is also expressed by its low coefficient of variation, much lower than those of other AO assays. Because of the assay advantages shown, and its versatility confirmed by this study as well, its wide application in the analysis of various beverages can be recommended.

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