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Colorimetric Sensing of Antioxidant Capacity via Auric Acid Reduction Coupled to ABTS Oxidation

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Cite This: *ACS Omega* 2024, 9, 11738−11746

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ABSTRACT: In this study, a simple and sensitive colorimetric assay has been developed for total antioxidant capacity measurement. The assay is based on the absorption measurement of the bluish-green oxidized product (ABTS$^{\cdot+}$) formed as a result of the oxidation reaction of the chromogenic reagent ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) with gold(III). However, in the presence of antioxidants, the ABTS oxidation process is effectively suppressed due to the reduction of gold(III) ions to the zerovalent state forming gold nanoparticles (AuNPs). Relatively lighter colors and a significant decrease in absorbance are observed depending on the total antioxidant capacity. Taking advantage of this situation, qualitative and quantitative total antioxidant capacity (TAC) measurements, with the naked eye and UV−vis spectroscopy, respectively, could be successfully performed. The assay is named "auric reducing antioxidant capacity" (AuRAC) because the gold(III) ion-reducing ability of antioxidants is measured. The AuRAC assay was applied to dietary polyphenols, vitamin C, thiol-type antioxidants, and their synthetic mixtures. Trolox equivalent antioxidant capacity (TEAC) values obtained with the AuRAC assay were found to be compatible with those of the reference CUPRAC (cupric reducing antioxidant capacity) assay. The AuRAC assay was validated through linearity, additivity, precision, and recovery, demonstrating that the assay is reliable and robust. Compared to the simple TAC assays in the literature based on AuNP formation with subsequent surface plasmon resonance (SPR) absorbance measurement, this indirect assay has a smoother linear range starting from lower antioxidant concentrations. This method displays much higher molar absorption coefficients for antioxidant compounds than other conventional single electron transfer (SET) assays because 3-e$^-$ reduction of trivalent gold (i.e., Au(III) → Au(0)) produces three chromophore cation radicals (ABTS$^{\cdot+}$) of the assay reagent. The sensor has been successfully applied to complex matrices, such as tea infusions and pharmaceutical samples. The AuRAC assay stands out with its high molar absorptivity connected to enhanced sensitivity as well as its potential to convert into a paper-based colorimetric sensor.
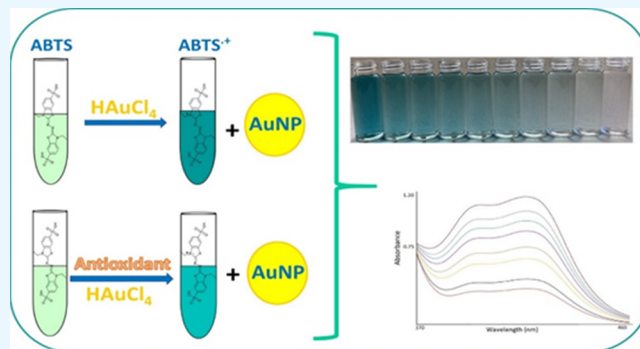
1. INTRODUCTION

Oxidative stress, which causes the emergence and spread of degenerative diseases in organisms, results from the imbalance between reactive species and antioxidants. By definition, antioxidants are such compounds that, even at relatively low concentrations relative to those of the oxidizable substrates, retard or prevent oxidative conversions in their media.[1,2] Studies in the literature show that antioxidants play an important role in maintaining human health and preventing and treating diseases due to their ability to reduce oxidative stress.

Measuring the antioxidant activity/capacity of foods and biological samples is extremely important to estimate the effectiveness of food antioxidants in preventing and treating oxidative stress-related disease.[3] Therefore, many antioxidant capacity and activity methods have been developed and tested in the literature. Although these methods are generally divided into two categories as hydrogen atom transfer (HAT) reaction and electron transfer (ET) reaction-based methods, ET and HAT mechanisms often operate together in various examples.[4] The

most widely used ET-based spectrophotometric total antioxidant capacity (TAC) assays are Folin−Ciocalteu,[5] ABTS/TEAC (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid/Trolox equivalent antioxidant capacity),[6] CUPRAC (Cupric Reducing Antioxidant Capacity),[7] and FRAP (Ferric Reducing Antioxidant Power).[8] Antioxidant activity assays such as oxygen radical absorbance capacity (ORAC),[9] total peroxyl radical trapping antioxidant parameter (TRAP)[10] and total oxyradical scavenging capacity (TOSC)[11] are known as HAT-based assays, whereas certain radical scavenging assays are considered to operate in mixed-mode (ET-HAT).

In recent years, nanoparticle-based methods have been developed for the determination of the total antioxidant capacity. These methods are usually based on the formation or growth of noble metal nanoparticles. Antioxidant compounds can reduce Au(III) to AuNPs in colloidal dispersion, consistent with their electron-donating affinity. By using these AuNPs as colored probes due to their localized surface plasmon resonance (LSPR) absorption, antioxidant capacity determination is performed.[12] Similarly, noble metal can be selected as silver so as to produce silver nanoparticles (AgNPs) by the reduction of Ag$^+$ to AgNPs with antioxidants, and again there are two options, namely formation or growth of AgNPs.[13−16] If the second option is selected, the enlargement of preformed AgNPs gives rise to enhanced LSPR absorption dependent on the polyphenol concentration with a better linear response. Chou et al.[17] performed a green synthesis of AuNPs using natural antioxidants found in fruit juices. The colorimetric response of AuNPs was dependent on the concentration of antioxidants tested. Thus, a colorimetric method based on the formation of AuNPs was developed to evaluate the total antioxidant capacity of fruit juices. Scroccarello et al.[18] determined the sugar content and antioxidant capacity of apples through the formation of metal nanoparticles. Sugar content was determined by silver nanoparticle formation, while antioxidant capacity was determined by gold nanoparticle formation. The absorbance of AuNPs formed in the presence of antioxidants was measured and recorded at 540 nm. In another study, a new paper-based antioxidant activity assay was developed, again using polyphenol-mediated growth of AuNPs on the paper surface.[19] Determination of antioxidant capacity based on the analyte-driven formation of AuNPs on paper-based devices relies on the direct reduction of gold ions to Au(0) by phenolic acids in the paper matrix. The intensity of the characteristic red color, indicative of the localized surface plasmon resonance properties of Au nanoparticles, depends on the reducing power of the sample solution, which is proportional to the antioxidant capacity. Ma et al.,[20] designed a new colorimetric sensor for the detection of heparin using gold nanoparticles. AuNPs tend to aggregate in the presence of poly(diallyldimethylammonium chloride) and tend to shift to red in their UV−vis absorption spectra. Heparin can bind poly(diallyldimethylammonium chloride) to form a stable complex, thus effectively preventing the aggregation of AuNPs. Nanoparticles of transition metal oxides such as cerium are also used among recently developed nanotechnology-based antioxidant capacity assays. Sharpe et al.[21] used immobilized ceria nanoparticles that change color after interacting with antioxidants through redox and surface chemistry reactions in the method they developed. In another study using cerium nanoparticles, the total antioxidant capacity of plant and food extracts was determined by utilizing the oxidase-like behavior of cerium nanoparticles. Poly(acrylic acid) sodium salt-coated cerium oxide nanoparticles oxidize tetramethyl benzidine, a peroxidase substrate, to a blue charge transfer complex in a slightly acidic solution. Antioxidants reduced the color intensity of the nanoceria suspension and were indirectly determined by the absorbance difference. Unlike established "nanocera methods", in this method, instead of taking advantage of the natural color change of nanoceria when in contact with antioxidants, sensitivity is increased by using an indirect colorimetric method with TMB (3,3′,5,5′-tetramethylbenzidine).[22]

In this study, a simple and sensitive ET-based antioxidant capacity assay was developed by utilizing the reduction of Au(III) with antioxidant compounds. Because the gold(III) ion-reducing ability of polyphenols is measured, the method is named "Auric Reducing Antioxidant Capacity", abbreviated as the AuRAC assay. While the reactive aromatic hydroxyl groups of polyphenols were oxidized to the corresponding quinones, Au(III) ions were reduced to a zerovalent state, forming AuNPs. ABTS was used as a chromogenic reagent that was oxidized to a blue-green oxidation product with the remaining unreduced Au(III) in the reaction medium. As a result, the absorbance of the oxidized-ABTS product (ABTS$^{.+}$) shows maximum light absorption at 412 nm which decreases with increased antioxidant capacity. We developed a novel approach to qualitatively and quantitatively determine antioxidant capacity by the naked eye and UV−visible spectroscopy, respectively. AuRAC assay was applied to various antioxidant compounds (dietary polyphenols, vitamin, and thiol-type antioxidants), their synthetic mixtures, and real samples with higher sensitivity owing to the fact that formation of the assay chromophore (ABTS$^{.+}$) involves 3-e$^-$ reduction of Au(III) to Au(0) compared to the single electron transfer (SET) of conventional TAC assays.

2. METHODS

2.1. Chemicals. The following chemical substances of the analytical reagent grade were supplied from the corresponding sources: tetrachloroauric acid (HAuCl$_4$, 99.99%) solution (30% by wt, in dilute HCl), L-glutathione reduced (GSH), N-acetyl-L-cysteine (NAC), quercetin (QR), acetic acid, sodium acetate, and ethanol were purchased from Sigma-Aldrich. Gallic acid (GA), rutin, L-methionine (MET) and were purchased from Sigma. Neocuproine, L-ascorbic acid (AA), Trolox (TR), rosmarinic acid (ROS), copper(II) chloride (CuCl$_2$), and ammonium acetate (NH$_4$Ac) were purchased from Aldrich. L-cysteine (CYS), DL-homocysteine (HCYS), 1,4-dithioerythritol (DTE), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), epicatechin (EC), catechin (CAT) and epicatechin gallat (ECG) were purchased from Fluka. The pharmaceutical samples of NAC effervescent tablet (600 mg NAC) and GSH tablet (250 mg GSH) and tea samples were purchased from a local drugstore and market.

2.2. Apparatus. Spectrophotometric measurements were performed with a Shimadzu UV-1900 spectrophotometer. X-ray photoelectron spectroscopy (XPS) was conducted using a K-Alpha spectrometer (Thermo Fisher, USA) employing a monochromated Al Kα X-ray source ($h\nu$ = 14686.6 eV). The wet-STEM images of gold nanoparticles formed as a result of the reduction of Au(III) by ABTS in the reaction medium were obtained using a Thermo Scientific Quattro scanning electron microscope (USA). The particle size analysis of the reaction mixture was determined by a dynamic light scattering (DLS) instrument (HORIBA SZ-100) (Japan).

2.3. Preparation of Solutions. ABTS and gold(III) solutions were prepared in distilled water at 1.0 mM concentration. Polyphenolic compounds were freshly prepared in absolute ethanol, and thiol-type antioxidant solutions and ascorbic acid were prepared in distilled water. All antioxidant stock solutions were prepared at a 1.0 mM concentration and diluted to the desired concentration.

Synthetic antioxidant mixtures were prepared at the final concentrations listed below:

Mix 1: 5×10^{-5} M CAT + 5×10^{-5} M GA + 5×10^{-5} M QR
Mix 2: 4×10^{-5} M QR + 5×10^{-5} M GSH + 1×10^{-4} M NAC
Mix 3: 4×10^{-5} M CAT + 4×10^{-5} M AA + 1×10^{-4} M CYS

Mix 4: 4×10^{-5} M ROS + 8×10^{-5} M GA + 4×10^{-5} M CAT

The AuRAC method was applied by taking 0.1 mL of the synthetic antioxidant mixtures. Tea infusions were prepared by steeping tea bags (2.0 g for black tea, 1.7 g for green tea) in a beaker containing 250 mL of freshly boiled water for 5 min. Tea infusions were filtered through a 0.45 μm PTFE filter after being cooled to room temperature and diluted (1:20) with distilled water for AuRAC and CUPRAC assays. The pharmaceutical samples (NAC effervescent tablet (600 mg NAC) and GSH tablet (250 mg GSH)) were prepared in 100 mL of distilled water and filtered through a 0.45 μm PTFE filter. Pharmaceutical samples were diluted 100 times with distilled water for the AuRAC and CUPRAC assays.

2.4. AuRAC Assay. For the AuRAC assay, 1.0 mL of acetate buffer (pH 3.6), $(3.6 - x)$ mL of distilled water, x mL of standard antioxidant solution (polyphenols, biothiols, etc.), or real sample solution and 0.1 mL of 1 mM Au(III) solution were added and 4 min were allowed for the reduction of Au(III) by antioxidants. A volume of 0.3 mL of 1 mM ABTS was added to this mixture and incubated for five more minutes at room temperature. After incubation, the absorbance of the reference solution (without antioxidant, A_0) and sample solution (A) was recorded at 412 nm against water. The standard calibration curve of each antioxidant compound was constructed in this manner as the ΔA ($A_0 - A$) versus concentration, and the molar absorptivities (ε) were determined from the slopes of the calibration lines.

2.5. CUPRAC Assay. One milliliter each of copper(II) chloride, neocuproine, and ammonium acetate solutions were added to a test tube. Then, x mL of antioxidant solution or real sample and $(1.1 - x)$ mL of water were added to the tube to reach a final volume of 4.1 mL. After 1/2 h incubation at room temperature, the absorbance at 450 nm was recorded against a reagent blank.[7]

2.6. Statistical Analysis. Descriptive statistical analyses were performed using Excel software (Microsoft Office 2002) for calculating the mean and standard error of the mean. Using SPSS software for Windows (version 13), the data were evaluated by two-way ANalysis Of VAriance (ANOVA).

3. RESULTS AND DISCUSSION

In this work, a novel antioxidant capacity assay was developed, based on the gold(III) ion-reducing ability of antioxidants. The chromogenic redox reagent used for the AuRAC assay was ABTS, which was oxidized with gold(III) (after reduction by antioxidants) in the reaction medium. Peroxidase substrates such as TMB, ABTS, and diazoaminobenzene can be directly oxidized to colored products by metal ions such as Ag(I) and Au(III). The use of noble metal ions that possess intrinsic oxidizing power, such as Ag(I) and Au(III), to directly oxidize colorless peroxidase substrates, can aid colorimetric assays by enhancing the signals due to colored oxidized forms of TMB or ABTS.[23] Thus, Chen et al.[23] were able to successfully oxidize the colorless ABTS by Au(III) to the bluish-green oxidized form of ABTS. Fazli et al.[24] and Jang and Roper[25] had shown that Au(III) is capable of oxidizing TMB to its blue-colored oxidized form, composed of a charge-transfer complex between oxidized and unoxidized TMB. On the other hand, the peroxidase-like activity of AuNPs,[26] normally formed as a result of Au(III) oxidation of a peroxidase-substrate dye, is expected to catalyze this type of redox reaction by reducing the activation energy. Garcia-Leis et al.[27] had shown that the interfacial and plasmonic properties of the formed noble-metal nanoparticles were

responsible for the catalytic effect on the redox reaction leading to ABTS$^{\cdot+}$ formation at acidic pH. Strong oxidizing agents like peroxydisulfate can oxidize ABTS to its radical cation (ABTS$^{\cdot+}$) with a first-order reaction at a significantly reduced activation energy, i.e., about 8 kcal/mol.[28] Likewise, the reduction potential of the Au(III)/Au(0) redox couple is far ahead of that of ABTS$^{\cdot+}$/ABTS. To summarize, the activation energy barrier of ABTS oxidation to ABTS$^{\cdot+}$ could be effectively overcome using gold(III) with a significant redox potential difference (responsible for thermodynamic favorability) and AuNP-catalyzed reaction having a low activation energy at an acidic pH of 3.6 (for kinetic favorability).

Gold(III) can oxidize ABTS to a bluish-green oxidation product (ABTS$^{\cdot+}$) having a strong absorption peak at 414 nm.[23] The standard reduction potential of the ABTS$^{\cdot+}$/ABTS redox couple is 0.68 V,[29] while that of Au(III)/Au(0) is 1.4 V (E^0 of AuCl$_4^-$/Au is 1.002 V due to selective chloride complexation of trivalent gold).[30] Thus, there is a sufficient difference between the redox potentials of the oxidant (Au(III)) and reductant (ABTS) in our work, providing the plentiful driving force for the assay reaction.

In the presence of antioxidants, Au(III) is reduced to its zerovalent state forming AuNPs, accompanied by the inhibition of ABTS oxidation giving rise to a diminished absorbance of the bluish-green ABTS$^{\cdot+}$ product. The antioxidant capacity was successfully determined by utilizing the decline in the absorbance value of the oxidation product, which showed a linear dependence on the amount of antioxidants.

XPS studies showed that gold(III) was reduced in the presence of the ABTS reagent or antioxidant compounds. Figure 1 shows Au 4f XPS spectra for the mixtures of gold(III) with the
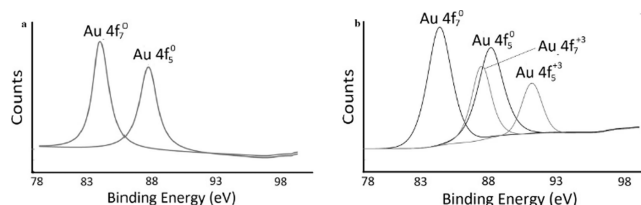

Figure 1. High-resolution Au 4f XPS spectrum of gold(III) mixtures with ABTS (a) and trolox (b).

ABTS reagent (Figure 1a) and Trolox (Figure 1b). XPS spectrum of the Au 4f core level can be characterized by pairs of peaks due to Au 4f$_{7/2}$ and Au 4f$_{5/2}$ spin–orbit coupling. While the positions of the first and most important pair (with binding energies of 84 and 87.3 eV) are related to Au in zero oxidation state (Au^0), those of the other pairs are related to Au$^+$ (with binding energies of 85.6 and 89.1 eV) and Au^{3+} (with binding energies of 87.3 and 90.4 eV).[31] XPS spectrum of the Au(III)-ABTS mixture shows the Au 4f$_{5/2}$ and Au 4f$_{7/2}$ doublet with binding energies of 87.6 and 84.0 eV, respectively, which are typical values for zerovalent Au, indicating almost all the Au(III) was successfully reduced by ABTS.[23] The spectrum of the mixture of Au(III) and Trolox (Figure 1b) indicates that Au(III) is reduced to the atomic state Au(0), while the remaining amount still exists as Au(III) in the reaction medium. XPS spectrum of the Au(III)-Trolox mixture shows the Au 4f$_{5/2}$ and Au 4f$_{7/2}$ doublet with binding energies of 87.6 and 84.0 eV for Au(0), 87.3, and 90.4 eV for Au(III), respectively. As expected, the remaining Au(III) in the reaction medium is reduced by ABTS added in the next step to form the colored oxidation

product. Due to the instability of the naked Au⁺ ion and its disproportionation to Au(III) and Au(0), no Au⁺ ion was found in this oxidation step in the reaction medium.[32]

Gold nanoparticles formed as a result of the reduction of Au(III) by ABTS or an antioxidant in the reaction medium were also visualized with STEM (Figure S1). The average size of AuNPs formed as a result of the reaction of Au (III) and ABTS was determined using the DLS instrument. The average size of the AuNPs was measured as 77.3 ± 0.6 nm (Figure S2). The nanoparticles exhibited a homogeneous distribution, and the polydispersity index was measured as 0.445 ± 0.005 for AuNPs.

Garcia-Leis et al.[27] reported a strong effect of silver plasmonic nanoparticles (Ag NPs) on ABTS, leading to the formation of ABTS·⁺. ABTS strongly interacts with Ag nanoparticles at low pH in a complex process that implies a two-step reaction. This reaction generates ABTS·⁺ via a catalytic process induced by the surface of silver nanoparticles. It has been reported that under appropriate conditions, ABTS molecules can trigger an AgNP etching process. First silver particles decompose and then larger silver nanostructures are formed by rearranging Ag clusters. The existence of both small silver clusters and large silver spheres was found to be consistent with the UV−visible spectrum, where no plasmon resonance was observed.[27]

Chen et al.[23] highlighted that the optimal pH value is 3.6 for the redox reaction between ABTS and Au(III). The oxidation of ABTS by Au(III) was more efficient under acidic conditions due to its greater solubility and higher oxidizing ability of Au(III) at low pH.[24]

The effect of the reaction time was investigated in two stages, namely, the redox reactions between Au(III)-antioxidant (10 μM TR) and the remaining Au(III)-ABTS. It was found that reaction times of 4 and 5 min, respectively, were found to be sufficient for the completion of these two oxidation reactions.

Since the developed method is based on the formation of colored oxidation products as a result of the reaction between Au(III) and ABTS, the concentration ratio of Au(III) and ABTS to be used in the reaction medium is of great importance. In order to determine the optimal concentrations of ABTS and Au(III), different concentration ratios ([ABTS]/[Au(III)]) in the range 2−6 were tested. Figure S3 shows that the maximum ΔA value was obtained when the ABTS concentration was three times that of the Au(III) concentration.

Figure 2a displays the UV−visible spectra of the ABTS oxidation product (ABTS·⁺) in the absence and presence of TR.
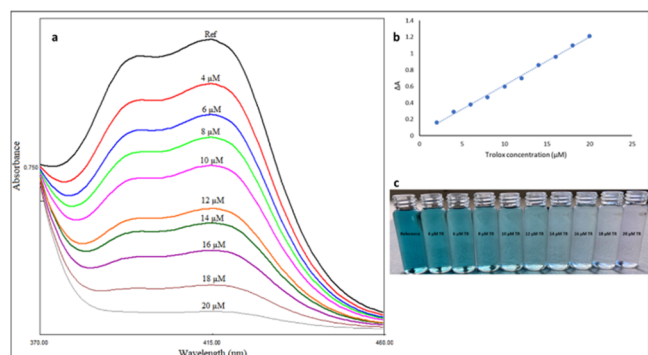

Figure 2. (a) UV−vis absorption spectra of the reaction mixtures with varying concentrations of TR in the range of 4−20 μM, (b) the calibration curve of TR with respect to the proposed assay, (c) photographs of Au(III)−ABTS solution in the absence and presence of different concentrations of TR in the range of 4−20 μM.

It can be observed that increasing Trolox concentrations from 4 to 20 μM gave rise to a decrement in the absorbance at 412 nm of the bluish green ABTS·⁺ product due to the consumption of some Au(III) in the reaction medium by the antioxidant. The linear equation for the calibration curve of TR was calculated as $\Delta A_{412} = 5.85 \times 10^4 c + 0.03$ ($r = 0.9985$) where c is the molar concentration (Figure 2b). The working concentration range and the limit of detection (LOD) were found as 1−20 and 0.41 μM, respectively. As opposed to methods merely relying on the formation of AuNPs,[33] the LOD is very low and there is no shift in the maximal absorption wavelength of the chromophore varying with antioxidant concentration. The shifts in absorption maxima of LSPR bands of methods targeting simply at AuNP formation[33] arise from the differential kinetics of various antioxidant phenolics in reducing Au(III) to Au(0) resulting in nanoparticles at different sizes. The optical absorption and scattering are largely dependent on the size of the nanoparticles. For a 20 nm AuNP, the total extinction is nearly all contributed by absorption. With increasing particle size (e.g., 40 nm), scattering begins to appear. It is known that the ratio of scattering to absorption for larger particle dimensions increases significantly.[34] In the AuRAC assay, the average size of the nanoparticles formed as a result of the reaction of ABTS and gold(III) was measured as 77 nm by DLS analysis. Because of particle size, the characteristic SPR band of AuNPs in the visible region was not observed. In the AuRAC assay absorbance measurement was made at the maximal light-absorption wavelength (412 nm) of bluish-green oxidation product (ABTS·⁺). In other words, the LSPR absorption of AuNPs does not cause an interference in the absorption measurement of the oxidation product. Moreover, perfect linearity cannot be achieved in methods merely relying on AuNP formation due to the same kinetic reasons, as observable in the sigmoidal curves.[35] In the absence and presence of antioxidants using the current method, the color difference among solutions depending on the amount of chromogenic oxidation product formed as a result of the ABTS−Au(III) reaction can be easily distinguished with the naked eye under daylight without any extra equipment (Figure 2c).

TEAC coefficients for the AuRAC assay of 14 individual antioxidants were calculated by using the molar absorption coefficient for TR ($\varepsilon = 5.85 \times 10^4$ L mol⁻¹ cm⁻¹). The TEAC coefficients of various antioxidant compounds found in the AuRAC assay are listed in Table 1 in comparison with the TEAC coefficients reported for the CUPRAC method.[36,37] As seen in the calibration curves of various antioxidants like TR (Table 1), the very high molar absorption coefficients arise from 3-e⁻ transfer of one Au(III) ion to 3 ABTS molecules to produce 3 radical cations of ABTS·⁺, unlike single electron transfer (SET) reagents used in conventional TAC assays. Au(III) has a sufficiently high reduction potential to oxidize both antioxidants and ABTS. Apak et al.[36] reported that the highest antioxidant capacities in the CUPRAC method were found in the order of rosmarinic acid, epicatechin gallate, quercetin, catechin, epicatechin, gallic acid, and rutin. A similar ranking was obtained for the antioxidant capacities found by the AuRAC assay for the studied antioxidant compounds (excluding thiol-type antioxidants). There was no significant difference between the precisions of the two methods ($P = 0.05$, $F_{exp} = 1.82$, $F_{table} = 4.75$, $F_{exp} < F_{table}$).

As is known from the literature,[7] the number and position of hydroxyl groups and the degree of conjugation of the molecule are directly related to antioxidant capacity as they are important

Table 1. Linear Equations, Linear Working Ranges, and TEAC Values for Antioxidants (AOX) with Respect to the AuRAC Assay As Compared to Those of the CUPRAC Assay

AOX	linear equation and correlation coefficient	TEAC$_{AuRAC}$	TEAC$_{CUPRAC}$	linear range (μM)
QR	$\Delta A = 15.51 \times 10^4 c - 0.055$ $r = 0.9975$	2.65	4.38[a]	0.5–9
GA	$\Delta A = 14.5 \times 10^4 c + 0.065$ $r = 0.9990$	2.48	2.62[a]	0.5–9
CAT	$\Delta A = 12.27 \times 10^4 c + 0.05$ $r = 0.9931$	2.10	3.09[a]	0.5–12
EC	$\Delta A = 13.79 \times 10^4 c - 0.180$ $r = 0.9920$	2.36	2.77[a]	1–15
ECG	$\Delta A = 18.9 \times 10^4 c - 0.059$ $r = 0.9963$	3.23	5.32[a]	0.4–5
rutin	$\Delta A = 6.64 \times 10^4 c + 0.007$ $r = 0.9917$	1.14	2.56[a]	1.8–20
ROS	$\Delta A = 25.2 \times 10^4 c + 0.160$ $r = 0.9930$	4.35	5.65[a]	0.2–5
AA	$\Delta A = 4.27 \times 10^4 c + 0.046$ $r = 0.9980$	0.73	0.96[a]	2–20
GSH	$\Delta A = 7.01 \times 10^4 c + 0.182$ $r = 0.9903$	1.20	0.57[b]	1–16
CYS	$\Delta A = 5.61 \times 10^4 c + 0.096$ $r = 0.9979$	0.96	0.39[b]	1.5–20
HCYS	$\Delta A = 7.92 \times 10^4 c - 0.036$ $r = 0.9968$	1.35	0.47[b]	1–16
DTE	$\Delta A = 8.50 \times 10^4 c + 0.085$ $r = 0.9974$	1.45	0.84[b]	1–20
NAC	$\Delta A = 6.19 \times 10^4 c + 0.068$ $r = 0.9978$	1.06	0.43[b]	1–20
methionine	$\Delta A = 3.83 \times 10^4 c - 0.100$ $r = 0.9968$	0.65	ND[b]	4–36

TEAC coefficients for AuRAC−CUPRAC assays; ($P = 0.05$, $F_{exp} = 1.82$, $F_{table} = 4.75$, $F_{exp} < F_{table}$). ΔA = the difference in absorbance, c = molar concentration, r = correlation coefficient. ND: not detected. [a]Data taken from ref 38. [b]Data taken from ref 37.

for efficient electron transfer. Medvidovic-Kosanovic et al.[39] investigated the electrochemical properties of three structurally different flavonoids with cyclic voltammetry and determined their antioxidant capacity with the Trolox equivalent antioxidant capacity (TEAC) assay. This study reports that a lower half-wave potential value ($E_{1/2}$) of the first oxidation wave also indicates a higher antioxidant capacity. Accordingly, quercetin has the lowest $E_{1/2}$ value (0.340 V), followed by catechin (0.391 V) and rutin (0.419 V) at pH 3.5. The TEAC assay yielded the same relative order of antioxidant capacities of the respective flavonoids obtained by cyclic voltammetry. The reason for the high antioxidant capacity observed for quercetin is that it has three important structural features such as the 5-hydroxy-4-keto group in A & C rings of flavonols, the 2,3-double bond connecting the two ring systems of flavonol via conjugation, and the 3′,4′-dihydroxy substitution of the B ring. The lower antioxidant capacity of catechin compared to quercetin can be explained by the lack of a 4-oxo group in the C ring. Rutin, also called quercetin-3-O-rutinoside, is known to have a lower antioxidant capacity than quercetin and catechin, as it has an O-rutinase substitution at the 3-position instead of −OH. O-rutinase substitution causes a decrease in antioxidant capacity by both decreasing the number of −OH groups in the molecule and increasing the steric tension in the molecule. TEAC values in the AuRAC assay were observed as 2.65 (5 OH), 2.10 (5 OH), and 1.14 (4 OH, -O-rutinose) for quercetin, catechin, and rutin (the values in parentheses showing the number of hydroxyl groups in the molecule of these compounds), respectively, in accordance

with these theoretical expectations. Salah et al.[40] determined the relative antioxidant activities of some flavanolic polyphenols, catechins, and catechin-gallate esters using the TEAC assay. Moreover, the order of antioxidant capacity of related antioxidants was measured as ECG (4.93) > QR (4.72) > GA (3.01) > EC (2.50) > CAT (2.40), again entirely consistent with the results of the AuRAC assay. The fact that the TEAC coefficient obtained for ECG is relatively higher than those for the related catechins may be due to the presence of trihydroxy benzoate (gallic acid) moiety.[40] Huang and Frankel[41] investigated the antioxidant activity of tea catechins in different lipid systems. According to this study, it was emphasized that gallic acid is a more effective antioxidant than EC and CAT, and also that EC is a better hydrogen atom donor than catechin on the basis of the one-electron redox potential. Grzesik et al.[42] compared the antioxidant properties of catechins with those of antioxidants including GSH and ascorbic acid by utilizing widely used methods such as peroxyl radical-induced fluorescein bleaching, ABTS/TEAC, and FRAP. In this study, it was reported that catechins have much higher antioxidant activity compared to GSH and ascorbic acid, and the antioxidant activity of both GSH and ascorbic acid is close to that of trolox. These results are in good agreement with those of the AuRAC assay.[42]

Li et al.[43] developed a sensitive colorimetric method for the determination of glutathione by using direct oxidation of ABTS with Ag(I). The molar absorption coefficient for glutathione was reported as 5.44×10^4 L mol^{-1}cm^{-1} by this colorimetric method. In this study, we used direct oxidation of ABTS with Au(III), and the molar absorption coefficient for glutathione was found to be 7.01×10^4 L mol^{-1}cm^{-1}. In two other studies where Ag$^+$-TMB[44] and I$^-$-H$_2$O$_2$-TMB[45] systems were used, the molar absorption coefficients for glutathione were reported as 11.48×10^4 and 3.54×10^4 L mol^{-1}cm^{-1}, respectively.

Thiols are compounds containing a sulfhydryl group (−SH) and have critical roles in many biological processes, such as combating oxidative stress and maintaining redox homeostasis. For these reasons, thiols are among the important antioxidant compounds.[46] AuRAC assay was applied to thiol-type antioxidants such as reduced glutathione, cysteine, homocysteine, N-acetyl cysteine, 1,4-dithioerythritol, and methionine. The antioxidant capacities of these biologically important antioxidant compounds were successfully determined by the AuRAC method. According to AuRAC results, the highest capacity among thiol-type antioxidants was observed in two −SH bearing DTE. This result is highly consistent with both the CUPRAC method results and the structural properties. TEAC coefficients decrease in the following order: DTE > HCYS> GSH > NAC > CYS > Met (Table 1). Methionine is a sulfur-containing thioether-type amino acid that occurs in proteins and its residues may act as endogenous antioxidants and important free radical scavengers in biological systems.[47,48] Güngör et al.[37] investigated the antioxidant capacity of methionine by the widely used spectrophotometric TAC assays such as CUPRAC, ABTS/TEAC, and FRAP, but an antioxidant capacity as TEAC coefficient could not be detected in all three methods, due to the fact that methionine lacks a free sulfhydryl: −SH group that is more easily oxidizable. Since Au(III) has higher oxidizing power than conventional assay reagents, the TEAC coefficient for methionine was determined as 0.65 with the AuRAC assay. The molar absorption coefficients obtained by the AuRAC assay for the studied antioxidant compounds were found to be higher than those found by the widely used ET-based spectrophoto-

metric TAC assays such as Folin−Ciocalteu,[5] ABTS/TEAC,[6] CUPRAC,[7] and FRAP.[8]

Precision and recovery of the AuRAC assay were investigated by spiking known amounts of antioxidants (QR and CAT) to a real sample. The relative standard deviations (RSD, %), indicative of the precision of the AuRAC assay, were calculated as 3.29 and 5.13%. The percentage recovery values of the added antioxidants were found as 105 and 97.5% for QR and CAT, respectively (Table 2).

Table 2. Precision and Recovery Values for Selected Antioxidants (AOX) Using the AuRAC assay (N = 3)

sample	AOX:QR	AOX:CAT
added AOX concentration (μM)	2.00	2.00
found AOX concentration added (μM), excluding original amount	2.10 ± 0.07	1.95 ± 0.10
REC%	105.0	97.50
RSD%	3.29	5.13

Data presented as (mean ± SD), where N = 3. TAC value of 1:20 diluted black tea infusion was 2.06 ± 0.05 and 2.61 ± 0.09 μM, as QR and CAT equivalents, respectively.

Total antioxidant capacities of ternary synthetic mixtures prepared as specified in Section 2.2 were calculated as μM TR equivalent by using the AuRAC assay. When these capacities are examined, it can be concluded that the sum of the individual capacities of the antioxidants in a synthetic mixture is an approximate estimate of the TAC, so there is no chemical deviation from Beer's law. As a result, there was no significant difference between the experimentally found capacity results and the theoretically expected capacity results at the 95% confidence level (P = 0.05, F_{exp} = 1.61, F_{table} = 10.13, F_{exp} < F_{table}). Thus, the AuRAC assay was validated (Table S1).

Tea infusions and pharmaceutical samples were used to test the applicability of the AuRAC assay to real matrices. The results found by the AuRAC assay were in agreement with those of the CUPRAC assay (Tables 3 and 4). Green and black teas have

Table 3. TAC Values of Tea Infusions Assayed by AuRAC and CUPRAC Assays

tea infusions	TAC$_{AuRAC}$ (μM TR)	TAC$_{CUPRAC}$ (μM TR)
black tea	8547 ± 280	8052 ± 130
green tea	9572 ± 340	9329 ± 243

Data presented as (mean ± SD), where N = 3.

Table 4. Biothiol Contents of Pharmaceutical Samples with Respect to the AuRAC and CUPRAC Assays

pharmaceuticals and their biothiol contents	found biothiol content with AuRAC assay (mg)	found biothiol content with CUPRAC assay (mg)
NAC effervescent (600 mg)	633 ± 20.4	640 ± 15
GSH tablet (250 mg)	249 ± 10.8	246 ± 9.6

Data presented as (mean ± SD), where N = 3.

different polyphenol contents due to their different manufacturing processes. While green tea is rich in catechin derivatives (epicatechin, epigallocatechin, epicatechin-3-gallate, and epigallocatechin-3-gallate), black tea contains thearubigins and theaflavins due to the fermentation process.[49] In the AuRAC method, the total antioxidant capacity of green tea was found to

be higher than that of black tea. This is supported by both the CUPRAC method and similar studies in the literature. For example, Lee et al.[50] studied the antioxidant activity of black tea versus green tea and pointed to the fact that green tea has more health benefits than an equal volume of black tea. Bener et al.[51] found the antioxidant capacity of green tea (1568 ± 76 μM TR/g sample) to be higher than that of black tea (1212 ± 45 μM TR/g sample) with heparin-stabilized gold nanoparticles-based CUPRAC colorimetric sensor. Anesini et al.[49] reported that green tea has a higher polyphenol content than black tea and there is a good correlation between antioxidant activities and polyphenol content. In addition, the AuRAC assay was applied to two pharmaceutical samples whose active ingredients were NAC and GSH. The percentage recovery values of the NAC effervescent tablet and GSH tablet were calculated as 105.5 and 99.6%, respectively.

The possible interference effect of some common ions (sodium, calcium, chloride, and citrate), sugars (glucose, fructose), and amino acids (glycine) for the AuRAC assay was examined. No interference effect was observed for the aqueous solutions of these interferent species (1000-fold (mol/mol) of trolox concentration, except for citrate (where 100-fold (mol/mol) of trolox concentration was tolerated)). The difference in absorbance at 412 nm in the presence of these interferent species is shown in Figure S4.

Table 5 summarizes the LOD values of gallic acid and glutathione (GSH) obtained by the AuRAC assay, colorimetric

Table 5. Performance Comparison for the Proposed Assay with Other TAC Assays in the Literature

methods	$\varepsilon_{Gallic\ Acid}$ (L mol^{-1} cm^{-1})	ε_{GSH} (L mol^{-1} cm^{-1})	reference
AuRAC	145,000	70,100	this work
conventional CUPRAC	43,900[a]	9470[b]	[a]Apak et al.[7]
ABTS/TEAC	50,100[a]	24,000[b]	[b]Güngör et al.[37]
CUPRAC sensor	50,600		Bener et al.[52]
Fe(III)-phen sensor	92,400	16,700	Bener and Apak[53]
CUPRAC-AuNP sensor	40,500	5600	Bener et al.[51]
SNPAC	22,700		Özyürek et al.[13]
Carregenan-SNP	65,500		Öztürk et al.[15]
SNP-based method	6000		Szydłowska-Czerniak et al.[54]
GSNP-AC	36,800		Beǧiç et al.[55]
PVA-SNP	10,700		Teerasong et al.[56]
RhNP-based method	2000		Gatselou et al.[57]

sensors, and nanoparticle-based TAC assays. According to the molar absorption coefficients listed in Table 5, the proposed AuRAC assay has higher sensitivity compared with other TAC tests.

The potential to convert the developed colorimetric method into a paper-based antioxidant sensor was examined. In this context, the first ABTS chromogenic reagent was dropped onto the paper and dried at room temperature. After drying, the paper impregnated with ABTS has acquired a pale green color as seen in Figure 3. When gold(III) solution was dropped onto paper impregnated with ABTS, the paper turned dark bluish-green due to the oxidation of the ABTS reagent (Figure 3a), whereas increasing concentrations of antioxidant proportionally bleached this color (Figure 3b,c). The intensity difference in
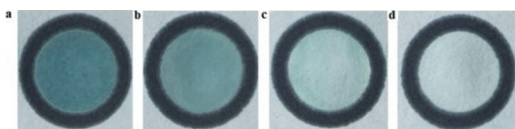
Figure 3. Photographs of paper-based antioxidant sensor. (a) Au(III) (b) Au(III)+ 5 μM TR (c) Au(III)+ 10 μM TR (d) paper impregnated with ABTS.

the dark green color is proportional to the antioxidant content of the sample. Thus, the potential to transform the proposed method into a paper-based, field-portable colorimetric kit format has been demonstrated.

4. CONCLUSIONS

In this study, a novel ET-based antioxidant capacity assay was described. It is based on the oxidation reaction of ABTS, used as a chromogenic reagent, with gold(III) to produce a bluish-green oxidized product (ABTS$^{•+}$) with an absorption peak at 414 nm. AuRAC assay is more sensitive than the methods based on the growth or formation of metal nanoparticles available in the literature, thanks to its high molar absorptivity. Although XPS spectra confirmed that Au(III) was selectively reduced to Au(0) in the presence of antioxidants, AuRAC is not dependent on AuNPs size or LSPR band shifts as the evaluation is made on the basis of differential ABTS$^{•+}$ formation. AuRAC assay was well correlated with the findings of the reference CUPRAC assay, and the applicability of the assay to food and pharmaceutical samples for total antioxidant capacity measurement with good accuracy and precision has been demonstrated. As a result, a novel colorimetric assay was developed for antioxidant capacity measurement in different sample matrices that could be used with a simple, reliable, and robust methodology. More fascinatingly, AuRAC testing is suitable for in situ detection as the color difference in the absence and presence of antioxidants allows visual detection of TAC without any additional measurement.

ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c09134.

> STEM images and particle size distribution of nano-particles; additional experimental details including concentration ratios of ABTS/Au(III) and potential interferents; and total antioxidant capacity of synthetic antioxidant mixtures (PDF)

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B.B.: Investigation, Methodology, Experimental work, Writing— original draft. R.A.: Conceptualization, Writing—review and editing.

Notes

The authors declare no competing financial interest.

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