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SNaP-C: Development of a Silver Nanoparticle Antioxidant Assay for the Selective Quantitative Analysis of Vitamin C in Beverages

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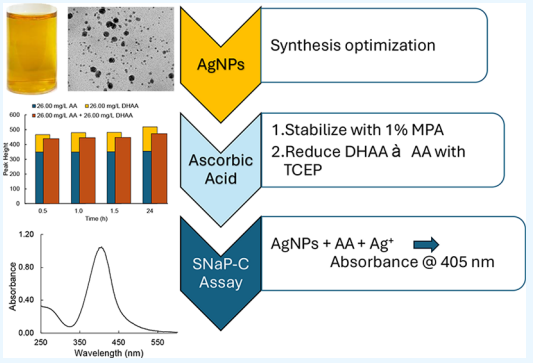
ABSTRACT: The development of a sensitive and selective silver nanoparticle assay for the quantitation of vitamin C (SNaP-C), as ascorbic acid (AA) and total ascorbic acid (TAA = AA + dehydroascorbic acid, DHAA), is described. Three assay parameters were investigated and optimized: (1) synthesis of silver nanoparticles (AgNPs) to produce a reliable enhanced localized surface plasmon resonance (LSPR) in the presence of specific added antioxidants; (2) ensuring long-term stability of AA and DHAA in aqueous solutions; and (3) SNaP-C assay conditions to allow for rapid analysis of samples (beverages) by monitoring the enhanced LSPR. The synthesis of AgNPs using soluble starch as a capping agent and D-arabinose as a reducing agent was optimized in a CEM Discover SP laboratory microwave. Given that AA and DHAA lack aqueous stability, these forms were stabilized via the addition of 1% (w/v) meta-phosphoric acid. To convert DHAA to AA, the reducing agent tris(2-carboxyethyl) phosphine hydrochloride (TCEP) was added with its concentration (2.5 mM) and reaction time (24 h) optimized. Using a Box−Behnken design for three factors, the SNaP-C assay reaction conditions of 1000 μL of AgNPs (in suspension) with 100 μL of beverage (or analytical standard) and 4.9 mL of water using the CEM Discover SP microwave were optimized, resulting in an incubation time of 90 °C for 6 min. Finally, a list of potential interferents that commonly respond to other antioxidant capacity assays, like the Folin−Ciocalteu, were investigated to demonstrate that the SNaP-C assay is selective and sensitive for ascorbic acid and gallic acid.



INTRODUCTION

A single universally accepted assay for the determination of antioxidant capacity has not been developed owing to differences in method procedure and standardization.[1−4] Numerous antioxidant assays have been developed and modified in the literature for broad application and sample type, but method parameters may not be fully evaluated.[5] Several comprehensive reviews highlight technical issues and lack of standardization in published methods.[2,5−7] Many currently employed assays for the analysis of antioxidant capacity (or total phenolic content or total reducing capacity) suffer from unclear mechanisms and poorly defined parameters that result in inconsistent measurements when replicated. Several literature reviews[2,5−8] describe the shortcomings of presently accepted assays that motivated the present work described here to develop a fully validated and defined assay for the analysis of vitamin C, which is predominantly ascorbic acid (AA) and dehydroascorbic acid (DHAA), in beverages.[9] Vitamin C has been of significant clinical and consumer interest since the COVID-19 pandemic,[10−13] with the growth of the functionalized beverages market expected to substantially increase.[14] Moreover, from an analytical perspective, the analysis of vitamin C in beverages is an interesting topic because of the lack of stability in aqueous solutions and conversion between forms AA and DHAA,[9] with several methods accepted for the analysis of AA and DHAA, including high-performance liquid chromatography methods or the AOAC Official Titration method 967.21 as reviewed by Spínola et al.[15] Recent manuscripts in which nanomaterials have been utilized for the detection of AA have not described issues of AA stability or measurement of TAA, though these nanomaterials have been very thoroughly characterized with demonstrated application to a limited number of biological or food samples.[16−19]

An ideal antioxidant assay with standardized parameters should meet the following criteria: 1) simple and adaptable; 2) have a defined end point using a suitable oxidant with a known mechanism; 3) demonstrate reproducibility; 4) utility for analysis of hydrophilic and lipophilic antioxidants; and 5) does not suffer from over- or underestimation because of the generation of new reactive species.[5] Here, we describe an assay using readily available instrumentation with chromophore

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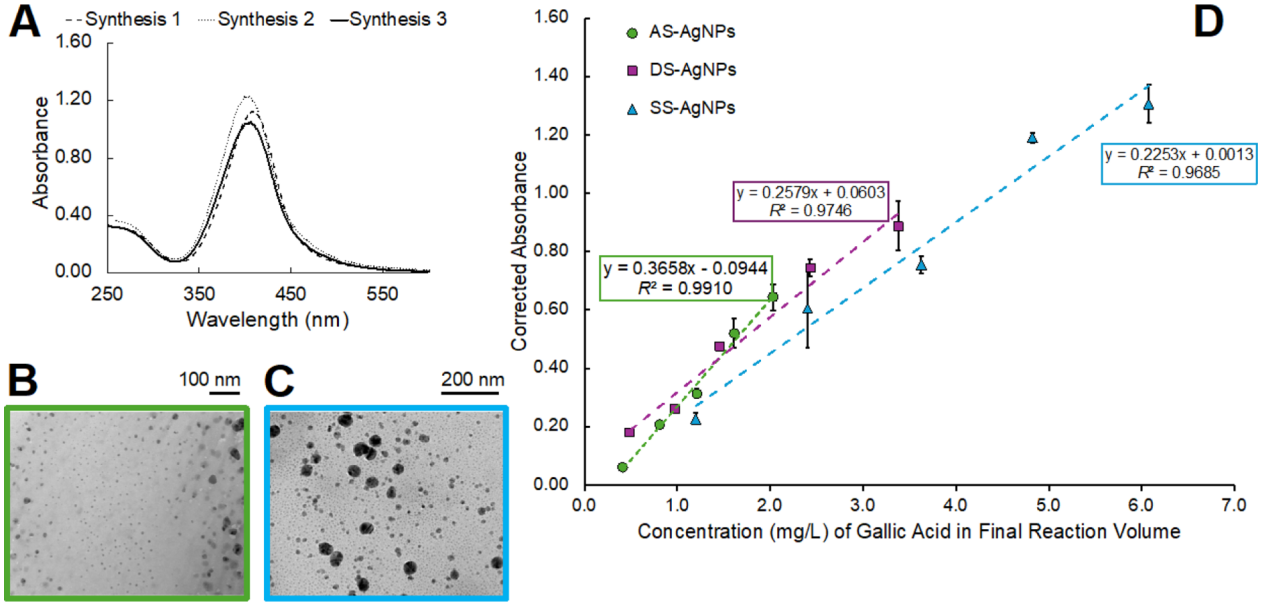

Figure 1. (A) Three overlaid UV/vis spectra demonstrating maximum absorbance at ∼407 nm to demonstrate consistency in the synthesis. Overlaid spectra of n = 48 batches of AS-AgNPs are included in Figure S3. These three syntheses of AS-AgNPs were pooled together and precipitated via centrifugation prior to imaging. TEM images of (B) AS-AgNPs versus (C) SS-AgNPs are included here as the SS-AgNPs were used in an antioxidant assay in our previous work.[21] (D) SNaP assay calibration curves using arabinose-starch (AS)-AgNPs, dextrose-starch (DS)-AgNPs, and soluble starch (SS) AgNPs with varying the initial concentrations of gallic acid, which ranged from 5 to 25 mg/L, where the final concentration of gallic acid in the reaction vessel was determined based upon required assay dilutions. The enhanced LSPR was monitored at 410 nm, and the signal of AgNPs (without addition of gallic acid) was subtracted from the AgNP signal with gallic acid. The AS-AgNPs demonstrated enhanced analytical sensitivity (m = 0.3658 AU/mg/L) and linearity (R^2 = 0.9910), LOD = 0.2 mg/L, and an analytical range of 5.0−25 mg/L GA, which had final diluted concentrations of 0.4 to 2.0 mg/L in the assay itself.

probes (silver nanoparticles, AgNPs) that absorb in the visible range. This work was inspired by the seminal report of the SNPAC assay[20] and modified by our group to utilize AgNPs in a laboratory microwave.[21] Those previous studies demonstrated that the enhanced localized surface plasmon resonance (LSPR) property of AgNPs could be utilized in a colorimetric assay that relies on the growth of preseeded AgNPs in the presence of an antioxidant such as gallic acid or other phenolic compounds. The work presented here focuses on the optimization of three components for the selective **s**ilver **na**no**p**article antioxidant capacity assay for the determination of vitamin **C** (SNaP-C): 1) the AgNP probe itself, 2) stabilization of AA and DHAA under assay conditions, and 3) the assay itself to meet the outlined criteria above. We present data concerning the validation and application of the SNaP-C assay for the selective and quantitative determination of AA and TAA in 31 beverages in a companion manuscript.[22] The work here is novel for describing a selective assay that is simple and robust and accurately quantifies AA and TAA using reproducibly synthesized AgNPs.

MATERIALS AND METHODS

Chemicals and Reagents. Standards and reagents for the preparation of the SNaP-C assay were as follows: D-arabinose (Sigma-Aldrich, St. Louis, MO), dextrose and soluble starch (Fisher Scientific, Fairlawn, NJ), and ultrapure silver nitrate (Acros Organics, Fairlawn, NJ). Analytical standards to assess the SNaP-C assay response included L-ascorbic acid (AA, Mallinckrodt Chemicals, Phillipsburg, NJ), L-dehydroascorbic acid (DHAA, Sigma-Aldrich, Steinheim, Germany), and gallic acid (Alfa Aesar, Fairlawn, NJ). The AA and DHAA standards were stabilized in a 1% (w/v) meta-phosphoric acid (MPA) aqueous solution. The solid MPA was from Sigma-Aldrich (Germany), while the tris(2-carboxyethyl)phosphine hydrochloride (TCEP) used to reduce DHAA to AA was from Alfa Aesar. Any AA analytical standards were reduced in the presence of 10 mM TCEP. All solutions were prepared in HPLC-grade organic-free 18 MΩ double-deionized water (Barnstead E-pure water system; Thermo Fisher Scientific, Asheville, NC). All other chemicals and reagents were purchased from Fisher Scientific unless otherwise stated.

Synthesis of AgNPs. As discussed in the Results and Discussion section, reagent amounts and reaction conditions were optimized for the synthesis of AgNPs. In brief, silver nitrate prepared in water (500 mg/L Ag^+) was incubated in a 35 mL microwave vessel in the presence of 75 mg of soluble starch (capping agent) and, if applicable, 30 mg of a reducing sugar (arabinose or dextrose). A Discover SP microwave (CEM Corporation, Charlotte, NC) was utilized with a 35 mL microwave vessel fitted with a Teflon-lined cap and stir bar. Once the synthetic parameters for the preseeded AgNPs of the SNaP-C assay were optimized, 100 μL of AgNPs (in suspension) was added to 4.9 mL of water and 100 μL of analytical standard to a 10 mL Pyrex Pressure MW vessel fitted with a Teflon-lined cap prior to microwave incubation. AgNPs synthesized here were characterized by UV−vis (Figure 1A) and TEM (Figure 1B,C), though our prior report includes additional (TEM, DLS) characterization.[23] Samples were prepared for low-resolution TEM by pelleting via centrifugation (30 min) using a Thermo Scientific Legend Micro 17 centrifuge operated at 17000 × g. The supernatant was removed using a blunt 22 1/2 gauge needle and saved for analysis by ISE for determination of the Ag^+ concentration.

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The remaining pellet was washed with 18 MΩ water multiple times to remove any excess Ag^+, starch, or arabinose.

HPLC-UV/Vis Analysis of Ascorbic Acid for the Determination of Optimal TCEP Concentration and Reaction Time. An Agilent 1100 high-performance liquid chromatograph (HPLC) fitted with a multiwavelength detector set to 254 nm was utilized to assess AA concentrations after incubating samples or analytical standards with the reducing agent TCEP for the SNaP-C assay. Injections (5 μL) onto a Phenomenex Kinetex column (XB-C18 100 Å, 250 × 4.6 mm, 5 μm) using an isocratic method that utilized solvent A (water +0.1%, v/v, phosphoric acid) and solvent B (methanol +1%, v/v, phosphoric acid) at 1.0 mL/min were employed to separate ascorbic acid from its stabilizing agent, MPA. Isocratic conditions of 20% B over a run time of 0 to 5 min were sufficient to separate AA (2.6 min) from MPA (2.3 min). A different HPLC gradient method utilizing the same column is described in our companion report to determine AA and TAA content in beverages.[22]

Determination of SNaP-C Assay Interferents. A range of potential interfering compounds was tested with the optimized SNaP-C assay. These compounds included the following: caffeic acid, ellagic acid (MP Biomedicals, Santa Ana, CA), oxalic acid, ferulic acid, vanillic acid, papain (Sigma-Aldrich, MO), succinic acid (Eastman Organic Chemicals, Rochester, NY), tartaric acid, maleic acid, citric acid, and limonene (Fisher Scientific). Other sugars from Fisher Scientific (fructose, melezitose, sucrose, and xylose) were also examined as potential interferents.

RESULTS AND DISCUSSION

Arabinose Is a Superior Reducing Agent in the Synthesis of AgNPs. In our previous work on adapting AgNPs to an antioxidant assay,[21] we demonstrated that the use of AgNPs made with soluble starch (SS) as a reducing and capping agent was a viable probe for the determination of antioxidant capacity in distilled spirits. This present work began with determining the effectiveness of soluble starch as a capping and reducing agent for synthesizing selective AgNPs for the determination of ascorbic acid content in food samples. In these first experiments of the present work, 15 mL of 500 mg/L Ag^+ was mixed with differing amounts of soluble starch: 75 mg, 150 mg, 300 mg, 600 mg, and 1500 mg. AgNPs were synthesized in a 35 mL Pyrex Pressure microwave vessel fitted with a Teflon-lined cap using a Discover SP microwave (CEM Corporation, Charlotte, NC) under the following conditions: a power setting of 300 W with high stirring, a temperature of 150 °C (15 min hold time), and a maximum pressure of 50 psi with air cooling.

The SS-AgNPs synthesized with 1500 mg of starch solidified within a few hours of synthesis. Within 2 days, 600 mg of SS-AgNPs also solidified (Figure S1). The SS-AgNPs at 150 mg and 300 mg had a darker amber color indicating increased spectroscopic scatter, which is not ideal for an assay. The SS-AgNPs synthesized with 75 mg of SS produced a low response at $\lambda_{405\ nm}$ (<0.1 AU), indicating a low concentration of AgNPs. This observation was confirmed by measuring free Ag^+ by a silver ion-selective electrode (Ag-ISE), where ∼90% of the original Ag^+ added to the MW vessel remained in solution.

Previous work in our laboratory suggested that the use of dextrose and arabinose as reducing agents would result in better size control of microwave-synthesized AgNPs, which we characterized through orthogonal approaches.[23] When mixed with soluble starch, dextrose-starch (DS)-AgNPs and arabinose-starch (AS)-AgNPs (Figure 1A) were reproducibly synthesized and exhibited high absorption maxima (Figure S2). TEM analyses indicated the presence of AgNPs (Figure 1B,C). The three types of AgNPs were then utilized in an antioxidant assay using gallic acid to evaluate their utility as probes (Figure 1D). In the present work, SS-AgNPs, which were prepared with half as much starch compared to our previous report,[21] were not as consistent when mixed with varying concentrations of gallic acid (Figure 1D). The DS-AgNPs performed comparably well to the SS-AgNPs as indicated by their assay sensitivity (m of fitted regression), but both classes were outperformed by the AS-AgNPs (Figure 1D), which demonstrated enhanced sensitivity of nearly 140% compared to the DS-AgNPs[21] and improved linearity (R^2 = 0.9910 for AS-AgNPs). In summary, the choice of AS-AgNPs for this assay is further supported by the reproducibility of optical properties across multiple syntheses (Figure 1A), with consistency in the resulting AS-AgNPs (Figure 1B) compared to our previous work that utilized SS-AgNPs (Figure 1C).

The AgNPs produced using 30 mg of arabinose and 75 mg of soluble starch were remarkably consistent over the 14 months that this work was carried out. Batches (n = 48) of AS-AgNPs were diluted 9-fold (Figure S3) to be characterized on the UV/vis where a maximum absorbance of 1.0 was desired (Figure S4) at λ_{max} of ∼405 nm. For these 48 batches, the mean absorption values recorded at 405, 410, 415, and 420 nm were compared with no statistically significant difference in the absorption between the four wavelengths. Broadening of the spectral peak may occur because of a change in the size and shape of the AgNPs, and such spectral changes were not optimal for the SNaP-C assay (Figure S5). When this observation was noted, a deep cleaning procedure of the Pyrex glassware for the MW, outlined in the Supporting Information, was required.

Acidic pH Is Required for the Synthesis of Small AgNPs for This Assay. Previous reports have indicated improved synthesis of AgNPs in alkaline conditions, especially when using dextrose as a reducing agent.[24−26] To this end, an early experiment was conducted in which ammonium hydroxide was added to adjust the pH to 9 when synthesizing DS-AgNPs versus an acidic solution (pH 4). These alkaline DS-AgNPs were prepared with 75 mg of soluble starch and 30 mg of dextrose in the microwave using the parameters above. These alkaline DS-AgNPs resulted in a very dark, cloudy solution, which prevented consistent analysis by transmission spectroscopy (Figure S6). Thus, the acidic pH was preferred for the synthesis of suspended AgNPs for this assay (Figure 1B and S2c). Gangwar et al. demonstrated that the rate of AgNP formation is independent of hydroxide ion concentration and that even a very small amount of hydroxide ion is enough for nucleation of AgNPs from Ag^+.[27]

Vitamin C, as AA and DHAA, Requires a Chemical Stabilizer and Reducing Agent Prior to SNaP-C Analysis. While vitamin C includes several unique compounds, the forms AA and DHAA (where total ascorbic acid, TAA = AA + DHAA) require the addition of chemical stabilizers because these forms degrade easily in aqueous solutions. While AA and DHAA are soluble in methanol and ethanol, these alcohols may be prooxidants,[28] which is not an ideal condition for this assay. The addition of 1% (w/v) metaphosphoric acid (MPA) and tris(2-carboxyethyl) phosphine hydrochloride (TCEP, Alfa Aesar, Waltham, MA) had been

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previously identified as important stabilizing and conversion agents, respectively.[29] In preliminary SNaP-C experiments, it was determined that AA standards in 1% (w/v) MPA produced linear, measurable, and consistent responses. However, because AA was still in an aqueous environment and subject to degradation, analytical standards were used for no more than 5 days postsynthesis in 1% (w/v) aqueous MPA.

The SNaP-C assay did not respond to DHAA, as is common for many assays or instrumental methods. Indeed, only LC/MS/MS can distinguish the two forms.[30] Therefore, a conversion agent was required to detect TAA (AA + DHAA) that would not interact with the AS-AgNPs of the assay. Optimal concentration of TCEP, necessary reaction time, and demonstration of analyte stability were determined through a series of experiments. In the first experiment to determine the optimal concentration of TCEP, we utilized HPLC fitted with a multiwavelength detector as described in our companion study.[22] In this experiment, 900 μL of watermelon juice cocktail samples was treated with 50 μL of varying concentrations of TCEP (1.0, 2.5, 5.0, and 10.0 mM) and 50 μL of 1% MPA. The samples were allowed to react for 24 h prior to analysis via HPLC. TAA concentrations were determined at various time points as a function of TCEP concentration, where 0.0 mM TCEP indicated the background AA concentration of the matrix (Table 1), to which the DHAA and TCEP concentrations were spiked. From these data, we determined that the addition of TCEP at the four studied concentrations resulted in a similar response, with noticeable degradation of the AA in the sample containing 0.0 mM TCEP. The mean (±std dev) background concentration of DHAA in these samples was 17.6 (±0.6) mg/L at 24 h incubation and 20.7 (±0.7) mg/L at 48 h.

Table 1. Original TAA Concentration (mg/L) of a Watermelon Juice Cocktail Sample with Varying Concentrations of TCEP, which Was Added as a Reducing and Stabilizing Agent, after 24 and 48 h Incubation[a]

	Original TAA concentrations (mg/L), accounting for dilution caused by addition of 1% MPA and TCEP, in the *unspiked* juice sample after incubation with various concentrations of TCEP		Original TAA concentrations (mg/L), accounting for dilution caused by spiking DHAA (58.7 mg/L) in 1% MPA and TCEP in *spiked* juice samples after 24 and 48 h incubation with determination of spike % Recovery = 100 × [$C_{spike} - C_{ctrl}$]/58.7 mg/L where C_{ctrl} = 17.6 mg/L for 24 h samples and 20.7 mg/L for 48 h samples	
TCEP Concentration (mM)	t = 24 h	t = 48 h	t = 24 h (% Recovery)	t = 48 h (% Recovery)
0.0	18.3	11.9	29.9 (16.2%)	22.1 (0%)
1.0	37.7	33.9	71.3[b] (78.0%)	72.0[b] (73.3%)
2.5	37.4	35.3	76.9 (88.1%)	78.5 (85.1%)
5.0	37.4	34.5	75.8 (86.4%)	76.9 (82.5%)
10.	38.9	35.7	75.2 (85.5%)	76.0 (81.4%)

[a]Additionally, a second set of samples was spiked with DHAA (mg/L) to assess the stability of this form in juice samples incubated with 1% MPA and varying concentrations of TCEP. [b]Spiked with 56.0 mg/L DHAA instead of 58.7 mg/L DHAA.

Interestingly, the increase in TAA (mg/L) for the watermelon juice sample spiked with DHAA and 0.0 mM TCEP (control matrix) could result from the presence of a natural reducing agent in the sample matrix itself. AA was spiked into the watermelon juice sample with a resulting recovery of 99.2%, so the lower percent recoveries of the DHAA shown here (Table 1) are at the lower limit of acceptability. However, other studies cite similarly low recoveries owing to an issue with the purity of commercially available DHAA, given its reactivity and lack of stability.[31,32] Based on these data, 2.5 mM TCEP was selected as the optimal concentration for converting DHAA to AA for the determination of TAA within a sample.

In another set of experiments, the optimal reaction time for the conversion of DHAA to AA by TCEP was evaluated. In these experiments, watermelon juice cocktail samples were treated with 10.0 mM TCEP and varying concentrations of DHAA, AA, and DHAA + AA (Figure S7). The samples were analyzed over a 48 h period by HPLC and compared to a control (unspiked) sample of watermelon juice cocktail. The conversion of the DHAA to AA was monitored (at 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 6.0, 24.0, and 48.0 h) and the % change in absorbance compared to a baseline measurement (0.0 h for the unspiked and AA-spiked samples; 0.5 h for the DHAA-spiked samples because TCEP must be allowed to convert DHAA to AA, which is the only form that is detectable by UV/vis).

The AA sample (spiked at 26.0 mg/L) did not change more than 2% over 24 h and less than 1% between the 24 and 48 h time points. The sample spiked with 26.0 mg/L AA + 26.0 mg/L DHAA changed 7% relative to the baseline signal of AA in a 24 h period and <1% between the 24 and 48 h time points. The three samples that were spiked with DHAA (26.0, 51.7, and 94.4 mg/L) had the greatest % change over the 48 h period, which was expected with the higher amounts of DHAA compared to the native concentration of AA (18.3 mg/L) in the sample. The majority of the DHAA was converted to AA within 24 h, with the 24 to 48 h period seeing <20% increase in the signal of AA by HPLC, which arises from the conversion of DHAA to AA. Thus, the 24 h incubation window was selected for optimal conversion of DHAA to AA.

The final experiment conducted was to assess the long-term storage stability of AA analytical standards stored with 2.5 mM TCEP (Table S1). Analytical standards were prepared in 1% (w/v) MPA and 2.5 mM TCEP and analyzed over an 8-day window by HPLC-UV/vis. The interday relative standard deviation (RSD, %) between analytical standards at each level of the curve ranged from 0.50% to 2.23% (note: the TAA calibration curve ranged from a low concentration of 16.7 to 499 mg/L). The mean (±std dev) of the slopes generated from seven individual calibration curves over the 8-day analysis window was 11.12 (±0.32) AU/(mg/L). For TAA determination, the stock solution and analytical standards can be stored at 4 °C and used for at least 8 days without degradation.

Putting AgNPs + Vitamin C (as AA and DHAA) together to Create an SNaP-C Assay: Optimization of the Assay Parameters. Given that the SNaP-C assay utilizes the Discover SP microwave reactor, the reaction temperature, reaction hold time, and dilution of the probe (AS-AgNPs) all required optimization. Our prior work utilized a temperature of 150 °C (3 min hold time) with SS-AgNPs,[21] but this high temperature was of concern owing to the possible degradation of AA. A Box–Behnken design for three factors (reaction temperature, reaction time, and ratio of AS-AgNPS to water) was used to optimize the microwave parameters using a TAA standard in 1% (w/v) MPA. Fifteen trials were completed with combinations of temperature (60, 90, and 150 °C), reaction

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hold times (3, 6, and 9 min), and ratios of AS-AgNPs to water containing 100 μL of TAA standard (500 μL of AgNPs to 5.40 mL of H_2O; 750 μL of AgNPs to 5.15 mL of H_2O; 1000 μL of AgNPs to 4.90 mL of water) within a total reaction volume of 6.0 mL. Single-factor ANOVA was used to assess statistical significance between the conditions (Table 2). The temperature of 150 °C resulted in the dissolution of AS-AgNPs as indicated by a resulting clear solution after microwaving (<20 mAU). None of the reaction condition combinations resulted in an oversaturation of the UV/vis detector. Reaction or hold time in the microwave and the volume of AS-AgNPs had no statistically significant main effect ($p = 0.9358$ and $p = 0.8591$, respectively), but these two parameters did have a statistically significant interactive effect ($p = 0.00032$). Temperature had a statistically significant main effect ($p < 0.0001$) and interactive effect with time ($p = 0.0001$) and volume of AS-AgNPs ($p = 0.0361$). The temperature of 90 °C was optimal. The volume of AS-AgNP suspension was kept at 1000 μL because the probe, which is the AgNPs themselves, would not be too dilute in the application of the assay for the analysis of beverage samples. The reaction times of 6 and 9 min were equivalent in their efficacy, but the 6 min time was favored to improve assay throughput.

Table 2. *P* Values by Single-Factor ANOVA for Assessing the Influence of Main and Interactive Effects in the Optimization of Microwave Parameters of the SNaP-C Assay Using a Box–Behnken Design for Three Factors

Effect	*P* value	*F* value (*F* critical)
Main effects		
Temperature	4.67×10^{-7}	62.1 (3.89)
Time	0.9358	0.0667 (3.89)
Volume of AS AgNPs	0.8951	0.154 (3.89)
Interactive effects		
Temperature × Time	0.0001	38.6 (4.15)
Time × Volume of AS AgNPs	0.0003	28.5 (4.15)
Temperature × Volume of AS AgNPs	0.0361	4.79 (4.15)

The SNaP-C Assay End Point Is Reached within the Microwave Hold Time. After the reaction conditions for the microwave were optimized, analytical standards of AA and consumer beverage samples were analyzed via the SNaP-C method. Here, a batch of samples was composed of calibration standards, a gallic acid analytical standard as a positive control (gallic acid is commonly used as the calibrant for antioxidant assays),[4] and three beverage samples (for the determination of AA and TAA after incubation with 2.5 mM TCEP). The resulting samples produced from the SNaP-C assay were analyzed four times within a 24 h period (0, 4, 18, and 24 h). There was no statistically significant difference in the resulting response by the SNaP-C assay over these time points, indicating that the reaction is completed after removal from the microwave reactor (raw data are provided in Table S2) as determined by paired *t*-tests (0 h vs 4 h, $p = 0.4741$; 0 h vs 18 h, $p = 0.2854$; 0 h vs 24 h; $p = 0.3894$).

High-Throughput SNaP-C Assay Is Simplified by Using an HPLC UV/Vis Detector over a Standard UV/Vis Instrument Fitted with a Cuvette. The SNaP-C assay blank commonly ranged from 0.5 to 0.7, which limited the concentration of antioxidants that could be incubated with the assay reagents to create an enhanced LSPR. We opted to utilize the multiwavelength detector of an Agilent 1100 HPLC. This detector has a path length of 10 mm, a fill volume of 13 μL, and a higher linear dynamic range than a typical benchtop UV/vis fitted with a cuvette. With a union fitting placed between the HPLC PEEK lines instead of an analytical column and with HPLC-grade water used as a solvent at 0.6 mL/min, the SNaP-C assay could be semiautomated using this flow-injection analysis approach. As no analytical column was used, each SNaP-C microwave-incubated analytical standard or sample could be transferred to an amber glass autosampler vial for analysis. 10 μL of the microwave reaction products could be injected every 1.0 min with the absorption peak height

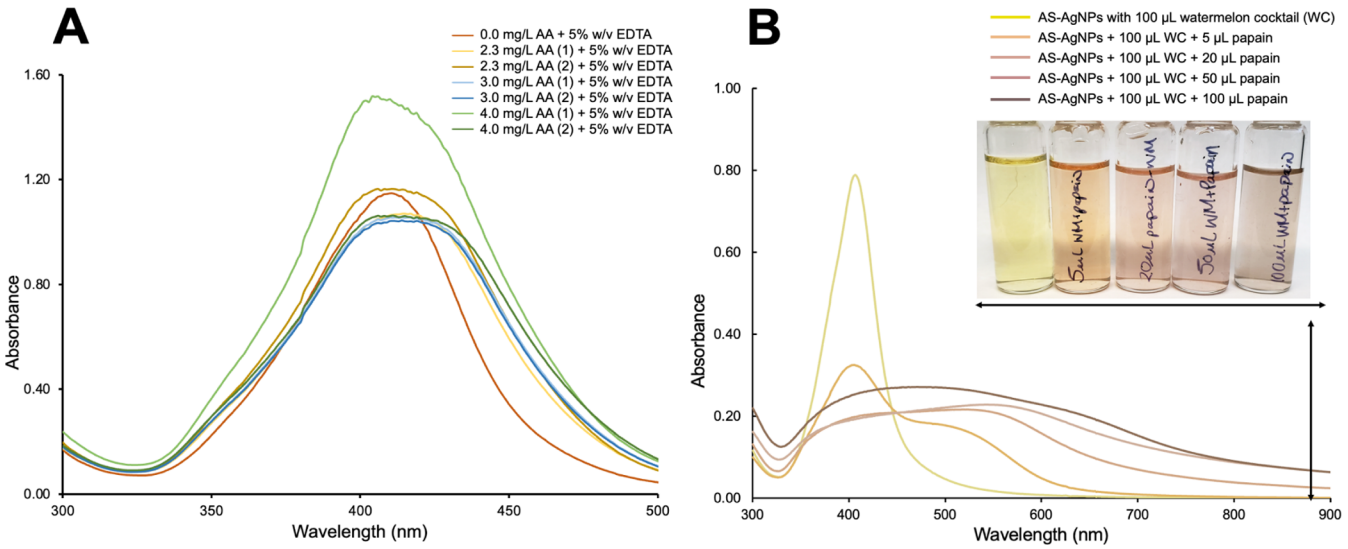

Figure 2. Many potential interferents that affect the assay response that either (A) chelate-free Ag^+ or (B) affect the AgNPs themselves. (A) Duplicate analyses of ascorbic acid (AA) analytical standards at three concentration levels in the presence of EDTA to chelate-free silver ions. These data demonstrated that free Ag^+ was required for the SNaP-C assay to produce a response when incubated with an antioxidant like AA. (B) UV–vis absorption spectra of SNaP-C assay samples in which aliquots of watermelon fruit cocktail were incubated with increasing volumes of the enzyme papain. The macerating enzyme caused a red shift in the LSPR of the enlarged AS-AgNPs prior to returning to the bulk state after 24 h.

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Table 3. Examination of Interfering Substituents and/or Known Antioxidants That Respond to the Folin−Ciocalteu Assay

Compound	MW (g/mol)	μM Concentration, Tested in SNaP-C (Normalized to GA concentration)	% Change from Assay Blank of AS-AgNPs (Normalized to GAE signal)	Normalized Reactivity with SNaP-C? (±10% of GA signal, with concentration correction)	GAE (molar) by Folin−Ciocalteu[39]
Phenolic compounds					
Caffeic acid	180.16	70.5 (2.07)	35 (0.31)	Yes	0.958
Ellagic acid	302.19	42.0 (1.23)	20 (0.30)	Yes	2.12
Ferulic acid	194.18	85.8 (2.52)	−160 (−1.2)	Yes	1.08
Gallic acid	170.12	34.1 (1.00)	54.5 (1.0)	Yes	1.00
Vanillic acid	165.15	76.9 (2.26)	−5 (−0.041)	No	-
Sugars					
Fructose	180.16	92.7 (2.72)	3 (0.020)	No	0.000
Glucose	180.16	92.7 (2.72)	0 (0)	No	0.000
Melezitose	504.4	33.1 (0.971)	0 (0)	No	-
Sucrose	342.3	48.8 (1.43)	−1 (−0.013)	No	0.000
Xylose	150.13	111 (32.6)	0	No	-
Carboxylic acids					
Citric acid	192.12	107.2 (3.14)	−3.0 (−0.018)	No	0.000
Maleic acid	116.07	158.0 (4.63)	−19 (−0.075)	No	-
Oxalic acid	90.03	217.7 (6.38)	−12 (−0.035)	No	0.000
Succinic acid	118.09	186.9 (5.48)	−14 (−0.047)	No	-
Tartaric acid	150.09	139.0 (4.08)	−19 (−0.086)	No	-
Others					
Ascorbic acid	176.12	28.4 (0.833)	87 (1.04)	Yes	0.620
Limonene	136.23	113.8 (3.34)	20 (0.059)	No	-
Papain	Enzyme	-	See Figure 2B	Yes	-
Trolox	250.29	75.9	213	Yes	0.525

recorded (mAU) at 405 nm for the bolus of the injected reaction mixture, which had a peak height maximum at the void time of the HPLC system (0.080 min).

The SNaP-C Assay Requires Free Ag^+ to Produce an Enhanced LSPR. The mechanism of the SNaP-C assay was investigated by removing free Ag^+ by two different methods and altering the incubation time and dilution parameters. In taking the preseeded AS-AgNPs in the presence of free Ag^+ and an antioxidant, Özyürek et al. demonstrated that the antioxidant operates as a secondary reducing agent to reduce Ag^+ to Ag^0 on the surface of preexisting AgNPs.[33,34] The formation of new AgNPs is probably minimized because antioxidants have various reduction potentials, which would result in a broad distribution of AgNP sizes and shifts in the LSPR.[34] The use of precapped AS-AgNPs allows for the selection of a specific monitoring wavelength (here, 405 nm) to assess assay response independently from antioxidant identity. This enhanced LSPR absorbance is linearly related to antioxidant concentration as the outermost layer of the AS-AgNPs likely thickens under the assay reaction conditions.[5,35]

In this work, we performed additional experiments to confirm and further elucidate the role of the free Ag^+ in the assay.[20] AA is highly reactive, pH-sensitive, and stoichiometrically reactive with redox-active transition metals.[36] In the first experiment, SS-AgNPs were pelleted by centrifugation (14,800 rpm; Thermo Scientific Legend 21 microcentrifuge) for 3 h. The pelleted AgNPs were removed from the microcentrifuge tube by using a syringe fitted with a blunt 22 1/2 gauge needle and resuspended in water in a volumetric flask. The concentration of $[Ag^+]$ before the AgNP synthesis and after isolation of the AgNPs by centrifugation was determined via Ag^+-ISE. Upon centrifugation, and with separation from the pelleted AgNPs, only 9% of the original $[Ag^+]$ remained to react with added antioxidants. When two antioxidants (gallic acid, 50 mg/L) and AA (49 mg/L) were incubated with the SS-AgNPs absent of free Ag^+, only a minimal response was noted (Figure S8), indicating that free Ag^+ is required for an enhanced LSPR that can be used as a quantifiable signal to assess AA concentration within a sample. To confirm these results, EDTA was added to chelate Ag^+ to prevent its participation in the SNaP-C assay. In this experiment, a stock solution of AA was made with 1% (w/v) MPA and 5% (w/v) EDTA. AA was then added to the suspended AS-AgNPs at varying concentrations, prior to microwave incubation and compared to the AS-AgNP blank, where no AA was added (Figure 2A). The resulting AgNPs incubated with AA and EDTA exhibited no assay response except at the highest concentration level. Exploring the kinetics and mechanism of this assay more fully is the current area of research interest.

Potential Interferents Abound. In antioxidant assays, interferences fall into three potential categories: additive, inhibitory, and augmenting.[37] When analyzing beverage samples, which we describe in our companion manuscript,[22] interferents were noted when they reacted with free Ag^+ in a mechanism other than enhancing the AgNP LSPR signal. The color of the resulting incubated AS-AgNPs of the SNaP-C assay after microwaving was a useful indicator to demonstrate that beverage components were affecting the assay response (Figure S9) and/or contributing to the creation of AgNPs with many different sizes/shapes (Figure S10). Citrus and pineapple products very commonly contain a component that results in blue-gray AgNPs upon being incubated with SNaP-C reagents; while the citrus products resulted in the creation of AgNPs with a broad range of sizes, the pineapple juice interferent caused the precipitation of Ag (*s*) after microwave incubation of the SNaP-C conditions. Indeed, previous work from our

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laboratory included a broad potential list of compounds in citrus fruits, of which aqueous extracts were used to produce AgNPs and may interfere with the assay.[38] Pineapple juice was another matrix type that has significant interferents wherein Ag (*s*) was produced after incubating the pineapple juice with the preseeded AgNPs. Macerating enzymes such as papain, a proteolytic enzyme, could possibly cause the precipitation that we observed. When we prepared a solution of papain and added it to the SNaP-C components and a watermelon fruit juice cocktail containing 18.3 mg/L endogenous AA, we observed a slow red shift prior to precipitation after allowing the microwaved samples to sit at room temperature for 24 h (Figure 2B).

Everette et al. provided an excellent example of surveying the assay response as gallic acid equivalents (GAE) for many potential interferents of the Folin−Ciocalteu assay.[39] Though testing the broad list of interferents as was done in that published report is beyond the scope of the present work, such interferents will be examined in future work in our laboratory in a similar experimental model. Nevertheless, some of these compounds were investigated here (Table 3) and have been investigated in our preliminary study in which SS-AgNPs were utilized to assess the antioxidant status of distilled spirits.[21] We examined a few of the compounds previously examined by others,[33,39] and we demonstrated similar nonreactivity for many compound classes (sugars, carboxylic acid-containing substances; Table 3). Interestingly, we demonstrated a lower antioxidant response compared with gallic acid after accounting for concentration differences for common antioxidants, including noted phenolic acids. Everette et al. demonstrated that caffeic acid, ellagic acid, and ferulic acid had molar GAE of 0.958, 2.12, and 1.08, respectively, when comparing reactivity via the Folin−Ciocalteu reagent.[39] In comparison, Özyürek et al., in describing their seminal work of the SNPAC assay for measurement of polyphenols, demonstrated a molar GAE of 0.85 for caffeic acid, while ellagic and ferulic acids were not examined in that study. In our first report of using a microwave-adapted SNaP method for antioxidant capacity determination of distilled beverages in which we used only soluble starch as a capping and reducing agent for the creation of the AgNPs, we found molar GAE of 0.49 and 1.24 for caffeic acid and ellagic acid, respectively.[21] In the present work, we find an even lower molar GAE for caffeic acid and ellagic acid, and a surprising no response from ferulic acid, which is a known potent antioxidant.[40] However, ferulic acid, like many phenolics such as caffeic acid and chlorogenic acid, does not have antioxidant activity in isolation but requires a co-antioxidant.[41] Additionally, ferulic acid, like other phenolic antioxidants, may chelate the free metal cations like Ag^+ that is required for the preseeded AgNP growth mechanism of the acidic SNaP-C assay. AgNPs could be created in the presence of ferulic acid under alkaline conditions.[42] Collectively, these data demonstrate that the SNaP-C assay allows for the selective and sensitive determination of AA and TAA, which accounts for the conversion of DHAA to AA, in beverages. Our application of the SNaP-C assay for such work is demonstrated in a companion report with validation by instrumental methods and comparison to the Folin−Ciocalteu and CUPRAC assays for those beverage samples.[22]

CONCLUSIONS

In conclusion, the development of a simple silver nanoparticle assay for vitamin C (SNaP-C) was demonstrated, where a defined end point was reached after 6 min under microwave conditions. The optimization of the assay included determining which type of AgNP (SS, DS, or AS) would have a sensitive and linear response to gallic acid, which is a common calibrator. Subsequently, the analytes AA and DHAA were stabilized in acidic conditions (1% MPA) with DHAA reduced to AA via TCEP to ensure accurate quantitation, as described in the companion manuscript.[22] The antioxidants ascorbic acid and gallic acid responded equivalently well to the assay using AS-AgNPs, while the responses to food interferents, especially other antioxidants, were largely minimized. We demonstrated that the presence of free Ag^+ was required for the enhanced surface plasmon resonance at 405 nm and that there were additional interferents to investigate that affect assay response. Current and ongoing work in our laboratory focuses on examining the kinetics and mechanism of the SNaP-C assay. Others have demonstrated that the growth of AgNPs follows first-order kinetics with regard to Ag^+ concentration, but the inclusion of tannic acid as a reagent has a complicated mechanism,[27] so further investigation is required.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c09746.

Examples of increasing amounts of soluble starch for optimizing the synthesis of silver nanoparticles (AgNPs; Figure S1); AgNPs synthesized with reducing sugars dextrose and arabinose (Figure S2); compiled UV/vis spectra for n = 48 batches of synthesized AgNPs produced over 14 months using arabinose (30 mg) and soluble starch (75 mg) with ultrapure silver nitrate (Figure S3); demonstration of dilution requirement of the synthesized AS-AgNPs (Figure S4); recommended cleaning procedures of glassware (text and Figure S5); synthesis of dextrose-starch AgNPs under alkaline conditions (Figure S6); demonstration of percent change in absorbance at 254 nm reflecting the conversion of DHAA to AA by TCEP in a beverage sample (watermelon juice content) with analysis by HPLC-UV/vis (Figure S7); demonstration of the need for free silver ion to ensure assay response (Figure S8); examples of unacceptable assay response (Figures S9 and S10); stability of total ascorbic acid standards over 8 days when analyzed by high-performance liquid chromatography/UV/vis detection (Table S1); analysis of ascorbic acids, gallic acid standard, and four commercial juice samples incubated with and without reducing agent TCEP (Table S2) (PDF)

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Notes

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■ REFERENCES

(1) Munteanu, I. G.; Apetrei, C. Analytical Methods Used in Determining Antioxidant Activity: A Review. *Int. J. Mol. Sci.* **2021**, *22*, 3380.

(2) Bibi Sadeer, N.; Montesano, D.; Albrizio, S.; Zengin, G.; Mahomoodally, M. F. The Versatility of Antioxidant Assays in Food Science and Safety—Chemistry, Applications, Strengths, and Limitations. *Antioxidants* **2020**, *9*, 709.

(3) Domínguez Díaz, L.; Fernández-Ruiz, V.; Cámara, M. An international regulatory review of food health-related claims in functional food products labeling. *J. Funct. Foods* **2020**, *68*, 103896.

(4) Olszowy-Tomczyk, M. How to express the antioxidant properties of substances properly? *Chem. Pap.* **2021**, *75*, 6157−6167.

(5) Apak, R. Current Issues in Antioxidant Measurement. *J. Agric. Food Chem.* **2019**, *67*, 9187−9202.

(6) Prior, R. L.; Wu, X.; Schaich, K. Standardized Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290−4302.

(7) Apak, R.; Özyürek, M.; Güçlü, K.; Çapanoğlu, E. Antioxidant Activity/Capacity Measurement. 1. Classification, Physicochemical Principles, Mechanisms, and Electron Transfer (ET)-Based Assays. *J. Agric. Food Chem.* **2016**, *64*, 997−1027.

(8) Huang, D.; Ou, B.; Prior, R. L. The Chemistry behind Antioxidant Capacity Assays. *J. Agric. Food Chem.* **2005**, *53*, 1841−1856.

(9) Yin, X.; Chen, K.; Cheng, H.; Chen, X.; Feng, S.; Song, Y.; Liang, L. Chemical Stability of Ascorbic Acid Integrated into Commercial Products: A Review on Bioactivity and Delivery Technology. *Antioxidants* **2022**, *11*, 153.

(10) Thomas, S.; Patel, D.; Bittel, B.; Wolski, K.; Wang, Q.; Kumar, A.; Il'giovine, Z. J.; Mehra, R.; McWilliams, C.; Nissen, S. E.; Desai, M. Y. Effect of High-Dose Zinc and Ascorbic Acid Supplementation vs Usual Care on Symptom Length and Reduction Among Ambulatory Patients With SARS-CoV-2 Infection: The COVID A to Z Randomized Clinical Trial. *JAMA Netw. Open* **2021**, *4*, No. e210369.

(11) Al Sulaiman, K.; Aljuhani, O.; Saleh, K. B.; Badreldin, H. A.; Al Harthi, A.; Alenazi, M.; Alharbi, A.; Algarni, R.; Al Harbi, S.; Alhammad, A. M.; et al. Ascorbic acid as an adjunctive therapy in critically ill patients with COVID-19: A propensity score matched study. *Sci. Rep.* **2021**, *11*, 17648.

(12) Moskowitz, A.; Huang, D. T.; Hou, P. C.; Gong, J.; Doshi, P. B.; Grossestreuer, A. V.; Andersen, L. W.; Ngo, L.; Sherwin, R. L.; Berg, K. M.; Chase, M.; Cocchi, M. N.; McCannon, J. B.; Hershey, M.; Hilewitz, A.; Korotun, M.; Becker, L. B.; Otero, R. M.; Uduman, J.; Sen, A.; Donnino, M. W. Effect of Ascorbic Acid, Corticosteroids, and Thiamine on Organ Injury in Septic Shock: The ACTS Randomized Clinical Trial. *JAMA* **2020**, *324*, 642−650.

(13) Schrotenboer, B. *Vitamin C by IV and an FBI raid. How hope, rather than proof, sent the antioxidant's sales soaring during COVID-19*. USA Today, 2020.

(14) *Anonymous Global Functional Beverages Market Is Expected to Reach USD 216.7 billion by 2028: Fior Markets*. GlobeNewswire, 2021.

(15) Spínola, V.; Llorent-Martínez, E. J.; Castilho, P. C. Determination of vitamin C in foods: Current state of method validation. *J. Chromatogr. A* **2014**, *1369*, 2−17.

(16) Wang, J.; Wang, L.; Su, X.; Yu, H. Core−Shell Quantum Dot-Based Honeycomb Films on Capillary Walls for Rapid In Situ Ascorbic Acid Detection. *ACS Appl. Nano Mater.* **2023**, *6*, 12231−12239.

(17) Sriram, B.; Kogularasu, S.; Wang, S.; Sheu, J. Deep Eutectic Solvent-Mediated Synthesis of Spinel Zinc Chromite Nanoparticles: A Simple Label-Free Electrochemical Sensor for Dopamine and Ascorbic Acid. *ACS Appl. Nano Mater.* **2023**, *6*, 17593−17602.

(18) Fu, W.; Ma, J.; Qiao, Z.; Xu, L.; Wang, L.; Ling, M.; Fu, X.; Li, G.; Han, C.; Zhang, J.; Jin, J. Ultrasound-Assisted Hydrothermal Synthesis of Highly Fluorescent Sulfur Quantum Dots for Fe3+ Ion and Ascorbic Acid Detection in Real Samples. *Langmuir* **2023**, *39*, 16349−16357.

(19) Nishan, U.; Ullah, I.; Gul, R.; Badshah, A.; Muhammad, N.; Khan, N.; Shah, M.; Asad, M.; Afridi, S.; Ullah, R.; Ali, E. A.; Ojha, S. C. Paracetamol-Mediated Synthesis of Silver Nanoparticles and Their Functionalization with Ionic Liquid for the Colorimetric Biosensing of Ascorbic Acid. *ACS Omega* **2023**, *8*, 44931−44941.

(20) Özyürek, M.; Güngör, N.; Baki, S.; Güçlü, K.; Apak, R. Development of a Silver Nanoparticle-Based Method for the Antioxidant Capacity Measurement of Polyphenols. *Anal. Chem.* **2012**, *84*, 8052−8059.

(21) Bukovsky Reyes, S. E. R.; Lowe, L. E.; Brandon, W. M.; Owens, J. E. Measurement of antioxidants in distilled spirits by a silver nanoparticle assay. *J. Instit. Brewing.* **2018**, *124*, 291−299.

(22) Rivera Lemon, E. N.; Lowe, L. E.; Owens, J. E. SNaP-C: Validation and Application of Silver Nanoparticle Antioxidant Capacity Assay for Ascorbic Acid and Total Ascorbic Acid Content in Beverages. *Food Chem.*, **2024**.

(23) Kahrilas, G. A.; Haggren, W.; Read, R. L.; Wally, L. M.; Fredrick, S. J.; Hiskey, M.; Prieto, A. L.; Owens, J. E. Investigation of Antibacterial Activity by Silver Nanoparticles Prepared by Microwave-Assisted Green Syntheses with Soluble Starch, Dextrose, and Arabinose. *ACS Sustainable Chem. Eng.* **2014**, *2*, 590−598.

(24) Meshram, S. M.; Bonde, S. R.; Gupta, I. R.; Gade, A. K.; Rai, M. K. Green synthesis of silver nanoparticles using white sugar. *IET Nanobiotechnol.* **2013**, *7*, 28−32.
(25) Durmazel, S.; Üzer, A.; Erbil, B.; Sayın, B.; Apak, R. Silver Nanoparticle Formation-Based Colorimetric Determination of Reducing Sugars in Food Extracts via Tollens. *Reagent. ACS Omega* **2019**, *4*, 7596−7604.
(26) Philip, D. Honey mediated green synthesis of silver nanoparticles. *Spectrochim. Acta, Part A* **2010**, *75*, 1078−1081.
(27) Gangwar, C.; Yaseen, B.; Kumar, I.; Singh, N. K.; Naik, R. M. Growth Kinetic Study of Tannic Acid Mediated Monodispersed Silver Nanoparticles Synthesized by Chemical Reduction Method and Its Characterization. *ACS Omega* **2021**, *6*, 22344−22356.
(28) Goldberg, D. M.; Hoffman, B.; Yang, J.; Soleas, G. J. Phenolic Constituents, Furans, and Total Antioxidant Status of Distilled Spirits. *J. Agric. Food Chem.* **1999**, *47*, 3978−3985.
(29) Chebrolu, K. K.; Jayaprakasha, G. K.; Yoo, K. S.; Jifon, J. L.; Patil, B. S. An improved sample preparation method for quantification of ascorbic acid and dehydroascorbic acid by HPLC. *Food Sci. Technol.* **2012**, *47*, 443−449.
(30) Boonpangrak, S.; Lalitmanat, S.; Suwanwong, Y.; Prachayasittikul, S.; Prachayasittikul, V. Analysis of Ascorbic Acid and Isoascorbic Acid in Orange and Guava Fruit Juices Distributed in Thailand by LC-IT-MS/MS. *Food Anal. Methods* **2016**, *9*, 1616−1626.
(31) Nováková, L.; Solich, P.; Solichová, D. HPLC methods for simultaneous determination of ascorbic and dehydroascorbic acids. *TrAC, Trends Anal. Chem.* **2008**, *27*, 942−958.
(32) Zhu, M.; Tang, J.; Tu, X.; Chen, W. Determination of Ascorbic Acid, Total Ascorbic Acid, and Dehydroascorbic Acid in Bee Pollen Using Hydrophilic Interaction Liquid Chromatography-Ultraviolet Detection. *Molecules* **2020**, *25*, 5696.
(33) Ozyurek, M.; Gungor, N.; Baki, S.; Guclu, K.; Apak, R. Development of a silver nanoparticle-based method for the antioxidant capacity measurement of polyphenols. *Anal. Chem.* **2012**, *84*, 8052−8059.
(34) Apak, R.; Demirci Çekiç, S.; Üzer, A.; Çelik, S. E.; Bener, M.; Bekdeşer, B.; Can, Z.; Sağlam, Ş.; Önem, A. N.; Erçağ, E. Novel Spectroscopic and Electrochemical Sensors and Nanoprobes for the Characterization of Food and Biological Antioxidants. *Sensors* **2018**, *18*, 186.
(35) Jiang, Z.; Liu, C. Seed-Mediated Growth Technique for the Preparation of a Silver Nanoshell on a Silica Sphere. *J. Phys. Chem. B* **2003**, *107*, 12411−12415.
(36) Shen, J.; Griffiths, P. T.; Campbell, S. J.; Utinger, B.; Kalberer, M.; Paulson, S. E. Ascorbate oxidation by iron, copper and reactive oxygen species: Review, model development, and derivation of key rate constants. *Sci. Rep.* **2021**, *11*, 7417.
(37) Singleton, V. L.; Orthofer, R.; Lamuela-Raventós, R. M. [14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods Enzymol.* **1999**, *299*, 152−178.
(38) Kahrilas, G. A.; Wally, L. M.; Fredrick, S. J.; Hiskey, M.; Prieto, A. L.; Owens, J. E. Microwave-Assisted Green Synthesis of Silver Nanoparticles Using Orange Peel Extract. *ACS Sustainable Chem. Eng.* **2014**, *2*, 367−376.
(39) Everette, J. D.; Bryant, Q. M.; Green, A. M.; Abbey, Y. A.; Wangila, G. W.; Walker, R. B. Thorough Study of Reactivity of Various Compound Classes toward the Folin−Ciocalteu Reagent. *J. Agric. Food Chem.* **2010**, *58*, 8139−8144.
(40) Urbaniak, A.; Szeląg, M.; Molski, M. Theoretical investigation of stereochemistry and solvent influence on antioxidant activity of ferulic acid. *Comput. Theor. Chem.* **2013**, *1012*, 33−40.
(41) Kadoma, Y.; Fujisawa, S. A Comparative Study of the Radical-scavenging Activity of the Phenolcarboxylic Acids Caffeic Acid, p-Coumaric Acid, Chlorogenic Acid and Ferulic Acid, With or Without 2-Mercaptoethanol, a Thiol, Using the Induction Period Method. *Molecules* **2008**, *13*, 2488−2499.
(42) Wang, H. Y.; Li, Y. F.; Huang, C. Z. Detection of ferulic acid based on the plasmon resonance light scattering of silver nanoparticles. *Talanta* **2007**, *72*, 1698−1703.

https://doi.org/10.1021/acsomega.4c09746
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