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Evaluation of Synergistic Antioxidant Potential of Complex Mixtures Using Oxygen Radical Absorbance Capacity (ORAC) and Electron Paramagnetic Resonance (EPR)

Tory L. Parker,[†] Samantha A. Miller,[‡] Lauren E. Myers,[‡] Fernando E. Miguez,[§] and Nicki J. Engeseth*,[‡]

[†]Department of Nutrition, Dietetics, and Food Science, Brigham Young University, S-135 ESC, Provo, Utah 84602, [‡]Department of Food Science and Human Nutrition, University of Illinois at Urbana−Champaign, 905 South Goodwin Avenue, 208 Bevier Hall, Urbana, Illinois 61801, and [§]Department of Crop Sciences, University of Illinois at Urbana−Champaign, 1206 West Gregory Drive, 1112 Institute for Genomic Biology, Urbana, Illinois 61801

Previous research has demonstrated that certain combinations of compounds result in a decrease in toxic or pro-oxidative effects, previously noted when compounds were administered singly. Thus, there is a need to study many complex interactions further. Two *in vitro* techniques [electron paramagnetic resonance (EPR) and oxygen radical absorbance capacity (ORAC) assays] were used in this study to assess pro- and antioxidant capacity and synergistic potential of various compounds. Rutin, *p*-coumaric acid, abscisic acid, ascorbic acid, and a sugar solution were evaluated individually at various concentrations and in all 26 possible combinations at concentrations found in certain foods (honey or papaya), both before and after simulated digestion. EPR results indicated sugar-containing combinations provided significantly higher antioxidant capacity; those combinations containing sugars and ascorbic acid demonstrated synergistic potential. The ORAC assay suggested additive effects, with some combinations having synergistic potential, although fewer combinations were significantly synergistic after digestion. Finally, ascorbic acid, caffeic acid, quercetin, and urate were evaluated at serum-achievable levels. EPR analysis did not demonstrate additive or synergistic potential, although ORAC analysis did, principally in combinations containing ascorbic acid.

KEYWORDS: ORAC; EPR; synergistic; combination; fruit; papaya; honey; flavonoid; ascorbic acid; phenolic acid

INTRODUCTION

Oxidation can result in membrane damage and DNA mutation (*1*). Blocking oxidation is thus important for survival. While the body has many of its own defense mechanisms, foods rich in antioxidant nutrients also appear to decrease the risk for diseases that involve oxidative damage, such as cancer (*2*) and coronary disease (*3*). While epidemiological evidence supports the benefits of consuming more fruits and vegetables, there is little research examining the benefits of combinations of components found in them. To clarify the role that antioxidant nutrients play in disease prevention, it is critical that we better understand how they interact, whether antagonistic, additive, or synergistic.

A recent review (*4*) discussed potential benefits and risks of combining drugs, as well as combinations including food phytochemicals, noting that high doses of one substance could be toxic or pro-oxidative. Combinations have the potential to decrease both of these risks. Combinations of α-tocopherol and/or ascorbic acid with caffeic acid, catechin, epicatechin, myricetin, gallic acid, quercetin, and rutin had greater antioxidant activity than any of the compounds alone in a Fe^{2+}-induced lipid oxidation system (*5*). Suppression of superoxide and nitric oxide generation in inflammatory cells was achieved when different types of chemicals with different action mechanisms were combined in low concentrations (*6*). However, the interactions were complex and could be antagonistic, additive, and/or synergistic, depending upon the chemical and the conditions. A combination of a green tea extract, quercetin, and folic acid synergistically prevented H_2O_2-induced cellular damage significantly better than any compound alone (*7*). Research examining combinations is fairly new; there are many complex interactions that require further study.

To begin to understand the ramifications of consuming a complex diet, a three-part analysis was designed to explore combinations of five compounds that can be found in antioxidant-rich foods previously studied in our laboratory, both before and after acid/base digestive processes, and four compounds that have been quantified in human serum. Two *in vitro* techniques were used to assess these interactions: oxygen radical absorbance capacity (ORAC) assay, used to test the hydrogen radical donating capacity, and electron paramagnetic resonance (EPR), used to analyze hydroxyl radical quenching capacity.

*To whom correspondence should be addressed. Telephone: 217-244-6788. Fax: 217-265-0925. E-mail: engeseth@illinois.edu.

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MATERIALS AND METHODS

Materials. Ascorbic acid, Trolox, dextrose, sucrose, ferrous sulfate, and disodium ethylenediaminetetraacetic acid (EDTA) were purchased from Fisher Scientific (Fairlawn, NJ). Fluorescein, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN), fructose, rutin, *cis*,*trans*-abscisic acid, *p*-coumaric acid, caffeic acid, quercetin, and urate were acquired from Sigma Chemical (St. Louis, MO). 2,2′-azobis(2-amidino-propane)dihydrochloride (AAPH) was obtained from Wako Chemical (Richmond, VA).

Sample Preparation. *Food Concentrations*. Rutin (9.8 μM as quercetin in soy honey) (*8*), *p*-coumaric acid (73 μM in buckwheat honey) (*8*), abscisic acid (45 μM in tupelo honey) (*8*), and a sugar solution matching papaya sugar concentrations (130 mM fructose, 140 mM glucose, and 130 mM sucrose in papaya) (*9*) were prepared in phosphate-buffered saline (PBS, pH 7.4) and stored at −20 °C when not in use. Ascorbic acid (4.26 mM in papaya) (*10*) was made fresh daily. Concentrated stock solutions of phenolics were prepared (40−80×) to facilitate preparing combination solutions. The 5 individual solutions and all 26 possible combinations were prepared. Rutin replaced quercetin for enhanced solubility in PBS.

Digestion. The 5 individual solutions and all 26 combinations were prepared at food concentrations in PBS. Simulated human digestion was carried out as described previously (*11*). Digestive enzymes were not included because their common substrates were not present in our samples and the protein presence would interfere with the antioxidant analyses. Briefly, sufficient 1 M HCl was added to each sample to reach pH 2. Samples were incubated at 37 °C for 1 h, followed by the addition of 1 M $NaHCO_3$ until a pH of 7.5 was reached. This was followed by a second hour of incubation at 37 °C. Samples were analyzed immediately following the simulated intestine incubation.

Serum Concentrations. Caffeic acid (0.5 μM) (*12*), quercetin (5 μM) (*13*), and urate (350 μM) (*14*) were prepared in PBS and stored at −20 °C when not in use. Ascorbic acid (150 μM) (*12*) was made fresh daily. The 4 individual solutions and all 11 combinations were prepared. Concentrated stock solutions were prepared (12−4000×) to facilitate weighing and mixing for combination trials. Quercetin and urate required a few drops of 1 M NaOH and heat (sub-boiling) for initial solubility. Subsequent dilution restored neutral pH while retaining solubility because of the low concentration. Caffeic acid required only heat for solubilization.

EPR. The EPR protocol was based on ref *15*. Stock solutions of POBN (10 mM) and EtOH (1 M), $FeSO_4 \cdot H_2O$ (10 mM) and 2NaEDTA (12 mM), and H_2O_2 (10 mM) were each prepared in 10 mL of PBS the day of the experiment. For controls, 300 μL of POBN/EtOH, 550 μL of PBS, 100 μL of Fe/EDTA, and 50 μL of H_2O_2 were sequentially added to Eppendorf tubes. For food, digestion, and serum concentration samples, 300 μL of POBN/EtOH, 500 μL of sample, 50 μL of PBS, 100 μL of Fe/EDTA, and 50 μL of H_2O_2 were sequentially added. For tests of the concentration effect, varying volumes of sample and PBS to bring the total volume to 1 mL were added, with the other three components as above. A quartz flat cell (SP Industries, Inc., Wilmad-labglass, Buena, NJ) was marked for consistent placement in the instrument, filled with sample, wiped dry, and inserted into the EPR machine. Scans began 3 min after the addition of H_2O_2.

Spectra were recorded at X-band (~9.5 GHz) on a Varian E-122 spectrometer with a TE102 cavity (Palo Alto, CA). Other parameters were as follows: frequency, 9.51 GHz; field center, 3390; scan range, 80; receiver gain, 2000−50 000; modulation amplitude, 1; power, 20 mW; number of scans, 5; scan time, 30 s; time constant, 0.032 s. Peak height was measured from the maximum to the minimum value of the first peak in the second doublet. Larger peaks represent increased POBN spin trap formation, while smaller peaks represent decreased POBN spin trap formation. The peak width was also evaluated but found to be insignificant compared to the peak height.

To confirm optimized experimental conditions, various concentrations of iron were tested to maximize the EPR signal. Unlike ref *15*, which found that 100 μM was optimum, these experiments indicated that 1000 μM maximized the EPR signal. Also, a series of systematic variations of POBN, EtOH, and H_2O_2 concentrations resulted in the determination that the effects of each variation was minor compared to changes in the iron concentration.

After the EPR value was calculated for each individual solution and combination then a Fenton control was subtracted, additive and synergistic potential mean estimates were determined using a mixed model (SAS Institute, Inc., Cary, NC). The estimates compared the sum of the EPR values of the compounds assayed separately to the EPR value of the combination while taking into account the standard error of each.

$ORAC_{FL}$. $ORAC_{FL}$ assays were conducted as described previously (*16*) in a preheated (37 °C) BioTek FLx600 fluorometer (BioTek Instruments, Inc., Winooski, VT) using 96-well black side with clear bottom plates (Corning, Inc., Corning, NY). Data were collected using KC4 software (BioTek Instruments, Inc.). Parameters for experiments were as follows: emission wavelength, 515 nm (530/25); excitation wavelength, 493 nm (485/15); reading every minute for 80 min with shaking for 3 s at an intensity level of 3 before each reading. Reagent concentrations and plate organization were carried out as described previously (*16*).

ORAC values were calculated using the equation from the Trolox standard curve and adjusted for dilutions. After the ORAC value was calculated for each combination, additive and synergistic potential mean estimates were determined using a mixed model. The estimates compared the sum of the ORAC values of the compounds assayed separately to the ORAC value of the combination while taking into account the standard error of each.

Statistics. All analyses were performed in triplicate or quadruplicate. Removal of statistical outliers reduced the number of replicates in a few cases (indicated in the figures). The resulting raw data for both EPR and ORAC were processed in Microsoft Excel (Microsoft Corporation, Redmond, WA) to determine the peak height and area under the curve, respectively. The Fenton control was subtracted from EPR peak heights. Both EPR and ORAC results were separated into groups that did or did not contain ascorbic acid. The ORAC serum data was separated into urate and no urate groups. These separations were performed because of large differences in variance between the groups. Statistical significance was determined using analysis of variance [Fisher's least significant difference (LSD), SAS statistical software, SAS Institute, Inc., Cary, NC].

Combinations containing ascorbic acid had significantly higher variance than non-ascorbic-acid-containing combinations (or urate in the ORAC serum group); therefore, a mixed model that accounted for unequal variances among treatments was used (SAS statistical software, SAS Institute, Inc., Cary, NC). In this way, the linear contrasts used to test for additive or synergistic interactions accounted for the unequal variances by calculating an appropriate standard error.

RESULTS AND DISCUSSION

Compound Selection. Selected compounds for experiments (**Figure 1**) represent various classes of compounds found in honey or papaya that exhibit antioxidant activity, may exhibit pro-oxidant activity, or would be expected to do neither (*17*). This included a vitamin (ascorbic acid), a sugar solution, a phenolic acid (*p*-coumaric acid), a methyl-substituted phenolic acid (abscisic acid), and a flavonoid glycoside (rutin). These were selected because of their relatively high concentrations in honey and papaya. Also, because the experiment was designed to test all possible combinations, the number of combinations increased exponentially with each compound added. It was determined that five components would provide sufficient breadth to explore potential synergism.

Selected compounds (four) in serum experiments represent the same classes of molecules chosen for the food and digestion experiments above. Choices were limited by available data for serum-achievable levels and striving to match the time after consumption at which they reached the maximum serum concentration (*12*, *13*, *18*). Ascorbic acid was used as above. Quercetin was used as an appropriate flavonoid (replacing rutin above), and caffeic acid was used as an appropriate phenolic acid (to replace *p*-coumaric acid). No serum data were available for abscisic acid or other methyl-substituted phenolics; therefore, this class was not included. To replace the sugar solution, urate was chosen. Urate levels are increased after fructose consumption, which results in increased serum antioxidant capacity (*14*). Glucose (postprandial hyperglycemia) induces a pro-oxidant environment as it is metabolized (*19*). An increase in urate levels helps counteract the pro-oxidative effects of

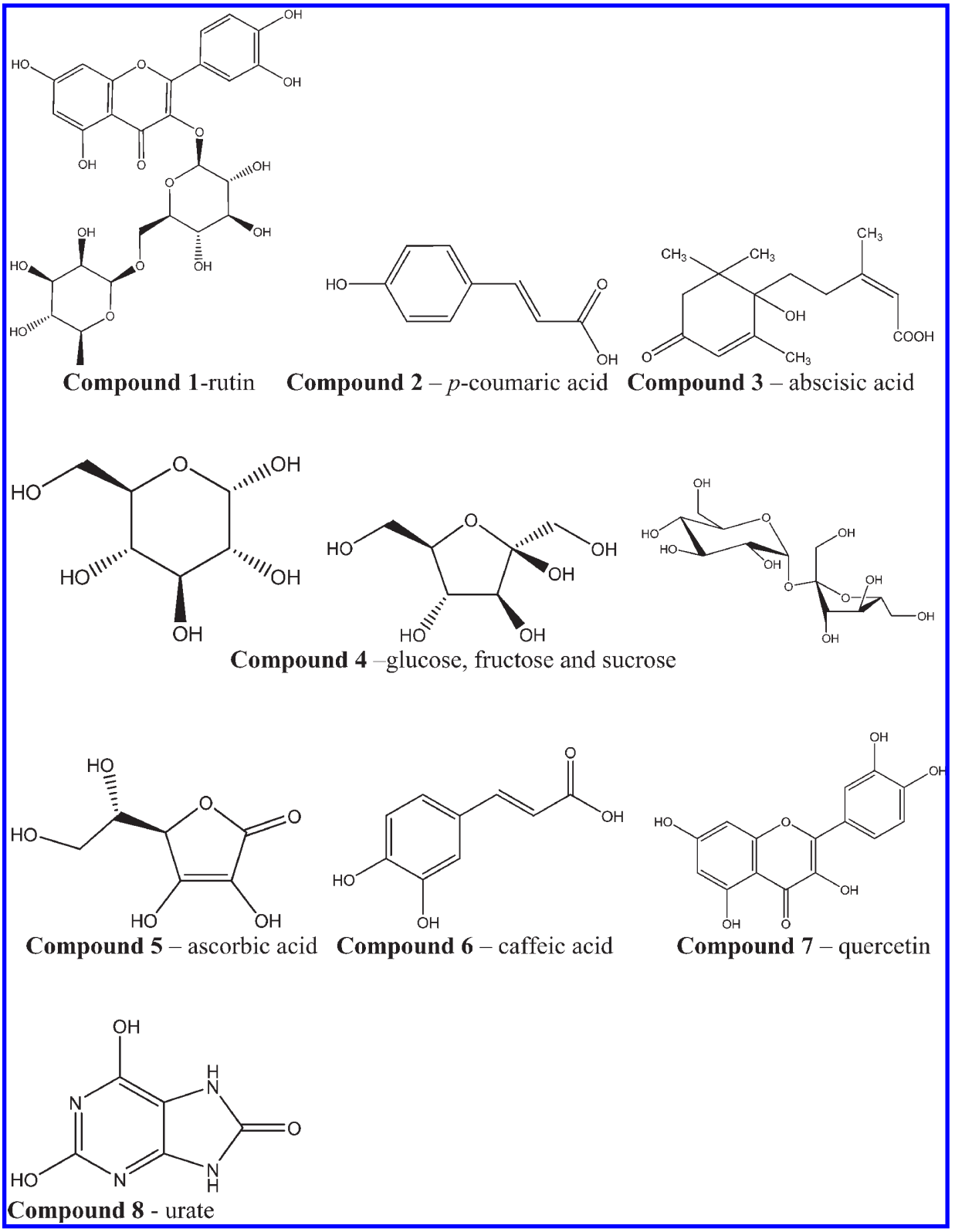

Figure 1. Structures and compound numbers used in combination experiments.

glucose metabolism. Thus, urate was chosen as a biologically relevant antioxidant that could interact in serum with the other chosen antioxidant compounds. The four selected compounds are expected to be at the specified concentration at the same time in human serum (*18*). Other components of serum, including potential oxidative effects of glucose metabolism were not directly accounted for in these studies; the aim was to evaluate whether the low serum-achievable concentrations would be sufficient to interact positively.

EPR—Effect of the Concentration on Pro- and Antioxidant Activity. The five compounds selected for the fruit concentration and digestion experiments were tested at various concentrations on the EPR spectrometer to observe the effect of the concentration on their pro- and antioxidant activities. We tested each of the five compounds at the following concentrations: from 0.2 μM to 2 mM (rutin, *p*-coumaric acid, and abscisic acid), from 0.2 μM to 8.5 mM (ascorbic acid), and from 0.2 μM to 130 mM (sugar

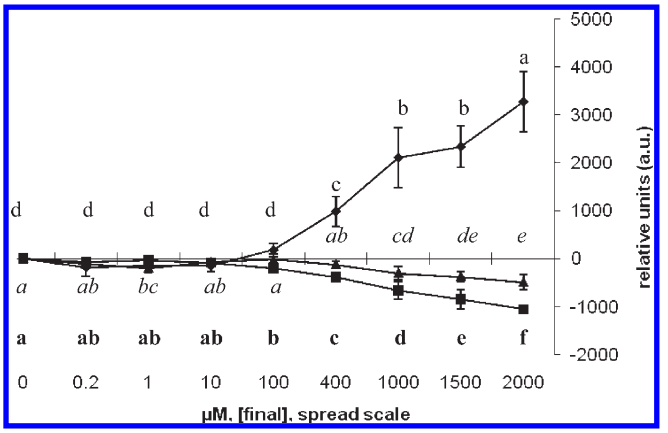

Figure 2. EPR signal intensities of rutin (**1**, unmodified font), *p*-coumaric acid (**2**, bold font), and abscisic acid (**3**, italic font) at various concentrations. Values are reported as mean ± standard deviation (SD). Values not sharing the same lowercase letter are significantly different ($p < 0.05$). $n = 3$.

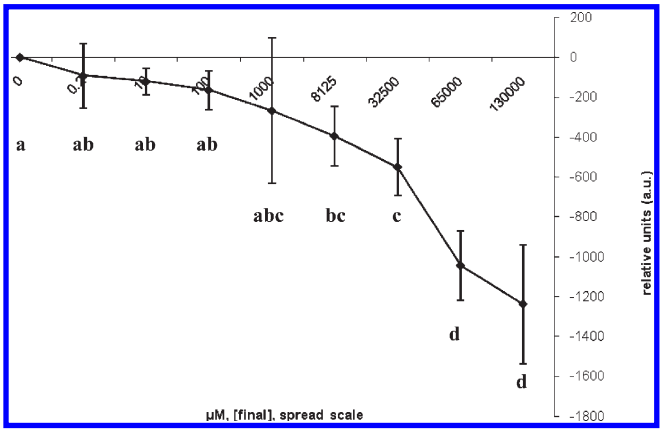

Figure 3. EPR signal intensity of a sugar solution matching concentrations of fructose, glucose, and sucrose found in papaya (*Carica papaya* L.) at various concentrations. Values are reported as mean ± SD. Values not sharing the same lowercase letter are significantly different ($p < 0.05$). $n = 3$.

solution) (**Figures 2**−**4**). Compounds used in the serum-achievable experiments (quercetin, caffeic acid, and urate) were excluded from this assay. Many supplements now contain concentrated extracts of the tested compounds; therefore, the concentrations tested would be biologically relevant for exposure to the digestive tract.

Rutin was significantly pro-oxidative at concentrations of 400 μM and above (**Figure 2**); however, at lower concentrations, it was not significantly different from the Fenton control. Despite the potential for electron sharing in its phenolic structure, at high concentrations, rutin radicals begin to interact with other rutin molecules, iron, and oxygen to a greater extent than rutin quenches hydroxyl radicals, giving a net result that is pro-oxidative (*17*). *p*-Coumaric acid exerted antioxidative capacity only at concentrations higher than 10 μM, although the averages for non-significant concentrations remained below zero, suggesting antioxidant activity. Antioxidant activity for *p*-coumaric acid up to 200 μM has been reported previously (*15*). Abscisic acid was significantly antioxidative at 1 and 1000 μM and above, although higher concentrations did not exhibit the same magnitude of antioxidant capacity as *p*-coumaric acid. Abscisic acid is not commonly studied as an antioxidant, although it is dominant among phenolic and substituted phenolic compounds in tupelo honey (*8*), which has an antioxidant capacity (ORAC) similar to

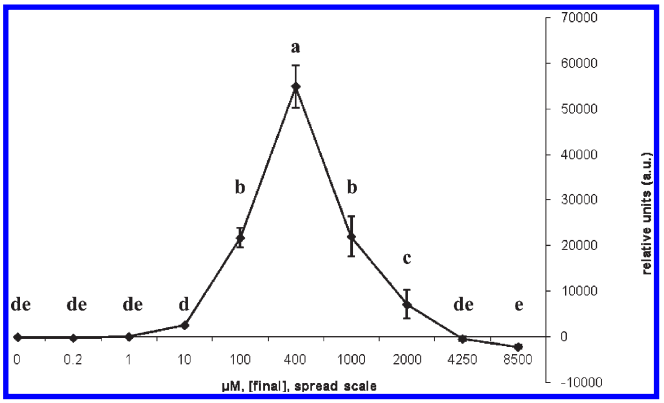

Figure 4. EPR signal intensity of ascorbic acid at various concentrations. Values are reported as mean ± SD. Values not sharing the same lowercase letter are significantly different ($p < 0.05$). $n = 3$.

clover honey (*20*). Clover honey has a higher overall phenolic acid and flavonoid content but little abscisic acid.

The papaya sugar mixture became increasingly antioxidative as the concentration increased (**Figure 3**), although only significantly above 1000 μM. Sugar exhibits antioxidant activity *in vitro* (*20*) but can have both antioxidant (fructose increases urate) (*14*) and pro-oxidant (glucose metabolism can produce radicals) (*19*) effects *in vivo*. Further work is needed to fully understand the biochemical implications of consuming varied ratios of sugars as well as the effects of sugar ratios in combination with phenolics.

Ascorbic acid (**Figure 4**) exhibited the most unique traits of the selected compounds, because of its ability to act as a redox reagent. As the concentration increased from 0.2 to 400 μM, the EPR signal increased rapidly (significantly different at 100 and 400 μM) because of the recycling of iron from Fe^{3+} to Fe^{2+}, thus significantly increasing the signal as more Fe^{2+} was available to react with hydrogen peroxide (eq 1). However, as the concentration of ascorbic acid increased beyond 400 μM, it began to donate hydrogen atoms to the hydroxyl radicals formed from the Fenton reaction to a greater degree than it recycled the Fe^{3+} (eq 2).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH \quad (1)$$

$$AH + \cdot OH \rightarrow A\cdot + H_2O \quad (2)$$

This resulted in the signal dropping just as quickly as it rose. All concentrations above 400 μM were significantly lower than the EPR signal at 400 μM, with 1000 and 2000 μM remaining significantly higher than the Fenton control. Another possibility is that the ascorbic acid was recycling the POBN spin trap directly at higher concentrations. However, the reduction potential of nitroxide compounds (similar in structure to POBN) is approximately −1500 mV (*21*). The reduction potential of ascorbic acid is 282 mV (*22*). To work as an antioxidant, a free-radical scavenger must have a lower reduction potential than the compound to which it would donate its electron. Thus, the ascorbic acid is reacting with the hydroxyl radicals (2310 mV) to reduce the EPR signal and not the POBN radical.

It was reported (*15*) that *p*-coumaric acid had a constant antioxidant effect (27% decrease from the control EPR signal) at concentrations from 0.2 to 200 μM using EPR. On a percentage basis, our results showed no more than a 9% decrease from the control signal in that concentration range and not all concentrations were significant (**Figure 2**). Epicatechin had antioxidant effects up to approximately 0.5 μM and then increasingly pro-oxidant effects up to 50 μM (*15*). No higher doses were measured.

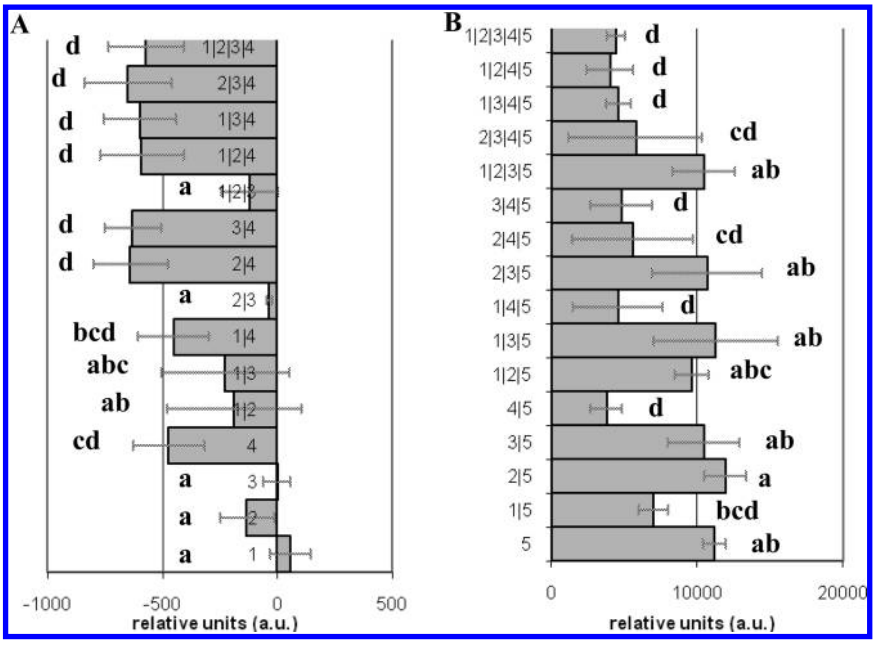

Figure 5. EPR signal intensities of combinations of rutin (**1**), *p*-coumaric acid (**2**), abscisic acid (**3**), a sugar solution matching papaya (**4**), and ascorbic acid (**5**) at concentrations found in honey and papaya. Graph A includes all combinations not containing ascorbic acid. Lower relative unit values indicate decreased POBN spin trap formation. Values reported are the mean of the EPR signal intensity minus the Fenton control ± SD. Values not sharing the same lowercase letter are significantly different ($p < 0.05$). $n = 2$ for **2**|**3** and **1**|**5**, otherwise $n = 3$.

Gallic acid had a pro-oxidant effect at concentrations up to approximately 175 μM. Conversely, ref *23* found that caffeic acid, *o*-coumarin, 6,7-dihydroxy-4-methylcoumarin, and catechin all had antioxidant effects, listed in order of decreasing effect, at 4.8 mM. Scopoletin exhibited a pro-oxidant effect at this concentration. However, catechin was shown to have pro-oxidant effects at concentrations between 0.75 and 2.3 mM, with greater oxidation at lower concentrations. Because catechin would be expected to have similar redox properties of epicatechin, it is unclear why ref *15* found that increasing the concentration increased oxidation (although at micromolar levels), while ref *23* found the opposite (at millimolar levels). These conflicting results suggest that experimental conditions and the concentration are critical factors in determining when a compound might act as an antioxidant or pro-oxidant or switch from one to the other based on the concentration. The present results help clarify the disparity by measuring at both micro- and millimolar concentrations. Measuring these compounds individually in an *in vitro* system does not allow for extrapolation to what might be expected *in vivo*. However, these experiments clarify the properties of the compounds analyzed in a system that has biological relevance.

EPR Analysis. At concentrations found in honey and papaya, combinations containing sugar solution were significantly more antioxidant in nature than combinations without it (**Figure 5**). Rutin, *p*-coumaric acid, abscisic acid, and ascorbic acid combinations had no significant effect on EPR signal intensity compared to any of them alone. The phenolics found in honey and papaya range between 10 and 100 μM concentrations found in **Figure 2**. Thus, on their own, a large signal was not expected; in combination, the interactions were not significant. When analyzed for synergistic potential (**Table 1**), combinations containing the sugar solution and ascorbic acid reached significance. Similar means in **Table 1** suggest that rutin, *p*-coumaric acid, and abscisic acid did not contribute to the protective effect of the sugar solution and ascorbic acid combination.

After digestion (**Figure 6**), no specific chemical changes were measured because the goal was to explore the overall effect of acid and base treatment (as occurs in the digestive tract) on antioxidant capacity. Combinations containing sugar solution continued to be significantly more antioxidant in nature than combinations that did not contain sugar solution. Although the overall magnitude of the signal was higher, because of oxidative potentiation by bicarbonate (*24*), again the presence of rutin, *p*-coumaric acid, abscisic acid, and ascorbic acid did not result in any significant differences from any of them alone. To analyze combinations for synergistic potential (**Table 1**), the oxidative effect of bicarbonate was subtracted from each sample; otherwise, the analysis for synergistic potential resulted in false positives. Combinations containing sugar solution and ascorbic acid reached significance similar to the food data.

Figure 7 represents the effect of combinations based on serum achievable levels of the compounds chosen (*18*). Serum data indicate no combinations were significantly different from any of their components analyzed separately (**Figure 7A**). In the presence of ascorbic acid (**Figure 7B**), caffeic acid, quercetin, or urate did not result in significant changes. When tested for synergistic potential, no combinations were significantly different from the sum of their components. Possibly, concentrations of compounds in serum experiments accounted for the results. Urate had both pro- and antioxidant activities, depending upon the concentration, in a copper-induced low-density lipoprotein (LDL) oxidation assay (*25*). At concentrations used in our experiments, it may have a non-significant antioxidant effect. Surprisingly, ascorbic acid tended to prevent the pro-oxidant effects of urate in a copper-induced LDL assay (*26*), although not significantly. This is opposite of that experienced in the present ascorbic acid/urate combination. The concentration or assay differences may account for different results; more work is needed to explain this discrepancy.

ORAC Analysis. In contrast to EPR, the ORAC assay was significantly more responsive to complexity (**Figure 8**). In general, more complex combinations had significantly higher ORAC values than less complex combinations, with most having ORAC values that were additive. When analyzed for synergistic potential (**Table 1**), one combination of two (**2**|**4**), three

Table 1. Combinations with Synergistic Potential in Food and Digestion Experiments[a]

	EPR[b]				ORAC[c]			
	food		digestion		food		digestion	
combination[d]	mean ± SE	*p* value	mean ± SE	*p* value	mean ± SE	*p* value	mean ± SE	*p* value
2 + **4** versus **2**|**4**					80 ± 28	0.006		
4 + **5** versus **4**|**5**	−6924 ± 2120	0.002	−13945 ± 5058	0.008				
1 + **2** + **3** versus **1**|**2**|**3**					93 ± 35	0.009		
1 + **2** + **4** versus **1**|**2**|**4**					95 ± 33	0.006		
1 + **4** + **5** versus **1**|**4**|**5**	−6187 ± 2123	0.005	−12143 ± 5060	0.019				
2 + **3** + **4** versus **2**|**3**|**4**					94 ± 33	0.007		
2 + **4** + **5** versus **2**|**4**|**5**	−4984 ± 2123	0.022	−12502 ± 5060	0.016			308 ± 148	0.041
3 + **4** + **5** versus **3**|**4**|**5**	−5918 ± 2123	0.007	−13242 ± 5060	0.011				
1 + **2** + **3** + **4** versus **1**|**2**|**3**|**4**					91 ± 38	0.019		
1 + **2** + **3** + **5** versus **1**|**2**|**3**|**5**					339 ± 100	0.001		
2 + **3** + **4** + **5** versus **2**|**3**|**4**|**5**	−4767 ± 2125	0.029	−12497 ± 5063	0.016	313 ± 100	0.003	320 ± 153	0.04
1 + **3** + **4** + **5** versus **1**|**3**|**4**|**5**	−6151 ± 2125	0.005	−11329 ± 5063	0.029	431 ± 100	<.001		
1 + **2** + **4** + **5** versus **1**|**2**|**4**|**5**	−6607 ± 2125	0.003			503 ± 100	<.001		
1 + **2** + **3** + **4** + **5** versus **1**|**2**|**3**|**4**|**5**	−6193 ± 2127	0.005	−11112 ± 5066	0.032	437 ± 102	<.001	393 ± 158	0.015

[a] Means represent the difference between the combination and the sum of the components ± standard error (SE). [b] Lower means indicate decreased POBN spin trap formation in the combination. [c] Higher means indicate greater resistance to AAPH-induced radicals in the combination. [d] **1**, rutin; **2**, *p*-coumaric acid; **3**, abscisic acid; **4**, sugar mixture; **5**, ascorbic acid. $n = 3$ for each compound or combination. $p < 0.05$ was used to determine significance.

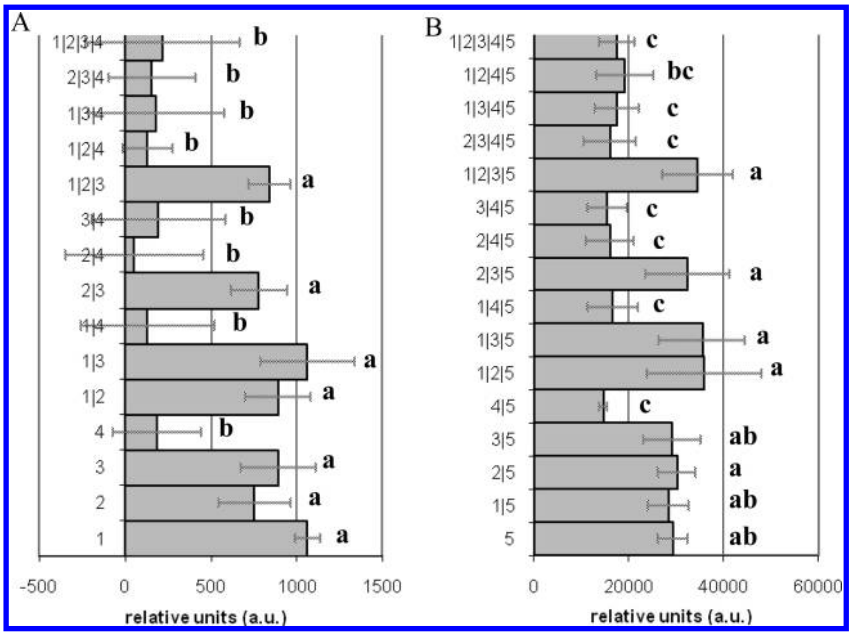

Figure 6. EPR signal intensities of combinations of rutin (**1**), *p*-coumaric acid (**2**), abscisic acid (**3**), a sugar solution matching papaya (**4**), and ascorbic acid (**5**) at concentrations found in honey and papaya after simulated digestion. Graph A includes all combinations not containing ascorbic acid. Lower relative unit values indicate decreased POBN spin trap formation. Values reported are the mean of the EPR signal intensity minus the Fenton control ± SD. Values not sharing the same lowercase letter are significantly different ($p < 0.05$). $n = 3$.

combinations of three (**1**|**2**|**3**, **1**|**2**|**4**, and **2**|**3**|**4**), and all combinations of four or five were significantly more antioxidant in nature than the sum of their components.

Figure 9 represents the effect of simulated digestion treatment on the same combinations. While the trend is similar to **Figure 8**, the overall magnitude of the average ORAC of many combinations was higher. The only difference between assays was the presence of bicarbonate; we speculate that it may have had an antioxidant effect in some combinations. More work is needed to derive why this might have occurred. Despite the increased magnitude, the results appear to be additive as in the food analysis. When analyzed for synergistic potential, far fewer combinations reached significance (**Table 1**). Recent reports (*5*, *7*) are in agreement with our results, showing the synergistic antioxidant potential of combinations.

Unlike the EPR serum data, many ORAC serum combinations were significantly higher than their components (**Figure 10**). As in the ORAC food and digestion experiments, the combinations appear to be additive in their effects. In contrast to all other data, these serum data were separated into urate- and non-urate-containing groups, because urate resulted in a significantly higher ORAC value and had a larger variance. When analyzed for synergistic potential, **5**|**6**, **5**|**8**, **5**|**7**|**8**, **6**|**7**|**8**, and **5**|**6**|**7**|**8** all had significantly higher ORAC values than the sum of their components (**Table 2**). These results contrast with EPR serum results; because of the redox activity of ascorbic acid, it is possible that the concentrations of ascorbic acid, quercetin, or urate were not as able to overcome the iron recycling capacity (and thus increased signal) of ascorbic acid. Ascorbic acid exhibits only antioxidant activity in the ORAC assay; therefore, the effects of quercetin and

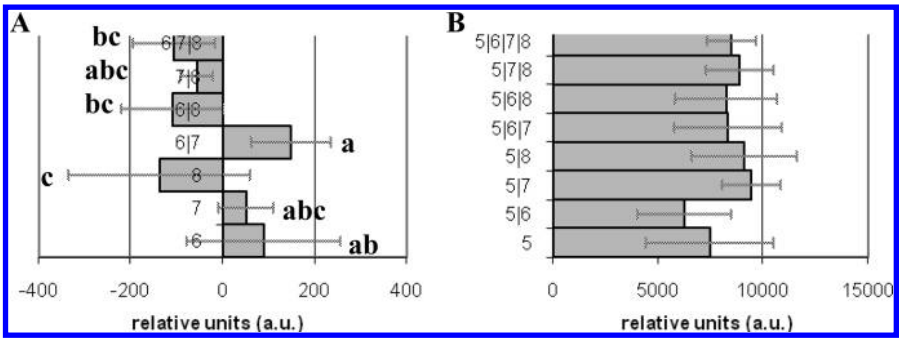

Figure 7. EPR signal intensities of combinations of ascorbic acid (**5**), caffeic acid (**6**), quercetin (**7**), and urate (**8**) at concentrations found in serum. Graph A includes all combinations not containing ascorbic acid. Lower relative unit values indicate decreased POBN spin trap formation. Values reported are the mean of the EPR signal intensity minus the Fenton control ± SD. Values not sharing the same lowercase letter are significantly different ($p < 0.05$). No combinations containing ascorbic acid were significantly different. $n = 3$.

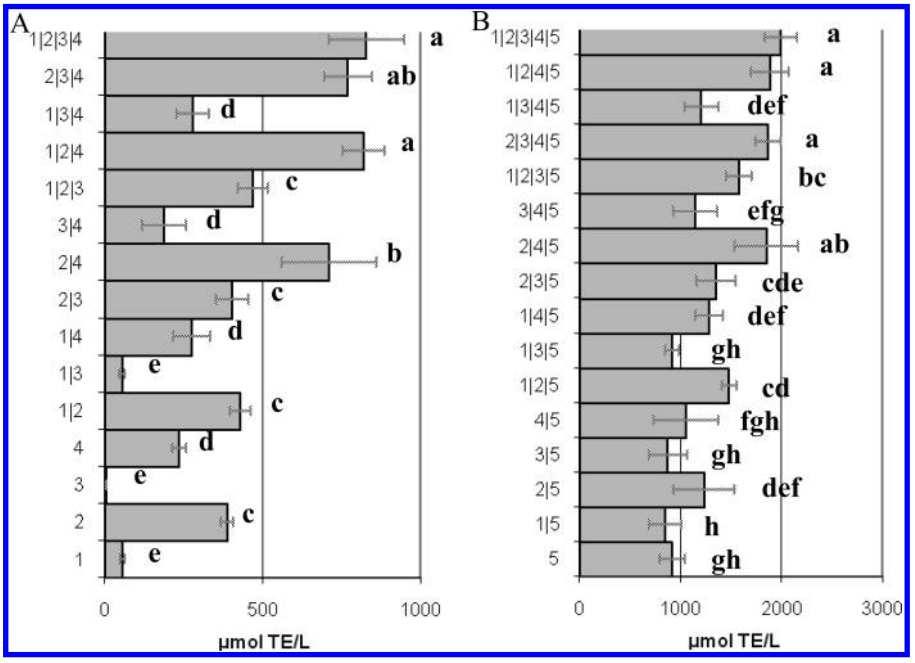

Figure 8. ORAC of combinations of rutin (**1**), *p*-coumaric acid (**2**), abscisic acid (**3**), a sugar solution matching papaya (**4**), and ascorbic acid (**5**) at concentrations found in honey and papaya. Graph A includes all combinations not containing ascorbic acid. Higher values indicate greater resistance to AAPH-induced radicals. Values reported are µmol of tocopherol equivalent (TE)/L ± SD. Values not sharing the same lowercase letter are significantly different ($p < 0.05$). $n = 2$ for **1**|**3**, otherwise $n \geq 3$.

urate may have been more apparent. As mentioned earlier, urate may have both pro- and antioxidant activities depending upon the concentration and assay (*25*, *26*).

EPR versus ORAC. Overall, digestion appeared to have little effect on EPR results (**Figures 5** and **6**) other than the magnitude, which can be attributed to the presence of bicarbonate. The results of the ORAC assay (**Figures 8** and **9**) were also similar other than magnitude, which may or may not be due to bicarbonate. However, while the synergistic potential of EPR results remained unchanged after digestion (**Table 1**), far fewer ORAC combinations retained synergistic potential after digestion. EPR serum concentration combinations (**Figure 7**) were not significant, while ORAC results (**Figure 10**) appeared to be additive and a number of combinations demonstrated synergistic potential (**Table 2**). Sugars were the greatest contributor to differences in the EPR system, while all components appeared to contribute to the ORAC results. Both the food and digestion EPR results agree in direction with ORAC results, with mechanistic differences between assays likely accounting for differences between results. These results have implications for the value of food that contains these combinations, especially when metals such as iron or copper are present from other components of a meal or a supplement. In ORAC, no transition metals are added; therefore, ascorbic acid contributes primarily as a hydrogen donor. Both of these scenarios are likely to occur in a mixed diet or physiologically, where iron is generally tightly sequestered but can become more available during oxidative stress (*27*). These differences demonstrate the benefit of applying more than one method to fully understand interactions. They also demonstrate the many complex scenarios and interactions that could occur *in vivo*. Further work is needed to fully elucidate the physiological ramifications before and after digestion of food and after absorption.

Biological Relevance. Selected components have potential for interaction in food, in the digestive system before absorption, and in serum. In serum, the interactions become most complex. Oxidation can result because of metabolism of glucose (*28*). The concentration of other compounds not found in food can be altered, such as the effect of fructose consumption on urate levels (*14*). Redox interactions occur between ascorbic acid and tocopherol at the membrane interface (*29*). Some flavonoids can

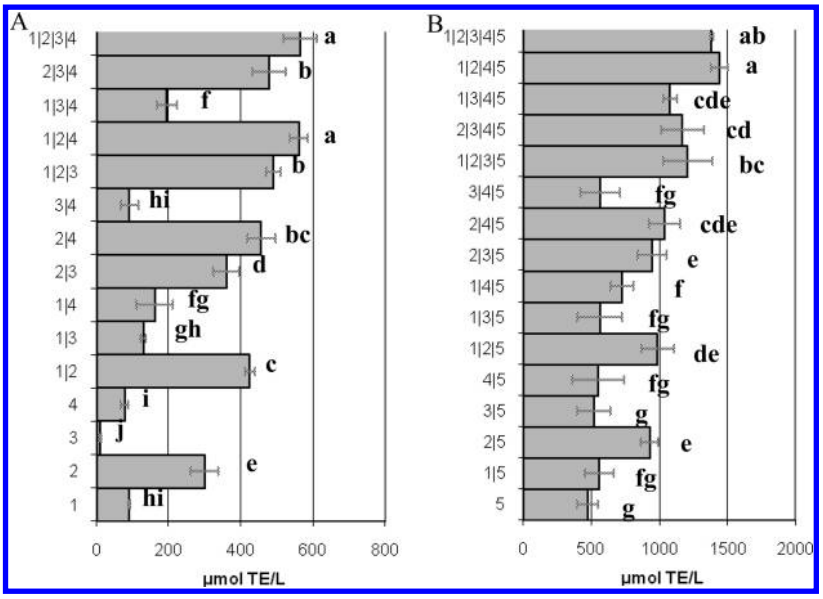

Figure 9. ORAC of combinations of rutin (**1**), *p*-coumaric acid (**2**), abscisic acid (**3**), a sugar solution matching papaya (**4**), and ascorbic acid (**5**) at concentrations found in honey and papaya after simulated digestion. Graph A includes all combinations not containing ascorbic acid. Higher values indicate greater resistance to AAPH-induced radicals. Values reported are μmol of TE/L $\pm$ SD. Values not sharing the same lowercase letter are significantly different ($p < 0.05$). $n \geq 3$.

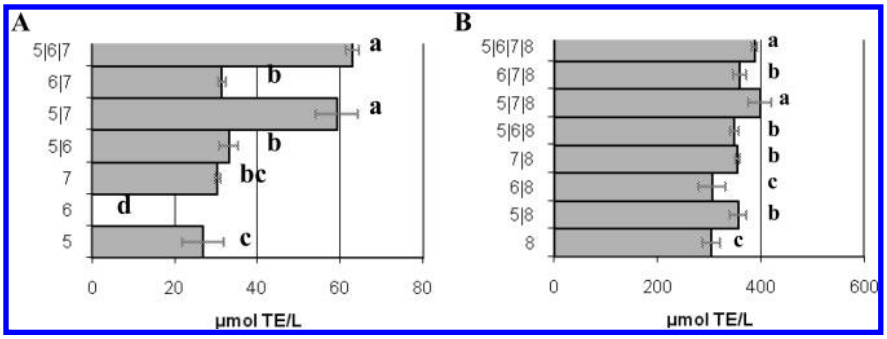

Figure 10. ORAC of combinations of ascorbic acid (**5**), caffeic acid (**6**), quercetin (**7**), and urate (**8**) at concentrations found in serum. Graph A includes all combinations not containing urate. Higher values indicate greater resistance to AAPH-induced radicals. Values reported are μmol of TE/L difference $\pm$ SD. Values not sharing the same lowercase letter are significantly different ($p < 0.05$). $n = 4$.

chelate and, thus, reduce the availability of transition metals (*30*). Finally, nutrients can affect genetics (*31*). While these experiments were not able to test all possible interactions, they demonstrate that interactions occur and that, in the situations we tested *in vitro*, those interactions were additive and had some potential for synergy. Although these results cannot be extrapolated directly to human biology, they point to the need to examine interactions in more detail in more biologically relevant ways. During oxidative stress, free iron and hydrogen peroxide production can increase while GSH decreases (*22*). Increased iron and hydrogen peroxide increases their interaction and formation of hydroxyl radicals (*27*). As GSH levels decrease, glutathione peroxidase is less able to remove hydrogen peroxide formed from metabolism or superoxide dismutase activity. The presence of antioxidants, chelating flavonoids, and reducing agents can inhibit these processes, all of which appear to work better together based on our results.

Our approach was to use individual dietary components that were lacking in complexity. It was not expected to encounter major chemical changes after digestion; however, it was expected that ascorbic acid may gain two electrons, thus compromising its potential antioxidant capacity. Also expected would be cleavage of glycosidic linkages in rutin, potentially yielding an effective antioxidant, quercetin. Sucrose hydrolysis to glucose and fructose would also be possible. It was the goal to see if companion molecules in the mixtures would protect each other. Because these chemical changes (e.g., hydrolysis) were not monitored, we can only speculate that our results reflect some of these potential changes.

Table 2. Combinations with Synergistic Potential in the Serum ORAC Experiment[a]

combination	mean $\pm$ SE	*p* value
5 + **6** versus **5**|**6**	6.1 $\pm$ 2.6	0.021
5 + **8** versus **5**|**8**	24.9 $\pm$ 11.2	0.031
5 + **7** + **8** versus **5**|**7**|**8**	36.5 $\pm$ 11.3	0.002
6 + **7** + **8** versus **6**|**7**|**8**	25.2 $\pm$ 11.3	0.031
5 + **6** + **7** + **8** versus **5**|**6**|**7**|**8**	26.5 $\pm$ 11.4	0.025

[a] Means represent the difference between the combination and the sum of the components $\pm$ standard error. Higher means indicate greater resistance to AAPH-induced radicals in the combination. **5**, ascorbic acid; **6**, caffeic acid; **7**, quercetin; **8**, urate. $n = 4$ for each compound or combination. $p < 0.05$ was used to determine significance.

Both EPR and ORAC experiments resulted in certain combinations having synergistic potential. The EPR food and digestion results were significant for those combinations containing the

sugar solution, with synergistic potential in compounds containing both the sugar solution and ascorbic acid. ORAC food and digestion results were additive, with some combinations having synergistic potential. At serum concentrations, EPR results revealed no significant differences between individual components and combinations and no synergistic interactions, perhaps because of the redox activity of ascorbic acid and/or pro- and antioxidant actions of urate. Serum ORAC values were additive, with many combinations having synergistic potential. These conflicting results will require further study and demonstrate the potential for differing results depending upon the test system selected. There is a benefit to interactions, although more work is needed to incorporate the complexities of food, digestion, and serum, as well as *in vivo* biochemistry.

ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-amidino-propane)dihydrochloride; EPR, electron paramagnetic resonance; ORAC, oxygen radical absorbance capacity; POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone; TE, tocopherol equivalent.

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