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Synergistic, Additive, and Antagonistic Effects of Food Mixtures on Total Antioxidant Capacities

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Different foods possess different bioactive compounds with varied antioxidant capacities. When foods are consumed together, the total antioxidant capacity of food mixtures may be modified via synergistic, additive, or antagonistic interactions among these components, which may in turn alter their physiological impacts. The main objective of this study was to investigate these interactions and identify any synergistic combinations. Eleven foods from three categories, including fruits (raspberry, blackberry, and apple), vegetables (broccoli, tomato, mushroom, and purple cauliflower), and legumes (soybean, adzuki bean, red kidney bean, and black bean) were combined in pairs. Four assays (total phenolic content, ferric reducing antioxidant power, 2,2-diphenyl-1-picrylhydrazyl, radical scavenging capacity, and oxygen radical absorbance capacity) were used to evaluate the antioxidant capacities of individual foods and their combinations. The results indicated that within the same food category, 13, 68, and 21% of the combinations produced synergistic, additive, and antagonistic interactions, respectively, while the combinations produced 21, 54, and 25% synergistic, additive, and antagonistic effects, respectively, across food categories. Combining specific foods across categories (e.g., fruit and legume) was more likely to result in synergistic antioxidant capacity than combinations within a food group. Combining raspberry and adzuki bean extracts demonstrated synergistic interactions in all four chemical-based assays. Compositional changes did not seem to have occurred in the mixture. Results in this study suggest the importance of strategically selecting foods or diets to maximum synergisms as well as to minimum antagonisms in antioxidant activity.

KEYWORDS: Synergism; additive interaction; antagonism; antioxidant capacity; TPC; FRAP; ORAC; DPPH; HPLC; fruits; vegetables; legumes; dietary antioxidant; food category

INTRODUCTION

Oxidative damage plays an important role in the development of several chronic diseases including cardiovascular disease, diabetes, and cancer (*1*−*3*). The human body possesses a sophisticated and cooperative array of antioxidant defense systems to help prevent accumulation of this damage and thereby delay or prevent the onset of several chronic diseases. Many environmental and lifestyle factors and the normal process of aging can introduce an imbalance between the antioxidative defense and the free radical pressure from excess reactive oxygen species (ROS) and reactive nitrogen species (RNS) (*4*). Results from epidemiological studies have shown a good correlation between the consumption of plant-based foods such as fruits, vegetables, whole grains, and legumes and the reduced risk of the aforementioned chronic diseases (*5*−*7*).

A substantial body of research has investigated the antioxidant properties of plant foods and showed the importance of polyphenolic compounds, such as phenonic acids and the many subgroups of flavonoids (flavanols, flavanones, flavones, flavonols, isoflavones, and anthocyanins) (*8*−*10*). Many studies have also attempted to investigate whether certain health benefits can be linked to the antioxidant activity *in vitro* and *in vivo* (*11*). Recent dietary intervention studies have shown that diets based on high total antioxidant capacities yield more positive health outcomes, such as improvements on endothelial function (*12*) and liver function in healthy populations (*13*), as compared to diets of low total antioxidant capacity. Despite these studies, results are still inconclusive.

Many plant-based foods are good sources of unique phytochemical antioxidants, which may exert different health-promoting effects. For example, isoflavones in certain legumes possess relatively weak antioxidant capacity as compared to other polyphenols in fruits but may act as weak estrogens, thereby competing with endogenous sex hormones and possibly decreasing the risk of breast and prostate cancer (*14*, *15*). Structurally diverse phytochemicals may possess similar, overlapping, or different but complementary effects in their antioxidant activities. A combination of different plant-based foods may exhibit additive, synergistic, or antagonistic interactions among their different phytochemicals.

An additive effect refers to a food combination that provides the sum of the effects of the individual components; a synergistic effect occurs when the effect is greater than the sum of individual

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components, and antagonism occurs when the sum of the effects is less than the mathematical sum that would be predicted from individual components. We were particularly interested in identifying synergistic interactions as a possible way to minimize disease-associated oxidative stress.

Researchers have already realized that approaches from single antioxidant are not adequate to assess the health benefit of food mixtures, as the bioactive constituents in edible plants are always ingested in the form of natural mixtures (*16*). When in a mixture, interactions between phytochemicals such as flavonoids in a particular plant can contribute significantly to the ability of natural plant extracts to protect human health or mitigate disease damage, because the responsible bioactive compounds seldom work independently (*16*). Interactions between antioxidative food components are important, and the ultimate results *in vivo* depend on many factors, including *in vitro* activities, food processing, and metabolism in human. However, the majority of investigations are still limited to *in vitro* tests on purified antioxidant mixtures (*17*, *18*), different compounds in a specific food (*19*), or similar foods within the same fruits or vegetables category such as those reported by Zafra-Stone *et al.* who found that a combination of wild bilberry, cranberry, elderberry, raspberry, and strawberry exhibited higher antioxidant capacity when compared with the individual berries (*20*). It was not clear as to how the phytochemicals in these studies interact with each other and how the interactions lead to synergistic effects. More work is needed to investigate different types of interactions within and across food categories as well as to identify mixtures that hold synergistic interactions that can ultimately lead to the development of functional foods.

For this purpose, a total of 11 foods were selected, including three fruits (raspberries, blackberries, and apples), four vegetables (broccoli, tomatoes, mushrooms, and purple cauliflower), and four legumes (soybeans, adzuki beans, red kidney beans, and black beans), in this study. Many of these are listed in the U.S. Department of Agriculture list of 20 top antioxidant foods (*21*). There is no information available whether any synergistic effects are evident among these foods.

On the other hand, an assessment of synergistic effects, while a laudable goal, is made more complicated and difficult because the different assays available are based on different chemical mechanisms (*22*, *23*). The specificity and sensitivity of a single method do not guarantee a reliable assessment of all types of dietary antioxidants; therefore, a combination of several tests is considered a more accurate measure of the antioxidant activity (*24*). Therefore, we used four different assays that included total phenolic content (TPC), ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity, and oxygen radical absorbance capacity (ORAC). These commonly used assays reflect two possible mechanisms, hydrogen atom transfer (HAT) and single electron transfer (SET), which are relevant mechanisms for dietary antioxidants used in the human body (*22*, *25*). The ORAC assay uses the former mechanism, while other three assays are based on the latter. A combination containing antioxidant compounds that can contribute HAT and SET systems should result in a more powerful antioxidant response.

In this study, crude methanol extracts of selected fruits, vegetables, and legumes were evaluated for their antioxidant capacity individually and then in combination using four different *in vitro* models. The methanol extracts were used since preliminary studies showed that the methanol extracts had the highest antioxidant activity for most fresh plant foods. The possible mechanism(s) responsible for the observed synergism was investigated.

MATERIALS AND METHODS

Chemicals and Reagents. Ascorbic acid, 2,4,6-tripyridyl-s-triazine (TPTZ), and the Folin−Ciocalteu reagent (FCR) were purchased from Sigma Chemical Co. (St. Louis, MO). DPPH, 2,2′-azobis (2-methylpropanimidamide) dihydrochloride (AAPH), and 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox) were obtained from Sigma-Aldrich (Oakville, ON, Canada). Ferric chloride ($FeCl_3$) and sodium acetate were from Aldrich Chemical Co. (Milwaukee, WI). All solvents were of high-performance liquid chromatography (HPLC) grade and purchased from Caledon Laboratories Ltd. (Georgetown, ON, Canada).

Sample Collection and Preparation. *Source of Samples*. Fresh commercially available fruits apple (*Malus domestica*), raspberry (*Rubus strigosus*), and blackberry (*Rubus fruticosus*) and vegetables broccoli (*Brassica oleracea*), and tomato (*Solanum lycopersicu*) were purchased from a local grocery store (Metro, Guelph, Ontario, Canada, May−August, 2009). White button mushroom (*Agaricus bisporus*) and purple cauliflower (*B. oleracea*) were harvested from the Vineland Station and Muck Station of the University of Guelph (October, 2009), respectively.

Selected commercially available dry leguminous seeds including black beans (*Phaseolus vulgaris*), adzuki beans (*Vigna angulariz*), and red kidney beans (*P. vulgaris*) were purchased from a local grocery store (Bulk Barn, Guelph, Ontario, Canada, 2008). Soybean seeds (*Glycine max*) (Cv. S96 3-1450) harvested from the Elora Experimental field of the University of Guelph (Ontario, Canada) were gift from Dr. Istvan Rajcan (harvested in 2008, University of Guelph).

Sample Preparation and Extraction. Fresh berries (raspberries and blueberries, ca. 1000 g) were first divided into 200 g subsamples. One of the subsamples (200 g) was then blended at high speed for 1 min in a Warring 7009 L blender (Torrington, CT). Fifty grams of the slurry was mixed with 80% methanol in a total volume of 250 mL and shaken at room temperature for 4 h on a G24 environmental incubator shaker (New Brunswich Scientific, Edison, NJ). The suspension was centrifuged at 3000*g* for 15 min at room temperature, and the supernatant was filtered through a 0.45 μm PVDF filter (Whatman, Sanford, ME) into amber glass flask, topped to a final volume of 250 mL with 80% methanol, and then used in all assays. Similarly, a representative subsample (50 g) of apple and tomato slices with skin (from four apples/tomatoes, each cut into 16 equal pieces), 50 g of broccoli and cauliflower florets (from ca. 1 kg, cut in 5−10 cm^3), and 50 g of mushroom heads (from 1 kg; size, 5 cm diameter; cut in quarters) were ground separately in a Warring blender and extracted with 80% methanol in a total volume of 250 mL, filtered, topped to 250 mL, and analyzed. All extracted samples were stored at 4 °C for <2 days before analysis.

The moisture contents of fresh fruits and vegetables were calculated by the percentage of weight loss from the sample in an isotemp vacuum oven (Fisher Scientific, Toronto, Ontario, Canada). Specifically, 50 g representative fresh samples were dried in the oven maintained at 30 Hg (635 mm) vacuum and 70 °C, as a single layer on a tray, until a constant weight was reached.

Leguminous seeds (250 g) were ground in a Retsch MM2000 ball mill (GmbH & Co. Haan, Germany) before extraction. From each ground sample, a 50 g aliquot was extracted with 80% methanol in a total volume of 250 mL at room temperature for 12 h on a VWR-Rocking platform Shaker (VWR, Batavia, IL). The suspension was centrifuged at 3000*g* for 30 min at room temperature, and the supernatant was filtered through a 0.45 μm PVDF filter (Whatman) after it was collected in amber glass vials, and made up to 250 mL with 80% methanol. All extracted samples were stored at 4 °C for <2 days before analysis.

Antioxidant Capacity Evaluation. The 80% methanol extracts of individual foods and mixtures were evaluated using four antioxidant assays. For the different mixtures, the 80% methanol extracts were mixed in pairs at a 1:1 (v/v) ratio. For assays of all individual extracts, they were diluted 2-fold with 80% methanol before testing.

Total Phenolic Content (TPC) Assay. The TPC was determined by a modified Folin−Ciocalteu method described previously (*26*, *27*). Briefly, 0.2 mL of the extract was mixed with 1 mL of the FCR and 0.8 mL of 7.5% sodium carbonate. The mixture was gently shaken and allowed to stand at room temperature for 30 min, and the absorbance was read at 765 nm. A gallic acid standard curve was prepared with a concentration range from 50 to 250 μg/mL. The TPC in the various crude extracts or extract combinations was expressed as micrograms of gallic acid equivalent (GAE) per gram of sample. All tests were done in triplicate.

FRAP Assay. The FRAP assay followed the methods of Benzie *et al.* and Tsao *et al.* (*28*, *29*). Briefly, 10 μL of crude extract or extract combinations was mixed in the well of a 96-well plate with 300 μL of freshly prepared FRAP reagent. The FRAP reagent is a mixture of 300 mM acetate buffer, pH 3.6 (3.1 g sodium acetate and 16 mL glacial acetic acid per liter of buffer solution), 10 mM TPTZ in 40 mL of 40 mM HCl, and 20 mM $FeCl_3$ at 10:1:1 (v/v/v). The plate was kept at 37 °C, and the absorbance was read at 593 nm immediately after mixing and at 4 min intervals for 12 min with a visible-UV microplate kinetic reader. All samples were tested in triplicate. The final FRAP value of the samples was calculated on the basis of 500 μM ascorbic acid being equivalent to a 1000 μM FRAP value.

DPPH Radical Scavenging Capacity Assay. The DPPH radical scavenging capacity assay was based on the modified method previously described (*30*). Ten microliters of each crude extract or extract combination in methanol was serially diluted and was added to 250 μL of DPPH (2.5 μM) in methanol in a 96-well plate and shaken vigorously. After incubation at room temperature for 30 min, the absorbance of the remaining DPPH was determined in a microplate reader at 517 nm, and the radical scavenging activity of each sample was expressed as EC_{50} (concentration of sample extract necessary to scavenger initial concentration of free radical DPPH 50%), which is defined as the concentration of sample that decreased the initial DPPH radical concentration by 50%. The mean values were obtained from triplicate determinations.

ORAC Assay. Basically, the ORAC assay measures a fluorescent signal from a probe that decreases or is "quenched" in the presence of a ROS generator (*31*). The extract, the phosphate buffer, and the fluorescein solution were mixed and placed in the wells of the microplate. The mixture was preincubated for 15 min at 37 °C. A free radical generator AAPH solution (25 μL; 153 mM) was added. A multidetection microplate reader recorded the fluorescence every minute for 60 min with an emission and excitation of 535 and 485 nm, respectively. A standard curve was generated with a trolox concentration range from 6.25 to 100 μM. The ORAC values were calculated as the area under the curve (AUC) and expressed as micromoles of trolox equivalent (TE) per gram of fresh fruit and vegetable or dry weight of legume. Three replicate assays were performed for each sample.

HPLC Analysis. The HPLC method (*32*) with modifications was used to monitor the changes among the phytochemical profiles of individual foods and their mixtures. The 80% methanol extracts of two foods were mixed at a 1:1 (v/v) ratio and filtered through 0.45 μm PVDF filter (Whatman) before HPLC analysis.

An HPLC system (Agilent Technology 1100 Series, Palo Alto, CA) was equipped with a quaternary pump, an inline degasser, a thermostatic autosampler, and a diode array detector (DAD). A Phenomenex Luna 5 μ column (250 mm × 4.60 mm) with a C18 guard column (Torrance, CA) and a binary mobile phase of water/acetic acid (98:2, v/v) (solvent A) and water/acetonitrile/acetic acid (78:20:2, v/v/v) (solvent B) were used with the following gradient program: 0−55 min, 100−20% A; 55−70 min, 20−10% A; 70−80 min, 10−5% A; and 80−100 min, 5−0% A. The flow rate was 1.2 mL/min, and the injection volume was 20 μL for individual foods and 40 μL for their mixture. The DAD collected data from 190 to 700 nm.

Statistical Analysis. All analyses were performed in triplicate. The data were expressed as means ± standard errors, and one-way analysis of variance (ANOVA) was performed to test for significant differences among the means by Statistix software (V2.0, Analytical Software, Tallahasse, FL). Differences among means at $p \leq 0.05$ were considered significant.

RESULTS AND DISCUSSION

Antioxidant Profiles of Individual Foods. Eleven selected foods were evaluated individually by TPC, FRAP, ORAC, and DPPH, and their antioxidant potentials were compared. The results demonstrate that different foods exhibit different antioxidant potentials within the same food category (**Figure 1**). In the fruit group, blackberries had a significantly higher antioxidant capacity in the four assays, followed by raspberries and apples ($p < 0.05$). These results are consistent with the measurements made by other investigators (*21*). In the vegetable group, purple cauliflower

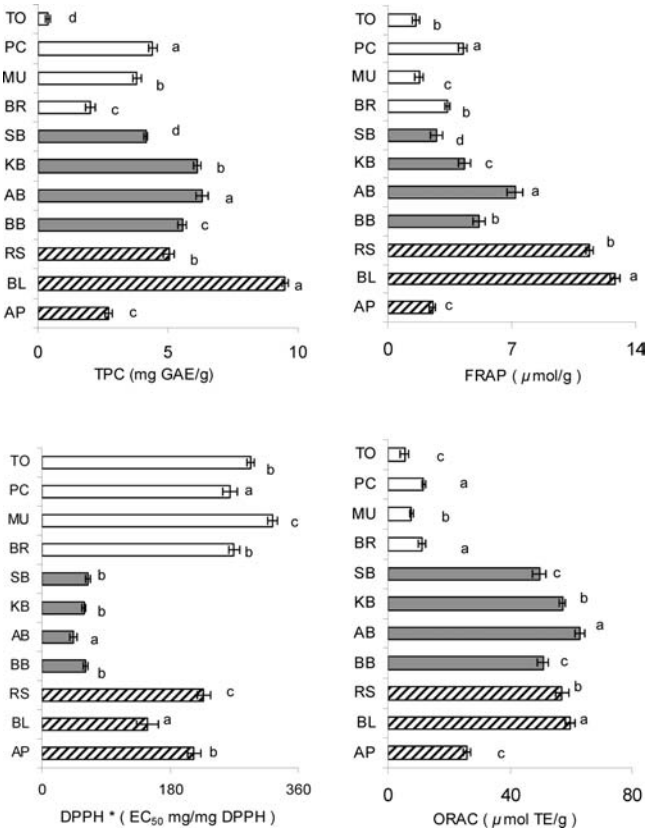

Figure 1. Comparison of antioxidant activities of individual foods as measured by TPC, FRAP, DPPH, and ORAC. In each assay, means followed by different letters indicate significant difference by ANOVA ($p < 0.05$) within the same food category. Open bars, vegetables; filled bars, leguminous seeds; and hatched bars, fruits. *For DPPH, lower EC_{50} values indicate higher antioxidant capacities. TO, tomato (70% moisture); PC, purple cauliflower (61% moisture); MU, mushroom (76% moisture); BR, broccoli (60% moisture); SB, soy bean; RK, red kidney bean; AB, adzuki bean; BB, black bean; RS, raspberry (60% moisture); BL, black berry (61% moisture); and AP, apple (58% moisture).

demonstrated the highest antioxidant capacity as compared with other vegetables. Specifically, the antioxidant capacities of the four vegetables were in the following order from highest to lowest: TPC, purple cauliflower > mushroom > broccoli > tomato; FRAP, purple cauliflower > broccoli > mushroom > tomato; DPPH, purple cauliflower = broccoli > tomato > mushroom; and ORAC, purple cauliflower = broccoli > mushroom > tomato. The relatively high TPC value in mushrooms has been reported by other researchers (*33*). In leguminous foods, the TPC and ORAC values were found in the following order from highest to lowest: adzuki beans > red kidney beans > black beans > soybeans. The FRAP and DPPH values follow the same pattern with the exception that black beans had higher values than red kidney beans. Previous researchers reported the same finding in which the 70% methanol extract of red kidney beans showed higher TPC and ORAC values than the 70% methanol extract of black beans; opposite observations were found in their DPPH and FRAP values (*34*).

Variability is common in the evaluation of antioxidant capacities among some fruits, vegetables, and legumes measured by different investigators even when the same assay was employed. The variance could be due to changes in phytochemical compositions, which are effected by cultivars, growing region, harvest season, maturity stage, storage conditions, as well as the part of the foods tested (for example, apples with and without the peel) (*35*, *36*). The variance could also be attributed to different extracting

solvents used. For example, 80% methanol was used in the present study, which may result in different results from those reported by others using different solvents, particularly for dry legume samples. Beans may contain methanol insoluble compounds that could have potential antioxidant capacity.

The results also demonstrate that different food categories exhibit different antioxidant potentials. The average antioxidant capacity derived from individual foods was calculated and compared among these food categories (**Figure 2**). The vegetable group had the lowest antioxidant capacity as compared with the fruits or legumes in all four assays. Leguminous foods exhibited the highest antioxidant capacity in ORAC and DPPH assays indicating that the legumes have a strong ability to scavenge free radicals. The fruits had the highest TPC and FRAP values demonstrating their high content of phenolics as well as outstanding reducing power. Results in this study are reported and discussed based on the natural forms of the foods; that is, data are expressed in fresh weight of fruits and vegetables and dry weight of the leguminous seeds. Water or moisture contents of the individual foods are listed in the legend of **Figure 1**.

Antioxidant Capacity in Food Combinations. Fruits and vegetables contain high concentrations of different flavonoids including flavanols, flavanones, flavones, flavonols, and anthocyanins. In legumes, while some flavonols and antocyanins have been found in different beans, isoflavones are the most abundant in soybean (*27*, *29*, *37*−*39*). Compositional changes of and interactions among these and other phytochemicals are not known when different foods are mixed together. Also unclear is whether or not mixing different groups of foods will lead to changes in total antioxidant capacity.

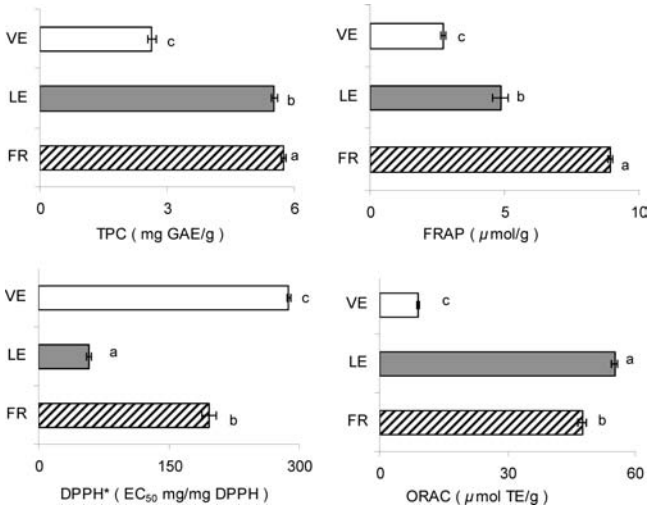

Figure 2. Comparison of antioxidant capacities by food category. In each assay, means followed by different letters indicate significant difference by ANOVA ($p < 0.05$). VE, vegetables; LE, legumes; and FR, fruits. *For DPPH, lower values indicate higher antioxidant capacities.

To identify specific combinations of foods that exhibit synergistic interactions, individual food extracts were mixed in pairs as described earlier, and four antioxidant assays were employed to evaluate their antioxidant capacities. In total, 55 combinations were tested, and the observed antioxidant capacities of the mixtures were recorded. The observed antioxidant capacity of each mixture was compared with the expected value, which is the mathematical sum of the antioxidant capacity derived from the individual extracts analyzed at 2-fold dilution. Therefore, all comparisons were based on the same total weight of the pair; for example, the antioxidant capacity (observed value) of a 1 g mixture of apple and raspberry (0.5 g each when mixed at 1:1 v/v ratio) was compared with the mathematical sum of the antioxidant capacity (expected value) of 0.5 g of apple and that of 0.5 g of raspberry (2-fold dilution). If the observed values were significantly higher than the expected value derived from of the same pair of individual foods ($p < 0.05$), a synergistic interaction was considered to have occurred in the mixture. The opposite result, meaning that the observed value was significantly lower than the expected value, was defined as an antagonistic interaction. No significant difference between the two values indicated an additive interaction. The approach was used for all assays except for the DPPH assay. If the observed DPPH values were the same, lower or higher than the expected DPPH values, they were considered as additive, synergistic, and antagonistic interactions, respectively, because a higher DPPH value (EC_{50}) represents a lower antioxidant capacity.

Both observed and expected values of 55 combinations from the four assays are summarized in **Tables 1**−**6**. All types of interactions, that is, synergistic, additive, and antagonistic interactions, were observed. This indicates that simply combining foods did not guarantee that the antioxidant capacity would be equal to the expected value (additive interactions). The total antioxidant capacity of food combinations may increase through synergistic interactions or decrease through antagonistic interactions when combined. For instance, blackberries and adzuki beans had the highest antioxidant capacity among all 11 individual foods that we tested. However, the combination of blackberries and adzuki beans did not result in the highest antioxidant capacity of all 55 combinations (**Table 6**). This implies that when two foods, for example, blackberries and adzuki beans, are consumed at the same time, the ultimate total antioxidant capacity may not depend on those of the individual foods. In other words, food−food interactions can potentially play a role in determining the final total antioxidant capacity of food combinations.

Each combination was evaluated for synergistic, additive, and antagonistic interaction in four different assays (**Tables 1**−**6**). The numbers of each type of interactions within the 55 combinations were added together across the four antioxidant assays. The percentage of each type of interaction was further divided into those occurring within a food category and those occurring across categories (**Figure 3**). The reason to pool results across all assays was to capture contributions of all types of antioxidants in food

Table 1. TPC, FRAP, DPPH, and ORAC Values of Combinatorial Fruits[a]

food combinations		TPC (mg GAE/g)		FRAP (μmol/g)		DPPH EC $_{50}$ (mg/mg DPPH)		ORAC (μmol TE/g)	
RS + AP	O	4.58 ± 0.22*	Sy	6.86 ± 0.05	Ad	214.98 ± 2.93	Ad	42.70 ± 0.58	Ad
	E	3.87 ± 0.07		6.89 ± 0.13		220.51 ± 7.25		41.46 ± 1.31	
RS + BL	O	8.31 ± 0.27*	Sy	11.45 ± 0.38*	An	182.77 ± 5.29	Ad	61.43 ± 1.04	Ad
	E	7.27 ± 0.16		12.13 ± 0.11		187.53 ± 10.39		60.12 ± 2.45	
AP + BL	O	5.87 ± 0.14	Ad	7.54 ± 0.27	Ad	202.01 ± 8.64*	An	43.17 ± 0.58	Ad
	E	6.10 ± 0.04		7.68 ± 0.18		180.94 ± 11.99		42.74 ± 0.22	

[a] Data are expressed as means ± standard errors ($n = 3$). The asterisk indicates a significant difference between observed value and expected value ($p < 0.05$). RS, raspberry; AP, apple; BL, blackberry; O, observed value; E, expected value; Sy, synergistic interaction; Ad, additive interaction; and An, antagonistic interaction.

Table 2. TPC, FRAP, DPPH, and ORAC Values of Combinatorial Vegetables[a]

food combinations		TPC (mg GAE/g)		FRAP (μmol/g)		DPPH EC $_{50}$ (mg/mg DPPH)		ORAC (μmol TE/g)	
BR + TO	O	1.42 ± 0.05*	Sy	2.27 ± 0.11*	An	281.00 ± 11.99	Ad	8.55 ± 0.23	Ad
	E	1.19 ± 0.09		2.46 ± 0.05		281.72 ± 6.23		8.23 ± 0.27	
BR + MU	O	3.04 ± 0.07	Ad	2.25 ± 0.25	Ad	292.26 ± 5.92	Ad	9.74 ± 0.68	Ad
	E	2.89 ± 0.16		2.57 ± 0.11		297.34 ± 2.63		9.35 ± 0.18	
BR + PC	O	3.40 ± 0.11	Ad	3.67 ± 0.18	Ad	278.85 ± 5.92*	An	11.30 ± 0.09	Ad
	E	3.20 ± 0.18		3.80 ± 0.17		267.53 ± 1.80		11.32 ± 0.11	
TO + MU	O	2.06 ± 0.05	Ad	1.37 ± 0.11*	An	303.37 ± 4.39	Ad	6.45 ± 0.23	Ad
	E	2.09 ± 0.09		1.67 ± 0.05		308.96 ± 1.82		6.61 ± 0.04	
TO + PC	O	3.02 ± 0.14*	Sy	2.78 ± 0.07	Ad	282.72 ± 8.80	Ad	10.32 ± 0.32*	Sy
	E	2.40 ± 0.09		2.89 ± 0.13		279.14 ± 2.59		8.58 ± 0.31	
MU + PC	O	3.96 ± 0.02	Ad	2.65 ± 0.18*	An	305.09 ± 3.33*	An	10.20 ± 0.50	Ad
	E	4.10 ± 0.16		3.00 ± 0.16		294.76 ± 8.32		9.71 ± 0.40	

[a] BR, broccoli; TO, tomato; MU, mushroom; and PC, purple cauliflower.

Table 3. TPC, FRAP, DPPH, and ORAC Values of Combinatorial Legumes[a]

food combinations		TPC (mg GAE/g)		FRAP (μmol/g)		DPPH EC $_{50}$ (mg/mg DPPH)		ORAC (μmol TE/g)	
SB + AB	O	5.35 ± 0.11	Ad	4.58 ± 0.13	Ad	57.81 ± 2.00*	An	64.18 ± 0.65*	Sy
	E	5.23 ± 0.13		4.96 ± 0.38		54.48 ± 1.98		56.26 ± 1.75	
SB + RK	O	5.54 ± 0.34	Ad	3.31 ± 0.07	Ad	63.71 ± 1.82	Ad	52.11 ± 0.70	Ad
	E	5.14 ± 0.09		3.53 ± 0.32		62.01 ± 1.98		53.40 ± 1.17	
SB + BB	O	5.29 ± 0.09	Ad	3.87 ± 0.50	Ad	67.98 ± 3.13*	An	50.87 ± 0.59	Ad
	E	4.85 ± 0.11		3.94 ± 0.34		63.43 ± 2.30		50.20 ± 0.99	
AB + RK	O	6.41 ± 0.16	Ad	5.39 ± 0.11	Ad	57.34 ± 5.44	Ad	59.20 ± 0.18	Ad
	E	6.20 ± 0.18		5.75 ± 0.38		51.83 ± 2.36		60.04 ± 1.04	
AB + BB	O	6.28 ± 0.02*	Sy	5.48 ± 0.23*	An	59.87 ± 8.06	Ad	57.38 ± 0.36	Ad
	E	5.92 ± 0.11		6.16 ± 0.40		53.25 ± 2.74		56.86 ± 0.54	
RK + BB	O	6.19 ± 0.12*	Sy	4.19 ± 0.16*	An	65.96 ± 2.99	Ad	56.06 ± 0.67	Ad
	E	5.84 ± 0.09		4.73 ± 0.34		60.77 ± 2.97		53.99 ± 0.54	

[a] SB, soy bean; AB, adzuki bean; RK, red kidney bean; and BB, black bean.

Table 4. TPC, FRAP, DPPH, and ORAC values of combinatorial fruits and vegetables

food combinations		TPC (mg GAE/g)		FRAP (μmol/g)		DPPH EC $_{50}$ (mg/mg DPPH)		ORAC (μmol TE/g)	
RA + BR	O	3.91 ± 0.07*	Sy	6.43 ± 0.38*	An	253.47 ± 8.80	Ad	35.87 ± 2.39	Ad
	E	3.52 ± 0.14		7.38 ± 0.07		248.60 ± 6.28		34.00 ± 0.94	
RA + TO	O	2.81 ± 0.07	Ad	6.39 ± 0.14	Ad	262.66 ± 3.33	Ad	32.33 ± 0.38	Ad
	E	2.71 ± 0.11		6.48 ± 0.11		260.22 ± 5.44		31.26 ± 1.08	
RA + MU	O	4.47 ± 0.22	Ad	5.91 ± 0.36 *	An	277.74 ± 2.56	Ad	36.35 ± 0.47*	Sy
	E	4.42 ± 0.18		6.59 ± 0.05		275.84 ± 1.66		32.40 ± 1.01	
RA + PC	O	5.12 ± 0.16*	Sy	6.85 ± 0.43*	An	248.31 ± 11.65	Ad	35.58 ± 0.36	Ad
	E	4.72 ± 0.14		7.82 ± 0.04		246.02 ± 2.88		34.35 ± 1.39	
AP + BR	O	2.87 ± 0.11*	Sy	2.41 ± 0.20*	An	240.29 ± 4.39	Ad	18.37 ± 0.29	Ad
	E	2.53 ± 0.05		2.94 ± 0.08		242.01 ± 7.40		18.42 ± 0.11	
AP + TO	O	1.25 ± 0.13*	An	2.12 ± 0.22	Ad	261.51 ± 5.76	Ad	16.85 ± 0.18*	Sy
	E	1.55 ± 0.67		2.03 ± 0.10		253.62 ± 6.84		15.68 ± 0.27	
AP + MU	O	2.71 ± 0.09*	An	2.29 ± 0.16	Ad	250.61 ± 8.80*	An	17.12 ± 0.23	Ad
	E	3.25 ± 0.02		2.14 ± 0.07		269.25 ± 2.88		16.82 ± 0.36	
AP + PC	O	3.89 ± 0.11*	Sy	2.99 ± 0.29*	An	208.25 ± 7.25*	Sy	19.51 ± 0.94	Ad
	E	3.56 ± 0.07		3.37 ± 0.11		239.43 ± 3.33		18.78 ± 0.63	
BL + BR	O	6.66 ± 0.23*	Sy	8.20 ± 0.16	Ad	258.06 ± 14.40*	An	35.03 ± 0.34	Ad
	E	5.74 ± 0.13		8.11 ± 0.14		209.03 ± 10.08		35.28 ± 0.38	
BL + TO	O	5.56 ± 0.18*	Sy	5.97 ± 0.09*	An	230.40 ± 4.99	Ad	32.63 ± 0.34	Ad
	E	4.94 ± 0.07		7.20 ± 0.16		220.65 ± 9.90		32.54 ± 0.20	
BL + MU	O	6.49 ± 0.20	Ad	5.82 ± 0.31*	An	244.30 ± 10.39	Ad	33.57 ± 0.32	Ad
	E	6.65 ± 0.14		7.30 ± 0.18		236.27 ± 5.99		33.67 ± 0.22	
BL + PC	O	7.69 ± 0.27*	Sy	7.04 ± 0.11*	An	196.70 ± 11.65	Ad	35.78 ± 0.11	Ad
	E	6.95 ± 0.13		8.54 ± 0.22		206.45 ± 5.76		35.63 ± 0.29	

combinations, since no single assay represents the actual total antioxidant activity. The results indicated that combining foods within and across categories resulted in different degrees of synergism, additive effects, and antagonism (**Figure 3**). Within the same food category, 13, 68, and 21% of the tested combinations showed synergistic, additive, and antagonistic interactions, respectively, while across food categories 21, 54, and 25% of the tested combinations demonstrated synergistic, additive, and antagonistic interactions, respectively (**Figure 3**).

In terms of synergistic interactions, food combinations across categories had a higher chance of demonstrating synergistic interactions as compared to that of combinations within a category.

Table 5. TPC, FRAP, DPPH, and ORAC Values of Combinatorial Fruits and Legumes

food combinations		TPC (mg GAE/g)		FRAP (μmol/g)		DPPH EC $_{50}$ (mg/mg DPPH)		ORAC (μmol TE/g)	
RS + SB	O	4.66 ± 0.07	Ad	6.81 ± 0.59*	An	164.59 ± 5.99*	An	58.73 ± 1.19*	Sy
	E	4.60 ± 0.13		7.06 ± 0.23		145.88 ± 2.18		53.32 ± 1.94	
RS + AB	O	8.14 ± 0.27*	Sy	11.10 ± 0.23*	Sy	103.23 ± 10.39*	Sy	64.88 ± 0.97*	Sy
	E	5.67 ± 0.22		9.29 ± 0.31		135.70 ± 5.31		59.98 ± 1.80	
RS + KB	O	5.90 ± 0.36	Ad	7.08 ± 0.43*	An	137.17 ± 12.69	Ad	56.16 ± 0.70	Ad
	E	5.58 ± 0.18		7.86 ± 0.25		143.23 ± 2.20		57.11 ± 1.57	
RS + BB	O	5.40 ± 0.16	Ad	6.59 ± 0.47*	An	147.60 ± 2.88	Ad	61.42 ± 1.39*	Sy
	E	5.30 ± 0.13		8.27 ± 0.25		144.65 ± 2.41		53.82 ± 0.25	
AP + SB	O	3.75 ± 0.07*	Sy	2.20 ± 0.54	Ad	155.67 ± 4.39*	An	38.40 ± 0.94	Ad
	E	3.43 ± 0.09		2.62 ± 0.13		139.28 ± 3.74		37.74 ± 1.99	
AP + AB	O	4.86 ± 0.05*	Sy	3.94 ± 0.18*	An	133.30 ± 3.58	Ad	48.51 ± 1.26*	Sy
	E	4.51 ± 0.05		4.84 ± 0.20		129.11 ± 6.89		44.40 ± 0.81	
AP + KB	O	4.33 ± 0.11	Ad	2.75 ± 0.45*	An	169.75 ± 8.80	Ad	34.57 ± 0.97*	An
	E	4.41 ± 0.04		3.42 ± 0.14		166.15 ± 2.25		41.53 ± 0.79	
AP + BB	O	4.15 ± 0.07	Ad	4.11 ± 0.11*	Sy	139.92 ± 10.12	Ad	38.34 ± 0.76*	Sy
	E	4.13 ± 0.14		3.82 ± 0.14		138.05 ± 4.07		35.34 ± 0.35	
BL + SB	O	7.32 ± 0.05*	Sy	7.77 ± 0.18	Ad	107.73 ± 4.19	Ad	54.82 ± 0.27	Ad
	E	6.83 ± 0.07		7.80 ± 0.04		106.31 ± 7.02		54.60 ± 1.22	
BL + AB	O	8.38 ± 0.07*	Sy	10.10 ± 0.20	Ad	105.24 ± 9.25	Ad	61.94 ± 1.67	Ad
	E	7.89 ± 0.16		10.02 ± 0.11		96.13 ± 10.13		61.25 ± 0.88	
BL + KB	O	8.16 ± 0.11*	Sy	8.45 ± 0.18	Ad	127.71 ± 5.76*	An	58.25 ± 0.90	Ad
	E	7.81 ± 0.13		8.59 ± 0.05		103.66 ± 7.16		58.39 ± 0.45	
BL + BB	O	7.43 ± 0.11	Ad	8.71 ± 0.31	Ad	116.99 ± 3.67*	An	55.45 ± 0.49	Ad
	E	7.53 ± 0.07		9.00 ± 0.05		103.66 ± 4.64		55.19 ± 0.92	

Table 6. TPC, FRAP, DPPH, and ORAC Values of Combinatorial Vegetables and Legumes

food combinations		TPC (mg GAE/g)		FRAP (μmol/g)		DPPH EC $_{50}$(mg/mg DPPH)		ORAC (μmol TE/g)	
BR + SO	O	1.85 ± 0.13*	An	2.19 ± 0.05*	An	189.76 ± 5.76*	An	29.56 ± 0.81	Ad
	E	2.86 ± 0.05		3.05 ± 0.20		167.39 ± 3.15		30.29 ± 1.06	
BR + AB	O	4.04 ± 0.14	Ad	4.57 ± 0.19*	An	162.87 ± 10.12	Ad	39.73 ± 0.83*	Sy
	E	4.15 ± 0.18		5.27 ± 0.22		157.20 ± 5.45		34.94 ± 0.67	
BR + RD	O	4.77 ± 0.09*	Sy	3.73 ± 0.11	Ad	171.61 ± 10.08	Ad	31.41 ± 0.70*	An
	E	4.12 ± 0.20		3.84 ± 0.18		164.73 ± 2.52		36.11 ± 1.60	
BR + BB	O	4.26 ± 0.14*	Sy	4.02 ± 0.16	Ad	198.14 ± 8.87*	An	31.77 ± 1.37	Ad
	E	3.77 ± 0.02		4.25 ± 0.20		166.47 ± 2.25		30.88 ± 1.13	
TO + SO	O	2.33 ± 0.05	Ad	1.61 ± 0.11*	An	170.17 ± 2.00*	Sy	28.32 ± 0.11	Ad
	E	2.27 ± 0.04		2.15 ± 0.14		179.00 ± 1.85		27.55 ± 1.03	
TO + AB	O	3.26 ± 0.13	Ad	4.45 ± 0.10	Ad	173.11 ± 6.10	Ad	37.03 ± 1.58*	Sy
	E	3.33 ± 0.11		4.37 ± 0.20		168.82 ± 4.88		34.19 ± 0.68	
TO + KB	O	3.17 ± 0.05	Ad	2.80 ± 0.19	Ad	194.78 ± 4.66*	An	31.81 ± 0.56	Ad
	E	3.25 ± 0.09		2.95 ± 0.14		176.34 ± 1.66		31.33 ± 0.47	
TO + BB	O	2.94 ± 0.05	Ad	3.45 ± 0.07	Ad	193.28 ± 3.71*	An	27.17 ± 0.58	Ad
	E	2.97 ± 0.07		3.35 ± 0.16		177.76 ± 1.78		28.14 ± 0.95	
MU + SB	O	3.62 ± 0.13*	An	2.22 ± 0.18	Ad	201.29 ± 7.61	Ad	24.74 ± 0.41*	An
	E	3.98 ± 0.09		2.26 ± 0.14		194.62 ± 4.05		28.68 ± 1.08	
MU + AB	O	5.69 ± 0.29*	Sy	3.66 ± 0.18*	An	191.08 ± 7.18	Ad	34.35 ± 1.87	Ad
	E	5.04 ± 0.20		4.47 ± 0.19		184.44 ± 2.65		35.33 ± 0.79	
MU + KB	O	5.12 ± 0.18	Ad	2.29 ± 0.20*	An	197.49 ± 8.77	Ad	31.73 ± 1.21	Ad
	E	4.96 ± 0.16		3.05 ± 0.13		191.97 ± 4.59		32.46 ± 0.58	
MU + BB	O	4.54 ± 0.14	Ad	3.22 ± 0.24	Ad	210.72 ± 4.39*	An	28.15 ± 0.43	Ad
	E	4.67 ± 0.07		3.45 ± 0.14		193.39 ± 4.88		29.28 ± 0.85	
PC + SO	O	4.03 ± 0.06*	An	2.98 ± 0.20	Ad	171.68 ± 6.70	Ad	32.87 ± 0.49*	Sy
	E	4.28 ± 0.05		3.18 ± 0.18		164.81 ± 5.72		30.65 ± 0.69	
PC + AB	O	5.39 ± 0.13	Ad	4.41 ± 0.31*	An	158.85 ± 7.25	Ad	36.69 ± 0.25	Ad
	E	5.35 ± 0.18		5.70 ± 0.14		154.62 ± 3.96		37.30 ± 0.90	
PC + KD	O	5.48 ± 0.22	Ad	4.01 ± 0.15	Ad	182.04 ± 5.76*	An	33.68 ± 0.41	Ad
	E	5.27 ± 0.13		4.18 ± 0.13		162.15 ± 6.25		34.43 ± 0.83	
PC + BB	O	5.38 ± 0.11*	Sy	4.30 ± 0.14*	An	171.75 ± 6.50	Ad	33.87 ± 0.54*	Sy
	E	4.98 ± 0.02		4.68 ± 0.13		163.57 ± 6.53		31.24 ± 0.68	

The specific combinations of fruit and legume were more likely to demonstrate synergistic antioxidant capacity than other food category combinations (**Figure 4**).

It should be noted that while the majority of the combinations had an additive effect, the number of antagonisms was approximately equal to the number of synergisms when combining food within or across food categories (**Figure 3**). These antagonistic effects may have negative health implications for individuals attempting to increase antioxidant intake through consuming food mixtures. Pharmacologic researchers have paid close attention to the antagonistic interactions between nutrients and drugs (*40*). However, food−food interactions have not received

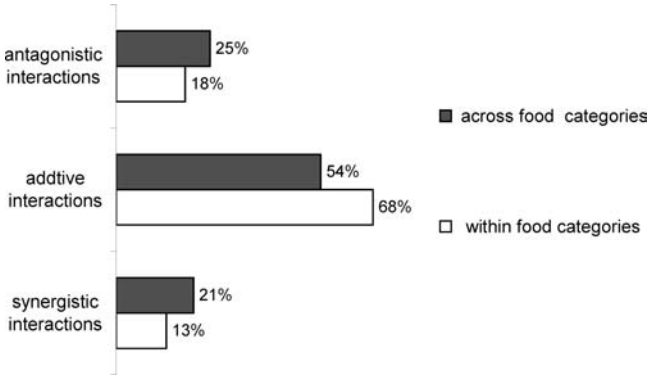

Figure 3. Different interactions (% in all three interactions) observed when combining foods within and across categories.

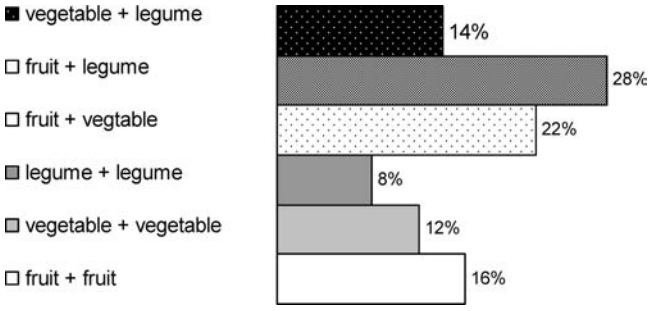

Figure 4. Percent synergistic interactions by food combination patterns.

particular interests from researchers in food and nutrition. Both synergistic and antagonistic interactions between foods are important, particularly for functional foods that contain elevated levels of bioactive components.

Exploration of Possible Mechanisms Behind Synergism. Only one food combination, raspberry and adzuki bean, demonstrated a synergistic response in all four assays (**Figure 5**). This combination was considered the strongest candidate for potential in vivo antioxidant synergism as it most likely contains components that act through more than one antioxidant mechanism. Such observations have not been reported elsewhere. Most of the reported data showed synergism using one assay method or only one food category, that is, fruit−fruit mixtures (*20*).

While composition and identification of the foods are not an objective of this study, to explore the possible mechanisms behind this synergism, the phytochemical compositions of the crude methanolic extracts of raspberries and adzuki beans were investigated separately and in combination by HPLC. Multiple wavelengths (280, 320, 360, and 520 nm) were used to simultaneously monitor the polyphenolic content as we reported previously (*27*). This method provides a fuller picture of the phytochemical composition; therefore, any compositional changes as shown in the chromatogram of a mixed extract may help explain, at least in part, the mechanism responsible for the synergistic effect.

We paid particular attention to changes in the phenolic profiles of the individual and mixed extracts, because phenolics have been reported as major contributors to the total antioxidant capacity of plant-based foods (*41*, *42*). The positive correlation between the total phenolic content and the antioxidant capacity of foods has been previously reported (*11*). Apparent synergisms demonstrated by an increase in the value in the TPC assay may correlate with changes in the phytochemical profiles as measured by HPLC. Contrary to our expectations, no new peaks appeared in the chromatogram of raspberry and adzuki bean extract mixture as compared to the individual chromatograms; no existing peaks

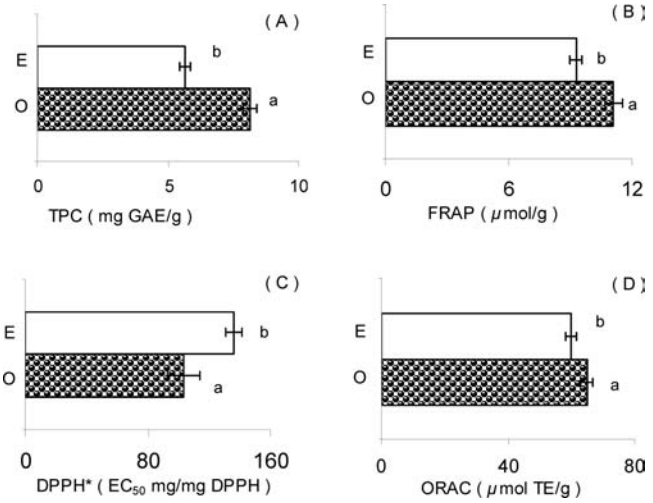

Figure 5. Synergistic antioxidant interactions exhibited between raspberries and adzuki beans in four antioxidant assays. In each assay, means followed by different letters indicate significant difference by ANOVA ($p < 0.05$). *For DDPH assay, lower values indicate higher antioxidant capacities: (**A**) antioxidant capacity by TPC, (**B**) antioxidant capacity by FRAP, (**C**) antioxidant capacity by DPPH, and (**D**) antioxidant capacity by ORAC. E, expected value: a mathematical sum of the antioxidant capacity derived from the individual foods in the mixtures at 2-fold dilution; and O, observed value.

disappeared either, at all wavelengths monitored. **Figure 6** shows the chromatographic profiles of polyphenols in individual and mixed raspberry and adzuki bean extracts at 320 nm, at which the largest number of peaks were observed. Our observations suggest that the synergistic response was likely a result of the combinatorial effect of the existing phytochemicals therein, rather than the formation of new compounds or disappearance of some known compounds.

However, the above-mentioned phenomena with the crude extracts are somewhat different from what was observed in combinations of pure compounds (*18*). In a study of 12 phenolic compounds commonly found in fruits and vegetables, individual and combinations of two or three phenolics were evaluated for their antioxidant capacities in the ABTS radical scavenging abililty assay system, and no synergistic antioxidant effect was found (*18*). Therefore, the synergistic antioxidant response in food combinations may not necessarily only arise from the phenolic compounds interacting with each other but possibly from interactions with other phytochemicals. Further studies on the mechanisms of the sygergistic interaction of raspberry and adzuki bean will need to be carried out, particularly on other phytochemical species.

In conclusion, the current study presents data from four chemical-based assays evaluating three possible interactions, synergistic, additive, and antagonistic, which occurred when fruit, vegetable, and legume extracts were combined. Combining foods across food categories was more likely to create an antioxidant synergism. A combination of a fruit (raspberries) and a legume (adzuki beans) is one good example of such synergism. These results could be useful for developing functional foods with enhanced antioxidant capacity and for individuals who wish to maximize dietary antioxidant intake from selected foods or diet combinations.

Our findings also showed that antioxidant interactions not only can result in positive effects but also could produce negative effects on the total antioxidant capacities of foods or diets. More studies need to be done to strategically select appropriate foods and food combinations for synergisms, as well as to avoid antagonisms. It is also understood that ultimately both the synergistic and the

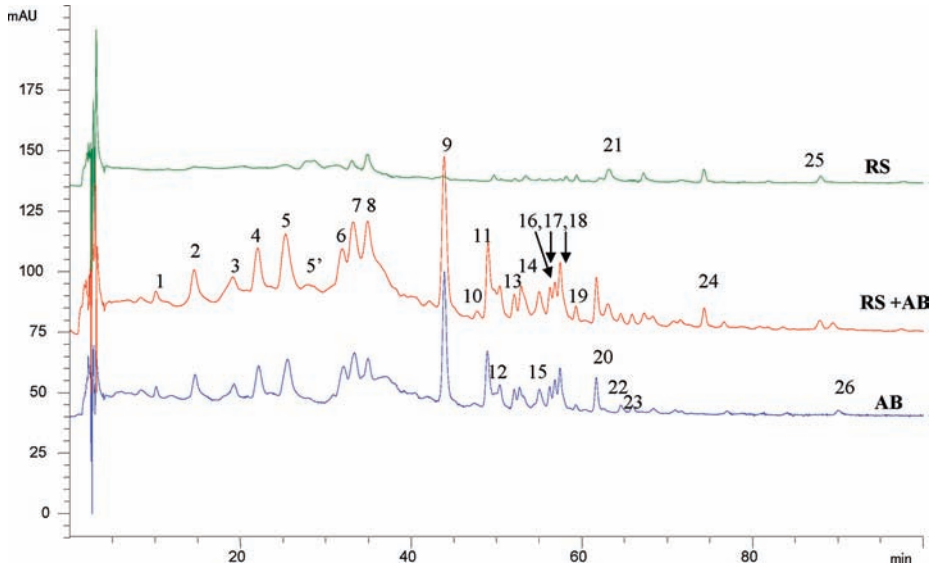

Figure 6. HPLC profiles of the phytochemical profiles in the methanol extracts of raspberries and adzuki beans and their mixture, as monitored at 320 nm. Column: Phenomenex Luna 5 μ (250 mm $\times$ 4.60 mm) with a C18 guard column. Mobile phase: a binary mobile phase of water/acetic acid (98:2, v/v) (solvent A) and water/acetonitrile/acetic acid (78:20:2, v/v/v) (solvent B) was used with the following gradient program: 0−55 min, 100−20% A; 55−70 min, 20−10% A; 70−80 min, 10−5% A; and 80−100 min, 100% B. The flow rate was 1.2 mL/min. RS, raspberries; RS + AB, mixture of raspberries and adzuki beans; and AB, adzuki beans. Peaks were tentatively identified by matching UV spectra with the standards and by comparing with those reported in ref *31*. Peaks: 1, unknown; 2, gallic acid; 3, unknown phenolic acid; 4, ferulic acid; 5, 6, and 8, cinnamic acid derivatives; 7, procyanidin dimer; 5′, peonidin-3-glucoside; 9, *p*-coumaric acid; 10, quercetin glycoside; 11−19 and 21−23, quercetin/kaempferol derivatives and cinnamic acid derivatives; 20, kaempferol glycoside; and 24−26, unknown.

antagonistic effects observed in the in vitro chemical models need be confirmed in biological systems. For these reasons, future studies will focus on the outcome of investigating effects using biological matrix such as cultured cells and animals and compare these in vivo activities with the outcome of the TPC, FRAP, DPPH, and ORAC assays. Nutrigenomic approaches examining specific molecular targets will also be done to help unlock the mechanisms of the different interactions.

ABBREVIATIONS USED

FCR, Folin−Ciocalteu reagent; FRAP, ferric reducing antioxidant power; ORAC, oxygen radical absorbance capacity; APPH, 2,2′-azobis (2-methyl-propanimidamide) dihydrochloride; DPPH, 2,2-diphenyl-1-picrylhydrazyl; TPTZ, 2,4,6-tris(2-pyridyl)-s-triazine; trolox, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; EC_{50}, concentration of sample extract necessary to scavenger initial concentration of free radical DPPH 50%; ANOVA, one-way analysis of variance; HAT, hydrogen atom transfer; SET, single electron transfer; TE, trolox equivalent; GAE, gallic acid equivalent; AUC, area under the curve; DAD, diode array detector; *p*, probability value.

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