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Assays for Hydrophilic and Lipophilic Antioxidant Capacity (oxygen radical absorbance capacity ($ORAC_{FL}$)) of Plasma and Other Biological and Food Samples

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Methods are described for the extraction and analysis of hydrophilic and lipophilic antioxidants, using modifications of the oxygen radical absorbing capacity ($ORAC_{FL}$) procedure. These methods provide, for the first time, the ability to obtain a measure of "total antioxidant capacity" in the protein free plasma, using the same peroxyl radical generator for both lipophilic and hydrophilic antioxidants. Separation of the lipophilic and hydrophilic antioxidant fractions from plasma was accomplished by extracting with hexane after adding water and ethanol to the plasma (hexane/plasma/ethanol/water, 4:1:2:1, v/v). Lipophilic and hydrophilic antioxidants were efficiently partitioned between hexane and aqueous solvents. Conditions for controlling temperature effects and decreasing assay variability using fluorescein as the fluorescent probe were validated in different laboratories. Incubation (37 °C for at least 30 min) of the buffer to which AAPH was dissolved was critical in decreasing assay variability. Lipophilic antioxidants represented 33.1 ± 1.5 and 38.2 ± 1.9% of the total antioxidant capacity of the protein free plasma in two independent studies of 6 and 10 subjects, respectively. Methods are described for application of the assay techniques to other types of biological and food samples.

KEYWORDS: $ORAC_{FL}$; phenolics; antioxidant; free radical; lipophilic; hydrophilic; blueberry; fruit juices

INTRODUCTION

There is increasing interest in the use and measurement of antioxidant capacity in the food and pharmaceutical industries and in clinical studies. This interest is derived from the overwhelming evidence of the importance of reactive oxygen species (ROS) in the aging process and the pathogenesis of many diseases in which ROS are involved. Several methods for measuring antioxidant capacity in vitro have been developed and reviewed (*1*). However, the oxygen radical absorbance capacity (ORAC) method, with some modifications that have been made over time, is becoming a widely used method for assessing antioxidant capacity in biological samples and foods. A limitation of this method has been the inability to determine both hydrophilic and lipophilic antioxidants. The ORAC method is based on the inhibition of the peroxyl-radical-induced oxidation initiated by thermal decomposition of azo-compounds, like 2,2′-azobis(2-amidino-propane) dihydrochloride (AAPH). This peroxyl-radical-induced oxidative reaction was first studied by Glazer et al. (*2*) and Ghiselli et al. (*3*), and the basic principles have subsequently been utilized as a basis for the current methodology (*4*−*8*). Thus, the ORAC assay utilizes a biological relevant radical source and is the only method that combines both inhibition time and degree of inhibition into a single quantity. The ORAC method has recently been adapted to use fluorescein as the fluorescent probe (*9*) and has been adapted to a high-throughput assay format (*6*).

In clinical studies where analysis of antioxidant status is important, the current ORAC procedures have been used to evaluate the hydrophilic antioxidants, but a good method for assay of lipophilic antioxidants in plasma or other biological samples has not been available. In early studies (*4*), acetone extraction was used for the lipophilic antioxidants, but the results were variable, and other solvent systems are preferred for

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extraction of the lipophilic components. However, organic solvents are not compatible with the aqueous ORAC assay system. Naguib (*10*) developed an organic solvent system for assay of lipophilic antioxidants using BODIPY 581/591 or BODIPY 665/676 as the fluorescent probe and 2,2′-azobis-2,4-dimethylvaleronitrile (AMVN) as the peroxyl radical source. Aldini et al. (*11*) modified Naguib's method to measure the lipid compartment of plasma. However, the BODIPY dye may be subject to photobleaching (*12*) after exposure to excitation light, which may limit its use in a quantitative assay. The method to be described for lipophilic antioxidants is based upon that of Huang et al. (*12*), using randomly methylated β-cyclodextrin (RMCD) as a solubility enhancer.

The objective of this study was to (1) develop extraction conditions to determine both lipophilic and hydrophilic antioxidant capacity of plasma and other biological and food samples, (2) demonstrate its application in experimental samples, and (3) to test methods in multiple labs.

MATERIALS AND METHODS

Chemicals and Apparatus. Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and ascorbic acid were obtained from Aldrich (Milwaukee, WI). The 2,2′-azobis(2-amidino-propane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA). Randomly Methylated β-Cyclodextrin (RMCD) (Trappsol) (pharmacy grade) was obtained from Cyclodextrin Technologies Development Inc. (High Springs, FL 32643). Fluorescein (FL) (Na salt) was obtained from Aldrich (Milwaukee, WI). We have used two different models of fluorescent microplate readers (FLUOstar Galaxy, and FLUOstar Optima, BMG Labtechnologies, Duraham, NC). Fluorescence filters with an excitation wavelength of 485 nm and an emission wavelength of 520 nm were used in both instruments. The 96 well FLUOTRAC 200 black microplates (part # 655076) were purchased from Greiner America, Inc (Lake Mary, FL), and 48 well microplates (Falcon No. 3230) were purchased from VWR (St. Louis, MO). Clear polystyrene 96-well plates (Nunc) were purchased from Fisher Scientific, Atlanta, GA.

Microplate Assay. A stock fluorescein solution (Stock #1) was prepared by dissolving 0.0225 g of FL in 50 mL of 0.075 M phosphate buffer (pH 7.0). A second stock solution was prepared by diluting 50 μL of stock solution #1 in 10 mL of phosphate buffer. A 320-μL portion of solution #2 was added to 20 mL of phosphate buffer, of which 400 μL was added to each well. This provides 7.5 nmoles of fluorescein per well, or a final concentration of 14 μM.

A stock standard of Trolox (500 μM) was aliquoted into small vials for storage at −70 °C until use. In the standard assay, 40 μL Trolox calibration solutions (6.25, 12.5, 25, 50 μM) in phosphate buffer (0.075 M, pH 7.0) were pipetted into appropriate wells. A new set of stock Trolox vials were removed from the freezer daily for use. Early studies were completed using a 96-well black plate in which excitation/emission was from the top of the plate. We used no more than 40−50 wells of the microplate, due to the cycle time required for pipetting and reading. We found that reproducibility was improved by reading from the bottom and using a 48-well plate in which the reagent volumes were doubled. However, good results have been obtained using a multichannel liquid handling system coupled with a fluorescence microplate reader in 96-well format (*6*). Methods for the 96-well format were similar, except that the total volume was reduced by 50%.

The FLUOstar plate reader is equipped with an incubator and two injection pumps; the temperature of the incubator was set to 37 °C. The rate of peroxyl radical production from AAPH is temperature sensitive, so timing and handling of the AAPH solution is critical. Thus, a new AAPH solution was prepared for each run. Old FL and AAPH solutions were flushed from the syringes, and the syringes were primed with new FL and AAPH before starting the run. In addition, to optimize the signal amplification to give maximum sensitivity, a gain adjustment was always performed by manually pipetting 400 μL of FL into a designated well before starting the program. In cycle 2, the instrument read the fluorescence in each of the wells and pipetted 400 μL of FL from pump #1 into the respective wells, to give a final FL concentration of 14 μM, followed by reading the fluorescence.

During cycle 4, pump 2 was programmed to inject 150 μL of AAPH (271.17 g/mole; 8.6 mg/mL; 31.7 mM) into the respective wells to give a final AAPH concentration of 4.8 mM (4.7 μmole/well). The plate contents were mixed with shaking for 8 s following each injection and/or reading. In initial studies, we compared the effects of different amounts of AAPH on the Trolox standard curves by using 40 μL (12.8 μmol) or 10 μL (3.2 μmol) of the AAPH solution in the assay.

Plasma Extraction. Plasma or serum samples that had been stored at −70 °C were thawed slowly, mixed well on a vortex, and centrifuged if needed. Plasma or serum extraction was based upon the procedure published by Aebischer et al. (*13*). One hundred microliters of plasma or serum was transferred to a glass tube, 200 μL of ethanol and 100 μL of water was added and mixed, and then 400 μL of hexane was added, followed by mixing. The mixture was left to sit for 1−2 min or until two layers appeared, followed by centrifugation for 5 min at 14 000 rpm. The hexane layer was removed and added to a separate amber tube. An additional 400 μL of hexane was added to the original tube, mixed, left to settle for 2 min, and then centrifuged for 5 min at 14 000 rpm. The hexane layer was removed and combined with the first extract. The combined hexane extracts were dried down under nitrogen flow in preparation for lipophilic $ORAC_{FL}$ analysis. Any hexane remaining following hexane extraction of the aqueous plasma sample was removed by drying under nitrogen. Then 400 μL of 0.5 M perchloric acid was added to precipitate the protein. The sample was then centrifuged for 5 min at 14 000 rpm. From the supernatant, 160 μL was added to 840 μL of phosphate buffer and mixed.

Extraction of Food Samples. A freeze-dried food sample (1 g) was extracted in a 15-mL screw-cap tube with 2 × 10 mL of hexane, followed by centrifugation and removal of the hexane layer. The hexane fractions were combined. Residual hexane was evaporated, and then the residue was extracted with 10 mL of acetone/water/acetic acid, (70:29.5:0.5, v/v/v). After adding solvent, the tube was vortexed for 30 s, followed by sonication at 37 °C for 5 min. The tube was inverted once in the middle of the sonication step to suspend the samples. Then, the tube remained at room temperature for 10 min with occasional shaking. The tube was centrifuged at 3500 rpm for 15 min. The supernatant was removed and transferred to a volumetric flask and diluted to 25 mL total volume. The combined hexane fraction was dried under nitrogen flow.

Lipophilic $ORAC_{FL}$ Assay. For the lipophilic antioxidant assay, the dried hexane extract was dissolved in 250 μL of acetone and then diluted with 750 μL of a 7% RMCD solution (50% acetone/50% water, v/v). Any further dilution was with the 7% RMCD solution. The 7% RMCD solution was used as a blank and to dissolve the Trolox standards for the lipophilic assay. For plasma lipophilic analysis, 40 μL of this solution was added to the 48 well microplate. Four hundred μL of fluorescein solution was added by injectors in the microplate reader, followed by 150 μL of AAPH (17.2 mg/mL, 9.4 μmol/well); readings were initiated immediately.

Hydrophilic $ORAC_{FL}$ Assay. Any further dilution of the hydrophilic fraction (acetone/water/acetic acid extract) was made with phosphate buffer. A 40-μL portion of the diluted sample was added to a well in a 48 well microplate. The fluorescein solution and AAPH were added in the same manner as that for the lipophilic assay, except that only 75 μL of the AAPH was added to the assay mixture.

Human Clinical Protocol. *Study 1.* Six female subjects (ages, 60−71 yrs) were given 189 g of lowbush blueberries, and blood samples were obtained before and at 1, 2, and 4 h after consumption in heparinized tubes. Following centrifugation, the plasma was stored at −70 °C until analysis.

Study 2. Fasting blood samples were obtained from 10 healthy nonsmoking adult females (age 22−40 yrs). Appropriate approvals were obtained from the Institutional Review Boards and informed consent was obtained from participating subjects. Changes in plasma antioxidant capacity were calculated by subtracting the baseline before the meal from each time point following the meal.

Calculations. The final $ORAC_{FL}$ values were calculated by using a regression equation ($Y = a + bX$, linear; or $Y = a + bX + cX^2$,

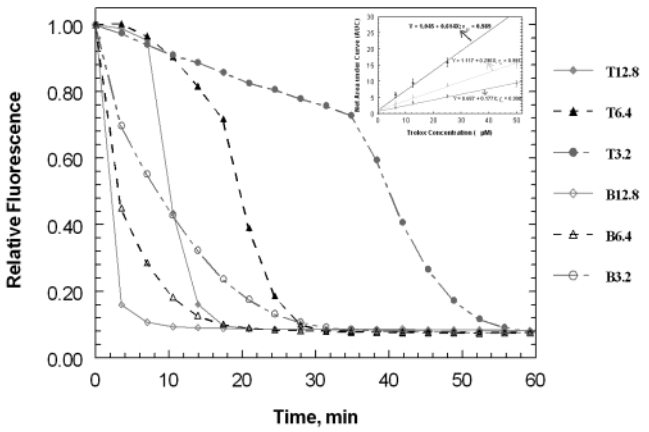

Figure 1. Effect of amount of AAPH concentration (3.2, 6.4, and 12.8 μmol per assay) on relative fluorescence versus time of 25 μM Trolox (T) and blanks (B). The insert presents the linear plot of the net area under the curve (AUC) versus Trolox concentrations for the three levels of AAPH (3.2, 6.4, and 12.8 μmol per assay). Data in the inset is pooled from multiple runs on 3–6 separate days.

quadratic) between Trolox concentration (Y) (μM) and the net area under the FL decay curve (X). Linear regression was used in the range of 6.25−50 μM Trolox. Data are expressed as micromoles of Trolox equivalents (TE) per liter or per gram of sample (μmol TE/g or μmol TE/L). The area under curve (AUC) was calculated as

$$\text{AUC} = (0.5 + f_5/f_4 + f_6/f_4 + f_7/f_4 + ... + f_i/f_4) \times \text{CT} \quad (1)$$

where f_4 = initial fluorescence reading at cycle 4, f_i = fluorescence reading at cycle i, and CT = cycle time in minutes.

The net AUC was obtained by subtracting the AUC of the blank from that of a sample.

The data were analyzed by Microsoft Excel (Microsoft, Roselle, IL), to apply eq 1, to calculate the AUC.

RESULTS

48- Versus 96-Well Microplate Comparison. Two fruit juice samples were analyzed on the same instrument to directly compare 96- and 48-well microplates. The standard curve for the 96-well plates had a negative intercept (−6.48), whereas the intercept from the results from the 48-well plate was closer to the origin. We have consistently observed this negative intercept with the 96-well plate, but do not have an explanation for it. However, if the appropriate standard curve is used to calculate results, the means are similar using either a 96- or 48-well plate format. However, the coefficient of variation was higher in the 96-well plate (13.4%) versus the 48-well plate (6.3%). Also, the linear range of the assay seemed to be lower in the 96-well plates.

Effect of AAPH Concentration. Figure 1 presents the FL decay curves for the blank and 25 μM Trolox, using three different concentrations of AAPH (3.2 (10 μL), 6.4 (20 μL), and 12.8 (40 μL) μmol per assay). Increasing the amount of AAPH in the assay decreased the net area under the curve for the blank as well as the Trolox used in this example. A linear regression curve of three or four Trolox concentrations versus net area under the curve was obtained (see insert to **Figure 1**). All three concentrations of AAPH gave a good fit to the linear regression equation. **Table 1** presents the coefficient of variation for multiple assays for Trolox standards, gallic acid (area and net area) and the blank. As seen in Table 1, the coefficient of variation (CV) did not differ greatly between the 12.8 μmol and 3.2 μmol AAPH per assay for the blank or gallic acid (50 μM) but tended to be slightly lower for Trolox with 3.2 μmol AAPH in each well. With the higher concentrations of Trolox, the CV tended to be smaller.

Table 1. Coefficient of Variation (CV) (%) for Trolox Standards, Gallic Acid and Blank in $ORAC_{FL}$ Assay Using 48-Well Plate[a] and Two AAPH Concentrations

	AAPH in assay (μmol/well)	
	12.8	3.2
	Trolox, μM	
6.25	30.0 (14)[b]	13.6 (20)
12.5	17.3 (16)	13.8 (16)
25.0	9.3 (19)	8.3 (18)
50.0	10.3 (20)	7.1 (19)
overall CV ($x \pm$ SEM)	16.7 ± 4.8	10.7 ± 1.7
	gallic acid, 50 μM	
area CV (n)	6.3 (20)	6.7 (20)
net area CV (n)	8.2 (20)	9.6 (20)
blank CV (n)	5.2 (32)	6.9 (35)

[a] APH buffer solution not preincubated prior to assay. Excitation from top and emission read from bottom of plate. AAPH added in a volume of 10 μL. [b] Number of replicates presented in parentheses.

Table 2. Effect of Amount of AAPH in Assay on $ORAC_{FL}$ of Commercial Fruit Juices[a]

	$ORAC_{FL}$ (μmol TE/mL)		$ORAC_{FL}$[b]
juice	3.2 μmol AAPH	12.8 μmol AAPH	(μmol TE/mL)
blueberry[c]	32.7 ± 0.7		32.7 ± 0.7
dried plum[j]	21.0 ± 1.1		21.0 ± 1.1
Concord grape[d]	20.0 ± 0.6	19.9 ± 1.1	20.0 ± 0.5
red grape[d]	19.6 ± 1.2	18.7 ± 0.9	19.1 ± 0.6
cranberry/Concord grape[e]	14.9 ± 0.0	15.7 ± 0.4	15.3 ± 0.3
orange[g]	11.7 ± 0.2	10.0 ± 0.0	10.8 ± 0.5
strawberry[f]	11.1 ± 0.6	10.3 ± 0.7	10.7 ± 0.4
berry[f]	9.6 ± 0.4	8.3 ± 0.5	9.0 ± 0.4
apple[h]	4.6 ± 0.3	4.0 ± 0.3	4.3 ± 0.2
apple[j]	5.2 ± 0.1	4.7 ± 0.4	5.0 ± 0.2
white grape[f]	3.7 ± 0.2	3.1 ± 0.4	3.4 ± 0.2
white grape[d]	13.2 ± 0.4	11.6 ± 0.2	12.4 ± 0.4

[a] Data expressed as means ± SEM of 4–6 observations per juice. All samples were purchased at a local commercial supplier, except for blueberry juice, which was provided by the producer. [b] Data are pooled across wells containing 3.2 and 12.8 μmole AAPH. [c] Jasper Wyman and Son, Cherryfield, ME. [d] Welch's, Concord, MA. Vitamin C was added. [e] Ocean Spray Cranberries, Lakeville-Middleboro, MA. [f] Juicy Juice, Nestle USA, Beverage Division, Inc., Glendale, CA. [g] Perricone Juices, Beaumont, CA. [h] Mott's Inc., Stamford, CT. Vitamin C added. [i] Great Value. [j] Sunsweet, Yuba City, CA.

When we compared the $ORAC_{FL}$ of some commercial juices, similar results were obtained with 3.2 μmol versus 12.8 μmol of AAPH in the assay (**Table 2**). $ORAC_{FL}$ tended to be slightly higher (10.4 ± 2.3%; n = 10; mean ± SEM) with the lowest amount of AAPH in the reaction mixture based upon the 10 juices with data for both AAPH concentrations. Although there is not a strong reason to use one amount of AAPH over another, we have chosen to routinely use 4.7 and 9.4 μmol of AAPH in our hydrophilic and lipophilic $ORAC_{FL}$ assays, respectively.

Antioxidant Capacity of Fruit Juices. Data presented in **Table 2**, indicate that the antioxidant capacity of different fruit juices differs by almost 10-fold. The dark colored juices, in general, have the higher $ORAC_{FL}$. However, some commercial producers may add vitamin C (see two white grape juice products, **Table 2**), which alters this generalization concerning color and antioxidant capacity. Anthocyanins make a major contribution to antioxidant capacity of the dark colored juices, except dried plum, in which case, chlorogenic isomers are major contributors (*14*).

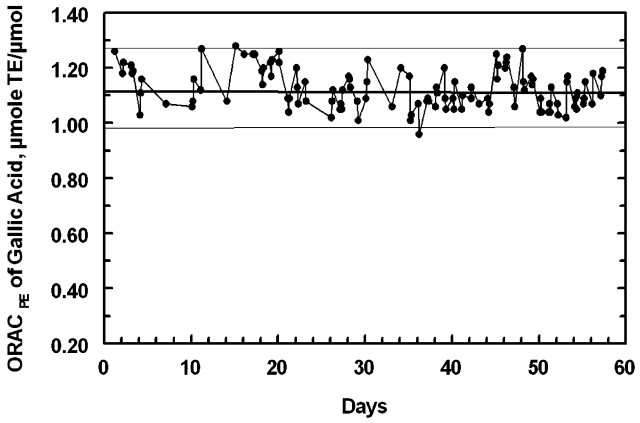

Figure 2. Ruggedness of hydrophilic $ORAC_{FL}$ method determined by using 50 μM gallic acid. The relative ORAC values were obtained over a period of 60 days with one to four separate analyses each working day. Mean ± SD = 1.12 ± 0.071; CV = 6.4%.

Precision and Factors Affecting Reproducibility. Figure 2 presents an illustration of the ruggedness of the assay using gallic acid (50 mM) as a quality control material. The same sample was analyzed over a 60 day period with 1 to 4 analyses on any given day. The mean ± SD for the gallic acid $ORAC_{FL}$ was 1.12 ± 0.071, and the CV was 6.4%.

Table 3 presents results of testing the repeatability of the blank $ORAC_{FL}$ net area, using different plate readers and different reading conditions and plates. These studies were initiated because of measurement inconsistencies we were observing, particularly after we purchased a second BMG microplate reader (OPTIMA). The results indicate that, as we observed earlier, a lower CV is obtained using the 48-well format compared to the 96-well format (See line 7 and lines 16−20, **Table 3**). The results included in **Table 3** for the OPTIMA excluded the values for the first two wells, as these were consistently higher and the CV ranged from 7.7 to 15.5 if these initial points were included in the calculations. Having the instrument set such that excitation and emission reading was from the bottom of the plate gave consistently lower CVs compared to bottom/top or top/top settings for excitation/emission, respectively. Near the end of some of these studies, we changed the procedure for preparing the AAPH solution and observed a large improvement in the reproducibility (**see lines 4−6 and 12−15**, **Table 3**). For this preparation, the buffer was incubated at 37 °C for at least 30 min, and then the AAPH was added to the warm buffer and the reaction started immediately. The CV was reduced to about 50% of other readings taken with 48-well plates and with excitation/emission set for the bottom of the plate (**compare lines 12−15 with 16−20**, **Table 3**). Similar effects were observed with both 48- and 96-well plates. A large decrease was also observed in the range of the observations, with some of the outliers observed with previous AAPH preparations compared to the latter being eliminated.

Table 3. Reproducibility of Blank Net Area in $ORAC_{FL}$ Assay Using Different Plate Readers and Plate Formats

microplate	reading mode	mean	sd	coeff of var (CV) %	range	Δ range/ mean
1. plate 1–96 well	XBB	4.807	0.534	11.1	4.04–7.93	80.9
2. plate 2–96-well	XBB	4.571	0.262	5.7	3.84–5.41	34.4
3. plate 3–96-well	XBB	4.545	0.395	8.7	3.98–5.92	42.7
mean CV				**8.5 ± 1.6**		
4. plate 1–96 well[c]	XBB	3.578	0.158	4.4	3.36–4.41	29.2
5. plate 2–96 well[c]	XBB	4.252	0.149	3.5	3.89–4.93	24.4
6. plate 3–96 well[c]	XBB	4.299	0.147	3.4	3.97–4.92	22.0
mean CV[c]				**3.78 ± 0.55**		
7. plate 1–96 well[b]	OBB	12.24	0.865	7.1	9.92–14.48	37.3
8. plate 1–96 well[d]	OTT	11.317	1.262	11.2	8.31–16.92	76.1
9. plate 2–96 well[d]	OTT	11.687	1.474	12.6	8.29–17.03	74.8
10. plate 1–96 well[f]	GTT	6.731	0.456	6.8	5.28- 8.22	43.7
11. plate 2–96 well	GTT	16.218	1.45	8.9	11.57–20.10	52.6
mean CV				**9.8 ± 1.6**		
12. plate 1–48 well[c]	GBB	14.478	0.299	2.1	14.02–15.30	8.8
13. plate 2–48 well[c]	GBB	14.017	0.408	2.9	13.51–15.24	12.3
14. plate 1–48 well[cd]	OBB	13.432	0.343	2.6	12.76–14.25	11.0
15. plate 2–48 well[cd]	OBB	13.320	0.399	3.0	12.43–14.21	13.3
mean CV				**2.6 ± 0.6**		
16. plate 1–48 well[e]	GBB	14.072	0.620	4.4	13.10–15.99	21.5
17. plate 2–48 well[e]	GBB	13.251	0.500	3.8	12.57–14.68	15.9
18. plate 1–48 well[d]	OBB	12.885	1.019	7.9	11.19–14.37	22.9
19. plate 2–48 well[d]	OBB	11.936	0.368	3.1	10.56–12.76	18.4
20. plate 3–48 well[d]	OBB	11.952	0.451	3.8	10.97–13.02	17.2
mean CV				**4.2 ± 1.4**		
21. plate 1–48 well[e]	GBT	19.677	1.017	5.2	17.60–21.40	19.3
22. plate 2–48 well[e]	GBT	19.515	0.897	4.6	17.49–21.20	19.0
23. plate 1–48 well[d]	OBT	10.654	0.639	6.0	9.64–12.08	22.9
24. plate 2–48 well[d]	OBT	10.246	0.516	5.0	9.35–11.35	19.5
mean CV				**5.2 ± 0.8**		
25. plate 1–48 well[e]	GTT	21.862	0.938	4.3	20.05–24.26	19.3
26. plate 1–48 well[e]	GTT	22.501	1.524	6.8	18.95–26.57	33.9
27. plate 1–48 well[d]	OTT	10.476	0.719	6.9	8.41–11.91	33.4
28. plate 1–48 well[d]	OTT	11.538	0.767	6.7	10.05–13.02	25.7
mean CV				**6.1 ± 1.1**		

[a] Instrument (X = high throughput assay conditions of Huang et al. (6); G = BMG Galaxy; O = BMG Optima) and method of reading (excitation, T = top, B = bottom; emission, T = top, B = bottom of plate). AAPH added per well was 4.7 μmole per well unless noted otherwise. AAPH buffer was not preincubated at 37 °C unless specifically noted. [b] Quartz 96-well microplate with black sides. [c] Buffer heated to 37 °C for 30 min, following which the solid AAPH was added and dissolved and assay started immediately. [d] Calculations based upon exclusion of the first two wells as they were consistently high and outliers. If the first two wells were included in the calculations, the CV ranged from 7.7 to 15.5. [e] AAPH, 20 μL; 2.35 μmole per well. [f] AAPH, 10 μL; 9.4 μmole per well.

Table 4. Hydrophilic and Lipophilic Antioxidant Capacity (AC) of Protein-Free Plasma from Fasting Human Subjects in Two Separate Studies[a]

antioxidant capacity (AC) (μmol Trolox equiv/L)	study 1[b]	study 2[c]
no. of subjects	6	10
total AC (*T*)	1807 ± 85.5	1909 ± 102
hydrophilic AC	1210 ± 68.6	1378 ± 96.0
lipophilic AC (*L*)	597 ± 34.8	531 ± 36.7
L/*T*, %	33.1 ± 1.5	28.2 ± 1.9

[a] Data presented as Means ± SEM. [b] Fasting blood samples were obtained from six healthy nonsmoking females (age 60–71 yrs). [c] Fasting blood samples were obtained from 10 healthy nonsmoking adult females (age 22–40 yrs).

Plasma Analysis. *Protein Precipitation Methods and Hydrophilic Antioxidant Extraction.* We have evaluated several methods for the precipitation of plasma proteins. A 1:1 ratio of plasma to either 0.5 or 1.0 M perchloric acid (PCA) gave a similar value for $ORAC_{FL}$ in the supernatant (1424 vs 1414). If a 20% solution of trifluoracetic acid (TFA) was used instead of 0.5 M PCA, the TFA results were about 21% lower (949 ± 17 vs 1194 ± 11 μM; $n = 16$).

We also evaluated the different components in the extraction method of Aebischer et al. (*13*). Increased total volume of the extraction mix relative to the plasma volume increased the amount of hydrophilic antioxidants extracted from plasma. However, total volume had less of an effect if ethanol was included in the extraction mixture.

Hexane Effects. Extracting the lipophilic components with hexane before analysis of the hydrophilic antioxidant fraction did not significantly alter the antioxidant capacity of the hydrophilic fraction (1604 ± 77 vs 1592 ± 178). Thus, it appeared that there was no carry over of the lipophilic components into the aqueous compartment, and one could obtain the same hydrophilic $ORAC_{FL}$ value, whether the samples were first extracted with hexane or not.

Blueberry Consumption and Plasma Antioxidant Capacity. The baseline plasma hydrophilic and lipophilic $ORAC_{FL}$ was 1210 ± 67 and 597 ± 35 (Study 1) and 1378 ± 96.0 and 531 ± 36.7 μmol TE/L (Study 2), from two female subject groups (**Table 4**). The changes from time 0 in plasma $ORAC_{FL}$ (Study 1) are presented in **Figure 3**. Plasma hydrophilic $ORAC_{FL}$ increased significantly ($p < 0.05$; Paired t test) at 1 h after the meal and then declined to below the baseline by 4 h after the meal. Lipophilic $ORAC_{FL}$ increased significantly at 2 h ($p < 0.05$; Paired t test) and remained above baseline at 4 h after the meal. The time of maximal increase in lipophilic $ORAC_{FL}$ varied by individual, with two individuals out of the six total reaching the highest level at each of the times (1, 2, and 4 h) after the meal.

Hydrophilic and Lipophilic $ORAC_{FL}$ of Food Samples. **Table 5** presents data from samples with a wide range of both hydrophilic (8.4−1191 μmol TE/g) and lipophilic (3.0−16.0 μmol TE/g) $ORAC_{FL}$. Pine nuts represent a food with relatively low total $ORAC_{FL}$, but relatively high (26%) lipophilic-to-total ratio. Brown sorghum bran contained a high total $ORAC_{FL}$ and the highest lipophilic $ORAC_{FL}$, but the lipophilic-to-total ratio was much lower (1.3%). Lipophilic $ORAC_{FL}$ for strawberries was equivalent to pine nuts, but the hydrophilic $ORAC_{FL}$ was much higher, giving a quite low ratio (0.84%) of lipophilic relative to total $ORAC_{FL}$.

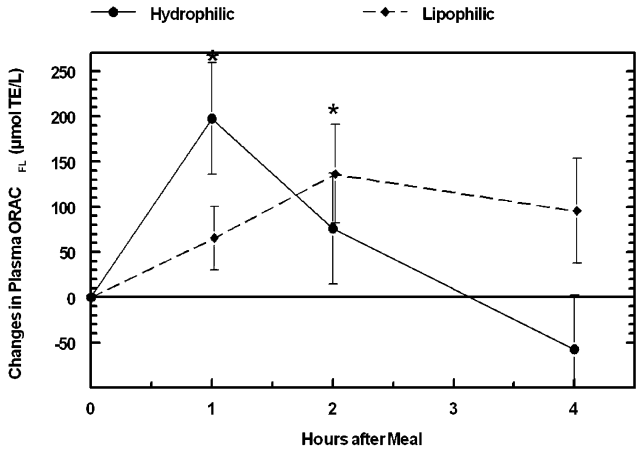

Figure 3. Changes in hydrophilic and lipophilic antioxidant capacity in plasma from six human subjects following consumption of 189 g of blueberries. Changes are expressed as μmol Trolox equiv/L. Significant ($p < 0.05$) increases observed in hydrophilic and lipophilic $ORAC_{FL}$ at 1 and 2 h, respectively.

Table 5. Hydrophilic and Lipophilic Antioxidant Capacity of Freeze-Dried Food Samples[a]

antioxidant capacity (AC) (μmol Trolox equiv/g DM)	pine nuts[b]	sorghum bran	strawberry[b]
number of samples	2	2	2
total AC (*T*)	11.41 ± 0.09	1206.5 ± 14.96	444.3 ± 3.01
hydrophilic AC	8.40 ± 0.06	1190.5 ± 15.34	440.6 ± 2.69
lipophilic AC (*L*)	3.01 ± 0.06	16.0 ± 0.38	3.74 ± 0.32
L/*T*, %	26.36 ± 0.33	1.33 ± 0.05	0.84 ± 0.07

[a] Data presented as means ± SD. [b] Freeze-dried samples.

DISCUSSION

This work extends previous results by Huang et al. (*12*), which provided validation for the assay of lipophilic antioxidants using RMCD. We now have developed conditions for extraction of plasma or serum and food samples, whereby both the lipophilic and hydrophilic $ORAC_{FL}$ can be determined on the same sample to assess "total antioxidant capacity" of the sample. In addition, we have tested and validated additional parameters related to the $ORAC_{FL}$ assay including effects of temperature, AAPH concentration and repeatability.

The effect of the amount of AAPH included in the assay was tested and found to have relatively small effects. Increased quantities of AAPH increased the reaction rate and thus decreased the run time and the corresponding area under the curve. From a practical point of view, this limits the upper amount of AAPH that can be included in the assay. Too little AAPH would extend the length of the reaction such that it would be impractical. However, within a reasonable range (3.2−12.8 μmol/well), there was no effect on the sample mean or on the coefficient of variation between samples (**Tables 2** and **3**).

We initiated a study of sources of variability in the $ORAC_{FL}$ when we were having difficulties obtaining similar results on two different fluorescence plate readers from BMG (Galaxy and Optima). In attempts to locate sources of assay variability, a number of different parameters were evaluated. Instrument reading error was evaluated by analyzing the initial point in all wells. The CV for the initial point was 1.0% and 1.4% for the Optima and Galaxy, respectively, with excitation and emission set from the bottom of a 48-well plate. The CV increased to 7−9% if excitation and emission was set to any other combination. When we evaluated the variability of the net area of the

blank assay, the CVs were 4.9 and 4.1% for the Optima and Galaxy, respectively. The Optima instrument consistently gave erroneously high net areas for wells in the first seven positions of the reading sequence, which was not observed with the Galaxy. When the buffer was preincubated at 37 °C and the AAPH was dissolved in the buffer immediately before starting the reaction, the CV decreased to an average of 2.6% on the two instruments. However, with the Optima instrument, we still observed some wells that deviated by more than 3 standard deviations from the mean. The volume of AAPH added to initiate the reaction also seemed to affect variability, but only in the Optima instrument. Addition of 80 μL (up to 150μL) of AAPH produced a very low CV in rows B thru F in the 48-well plate, but the first 5 wells in row A had a net area that was approximately double the mean. When the volume of AAPH was decreased to 20 μL, the variability in the entire plate increased slightly, but wells in row A had a net area close to the mean; however, there were a few wells elsewhere in the plate that were consistently deviant from the mean. Part of the increased variability may be accounted for by pipetting errors with the 20-μL volume compared to the 150-μL volume. Preincubating the AAPH buffer solution clearly had the largest effect on decreasing variability, but using a total volume of 150 μL of AAPH also was of benefit. Data in **Tables 1−3** were obtained before making these improvements, so the reported variability represents a "worst case" scenario. Although data in **Table 3** does not clearly demonstrate what we believe to be a central issue related to variability, plate temperature control is critical in this assay.

$ORAC_{FL}$ analysis can be performed on plasma- or serum-containing protein or with protein removed. The protein fraction contributes significantly to the antioxidant capacity, which may mask responses, particularly if the interest is in small molecular weight antioxidants. Therefore, removal of protein is important. We observed in the extraction process with ethanol that some protein was precipitated, but it was not complete, and that acid treatment was needed to completely remove the protein. Perchloric acid (0.5 N) was found to be the best of the options tested.

Hexane extraction of plasma before analysis of hydrophilic $ORAC_{FL}$ did not alter the hydrophilic $ORAC_{FL}$ value obtained. This indicated that we could add the two values together for the lipophilic and hydrophilic assays and arrive at a measure of total antioxidant capacity of the sample. Addition of ethanol and water such that the final extraction solution contained 1 volume plasma in 8 volumes total was determined to be important in order to get maximal extraction relative to a smaller total volume of extraction mix. A mixture of ethanol/plasma/H_2O/PCA of 2:1:1:4 (v/v/v/v) was found to be optimal based upon the combinations we tested.

The ORAC assay has been used to assess antioxidant capacity of fruits and vegetables, dietary supplements, nutraceuticals, juices, and wines, as well as plasma and urine samples from clinical trials (*8*, *15*−*18*). Increases in plasma antioxidant capacity have been observed following a meal containing strawberries, red wine phenolics, or spinach (*16*) and following consumption of an increased number of daily servings of fruits and vegetables (*17*). However, measurements of any changes on lipophilic antioxidant capacity were not performed in these earlier studies, due to appropriate methods not having been developed. Our results indicate that the lipophilic components of plasma account for 28−33% of the total antioxidant capacity; however, the lipophilic component increased to 37% of the total following the blueberry meal. Following consumption of a single meal of blueberries, a response in both hydrophilic and lipophilic antioxidant capacity was observed 1−2 h after the meal. The blueberries were consumed without any additional fat in the meal. One might expect a larger increase in the lipophilic component following a complete meal containing fat and additional lipophilic antioxidants, and the absorption pattern might also be delayed more compared to the hydrophilic absorption pattern.

Kurilich et al. (*19*) extracted the lipophilic components from broccoli, using hexane, and then dried the hexane extract and dissolved it with DMSO. Because DMSO acts as an antioxidant in the ORAC assay, DMSO was included in the blank. Because of the low lipid content in broccoli, solubility in the aqueous system was not a problem, but materials with much higher lipid content will present problems without the use of something like RMCD to solubilize the lipids. Data are presented in **Table 5** on three diverse food samples with quite different amounts of lipophilic and hydrophilic $ORAC_{FL}$. We have analyzed over 90 different foods and 500 individual samples, with no complication with very diverse types of foods.

In summary, an $ORAC_{FL}$ method for lipophilic antioxidants in plasma or serum was developed and validated using fluorescein as the fluorescent probe. This method has the advantage that similar assay conditions and standards are used for both the hydrophilic and lipophilic $ORAC_{FL}$, such that the two values can be added together to obtain a total antioxidant capacity for the sample. Data are presented demonstrating an increase in plasma hydrophilic and lipophilic $ORAC_{FL}$ following consumption of 189 g of blueberries in human subjects. Although there is no single assay that will give a complete reflection of in vivo antioxidant status, the $ORAC_{FL}$ assay has several advantages, in that now one can determine both lipophilic and hydrophilic antioxidant capacity in biological samples with a single, biologically relevant free radical source.

ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-amidino-propane) dihydrochloride; AUC, area under curve; ORAC, oxygen radical absorbance capacity; FL, fluorescein; PCA, perchloric acid; RMCD, randomly methylated β-cyclodextrin; TFA, trifluoroacetic acid; Trolox, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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