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Microplate-Based Oxygen Radical Absorbance Capacity (ORAC) Assay of Hydrophilic and Lipophilic Compartments in Plasma*

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Methods have been developed to evaluate the total antioxidant capacity of foods and plasma but limitations are associated with their ability to determine precisely the contribution of lipophilic antioxidants in a lipid milieu as well as interactions among them. Thus, we modified the Oxygen Radical Absorbance Capacity (ORAC) assay to determine the peroxyradical scavenging ability of both hydrophilic and lipophilic compartments in plasma. The hydrophilic ORAC assay was performed in a phosphate buffer system utilizing 2,2´-azobis (2-amidinopropane) dihydrochloride as a peroxyradical generator and fluorescein as the target. The lipophilic ORAC assay was carried out in a dimethylsulfoxide :butyronitrile (DMSO/BN, 9:1 v/v) system using 2,2´-azobis (2,4-dimethyl valeronitrile) as a peroxyradical generator and BODIPY C11 581/591 as the target. Analyses were conducted in bovine serum supplemented with water - and lipid - soluble antioxidants and in human plasma. Albumin (0.5~5 g/dL) and uric acid (0.1~0.5 mmol/L) increased hydrophilic ORAC values in a dose-dependent fashion (R^2=0.97 and 0.98, respectively) but had no impact on lipophilic ORAC values. α-Tocopherol (15~200 μmol/L) increased lipophilic ORAC values in a dose-dependent fashion (R^2=0.94); neither α-tocopherol nor β-carotene had an impact on hydrophilic ORAC values. However, addition of β-carotene at physiological concentrations (0.23~1.86 μmol/L), either alone or in combination with other carotenoids, had no significant impact on lipophilic ORAC values. Thus, while assays of "total antioxidant capacity" in biological matrices would be a useful research and clinical tool, existing methods are limited by the lack of complete responsiveness to the full range of dietary antioxidants.

Key words: Oxygen Radical Absorbance Capacity (ORAC), Lipophilic antioxidants, Hydrophilic antioxidants, Microplate reader

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INTRODUCTION

The Oxygen Radical Absorbance Capacity (ORAC) assay has been employed widely in testing foods and beverages to provide an integrated and quantitative determination of "total antioxidant capacity" (TAC). The ORAC value is obtained by measuring the area under the curve (AUC) of the magnitude and time of inhibition of free radical attack on a fluorescent target molecule in reference to Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carbonyl acid) as a unit standard.[1] However, the ORAC and most other TAC assays, including the Trolox Equivalent Antioxidant Capacity (TEAC),[2,3] Total Radical Trapping Antioxidant Parameter (TRAP),[4] primarily measure protection afforded by hydrophilic antioxidants against oxidation in an aqueous milieu. The Ferric Reducing Antioxidant Power (FRAP) assay, another popular measurement of TAC, determines reducing capability of antioxidants, also in an aqueous milieu.[5]

The ORAC assay measures the loss of fluorescence of B-phycoerythrin[6] or fluorescein[7] targets as an indication of oxidative attack by peroxyradicals generated by 2,2´-azobis (2-amidinopropane) dihydrochloride (AAPH) during incubation at 37 ℃. However, the ORAC, TEAC, FRAP, and related TAC assays failed to show a correlation with lipophilic antioxidant activity, particularly in a plasma matrix.[8,9] Consequently, attempts have been made to develop assays designed to specifically determine TAC in a lipophilic matrix by using a lipid soluble radical

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initiator, 2,2´ azobis (4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN), and target, BODIPY C11 581/591.[10-13] These assays appear to provide satisfactory values for purified antioxidants added *in vitro*[10,12] and suggest the possibility of a method applicable to the determination of endogenous TAC in the lipophilc compartment of plasma.[11,14] Aldini *et al.*[11,14] claimed that their plasma oxidation assay could determine total antioxidant capacity contributed by both hydrophilic and lipophilic antioxidants as well as the interaction between them in the context of recycling and regenerating. In addition, Prior *et al.*[13] were able to determine antioxidant capacity of lipophilic constituents in protein free human plasma using a modified ORAC assay with the addition of randomized methylated β-cyclodextrin as a solubility enhancer in an aqueous system. However, as yet, no assay has been developed which selectively determines the TAC in a lipophilic milieu absent confounding by the hydrophilic milieu and its constituents. Such an assay would prove helpful in assessing the specific contribution of lipophilic antioxidants to quenching radicals, particularly in biological matrices such as plasma. Thus, we adapted the ORAC microplate reader assay to measure hydrophilic TAC ($ORAC_{total}$ and $ORAC_{pca}$) and to determine whether a specific lipophilic TAC ($ORAC_{lipo}$) method could be developed for plasma.

MATERIALS AND METHODS

1. Chemicals and Apparatus.

Fluorescent probes, sodium salt flourescein (FL) and C11-BODIPY 581/591 (BODIPY) were purchased from Sigma (St. Louis, MO, USA) and Molecular Probes (Eugene, OR, USA), respectively. Azo initiators 2,2´-azobis (2-amidinopropane) dihydrochloride (AAPH) and 2,2´-azobis (2,4-dimethyl valeronitrile) (AMVN) were obtained from Wako Chemicals (Richmond, VA, USA). Trolox was obtained from Aldrich (Milwaukee, WI, USA). All other chemicals and purified antioxidants were obtained from Sigma (St. Louis, MO, USA). Fetal bovine serum was purchased from Hyclone (Logan, UT, USA). A FLUOstar OPTIMA microplate reader equipped with automated liquid delivery device (BMG Labtechnologies, Inc., Durham, NC, USA) was used to conduct the ORAC assays. Black flat bottom 96 well microplates, polystyrene for water soluble and polypropylene for lipid soluble ORAC were purchased from Greiner Bio-one Inc. (Longwood, FL, USA).

2. Sample Preparation

Bovine serum samples were spiked with serial concentrations of α-tocopherol, ascorbic acid, β-carotene, bovine albumin or uric acid and subsequently used to evaluate contributions of each spiked antioxidants to TAC in ORAC assays. Serial dilutions of α-tocopherol and β-carotene were prepared using ethyl alcohol or tetrahydrofuran, respectively, and those of uric acid, bovine albumin, and ascorbic acid were prepared using phosphate buffered saline. In experiments using bovine serum, antioxidants were added into samples such that the final volume of solvent was 10% v/v, with the exception of albumin that was conducted at 50% v/v.

3. Antioxidant Determinations

Plasma concentrations of several antioxidants were determined to see their correlation with ORAC values. Since fasting blood samples were not required to evaluate the relationship between antioxidants and ORAC values, heparinized blood samples were collected form 16 non-fast volunteers. Concentrations of ascorbic acid, carotenoids and tocopherols in plasma were assayed according to the methods described by Behrens & Madere,[15] Yeum *et al.*,[16] and Bieri *et al.*,[17] respectively. Concentrations of albumin and uric acid in the same samples were determined by colorimetric procedures using a COMAS MIRA instrument (Roche Diagnostics, Germany).

4. Hydrophilic ORAC Assay

Phosphate buffer (0.75 M, pH ~7.0) was prepared as previously described[1] and used throughout the hydrophilic ORAC assays. FL stock solution (10 μmol/L) was prepared with phosphate buffer and aliquots were stored in brown cryogenic tubes at -80 ℃. FL working solutions were always shielded from light and diluted immediately before use with buffer to a final concentration of 200 nmol/L. Aliquots of a Trolox standard stock solution (100 μmol/L) in buffer were stored at -80 ℃ and diluted to 5~50 μmol/L for calibrating standard curves. Plasma samples for ORAC assay ($ORAC_{total}$) were diluted directly with buffer. ORAC assays of protein-precipitated plasma ($ORAC_{pca}$) were first treated with 0.5 M perchloric acid (PCA) (1:1 v/v) prior to dilution with buffer. Duplicate blanks, standards, and samples (50 μL each) were placed in a polypropyrene, black 96-well plate using reciprocal dosing order across the rows. The external wells of the plate were not used as reproducibility was affected due to variations in temperature control within the plate reader chamber. One hundred μL of FL working solution was added to each well with a multi-pipette. AAPH in buffer (37 ℃) (0.32 mol/L) was delivered by an automated syringe controlled by the plate reader. The prepared plate was placed in preheated (37 ℃) microplate reader and incubated for 10

min with 10 sec orbital shaking every 2 min prior to the addition of 25 μL AAPH. Fluorescence intensity was detected at 485/520 (ex/em) every 2 min for 60 min. Values for $ORAC_{total}$ and $ORAC_{pca}$ were calculated according to a modification of the method described by Cao *et al.*[6] Net areas under the FL decay curve of each standard and sample were calculated by subtracting AUC_{blank} from AUC_{sample}. The Trolox Equivalent (Trolox Eq.) of each sample was calculated with the Trolox standard curve from the net $AUC_{standard}$.

5. Lipophilic ORAC Assay

The $ORAC_{lipo}$ assay was conducted in an organic solvent system utilizing dimethyl sulfoxide (DMSO):butyronitrile (BN) (9:1 v/v). BODIPY stock solution (2 mmol/L) was prepared with DMSO with aliquots stored in brown cryogenic tubes at -20 ℃, and diluted to 8 μmol/L with DMSO/BN immediately before the assay. α-Tocopherol (5~200 μmol/L) prepared in DMSO/BN was used to determine the limit of detection for the assay. Aliquots of butylated hydroxytoluene (BHT) at 10 mmol/L, stored at -80 ℃, were serially diluted with DMSO/BN from 100~500 μmol/L to create a standard calibration curve. Two hundred μL of plasma or spiked bovine serum was mixed with ethyl alcohol (1:1 v/v) by vortexing in brown microtubes for 2 min followed by addition of double volume of hexane. After an additional 2 min of mixing, samples were centrifuged for 5 min at 10,000 g. The hexane layer (700 μL) was transferred to another microtube and evaporated to dryness under nitrogen. Subsequently, the dry residue was reconstituted with 175 μL of DMSO:BN (9:1 v/v), sonicated for 20 sec, vortexed for 1 min, and then used in the assay. Blanks (DMSO/BN), standards, and samples (75 μL each) were placed in 96 well microplates as described above. BODIPY (125 μL) was added to each well and the microplates were placed in the pre-incubated (37 ℃) microplate reader. Fifty μL of AMVN (0.5 mol/L), prepared in DMSO/BN, was added to initiate the assay in the same manner described above for hydrophilic ORAC assay. Changes in red and green fluorescence of BODIPY were determined at 580/600 (ex/em) and 485/520 (ex/em), respectively, every 2 min for a 60 min period. The net AUC of red and green fluorescence was calculated by the difference between AUC_{blank} and AUC_{sample} (or $AUC_{standard}$). Based on the BHT standard curve, the BHT equivalent (BHT Eq.) of each sample was calculated.

With florescence monitored every minute for 30 min at 580/600 (ex/em) at 41 ℃ in a microplate reader, we confirmed a self-quenching action by decreased fluorescence[18] but not photobleaching,[12] of BODIPY when the same concentration (10 μmol/L) was dissolved in octane, butyronitrile, or octane:butyrinitrile (9:1, v/v), but not in DMSO. Red fluorescence was decreased by 23, 9 and 19% for octane, butyronitrile, and octane:butyronitrile, respectively but, with DMSO, declining red fluorescence was not observed until after 30 min. Therefore, DMSO was chosen as the assay solvent with 10% butyronitrile added to increase the solubility of lipophilic compartment as suggested by Naguib.[10] Use of DMSO:butyronitrile (9:1, v/v) reduced red fluorescence loss to <5% during the 60 min assay. As BODIPY fluorescence was shifted from red to green with oxidation,[19] we used the AUC of the increasing green fluorescence for the $ORAC_{lipo}$ after confirming its stability over time and greater sensitivity to α-tocopherol and BHT. Further, the intensity of the green fluorescence provided a more linear relationship between $ORAC_{lipo}$ and BHT or α-tocopherol concentration than that observed when monitoring red fluorescence.

6. Statistical Analysis

Correlations between ORAC and endogenous antioxidants in human plasma were assessed with Pearson's correlation coefficients using SPSS for windows (version 11.0, SPSS Inc, Chicago). P values <0.05 were considered significant.

RESULTS

1. $ORAC_{total}$ and $ORAC_{pca}$ Assay Parameters

For $ORAC_{total}$ and $ORAC_{pca}$ assays, Trolox (5~50 μmol/L) in PBS provided a linear relationship between concentration and net AUC (R^2=0.99±0.01) and a sensitivity of ±5 μmol /L with photostability of FL and FL decay curves, so Trolox was used as the reference standard (Fig. 1). Inter

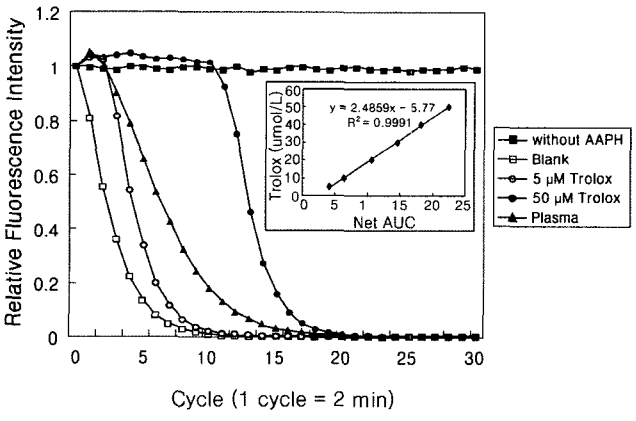

Fig. 1 AAPH-induced decay of fluorescein fluorescence in the presence of Trolox and plasma. Inset: Trolox standard curve.

- and intra - assay coefficient variations (CV) for $ORAC_{total}$ were 5% and 9% (n=5) and for $ORAC_{pca}$ were 5% and 4% (n=5), respectively.

2. $ORAC_{lipo}$ Assay Parameters

In the $ORAC_{lipo}$ assay, α-tocopherol (3.125~200 μmol/L) in DMSO/BN provided a linear relationship between concentration and AUC for red and green fluorescence (R^2=0.95±0.02 and 0.98±0.01, respectively). The detection limits of α-tocopherol for red and green fluorescence were 12.5 and 3.125 μmol/L, respectively, with a sensitivity of ±10 μmol/L. BHT also provided a linear relationship between net AUC and concentration such that a range of 50~500 μmol/L covered all tested plasma samples (R^2=0.98±0.02) (Fig. 2). Lower concentrations of BHT (12.5~50 μmol/L, R^2=0.99±0.01) were also detectable with a sensitivity of ±10 μmol/L. BODIPY maintained its green fluorescence for the entire assay period of 60 minutes as illustrated in the trace of the "without AMVN" (Fig. 2). Inter - and intra - assay CV for $ORAC_{lipo}$ were 8% (n=6) and 15% (n=6), respectively.

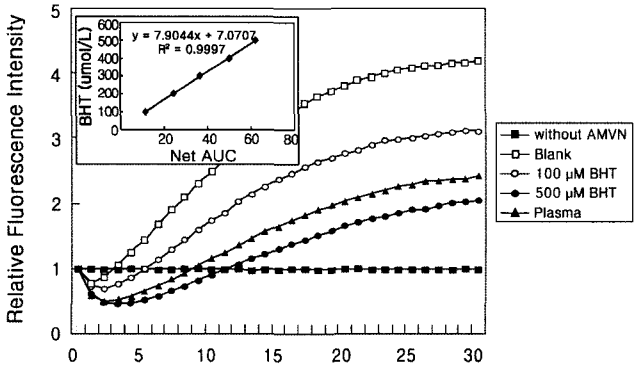

Fig. 2 AMVN-induced increase in BODIPY green fluorescence with BHT and in plasma. Inset: BHT standard curve.

3. ORAC Values of Antioxidant-Spiked Bovine Serum

Bovine serum was spiked with various concentrations of antioxidants for ORAC analyses. No effect was observed on $ORAC_{total}$ and $ORAC_{pca}$ values with the addition of the lipophilic α-tocopherol (0~200 μmol/L) or β-carotene (0~1.86 μmol/L), while a positive, dose-dependent relationship was found between α-tocopherol (0~200 μmol/L) and $ORAC_{lipo}$ (R^2=0.94) (Fig. 3). The addition of β-carotene at physiological concentrations (0~1.86 μmol/L) provided a modest positive correlation with $ORAC_{lipo}$ measured by red fluorescence intensity (R^2=0.55). The addition of

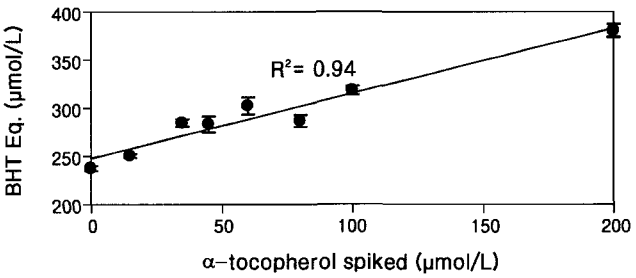

Fig. 3 $ORAC_{lipo}$ of bovine serum supplemented with α-tocopherol

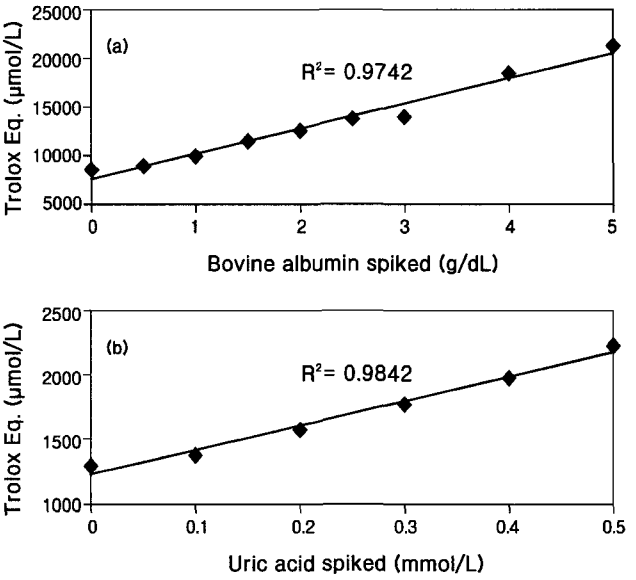

Fig. 4 $ORAC_{total}$ (a) and $ORAC_{pca}$ (b) of bovine serum supplemented with bovine albumin and uric acid.

the hydrophilic compounds albumin and uric acid to serum increased $ORAC_{total}$ (R^2=0.97) and $ORAC_{pca}$ (R^2=0.98), respectively, in a dose-dependent manner (Fig. 4) but had no effect on $ORAC_{lipo}$ (R^2=0.02, 0.07, respectively). The addition of ascorbic acid (0~200 μmol/L) produced a weak relationship to $ORAC_{total}$ (R^2=0.16) but there were no notable relationship with $ORAC_{pca}$ (R^2=0.17) and $ORAC_{lipo}$ (R^2=0.47).

4. Correlation between ORAC and Endogenous Antioxidants in Human Plasma

$ORAC_{total}$, $ORAC_{pca}$, and $ORAC_{lipo}$ values from 16 non-fasted, healthy volunteers were ranged from 7180~11234 μmol/L Trolox Eq., 654~1680 μmol/L Trolox Eq., and 79~460 μmol/L BHT Eq., respectively. However, no correlations were observed between plasma concentrations of lipophilic antioxidants such as α-tocopherol and β-carotene and the hydrophilic $ORAC_{total}$ and $ORAC_{pca}$ assays (Table 1). Three out of 16 volunteers showed their plasma α-tocopherol

Table 1. Correlations between selected antioxidants and ORACs (R^2) in human plasma (n=16)

	$ORAC_{lipo}$	$ORAC_{total}$	$ORAC_{pca}$
Total carotenoids[a]	0.14	0.01	0.03
all trans β-carotene[b]	0.19	0.00	0.08
α-tocopherol	0.43**	0.00	0.03
Reduced ascorbate	0.01	0.01	0.20
Uric acid	0.20	0.29*	0.54**
Albumin	0.00	0.29	0.05

[a] α-carotene+β-carotene+cryptoxanthin+lutein+lycopene+zeaxanthin
[b] n=13
* $P<0.05$, ** $P<0.01$

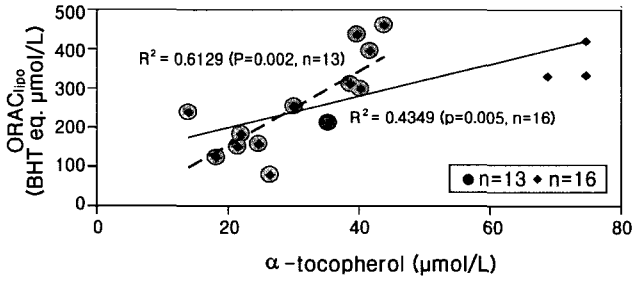

Fig. 5 Correlation between α-tocopherol concentrations and $ORAC_{lipo}$ in human plasma.

concentrations > 2 times than the mean α-tocopherol concentration (30.5 μmol/L) of other 13 volunteers. Fig. 5 shows the significant correlation between $ORAC_{lipo}$ and plasma α-tocopherol concentration in 13 and 16 volunteers, separately. However, no significant correlation was noted between $ORAC_{lipo}$ and plasma concentrations of carotenoids. Plasma uric acid was significantly correlated with $ORAC_{total}$ and $ORAC_{pca}$, but not with $ORAC_{lipo}$. Plasma albumin levels were significantly correlated with $ORAC_{total}$ but not with $ORAC_{lipo}$.

DISCUSSION

Since the introduction of the ORAC assay[6] and its subsequent widespread use,[20-23] several efforts have been made to improve this method as a measure of TAC in foods and beverages as well as in plasma and tissues. However, the poor response of the ORAC to lipophilic antioxidants and thus its inability to be a true measure of "total antioxidant activity" has stimulated the development of an assay that can better detect these compounds.[10-12,25] In addition, Aldini *et al.*[11] and Prior *et al.*[13] have reported techniques that attempted a single TAC assay for both hydrophilic and lipophilic antioxidant capacity, however, both methods are performed in an aqueous milieu and thus cannot directly examine lipophilic antioxidant actions. In the current trial, $ORAC_{lipo}$ was performed in the lipophilic environment and succeeded to show its sensitivity to α-tocopherol in plasma, independently of hydrophilic $ORAC_{total}$ and $ORAC_{pca}$.

The original ORAC method utilized B-phycoerythrin, a natural phycobiliprotein, as the hydrophilic fluorescent target of free radical attack. Because of the high product variability and photo-instability of B-phycoerythrin, synthetic fluorescein is now widely employed in ORAC assays.[7,13,22,23] Further, microplate readers have been introduced to replace the centrifugal analyzer instrumentation that has become obsolete.[13,24] Therefore, we employed fluorescein and microplate reader technique to determine $ORAC_{total}$ and $ORAC_{pca}$, and the values of volunteers in the current study were comparable to those reported in previous studies.[23,24]

In the combination of hydrophilic and lipophilic ORAC introduced by Prior *et al.*,[13] they used AAPH as a hydrophilic peroxyradical generator and fluorescein as the fluorescence target. Adapting the method developed by Naguib,[10] Aldini *et al.*[11] and the current study employed BODIPY as a fluorescence probe better suited to radical attack in a lipophilic milieu. However, the susceptibility of BODIPY to photobleaching may limit its reliability as a target in a TAC assay.[12] Further, decreasing BODIPY fluorescence over time in octane/butyronitrile may result from self-quenching due to its increasing concentration as the solvent evaporates.[10] A self-quenching property of BODIPY at high concentrations has been described for C11-BODIPY 581/591[18] and for C16-BODIPY.[26] We confirmed this self-quenching action of BODIPY, but not photobleaching, when it was dissolved in octane, butyronitrile or both solvents. The reduction in fluorescence intensity over time was prevented with DMSO as the solvent. Thus, DMSO plus butyronitrile (9:1, v/v) was employed as the working medium and BODIPY solvent for the $ORAC_{lipo}$ assay. Butyronitrile (10%) was added to increase the solubility of lipophilic constituents in the assay. Consistent with the original concept of the ORAC assay, we employed AUC quantification to combine both the time and degree of inhibition of the antioxidant action of the test compound into a single quantity.[27] However, $ORAC_{lipo}$ is the first such application of the ORAC method conducted exclusively in an organic solvent system, an approach that may ultimately best reflect the direct antioxidant capacity of these bioactive compounds in their actual lipophilic microenvironment.

The significant contributions of uric acid and albumin to the hydrophilic ORAC assay have been described[6,28] and were confirmed in our experiments. Consistent with other reports,[8,9,20] we also found the antioxidant capacity of lipophilic compounds such as α-tocopherol and carotenoids

was not evident in hydrophilic ORAC assays. Thus, we sought to determine whether the $ORAC_{lipo}$ assay might fill the need in the existing TAC assays by providing a method for ranking the potency of lipophilic antioxidants in plasma, a goal not fully addressed by others. Huang *et al.*[12] and Prior *et al.*[13] obtained a dose-dependent correlation with added tocopherols and using randomly methylated β-cyclodextrin *in vitro* but did not examine endogenous antioxidants in their system. Using human plasma, Aldini *et al.*[11] demonstrated oxidation of endogenous carotenoids in their plasma oxidation assay; however, their results were confounded by interactions occurring between antioxidants in the aqueous and lipid compartments in plasma.

When added to the organic solvent system of the $ORAC_{lipo}$, we found physiological concentrations of α-tocopherol produced a strong, linear relationship (R^2=0.98) between dose and AUC, similar to the results published by Huang *et al.*[12] Adding α-tocopherol to plasma resulted in a similar, but slightly attenuated correlation (R^2=0.94). Unlike α -tocopherol, neither exogenously added nor endogenous β-carotene had a significant positive effect on $ORAC_{lipo}$. These observations are in contrast to reports by Naguib[10,25] who found carotenoids quenched peroxyradicals and decreased the fluorescence of BODIPY 581/591 and BODIPY 665/676 and Miller *et al.*[2] and Cano *et al.*[3] who found carotenoids quench the $ABTS^{\bullet+}$ radical in the TEAC assay. However, it is important to note that the quenching activity of β-carotene on BODIPY and $ABTS^{\bullet+}$ in these experiments was obtained at concentrations of 20~476 μmol/L, from 10 to 400-fold higher than physiological concentrations tested in the $ORAC_{lipo}$ assay. We also tested plasma enriched with lycopene (0.09~1.86 μmol/L) and coenzyme Q10 (1~12 μmol/L), which was consistent with our results with β-carotene and failed to achieve any significant positive effect on $ORAC_{lipo}$ (data not shown). While examining results with lipophilic antioxidants in the $ORAC_{lipo}$ and related assays, it is worth noting the different mechanisms of their reactions, e.g., the chain breaking, lipid peroxidation quenching action of tocopherols versus the singlet oxygen scavenging activity of carotenoids.[12] These differences may underlie the responsiveness of the former and the insensitivity of the latter to the peroxyl radicals in the $ORAC_{lipo}$ system and warrant further investigation of the $ORAC_{lipo}$ with different radical generators.

While methods have been developed and modified to determine TAC *in vitro* and *in vivo*, limitations are associated with their ability to determine precisely the contribution of lipophilic antioxidants in a lipid milieu or the lipid compartment in which they exist in foods or cells.[14,30] The $ORAC_{lipo}$ described here addresses this need and succeeds in its sensitivity to α-tocopherol, but further modifications are required such that it displays similar responsiveness to physiological concentrations of carotenoids and other lipid-soluble antioxidants before it can be broadly applied as a measure of TAC with a lipid milieu.

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