Determination of Antioxidant potential using an Oxygen Radical Absorbance Capacity (ORAC) Assay with Synergy™ H4

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ORAC

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The oxygen radical absorbance capacity (ORAC) assay has emerged as a robust analytical method to determine the antioxidant potential of a range of substances found in neutraceutical, pharmaceutical and food products. ORAC relies on a common fluorescent probe, fluorescein, to monitor antioxidant activity which can be read on a microplate reader capable of detecting fluorescence. Here we show the ability to perform the assay in a 96-well microplate format amenable to higher throughput platforms. Intra-assay precision and accuracy were determined as well as quantification of the antioxidant capacity of several known compounds.

Introduction

Oxidative damage to living organisms has been associated with several disease states as well as aging[1,2]. The sources of reactive oxygen species (ROS) are varied and can lead to the formation of toxic compounds within organisms. ROS sources such as cellular respiration, interaction of biomolecules with ionizing radiation, and dedicated cellular pathways for ROS formation as a protective mechanism ensure chronic exposure of living organisms to ROS species. A proper balance must be maintained between oxidants and antioxidants to ensure the ubiquitous ROS species do not become deleterious to the organism.

There are several mechanisms that are responsible for sequestration and/or conversion of ROS species into harmless byproducts including antioxidant enzymes, proteins and antioxidants provided by diet. Disruption of any one of these mechanisms can result in an imbalance and ensuing damage to a number of critical components required to maintain cellular homeostasis. Thus, there is interest in the ability to accurately determine the antioxidant capabilities of foods, cosmetics, dietary supplements and pharmaceutical agents. These agents may help replace or enhance defective or deficient antioxidant mechanisms in organisms or be used for cosmetic purposes.

While several methods exist to measure total antioxidant capacity, ORAC has emerged as a low cost method suitable for high throughput automation in a microplate format[3,4].

The ORAC assay relies on free radical damage to a fluorescent probe, most commonly fluorescein, caused by an oxidizing reagent resulting in a loss of fluorescent intensity over time[5].

The resultant damage can then be correlated with the amount of oxidant present. Conversely, inhibition of oxidative damage to the fluorescent probe can be correlated with the antioxidant capacity of a compound acting as a free radical scavenger. Reactions containing antioxidants and blanks are typically run in parallel with a known equivalent of a ROS generator and fluorescent probe (Figure 1). Reactions are typically run to completion allowing the determination of the area under the resultant kinetic curve (AUC). Antioxidant protection can then be quantified by subtraction of AUC of the blank reaction from those reactions containing antioxidant. The resultant difference is considered to be the antioxidant protection conferred by the sample compound.

The antioxidant properties of Trolox® remain a popular standard against which the antioxidant capacity of a range of substances can be related[5]. Thus, ORAC results are commonly referred to as Trolox® equivalents (TE) as calculated from comparison to a Trolox® calibration curve. The AUC calculation combines both the inhibition time as well as inhibition percentage of free radical damage by the antioxidant into a single value[3]. Standardization of the assay has allowed the comparison of a wide range of compounds from a variety of sources and development of a quantitative database[6].

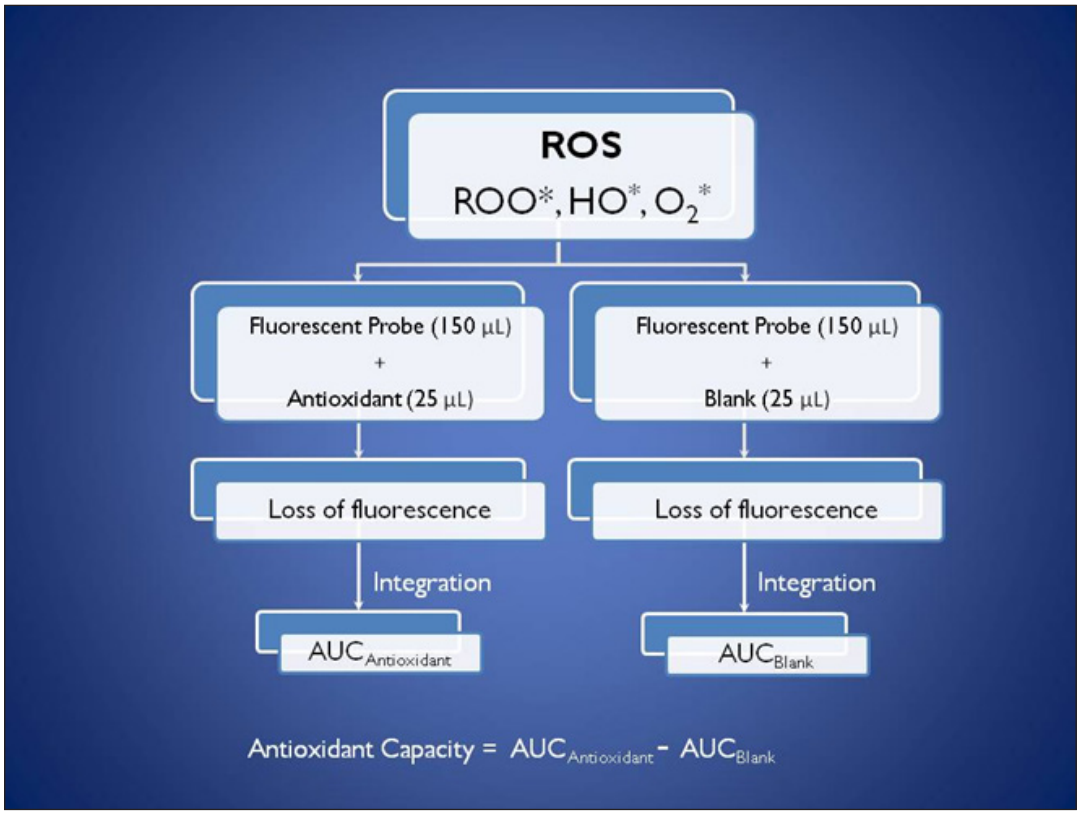

Figure 1. **Workflow of typical ORAC assay.** ROS generators are added to parallel reactions containing equivalent amounts of fluorescent probe. Reactions contain either an antioxidant or buffer blank. Loss of fluorescence due to oxidative damage to the probe is measured kinetically. The AUC is calculated as the integral of the area under the curve. The resultant antioxidant capacity is the difference between the AUC of the sample and that of the buffer blank (adapted from Huang, et al.).

Materials and Methods

Sodium Fluorescein was purchased from Life Technologies (Grand Island, NY). 2,2′-Azobis (2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®), gallic acid, Epigallocatechin gallate (EGCG), epigallocatechin (EGC), and quercetin dihydrate were purchased from Sigma-Aldrich (St. Louis, Mo). Nunc Micro-well™ plates were a gift of ThermoFisher Scientific (P/N260895, Nalge Nunc International, (Rochester, NY).

The ORAC assay was performed as described by Huang with the following modifications[3]. Briefly, AAPH (203.4g) was dissolved in 10 mL of 75 mM phosphate buffer (pH 7.4) to a final concentration of 75 mM and made fresh daily. A fluorescein stock solution (4 µM) was made in 75 mM phosphate buffer (pH 7.4) and stored wrapped in foil at 4 °C. Immediately prior to use, the stock solution was diluted 1:500 with 75 mM phosphate buffer (pH 7.4). The diluted sodium fluorescein working solution was made fresh daily. To all experimental wells, 150 µL of sodium fluorescein working solution was added. In addition, blank wells received 25 µL of 75 mM phosphate buffer (pH 7.4), while standards received 25 µL of Trolox® dilution and samples received 25 µL of sample. The plate was then allowed to equilibrate by incubating for a minimum of 30 minutes at 37 °C. Plate reader injector system priming with 5 mL of AAPH solution was performed just prior to addition to the preincubated microplate. Reactions were initiated by the addition of 25 µL of AAPH solution using the injectors of a Synergy™ H4 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT) for a final reaction volume of 200 µL. The fluorescence was then monitored kinetically with data taken every minute.

The fluorescent monochromators of the Synergy H4 Hybrid Multi-Mode Microplate Reader were used for all measurements. Excitation was performed at 485 nm with a 20 nm bandpass and emission was measured at 528 nm with a 20 nm bandpass. The plate reader was controlled by Gen5™ Data Analysis software (BioTek Instruments, Inc., Winooski, VT). Reactions were initiated by the addition of 25 µL of AAPH reagent (75 mM) followed by shaking at maximum intensity for 10 seconds. The fluorescence of each well was then measured from the bottom every 60 seconds using the autoscale option for gain optimization. ORAC values were calculated as described by Cao and Prior[4]. The AUC and the Net AUC of the standards and samples were determined using Gen5 Data Analysis Software using equations 1 and 2 respectively.

$$AUC = \left(\frac{R_1}{R_1}\right) + \left(\frac{R_2}{R_1}\right) + \left(\frac{R_3}{R_1}\right) + \ldots + \left(\frac{R_n}{R_1}\right) \quad \text{(Eq. 1)}$$

Where R_1 is the fluorescence reading at the initiation of the reaction and R_n is the last measurement.

$$\text{Net AUC} = AUC_{sample} - AUC_{blank} \quad \text{(Eq. 2.)}$$

The standard curve was obtained by plotting the Net AUC of different Trolox® concentrations against their concentration. ORAC values of samples were then calculated automatically using the Gen5 software to interpolate the sample's Net AUC values against the Trolox® standard curve.

Results and Discussion

Sensitivity of the assay to temperature fluctuations across the plate have been noted previously[5,7]. To determine intra-assay variability due to well positioning the precision of the assay was determined by kinetic analysis of blank wells consisting of sodium fluorescein working solution and buffer blank in all wells. AUC values were determined for each well and subject to statistical analysis (Table 1). Variability was noted in rows C and D as a decreased decay rate during initial experiments when the injector system was primed prior to a 30 minute plate incubation inside the reader (data not shown). Priming of the system immediately prior to AAPH reagent addition alleviated any variability. Using this method, consistent AUC values were achieved regardless of well position as indicated by a coefficient of variation of 2.2% across the assay plate.

Area Under the Curve (AUC)				
Normalized data	Count	Mean	Stdev	CV%
Std Nunc plate	96	4.71	0.10	2.18

Table 1. **Determination of intra-assay variability.** The precision of the ORAC assay was determined by analysis of the AUC of the kinetic plot of unprotected sodium fluorescein decay induced by oxidative damage.

To minimize assay read time, the sodium fluorescein and AAPH concentrations were optimized to ensure the reactions were completed (decay of fluorescent signal to background) within approximately 15 minutes in the absence of antioxidant. Kinetic curves of a 1:2 serial dilution of Trolox® demonstrate concentration dependent protection of fluorescein against oxidative degradation by AAPH (Figure 2).

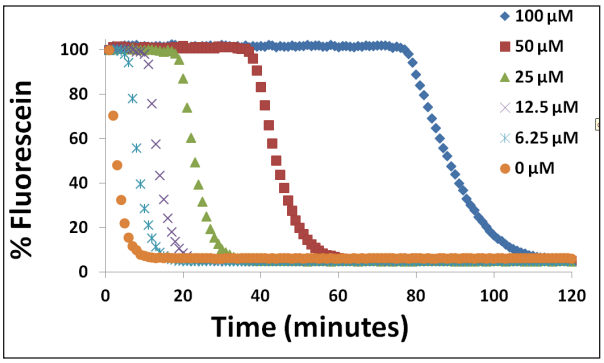

Figure 2. **Plots of Trolox Kinetic Curves.** Representative curves from ORAC assay of varying concentrations of Trolox antioxidant standards ranging from 0 to100 µM.

The net AUC was calculated as described above from the Trolox® kinetic curves and plotted against concentration resulting in a linear relationship as shown in Figure 3. Linear regression analysis resulted in a correlation coefficient (R^2) of 0.9998. The resultant standard curve can then be interpolated for detemination of antioxidant capacity of unknown samples and reported as Trolox® equivalents.

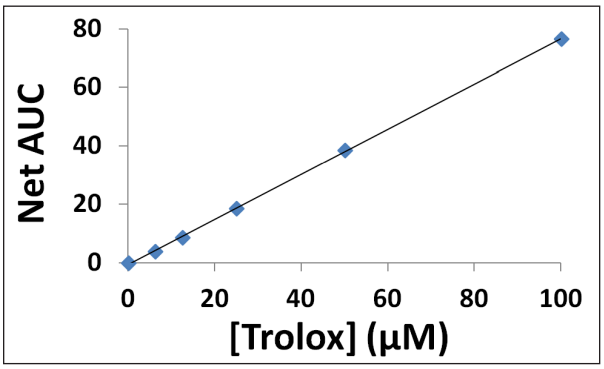

Figure 3. **Trolox Standard Curve.** The net AUC of varying concentrations of Trolox antioxidant standards ranging from 0 to100 µM are plotted vs. concentration. The subsequent calibration curve was used to interpolate the antioxidant capacity of various samples.

Several compounds known to have antioxidant properties were assayed by the ORAC method described above for determination of their antioxidant capacity. Each compound was subjected to a 6-point 1:2 serial dilution including a zero concentration point and assayed in triplicate. A Trolox® standard curve was included on each plate for antioxidant capacity determination and conversion to Trolox® equivalents. A Tris buffer titration was added as a negative control compound as well as several wells with assay buffer only. To determine the antioxidant capacity of compounds with known concentrations over a desired range the net AUC for each sample compound was calculated as above and plotted against the concentration as depicted in Figure 4. To determine Trolox equivalents of each sample range the ratio of the slope (m) of the linear

regression analysis of the compound to the slope of the linear regression of Trolox was used:

TE (range of concentrations) = $m_{compound}/m_{Trolox}$

The calculated Trolox equivalents can then be used for comparative analysis of the antioxidant capacity of the various samples tested (Table 2 and Figure 4). These determinants correlate well with those in the literature with the exception of EGC which also showed considerable variability when compared to values generated by the CODAS FARA II analogy[3].

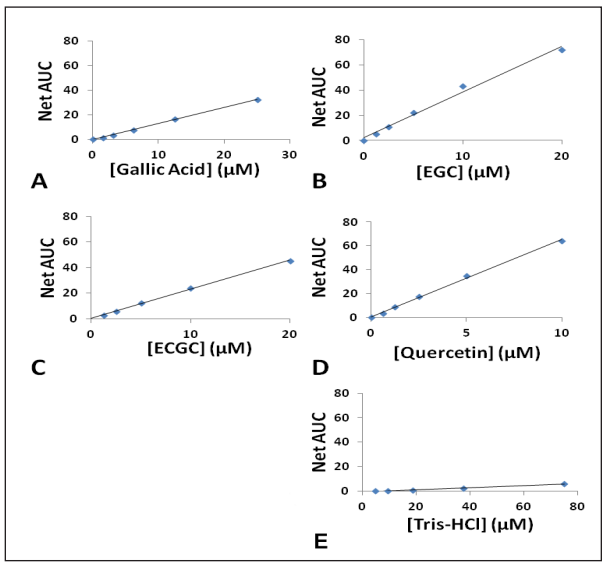

Figure 4. **Antioxidant Dose Response Curves.** The ORAC of several serial diluted compounds with known antioxidant properties, as well as Tris buffer, were measured and their Net AUC plotted against concentration. Each curve was subjected to linear regression analysis for comparison to a Trolox standard curve.

Antioxidant Capacity		
	Slope	TE[a]
Gallic Acid	1.69±0.04	N/A
EGC	4.71±0.26	2.34±0.12
EGCG	2.96±0.06	3.51±0.16
Quercetin	8.39±0.22	7.06±0.15
TRIS	0.07±0.01	N/A

[a] *ORAC results from pure chemicals expressed as Trolox® equivalents, Huang.*

Table 2. **Determination of Trolox® Equivalents.** Trolox® equivalents for compounds with known antioxidant properties were determined by the ratio of the slope of the linear regression curve of each with that of a Trolox® standard curve.

Conclusion

Determination of antioxidant capacity in a high sample throughput manner requires an assay with low cost and high precision and accuracy amenable to a 96-well or higher density microplate format. Here we show that the ORAC assay provides a method to determine the antioxidant capacity of several known antioxidants and subsequent conversion to the commonly accepted Trolox® equivalents for quantitative analysis. The assay uses common reagents, a standard 96-well microplate and a microplate reader capable of reading fluorescence. The ORAC assay provided excellent precision across all wells of the microplate tested along with the advantage of being capable of performing the assay in approximately 60 minutes.

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