AOAC Official Method 2012.23
Total Antioxidant Activity
Oxygen Radical Absorbance Capacity (ORAC) Using Fluorescein as the Fluorescence Probe
First Action 2012

Caution: Refer to the Material Safety Data Sheet for all chemicals prior to use. Follow all safety guidelines and use proper personal protective equipment. For perchloric acid, always work in fume hood.

A. Principle

The oxygen radical absorbance capacity (ORAC) method measures the capacity of antioxidants to protect the fluorescent probe from damage by free radicals. In this assay, 2,2′-azobis (2-amidino-propane) dihydrochloride (AAPH) is used as the source for the peroxyl radical, which is generated as a result of the spontaneous decomposition of AAPH at 37 ± 1°C. Fluorescein (FL) is the chosen target probe, whose loss of fluorescence is an indication of the extent of damage from its reaction with the peroxyl radical. The protective effect of the antioxidants is measured by assessing the longer fluorescence time/intensity area under the curve (AUC) of the sample compared to the blank (AUC), in which no antioxidant compounds are present. Trolox is used as the calibration standard. The ORAC assay provides a unique assessment since, as the reaction goes to completion, both the inhibition time and the inhibition degree are measured.

B. Apparatus

(**a**) *Microplate reader*.—Fluorescence microplate reader with an excitation filter at 485 ± 20 nm and an emission filter at 530 ± 25 nm, capable of automatic shaking and temperature control.

(**b**) *pH meter*.—Capable of measuring the pH of solutions to ±0.1 pH units.

(**c**) *Analytical balance*.—Capable of reading 0.1 mg.

(**d**) *Various glassware and pipets*.

C. Chemicals and Reagents

(**a**) *HPLC-grade water*.

(**b**) *FL sodium*.—Dye content 92.9%.

(**c**) *Potassium phosphate dibasic (anhydrous), K_2HPO_4.*

(**d**) *Potassium phosphate monobasic (crystals), KH_2PO_4.*

(**e**) *Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid)*.—CAS 53188-07-1.

(**f**) *AAPH*.

(**g**) *Acetone*.—ACS/HPLC grade.

(**h**) *Randomly methylated β-cyclodextrin (RMCD)*.—Cyclolab R&D (CDT, Inc., Alachua, FL) Cat. No. CY-2004.1.

(**i**) *Perchloric acid*.—0.5 N.

D. Working Reagents

(**a**) *KH_2PO_4 (0.75 M)*.—Weigh 102.07 g potassium phosphate monobasic and dissolve in approximately 800 mL distilled water. Quantitatively transfer to a 1 L volumetric flask and bring to volume with distilled water. Stir 1 h and then filter. Transfer to a 1 L polyethylene bottle.

(**b**) *K_2HPO_4 (0.75 M)*.—Weigh 130.64 g of potassium phosphate dibasic and dissolve in approximately 800 mL distilled water. Quantitatively transfer to a 1 L volumetric flask and bring to volume with distilled water. Stir 1 h and then filter. Transfer to a 1 L polyethylene bottle.

(**c**) *ORAC stock buffer solution*.—Allow both 0.75 M buffers (K_2HPO_4 and KH_2PO_4) to come to room temperature before measuring. Measure 603 mL of the 0.75 M K_2HPO_4 and pour into a 1 L polyethylene bottle. Add 351 mL of 0.75 M KH_2PO_4. Shake well to mix.

(**d**) *Working ORAC buffer (75 mM)*.—Measure 900 mL distilled water and pour into a 1 L polyethylene bottle labeled "ORAC hydro only." Add 100 mL ORAC stock buffer solution, **D**(**c**). Mix well and then verify the pH (should be 7.0–7.4). Record pH on label.

(**e**) *Lipophilic ORAC buffer (1.4% RMCD)*.—Weigh 7.0 g RMCD into a 1 L beaker. Add 500 mL working ORAC buffer. Mix well. Transfer to a 500 mL polystyrene bottle.

(**f**) *AAPH solution*.—Weigh 0.828 g AAPH into a 50 mL conical tube. Add 20 mL working ORAC buffer. Cap and mix well by inversion until dissolved. Prepare fresh daily. Store in a cup with wet ice; refrigerate at 4–8°C.

(**g**) *FL concentrate (1.14×10^{-3} M)*.—Weigh 0.023 g FL. Quantitatively transfer to a 50 mL volumetric flask with working ORAC buffer. Bring to volume with buffer. Mix well. Aliquot 1 mL into labeled 1.5 mL microcentrifuge tubes. Stable for 3 years at –80°C.

(**h**) *FL stock solution (5.70 μM)*.—Measure 20 mL working ORAC buffer into a 50 mL conical tube. Remove 0.1 mL buffer; add 0.1 mL FL concentrate thawed at room temperature. Mix well. Wrap tube in foil. Stable for 1 month at 4–8°C.

(**i**) *FL working solution (11.12×10^{-2} μM)*.—Measure 200 mL of working ORAC buffer and pour into an amber bottle. Using a pipet, remove 3.90 mL buffer. Add 3.90 mL FL stock solution. Mix well and wrap bottle with foil. Stable for 2 weeks at 4–8°C.

(**j**) *Hydrophilic ORAC extraction solution*.—Acetone–distilled water (50 + 50). Measure 500 mL distilled water and pour into a 1 L beaker. Measure 500 mL acetone and add to the measured distilled water. Mix well. Pour solution into amber bottle with dispenser. Stable for 6 months at 18–25°C.

(**k**) *Lipophilic ORAC extraction solution*.—Hexane–ethyl acetate (75 + 25, v/v). Working in the hood, pour 750 mL hexane into a 1 L beaker. Pour 250 mL ethyl acetate into the same beaker. Mix well with stir bar. Transfer to a 1 L glass bottle with dispenser. Stable for 6 months at 18–25°C.

E. Standards

(**a**) *Trolox stock standard for hydrophilic ORAC (1000 μM)*.—Weigh 0.025 g Trolox. Quantitatively transfer to a 100 L volumetric flask with working ORAC buffer, **D**(**d**). Mix well to dissolve. Bring to volume with buffer. Aliquot 11 mL into 15 mL conical tubes.

(**b**) *Working Trolox standard for hydrophilic ORAC (100 μM)*.—Thaw Trolox stock standard (1000 μM). Mix thawed standard. Pipet 10 mL of stock Trolox into a 100 mL volumetric flask. Bring to volume with working ORAC buffer. Mix well. Aliquot 2.5 mL into labeled tubes.

(**c**) *Trolox stock standard for lipophilic ORAC (1000 μM)*.—Weigh 0.025 g Trolox. Quantitatively transfer to a 100 mL volumetric flask with lipophilic ORAC buffer. Mix well to dissolve. Bring to volume with buffer. Aliquot 11 mL into 15 mL conical tubes.

(**d**) *Working Trolox standard for lipophilic ORAC (100 μM)*.—Thaw Trolox stock standard (1000 μM) in RMCD. Mix thawed standard. Pipet 10 mL of stock Trolox into a 100 mL volumetric flask. Bring to volume with lipophilic ORAC buffer. Mix well.

Table 2012.23A. Summary of hydrophilic $ORAC_{FL}$ Trolox calibration curve [Y (μM) = a + bX(net area)]

Run No.	R^2	Slope (b)	Intercept (a)
1	0.9994	2.5368	–2.174
2	0.9993	2.7390	–4.690
3	0.9981	2.6947	–5.109
4	0.9973	2.5291	–3.846
5	0.9928	2.2331	1.361
6	0.9978	2.8868	–3.788
7	0.9981	2.6288	–3.012
8	0.9987	2.5297	–2.589
Average	0.9977	2.5846	–2.861
Acceptable criteria	≥0.9900	NA	NA

F. Procedure

(**a**) *Sample preparation for hydrophilic ORAC.*—Pure compounds are directly dissolved in acetone–water mixture (50 + 50, v/v) and diluted with 75 mM potassium phosphate buffer (pH 7.4) for analysis. Botanical ingredients are initially ground in a mechanical mill to produce a fine power. Then 0.5 g of the powders is accurately weighed and 20 mL acetone–water (50 + 50, v/v) extraction solvent is added. The mixture is shaken at 400 rpm at room temperature on an orbital shaker for 1 h. The extracts are centrifuged at 14 000 rpm for 15 min, and the supernatant is ready for analysis after appropriate dilution with buffer solution. For liquid samples, a 20 mL aliquot of sample is centrifuged for 15 min and the supernatant is ready for analysis after appropriate dilution. Blood plasma or serum is diluted 100- to 200-fold with pH 7.4 phosphate buffer before analysis. To measure the ORAC in nonprotein fraction, protein is removed using 0.5 N perchloric acid (1 + 1, v/v; plasma–acid), prepared in fume hood; the samples are then centrifuged at 14 000 × *g* for 10 min at 4°C. The supernatants are removed as the serum nonprotein fractions and appropriately diluted with pH 7.4 phosphate buffer before analysis.

Table 2012.23B. Hydrophilic $ORAC_{FL}$ net area corresponding to different concentrations of extracts from tea, blueberry, and grape skins

Concn, mg/L	Net area	ORAC[a]
Black tea leaves		
8	5.92	1586
16	10.81	1566
32	21.51	1629
Blueberry extracts		
5	5.73	2441
10	11.32	2635
20	22.98	2792
Grape skin extracts		
1.2	8.34	15675
2.4	15.63	15521
4.8	29.89	14714

[a] ORAC values are expressed as Trolox equivalents per gram. The RSD for average value of each sample is <15%.

Table 2012.23C. Lipophilic $ORAC_{FL}$ net AUC versus concentration[a]

Compound	Concn, μM	Net area	r^2
γ-Oryzanol	25	28.94	
	12.5	15.87	0.9979
	6.25	8.51	
	3.125	4.32	
γ-Tocopherol	100	28.45	
	50	14.83	0.9971
	25	7.78	
δ-Tocopherol	75	36.11	
	50	27.52	
	25	15.56	0.9668
	12.5	8.34	
	6.25	4.46	
α-Tocopherol	200	40.67	
	100	19.89	
	50	10.45	0.9990
	25	6.07	

[a] Regression equation is expressed as Y (net area) = kX (concentration) + intercept.

(**b**) *Sample preparation for lipophilic ORAC.*—Approximately 0.5 g of sample is dissolved in 20 mL acetone. An aliquot of sample solution is appropriately diluted with 7% RMCD solvent (w/v) made in 50% acetone–water mixture (v/v) and is shaken for 1 h at room temperature on an orbital shaker at 400 rpm. The sample solution is ready for analysis after further dilution with 7% RMCD acetone solvent.

(**c**) *Fluorescence microplate preparation.*—150 μL FL working solution is added to all wells. Then 25 μL of diluted buffer, standard, control, and samples are added to appropriate wells. The plate is incubated at 37°C for 30 min. A 25 μL amount of AAPH is added to all wells to make the final volume 200 μL in each well. Timing is critical for the addition of AAPH as the reaction begins immediately and the rate is temperature-dependent. A multichannel pipettor or use of a plate reader with a pipettor is ideal. The plate is immediately transferred to the plate reader and the fluorescence is measured every minute for 35 min. The fluorescence readings are referenced to the highest reading of wells in which no AAPH is added.

G. Calculations

The net AUC of the standards and samples is calculated. The standard curve is obtained by plotting Trolox concentrations against the average net AUC of the two measurements for each concentration. The final ORAC values are calculated using the regression equation between Trolox concentration and the net AUC and are expressed as micromole Trolox equivalent per liter for liquid samples or per gram solid samples. The AUC is calculated as

$$AUC = 0.5 + f_1/f_0 + ... f_i/f_0 + \ldots + f_{34}/f_0 + 0.5 \times (f_{35}/f_0) \quad (1)$$

where f_0 = initial fluorescence reading at 0 min and f_i = fluorescence

Table 2012.23D. Precision and accuracy of hydrophilic $ORAC_{FL}$

Trolox	QC1	QC2	QC3
Norminal concn, μM	20.00	40.00	75.00
	Run 1		
Intramean, μM	18.21	41.81	74.79
SD[a]	1.26	3.51	5.49
RSD, %[b]	6.90	8.40	7.34
Recovery, %	91.05	100.05	99.72
n	4	4	4
	Run 2		
Intramean, μM	21.33	42.79	76.18
SD	1.58	3.92	6.12
RSD, %	7.41	9.16	8.03
Recovery, %	106.65	107.03	101.57
n	4	4	4
	Run 3		
Intramean, μM	21.45	41.35	76.21
SD	1.37	3.21	5.19
RSD, %	6.39	7.76	6.81
Recovery, %	107.25	103.35	101.61
n	4	4	4
	Pooled runs		
Intermean, μM	20.33	41.98	75.72
SD	1.59	0.74	0.81
RSD, %	7.82	1.76	1.16
Recovery, %	101.65	104.95	100.96
n	12	12	12

[a] SD = Standard deviation.

[b] RSD = Relative standard deviation.

Table 2012.23E. Precision and accuracy of lipophilic $ORAC_{FL}$

Alpha-tocopherol	QC1	QC2	QC3
Nominal concn, μM	40	80	160
	Run 1		
Intramean, μM	41.69	84.48	175.40
SD[a]	2.87	2.12	5.67
RSD, %[b]	8.79	2.52	3.23
Recovery, %	104.25	105.60	109.62
n	4	4	4
	Run 2		
Intramean, μM	42.74	92.01	171.24
SD	2.65	5.12	10.86
RSD, %	6.20	5.57	6.34
Recovery, %	106.86	115.01	107.03
n	4	4	4
	Run 3		
Intramean, μM	39.0425	85.8525	167.21
SD	5.46	6.54	2.61
RSD, %	13.99	7.62	1.56
Recovery, %	97.60	107.31	104.50
n	4	4	4
	Pooled runs		
Intermean, μM	38.16	87.45	171.28
SD	3.66	6.89	6.38
RSD, %	9.66	5.24	3.71
Recovery, %	95.39	109.31	107.05
n	12	12	12

[a] SD = Standard deviation

[b] RSD = Relative standard deviation.

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The data were analyzed by a Microsoft Excel macro program (Microsoft, Roselle, IL) to apply Equation 1 to calculate the AUC. The net AUC is obtained by subtracting the AUC of the blank from that of a sample. The relative Trolox equivalent ORAC value is calculated as

$$\text{Relative ORAC value} = [(AUC_{sample} - AUC_{blank})/(AUC_{Trolox} - AUC_{blank})] \times (\text{molarity of Trolox/molarity of sample}) \quad (2)$$

H. Method Validation

(**a**) *Specificity*.—The purpose was to demonstrate whether the improved method is specific for antioxidants. This objective can be confirmed by obtaining positive results from a sample containing antioxidants and negative results from a same sample whose antioxidants have been destroyed. 100 μM gallic acid, 3% blueberry juice, and whole serum were chosen for specificity determination. After preincubation with 1.28×10^{-2} M AAPH and Fenton reagent ($H_2O_2 + Fe^{2+}$) at 37°C for 2 h, all three samples were found to have no free radical scavenging activities which resulted in no reading in the $ORAC_{FL}$ assay.

(**b**) *Linearity*.—For hydrophilic ORAC, the linear relationship between net area and antioxidant concentration was evaluated using Trolox, black tea leaves, blueberry extracts, and grape skin extracts at different concentrations. For lipophilic ORAC, the linearity was evaluated using γ-oryzanol, γ-tocopherol, δ-tocopherol, and α-tocopherol. Table **2012.23A** summarizes the correlation coefficient, slope, and intercept of the Trolox standard curve. Table **2012.23B** shows the net areas corresponding to the different concentrations of black tea leaves, blueberry extracts, and grape skin extracts, and the calculated ORAC values. Table **2012.23C** summarizes the net AUCs corresponding to the different concentrations and the linear coefficient (r^2) for four fat-soluble antioxidant compounds. All analyzed samples in the various forms demonstrate a good linear relationship between net area and concentration.

(**c**) *LOQ and LOD*.—The LOQ is the lowest concentration on the calibration curve, while the LOD is the lowest amount of

antioxidant that can be detected. In our experiment, the LOQ and LOD were determined to be 12.5 and 6.25 μM, respectively.

(**d**) *Precision and accuracy*.—Tables **2012.23D** and **E** summarize the precision and accuracy of the $ORAC_{FL}$ assay. The precision, which is expressed as relative standard deviation (RSD, %) for all quality control samples, was within ±15%. For hydrophilic ORAC, the accuracy of the method varies from 91 to 107% within individual batches, and from 101 to 105% between all the batches. For lipophilic ORAC, the accuracy of the method varies from 97.60 to 115.01% within individual batches, and from 95.39 to 107.05% between all batches.

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