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.

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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

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

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

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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.

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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.

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.

(**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

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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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ANTIOXIDANTS

AOAC SMPR 2011.011

Standard Method Performance Requirements for in vitro Determination of Total Antioxidant Activity in Foods, Beverages, Food Ingredients, and Dietary Supplements

1 Applicability

In vitro methods for determination of total (hydrophilic and lipophilic) antioxidant activity in foods, beverages, food ingredients, and dietary supplements.

2 Analytical Technique

Any analytical technique that meets the following method performance requirements is acceptable.

3 Definitions

Antioxidant activity.—The in vitro measurement of the total potential of a food, beverage, ingredient, or dietary supplement to inhibit or delay the oxidation of other compounds. Trolox activity will be used as the baseline unit of measurement to allow comparison between methods. If there is more than one *Official Method*[SM] adopted, either comparison factors should be provided to provide equivalent results for the same matrices or the scope statement should limit the applicability of the method.

Limit of detection (LOD).—The minimum concentration of a substance that can be measured and reported with 95% confidence that the antioxidant activity is greater than zero, and is determined from analysis of a low level of an antioxidant in a given matrix containing the antioxidant.

Limit of quantitation (LOQ).—The minimum analyte concentration for which quantitative results may be obtained with 95% confidence.

Repeatability precision.—Variation arising when all efforts are made to keep conditions constant by using the same instrument and operator, and repeating during a short time period. Expressed as the repeatability standard deviation (SD_r), or % repeatability relative standard deviation ($\%RSD_r$).

Reproducibility.—The SD or RSD calculated from among-laboratory data. Expressed as the reproducibility standard deviation (SD_R) or % reproducibility relative standard deviation ($\%RSD_R$).

Recovery factor.—The fraction or percentage of the analyte that is recovered when the test sample is analyzed using the entire method.

4 Method Performance Requirements

See Table 1.

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Approved by: AOAC Stakeholder Panel on Strategic Food Analytical Methods (SPSFAM). Final Version Date: March 21, 2012. Effective Date: March 22, 2012.
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Table 1. Method performance requirements[a]

Parameter	Level	Value
Analytical range	400–400,000[b]	
Limit of detection (LOD)[c]	133[b]	
Limit of quantitation (LOQ)[d]	400[b]	
Repeatability (RSD_r)[e]	400[b]	8.6%
	200,000[b]	3.4%
	400,000[b]	3.1%
Recovery factor	90–110%	
Reproducibility (RSD_R)[f]	400[b]	12.9%
	200,000[b]	5.1%
	400,000[b]	4.36%

[a] Concentrations apply to (*1*) foods as purchased; (*2*) foods as to be consumed; (*3*) beverages as to be consumed; (*4*) ingredients as purchased; (*5*) supplements as purchased.

[b] Units expressed as μmol trolox equivalents per 100 g. Trolox activity will be used as the baseline to allow comparison between methods. *Note*: The stated ranges may be adjusted based on the mechanics of the analytical method.

[c] Limit of detection (LOD) = Minimum concentration of a substance that can be measured and reported with 95% confidence that the antioxidant activity is greater than zero. In this table, units are expressed as μmol trolox equivalents per 100 g. Trolox activity will be used as the baseline to allow comparison between methods. *Note*: The stated LOQ may be adjusted based on the mechanics of the analytical method.

[d] Limit of quantitation (LOQ) = Level at or above which quantitative results may be obtained with a 95% degree of confidence. In this table, units are expressed as umol trolox equivalents per 100 g. Trolox activity will be used as the baseline to allow comparison between methods. *Note*: The stated LOQ may be adjusted based on the mechanics of the analytical method.

[e] Expected repeatability is 2/3 of the Horwitz-predicted %reproducibility (*see* footnote f).

[f] Expected reproducibility is based on the Horwitz equation for the listed concentrations. The Horwitz-predicted $\%RSD_R$ is based on the mass equivalent of one hydrogen and one electron (one hydride equivalent), which is transferred from the sample to the measuring agent. Therefore, for every micromole of trolox reacted, 1×10^{-8} g hydride equivalent per gram of sample are transferred. Thus, a unit of measurement factor of 10^{-8} was used for calculation using the Horwitz equation. *Note*: This approach may be adjusted depending on the method measurement system.

5 System Suitability Tests and/or Analytical Quality Control

Suitable methods will include blank check samples and check standards at the lowest point and midrange point of the analytical range, and a protocol to demonstrate suitability.

6 Reference Material(s)

Certified reference materials are available and should be used as appropriate.

7 Validation Guidance

Recommended level of validation: *Official Methods of Analysis*[SM].

8 Maximum Time-to-Result

No maximum time.

FOOD COMPOSITION AND ADDITIVES

Determination of Total Antioxidant Capacity by Oxygen Radical Absorbance Capacity (ORAC) Using Fluorescein as the Fluorescence Probe: First Action 2012.23

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An improved method for the measurement of oxygen radical absorbance capacity (ORAC) was developed and validated using fluorescein (3′,6′-dihydroxyspiro[isobenzofuran-1[3H], 9′[9H]-xanthen]-3-one) as a new fluorescence probe ($ORAC_{FL}$). Randomly methylated β-cyclodextrin (RMCD) was introduced as the water-solubility enhancer for lipophilic antioxidants. 7% RMCD (w/v) in 50% acetone–H_2O mixture sufficiently solubilized vitamin E compounds and other lipophilic phenolic antioxidants in 75 mM phosphate buffer (pH 7.4). Results indicated that fluorescein shows excellent photostability under the plate reader conditions. This $ORAC_{FL}$ was validated through linearity, precision, accuracy, and ruggedness. The validation results demonstrated that the $ORAC_{FL}$ assay is reliable and robust. The mean of intraday and interday CVs were <15%; for hydrophilic ORAC, LOD and LOQ are 5 and 6.25 μM, respectively; for lipophilic ORAC, LOD and LOQ are 6.25 and 12.5 μM, respectively. It is concluded that unlike other popular methods, the $ORAC_{FL}$ assay provides a direct measure of total antioxidant capacity against the peroxyl radicals.

The Expert Review Panel (ERP) on Strategic Foods Analytical Methods reviewed the method "Analytical Parameters of the $ORAC_{FL}$ Assay" during the AOAC INTERNATIONAL Annual Meeting on October 2, 2012. After evaluating the data available, the ERP agreed that the method meets standard method performance requirements, as articulated by the Stakeholder Panel on Strategic Foods Analytical Methods (1). The ERP granted the method First Action status, applicable to the measure of total antioxidant capacity (AC) of foods. The ERP will monitor the $ORAC_{FL}$'s performance, and after about 2 years, will recommend the method to the Official Methods Board for Final Action if the assay is found to be suitable.

The stakeholder panel recognized that the data for antioxidant capacity are used primarily as a marketing tool. These values are obtained from in vitro measurements and cannot be extrapolated to in vivo activities. While most food components contributing to the in vitro AC may have health benefits, it is generally recognized that they may not necessarily contribute as antioxidants in biological systems. The stakeholder panel also recognized the need for validated antioxidant methods to allow manufacturers a common basis for comparison. However, after considerable deliberation, the ERP determined that each of the different AC methods provides an independent, albeit nonequatable, indication of activity. Thus, the $ORAC_{PGR}$ (AOAC *First Action* **2012.03**) provides $ORAC_{PGR}$ values just as the $ORAC_{FL}$ provides $ORAC_{FL}$ values. Both are indicators of AC, neither is a true measure of AC, and the two are not equatable.

The same is true for other proposed methods to measure AC. The ERP determined that the crucial delimiter of the scope of the method is the measurement process, and it must be included in the title of the method. Thus, a direct comparison between methods is not valid. Comparison of the AC of different foods can only be valid if the same method and the same experimental conditions are used.

The $ORAC_{FL}$ assay was initially developed by Ou and Huang for hydrophilic AC measurement (2, 3). It was largely based on the early work of Cao and Prior (4). Later the $ORAC_{FL}$ assay was extended to measure lipophilic AC by introducing randomly methylated β-cyclodextrin (RMCD) as a solubility enhancer for lipophilic compounds (3). As such, the $ORAC_{FL}$ measures total AC contributed from hydrophilic and lipophilic antioxidants, and it has been one of the most widely used methods for total AC measurement (5). Sufficient data have been derived on foods, making it possible to utilize it in determining dietary intakes of $ORAC_{FL}$ and relate intake to health outcomes in several large epidemiology studies (6).

Submitted for publication March 25, 2013.
The method was approved by the Expert Review Panel on Strategic Food Analytical Methods as First Action.
The AOAC Stakeholder Panel on Strategic Food Analytical Methods invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
Corresponding author's e-mail: boxinou@gmail.com
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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 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**) *Fluorescein sodium*.—Dye content 92.9%.

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Acceptable criteria	≥0.9900	NA	NA

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

Natural products	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%.

(**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 (2,2′-azobis(2-amidino-propane) dihydrochloride*.

(**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, then filter. Transfer to a 1 L polyethylene bottle.

(**b**) *K_2HPO_4 (0.75 M)*.—Weigh 130.64 g 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, 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 0.75 M K_2HPO_4 and pour into a 1 L polyethylene bottle. Add 351 mL 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, 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 of 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**) *Fluorescein concentrate (1.14×10^{-3} M)*.—Weigh 0.023 g fluorescein. Quantitatively transfer to a 50 mL volumetric flask

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.

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

Trolox	QC1	QC2	QC3
Nominal concn, µM	20.00	40.00	75.00
Run 1			
Intra-mean, µM	18.21	41.81	74.79
SD	1.26	3.51	5.49
RSD, %	6.90	8.40	7.34
Recovery, %	91.05	100.05	99.72
n	4	4	4
Run 2			
Intra-mean, 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			
Intra-mean, 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			
Inter-mean, 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

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**) *Fluorescein 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 fluorescein concentrate thawed at room temperature. Mix well. Wrap tube in foil. Stable for 1 month at 4–8°C.

(**i**) *Fluorescein 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 fluorescein 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. 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.

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 14000 rpm for 15 min, and

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

α-Tocopherol	QC1	QC2	QC3
Nominal concn, µM	40	80	160
Run 1			
Intra-mean, µM	41.69	84.48	175.40
SD	2.87	2.12	5.67
RSD, %	8.79	2.52	3.23
Recovery, %	104.25	105.60	109.62
n	4	4	4
Run 2			
Intra-mean, 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			
Intra-mean	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			
Inter-mean, 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

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.

(**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 fluorescein 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

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

where f_0 = initial fluorescence reading at 0 min and f_i = fluorescence reading at time I.

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 **2012.23E** summarize the precision and accuracy of the $ORAC_{FL}$ assay. The precision, which is expressed as RSD for all QC 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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