JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effect of β-Cyclodextrin in Improving the Correlation between Lycopene Concentration and ORAC Values

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Lycopene, a lipophilic antioxidant, plays a crucial role in biological systems. It may play an important role in human biological systems by providing protection against cardiovascular disease and some cancers and by boosting the immune system. The oxygen radical absorbance capacity (ORAC) has been validated as an index of antioxidant activity for many hydrophilic antioxidants but not for lycopene. This study validates the ORAC assay for different concentrations of lycopene in the presence of β-cyclodextrin, a water-solubility enhancer. Lyc-O-Mato 6% extract was used as a source of lycopene for these experiments. Lycopene was extracted according to a standard spectrophotometric assay procedure in the presence of β-cyclodextrin at concentrations of 0, 0.4, 0.8, and 1.6%, and the antioxidant activity of lycopene was measured with the ORAC assay. Experiments were conducted in quadruplicate and statistical pooled correlations analyzed. Statistical analysis showed a very high correlation ($R^2 = 0.99$) between ORAC and ascorbic acid concentrations, validating this method. Lycopene concentration correlated poorly with ORAC ($R^2 = 0.33$) in the absence of β-cyclodextrin. Correlations improved with increasing levels of β-cyclodextrin ($R^2 = 0.58$ and 0.91 for 0.4 and 0.8% β-cyclodextrin, respectively). A very high β-cyclodextrin concentration (1.6%) decreased the correlation between ORAC and lycopene concentration. Inclusion of β-cyclodextrin in the ORAC assay improves correlation between ORAC and lycopene concentration, thus expanding the scope of the ORAC assay to include an additional fat-soluble antioxidant.

KEYWORDS: ORAC; lycopene; lipophilic antioxidants; β-cyclodextrin

Lycopene, a lipophilic antioxidant, may play a number of key health-promoting roles in the human body. Among these are cancer prevention, reduction of cardiovascular risks, and regulation of the immune system (*1*−*3*). Currently, the most extensive use of lycopene is as a coloring agent for the food, cosmetic, and pharmaceutical industries (*4*). However, the high antioxidant activity and bioavailability of lycopene make it very attractive as an ingredient in the functional food and nutraceutical products that are increasingly popular in today's food marketplace. Potential sources of lycopene and the levels present in different foods have been previously tabulated (*5*). The technology exists to extract and purify lycopene for use as a functional food ingredient. However, further research is needed to correlate lycopene concentration in a food product with antioxidant activity. Such knowledge is critical for determining effective and economical rates of lycopene addition. One standardized and well-established method for analyzing antioxidant activity as a function of concentration is the oxygen radical absorbance capacity (ORAC) assay. In the past, this method has been largely limited to water-soluble antioxidants. Modifying this method to work with lycopene and perhaps other fat-soluble antioxidants provides a powerful and convenient tool for quantifying the effectiveness of lycopene as an antioxidant in foods.

The ORAC assay originally was developed by Cao et al. (*6*). Ou et al. (*7*) enhanced the economy and repeatability of the method by substituting fluoresein for the phycoerythrin originally used as a florescent probe. This enhanced assay was termed $ORAC_{FL}$. Both the ORAC and $ORAC_{FL}$ assays employ hydrophilic reagents in an aqueous system. Even so, a number of recent studies have tested the suitability of this assay for use with lipophilic antioxidants. The basic challenge to be overcome when using the $ORAC_{FL}$ assay with lipophilic antioxidants is poor solubilization or dispersion of the antioxidant, resulting in poor assay validity. The use of cyclodextrins as solubility enhancers was first described by Szente et al. (*8*) during studies on fatty acid compounds. Cyclodextrins are ring molecules built from α-D-glucose units, and on the basis of the number of glucose units, they are named α-, β-, or γ-cyclodextrins. These doughnut-shaped compounds have the potential to bind a wide array of organic compounds into their central nonpolar cavities through hydrophobic interactions. Further extensive studies (*9*, *10*) on the application of cyclodextrins to carotenoid and fatty acid systems were carried out, and various potential cyclodextrin derivatives such as methylated β-cyclodextrin (β-CD), hydroxyl

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10.1021/jf048258m CCC: $30.25
Published on Web 02/09/2005

propylated β-cyclodextrin, and branched β-cyclodextrin were identified that improved the solubilization of lipophilic compounds. Recently, the $ORAC_{FL}$ assay has been adapted by Huang et al. (*11*) for use with vitamin E and other phenolic antioxidants using randomly methylated β-cyclodextrin as a solubility enhancer. In theory, these lipophilic antioxidants present solubility or dispersion problems similar to those of lycopene. Thus, a modified ORAC assay could potentially meet the need for an effective and reliable method for measuring the antioxidant activity of lycopene in foods. The goal of this study was to correlate the antioxidant activity of lycopene with lycopene concentration using the $ORAC_{FL}$ as modified by the use of β-cyclodextrin as a solubility enhancer.

MATERIALS AND METHODS

Reagents. Fluorescein, Trolox, β-CD, ascorbic acid, potassium phosphate, sodium phosphate, and sodium azide were purchased from Sigma (St. Louis, MO). 2,2′-Azobios(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals (Richmond, VA). Lyc-O-Mato 6% extract was obtained from LycoRed Natural Products Industries Ltd. (Beer-Sheva, Israel) as a source of lycopene for our experiments. Preliminary testing showed that the fluorescein used in our experiments underwent a decay of >90% fluorescence within 45 min in the presence of 0.332 g of AAPH in 10 mL of phosphate buffer (PB).

Lycopene Determination. Concentration of lycopene in the Lyc-O-Mato preparation was determined using a modification of the method of Sadler et al. (*12*). Acetone (25 mL), ethanol (25 mL), hexane (50 mL), and the lycopene concentrate were mixed in a foil-covered Erlenmeyer flask. An aqueous β-cyclodextrin solution (15 mL at 0, 0.4, 0.8, or 1.6%) was then added, the flask was sealed, and the mixture was agitated at ∼25 °C in a constant-temperature water bath/shaker for 15 min. The solution was allowed to equilibrate at room temperature for 15 min, and then ∼3 mL of the top hexane layer was sampled for spectrophotometric analysis. Absorbance at 503 nm was read on a Beckman DU series 500 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) with a 4.5 mL glass cuvette. Lycopene concentration was calculated as

$$C = (Ab_{503}/172) \times (50) \times (535.85) \times (1/SW)$$

where C = concentration of lycopene in mg/g of concentrate, Ab_{503} = absorbance of the hexane/lycopene solution measured at 503 nm, 172 = extinction coefficient of lycopene in hexane in kmol/cm, 50 = volume of hexane used for extraction in ml, 535.85 = molecular mass of lycopene in g/mol, SW = sample weight of lycopene concentrate in mg.

The actual measured concentration of lycopene in the extract was ∼166 mg/g.

Lycopene Solution Preparation. Approximately 11 mg of concentrate was weighed into a 250 mL foil-covered Erlenmeyer flask and extracted according to the lycopene determination method described above, yielding a stock solution of ∼68.0 μM lycopene in hexane. This was further diluted 2, 3, 4, 5, and 10 times in hexane to obtain approximately 34.0, 22.7, 17.0, 13.6, and 6.8 μM working lycopene solutions.

β-Cyclodextrin solutions (0, 0.4, 0.8, and 1.6%) were prepared in a 1:1 water/acetone mixture (v/v) and kept on a shaker for at least 1 h with heat until the solutions were completely dissolved. These solutions were introduced into the lycopene solutions during the extraction procedure (*12*) by replacing the water with the β-cyclodextrin solutions.

Ascorbic acid solutions (40 μM) were prepared by dissolving 0.705 mg of ascorbic acid in 100 mL of PB and diluting with water to get 20, 10, and 5 μM solutions. This served as a standard to evaluate the equipment for correlation between ascorbic acid antioxidant activity and concentration.

ORAC Assay. The ORAC was conducted using a Perkin-Elmer HTS-7000 microplate reader in Falcon brand 48-well clear plates at excitation and emission wavelengths of 485 and 535 nm, respectively. The reaction temperature used was 37 °C, as described in Ou et al. (*7*). Other than the antioxidant samples, all other reagents were prepared using PB of pH 7 as a diluent. The volume and final concentrations of freshly prepared $ORAC_{FL}$ reagents used in the 48-well clear plate were as follows: fluorescein, 160 μL/(0.125 g in 1000 mL of PB → 2 mL diluted to 125 mL of PB as diluted stock FL → 2 mL diluted stock FL diluted in 19 mL of PB and stored before use); 20 μM Trolox, 20 μL (standard wells); hexane, 20 μL (blank wells); lycopene extracts with β-cyclodextrin, 20 μL (sample wells); and AAPH, 20 μL/(0.332 g in 10 mL of PB). $ORAC_{FL}$ assays were run for 45 min (or 25 cycles) to generate relative fluorescence profiles over time. The analyzer was preset to record fluorescence every minute after the addition of AAPH. Results were expressed as relative fluorescence with respect to the initial reading. Final computation of results was made by taking the differences of areas under the decay curves between blank and sample and/or standard (Trolox). The assay was repeated for each of the five tested lycopene concentrations (34.0, 22.7, 17.0, 13.6, and 6.8 μM) using three β-cyclodextrin levels (0, 0.4, 0.8, and 1.6%), and all assays were replicated four times. Average values were calculated for the four replicates and average ORAC values expressed as micromoles of Trolox equivalent (TE). During all experiments, the time gap between the addition of AAPH and the start of fluorescence reading on the assay reader was kept as short as possible (∼15−20 s).

Experimental Design and Statistical Analysis. Our experiment was designed as a completely randomized design with a factorial treatment structure [3 × 5 (five levels of lycopene concentration by three levels of added β-cyclodextrin]. Experiments were conducted in quadruplicate; all extractions and analyses were replicated four times. For each replication, results from multiple $ORAC_{FL}$ assays were averaged. Means and standard deviations for $ORAC_{FL}$ values and Pearson's correlation coefficients for $ORAC_{FL}$ values and lycopene concentration were calculated using SAS (SAS, v. 7, Cary, NC, 1999) General Linear Means (GLM) and Means procedures. Means were tested for significant differences using Duncan's means separation test within the GLM procedure. Regression analysis was conducted, and linear model equations were generated using the SAS Regression procedure.

RESULTS AND DISCUSSION

Method Validation for Linearity. With ascorbic acid as a standard antioxidant, relative fluorescence (RF) trends were profiled and correlations obtained between ascorbic acid concentration and $ORAC_{FL}$ values. As in other studies, we observed a very high correlation (R^2 = 0.983) between ascorbic acid concentration and $ORAC_{FL}$ values, thereby validating our equipment and techniques (data not shown).

Stability of Lycopene. $ORAC_{FL}$ assays were run using both lycopene and, for comparison purposes, α-tocopherol without added AAPH to quantify possible intrinsic effects of our lipophilic antioxidant preparations on the oxidative decay of fluorescein. Lycopene concentration was also measured over time using the spectrophotometric method to quantify the inherent oxidative stability of our lycopene extracts over the time frame of a typical ORAC assay.

Figure 1 shows the RF versus time profiles obtained for ORAC assays conducted without the radical generator AAPH added. For assays containing lycopene, the net degradation seen over the course of an experimental run was ∼8.5%, and this remained consistent over several experiments. Compared to both blank and α-tocopherol samples, lycopene samples exhibited a slightly higher degree of degradation (∼8.5 versus <4.5%), but the relative fluorescence in all samples decayed by <10% during the 45 min ORAC assay period. Therefore, we concluded that lycopene did not itself significantly influence the oxidative decay of fluorescein in the absence of AAPH.

Results of spectrophotometric quantification of lycopene concentration in prepared extracts over time showed that degradation of lycopene during the time frame of our ORAC assay was insignificant (data not shown).

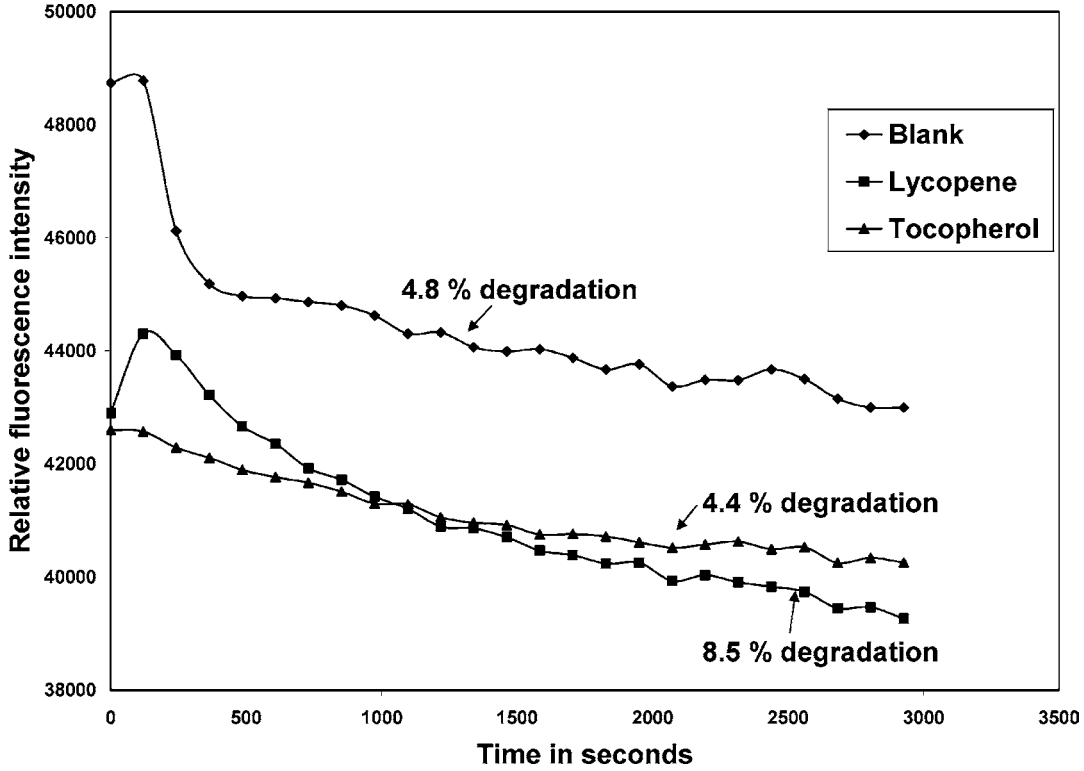

Figure 1. Fluorescence degradation kinetic profiles for lycopene and α-tocopherol without AAPH.

Table 1. Mean and Standard Deviation (SD) Information for Lycopene Concentration versus $ORAC_{FL}$ Values Obtained at Different Concentrations of Added β-Cyclodextrin (CD)

added CD concn (%)	lycopene concn (μM)	mean ORAC value (equiv μmol of Trolox)	SD of ORAC value	% rel error of ORAC value	*n*
0	6.8	0.523	0.0949	34.9	4
	13.6	0.675	0.1904	15.3	4
	17.0	0.846	0.3469	46.9	4
	22.7	0.902	0.0634	30.7	4
	34.1	0.977	0.1097	23.3	4
0.4	6.8	0.290	0.0360	20.67	4
	13.6	0.378	0.0325	21.8	4
	17.0	0.408	0.0249	6.1	4
	22.7	0.462	0.1007	18.6	4
	34.1	0.464	0.0145	14.7	4
0.8	6.8	0.310	0.0229	29.2	4
	13.6	0.350	0.0001	22.5	4
	17.0	0.395	0.0227	20.9	4
	22.7	0.408	0.0364	26.9	4
	34.1	0.492	0.0319	12.9	4

Lycopene Concentration and $ORAC_{FL}$ Correlation. Table 1 gives the mean and standard deviation information for lycopene concentration versus $ORAC_{FL}$ values obtained at different levels of β-cyclodextrin. The relative antioxidant activities of lycopene extracted with various levels of β-cyclodextrin, expressed as RF versus time, are shown in **Figures 2−4**. We observed a similar pattern in all of these profiles; an increase in lycopene concentrations reduced the rate of decay in the RF profiles. The pooled correlation of $ORAC_{FL}$ and lycopene concentration with β-cyclodextrin at 0, 0.4, and 0.8% is shown in **Figure 5**. Lycopene extracts had a very poor correlation with $ORAC_{FL}$ values in the absence of β-cyclodextrin ($R^2 = 0.331$). With inclusion of β-cyclodextrin at 0.4%, the correlation improved significantly ($R^2 = 0.58$, $p < 0.001$). This trend continued with increased β-cyclodextrin levels up to 0.8% ($R^2 = 0.91$, $p < 0.001$). At a β-cyclodextrin addition level of 1.6%, the correlation between lycopene concentration and $ORAC_{FL}$ declined significantly ($R^2 = 0.72$, data not shown). When averaged over all lycopene concentrations, there was no significant difference ($p > 0.05$) between mean $ORAC_{FL}$ values in the presence of 0.4 and 0.8% added β-cyclodextrin (0.0120 and 0.0077 equiv μmol of Trolox, respectively). However, mean $ORAC_{FL}$ values obtained in the absence of added β-cyclodextrin (029407 equiv μmol of Trolox) were significantly different ($p < 0.05$) from those measured when β-cyclodextrin was added.

Our demonstrated optimum level of β-cyclodextrin addition corresponded with the practical upper limit of β-cyclodextrin solubility that we observed. There was no problem dissolving the β-cyclodextrin at 0.4% in a water/acetone mixture with simple agitation; heat was required in addition to agitation at the 0.8% level, and at higher levels some degree of haze or sedimentation was seen.

One interesting result observed was that although the correlation between lycopene concentration and $ORAC_{FL}$ values improved significantly with the addition of β-cyclodextrin, overall relative fluorescence decay rates also increased, particularly at the higher lycopene concentrations (**Figures 2−4**). Previous studies have not shown that β-cyclodextrin functions as a pro-oxidant (*11*). This was supported by our observation of a proportionally greater change in decay rates between 0 and 0.4% β-cyclodextrin than between 0.4 and 0.8% β-cyclodextrin. Therefore, it seems likely that the apparent change in the rate of fluorescein oxidation was a result of the more complete dispersal of lycopene in the solution brought about by the addition of β-cyclodextrin. It may be that, in the absence of β-cyclodextrin, the nonpolar lycopene was forming micelles that served to entrap and protect the fluorescein from oxidation. This would explain both erratic and erroneously high $ORAC_{FL}$ values in the absence of β-cyclodextrin.

Our results demonstrating the effectiveness of β-cyclodextrin as a solubility enhancer for lycopene are similar to results

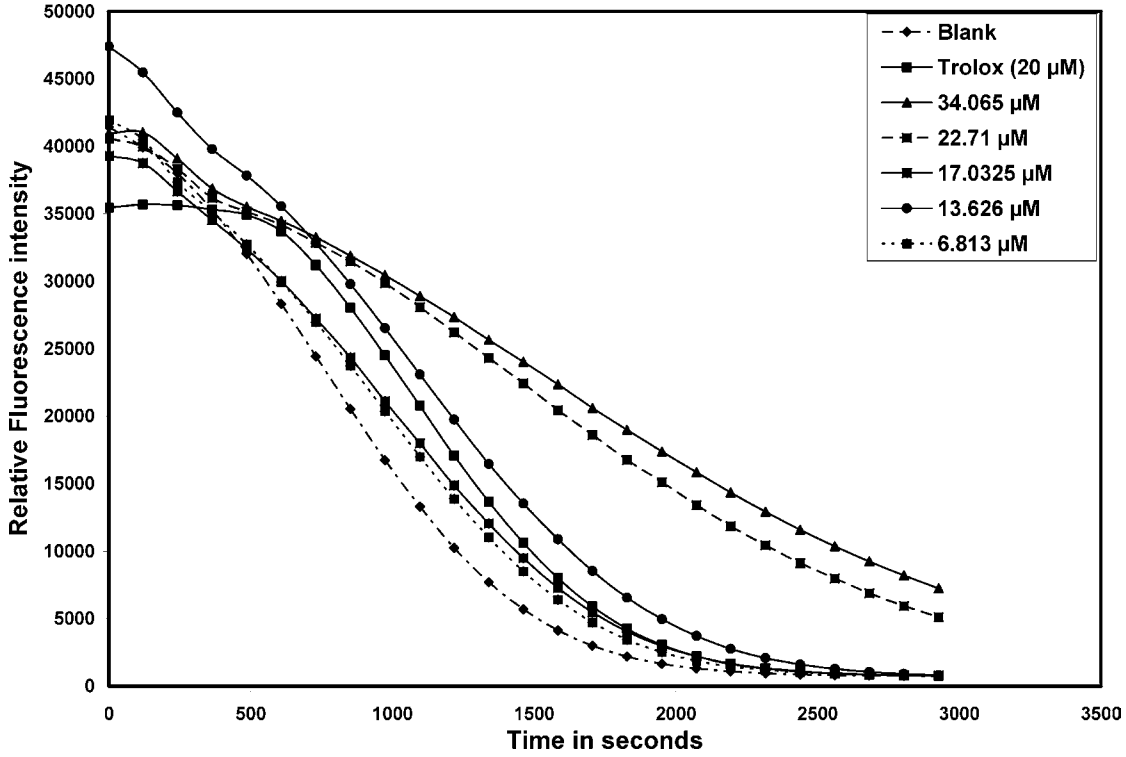

Figure 2. Fluorescence decay profiles induced by AAPH for lycopene at different concentrations in the absence of β-cyclodextrin.

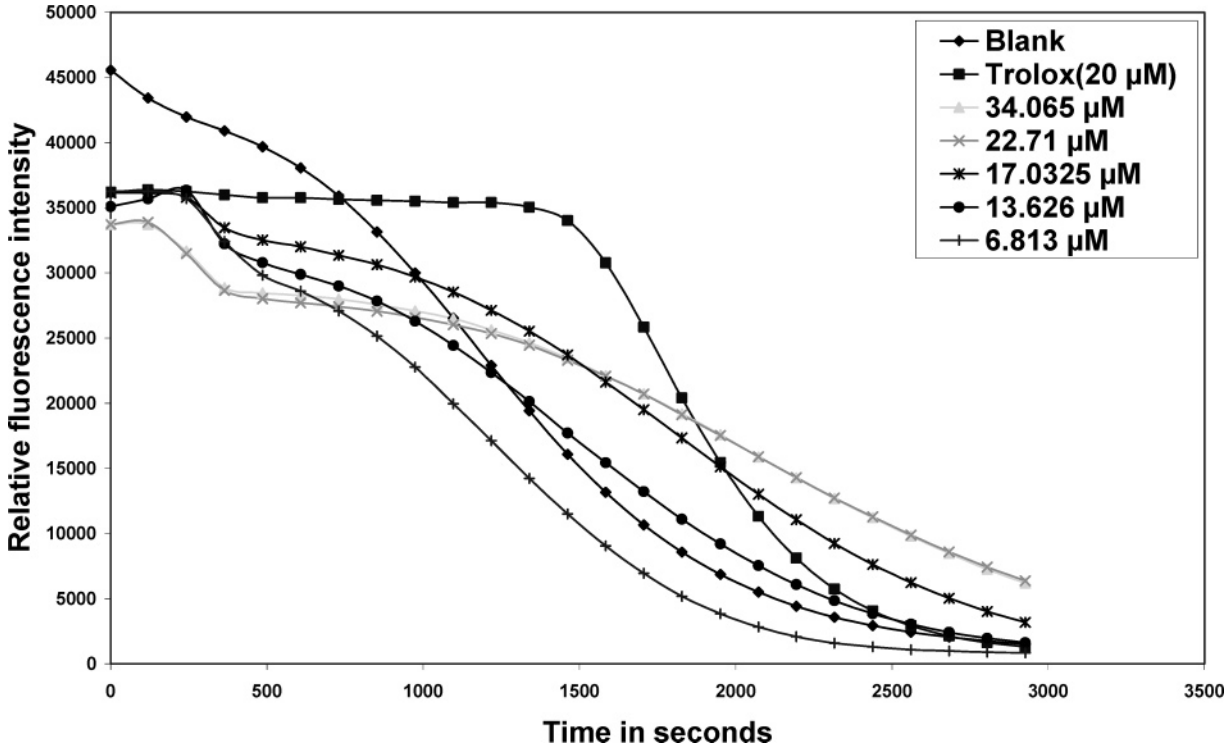

Figure 3. Fluorescence decay profiles induced by AAPH for lycopene at different concentrations in the presence of 0.4% β-cyclodextrin.

reported by other researchers using other lipophilic antioxidant compounds. Huang et al. (*11*) saw similar effects using randomly methylated β-cyclodextrin [7% in a (1:1) water/acetone mixture and in a 75 mM phosphate buffer at pH 7.4] as a solubility enhancer in $ORAC_{FL}$ assays on the lipophilic phenolic compounds α-tocopherol, δ-tocopherol, γ-tocopherol, and γ-oryzanol. Very high correlations ($R^2 > 0.97-0.99$) were reported for these compounds in the presence of β-cyclodextrin. This study was a sequel to that of Szente et al. (*9*), who ranked the solubilizing effectiveness of β-cyclodextrin and its derivatives in the following order: methylated β-cyclodextrin ≫ hydroxypropylated β-cyclodextrin = branched β-cyclodextrin. Similar studies on the effectiveness of β-cyclodextrin in solubilizing carotene/fatty acid compounds have been conducted with comparable results (*8*−*10*). Szente et al. (*9*) found that randomly methylated β-cyclodextrin added at a concentration of 10−40% enhanced the aqueous solubility of lipophilic antioxidants by as much as 1000-fold.

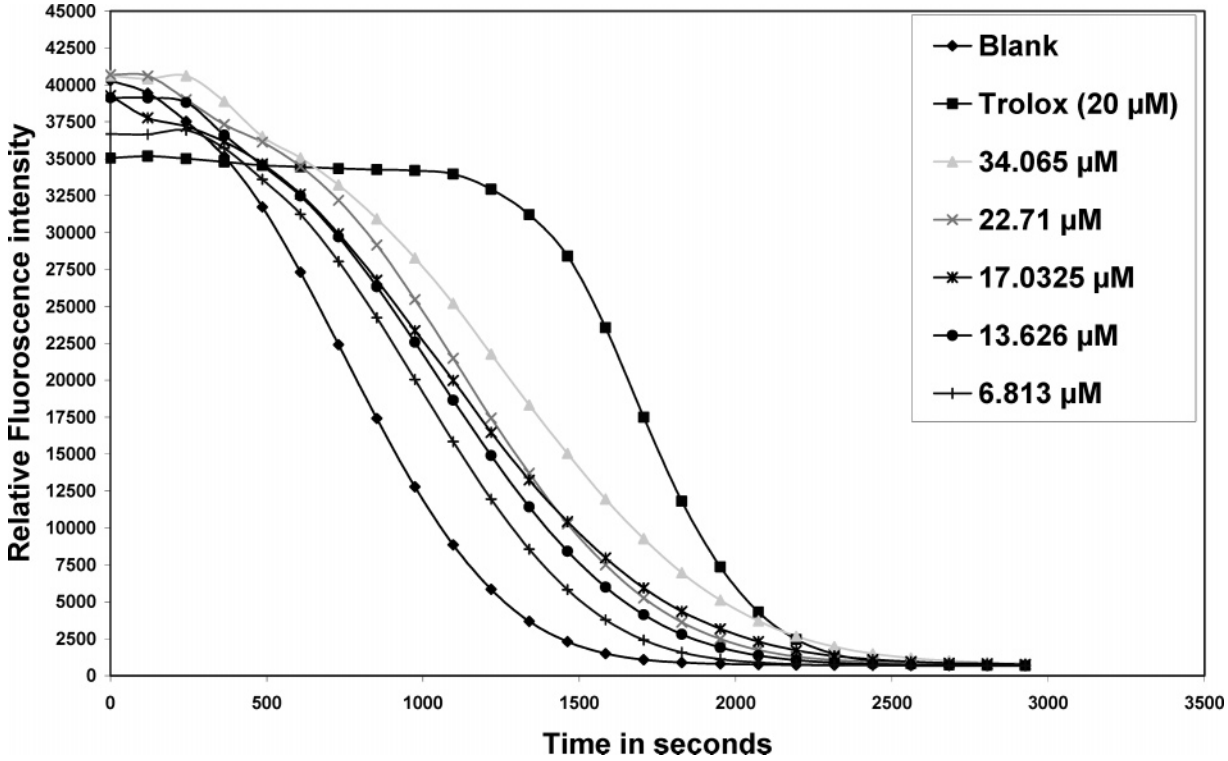

Figure 4. Fluorescence decay profiles induced by AAPH for lycopene at different concentrations in the presence of 0.8% β-cyclodextrin.

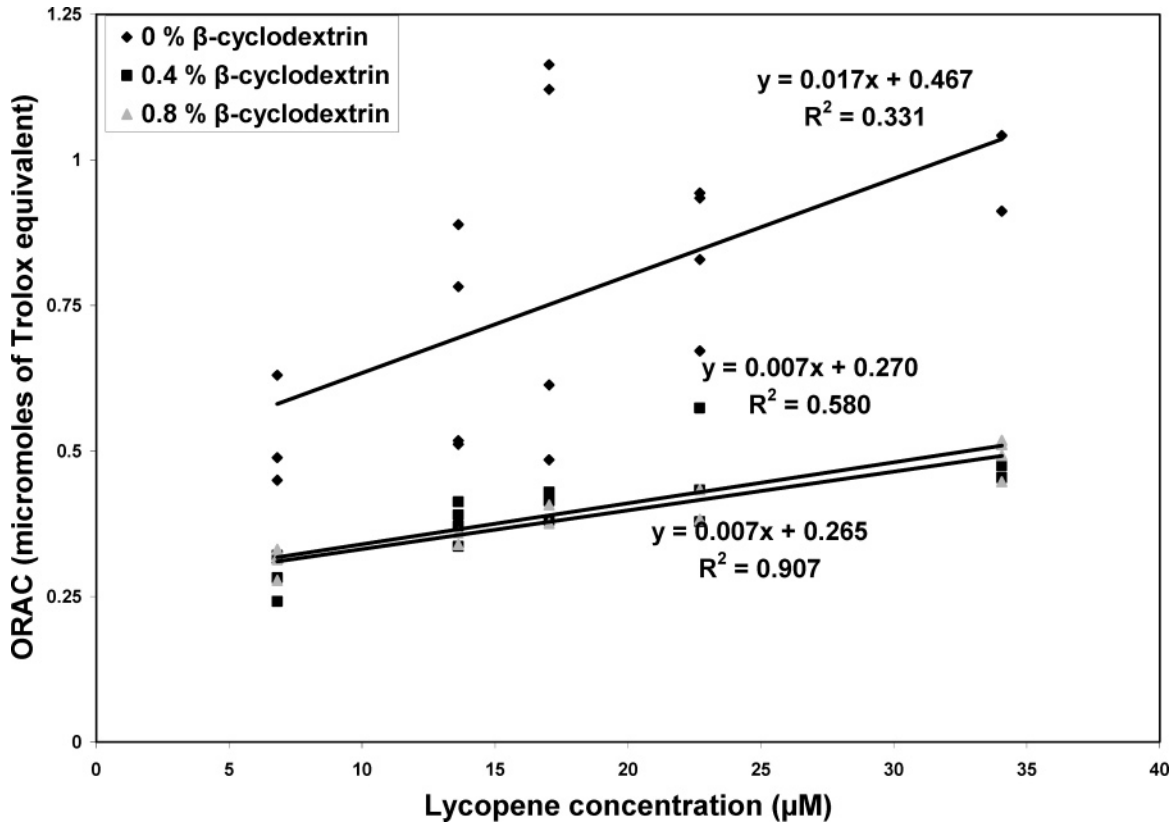

Figure 5. Lycopene and ORAC correlation derived for lycopene extractions with different levels of β-cyclodextrin.

Conclusion. Our results demonstrate that inclusion of β-cyclodextrin in the $ORAC_{FL}$ assay improves the correlation between $ORAC_{FL}$ and lycopene concentration, thus expanding the scope of the assay to include fat-soluble antioxidants. From our studies we infer that ~0.8% β-cyclodextrin is optimal for obtaining the highest correlation between $ORAC_{FL}$ and lycopene concentration. This opens up avenues for using the $ORAC_{FL}$ assay as a tool for evaluating lycopene antioxidant activity, thus enabling us to better understand the relationship between lycopene concentration and antioxidant activity in various food systems. Avenues for continued research include evaluating the effectiveness of randomly methylated β-cyclodextrin as a

lycopene-solubilizing agent and attempting to correlate $ORAC_{FL}$ values with lipophilic antioxidant compound concentrations in actual foodstuffs.

ACKNOWLEDGMENT

We thank the Department of Horticulture and Landscape Architecture and the Food and Agricultural Products Research and Technology Center for facilitating this research.

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Received for review October 20, 2004. Revised manuscript received January 3, 2005. Accepted January 5, 2005.

JF048258M