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ORAC-Fluorescein Assay To Determine the Oxygen Radical Absorbance Capacity of Resveratrol Complexed in Cyclodextrins

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The effect of the complexation of resveratrol with hydroxypropyl-β-cyclodextrins (HP-β-CDs) on the antioxidant capacity of the polyphenol is studied for the first time by means of the oxygen radical absorbance capacity (ORAC) method, using fluorescein (FL) as the fluorescent probe. The method is validated through its linearity, precision, and accuracy for measuring the ORAC of resveratrol in the absence or presence of cyclodextrins (CDs). The complexation of resveratrol in CDs increased the net area under the FL decay curve (net AUC) of resveratrol up to its saturation level, at which the polyphenol showed almost double the antioxidant activity it shows in the absence of CDs. The complexation constant (K_c) between resveratrol and HP-β-CDs was calculated by linear regression of the phase solubility diagram ($K_c = 18048\ M^{-1}$). The antioxidant activity of resveratrol was dependent on the complexed resveratrol because CDs acts as a controlled dosage reservoir that protects resveratrol against rapid oxidation by free radicals. In this way, its antioxidant activity is prolonged and only reaches its maximum when all the resveratrol is complexed.

KEYWORDS: Resveratrol; cyclodextrin; ORAC assay; antioxidant; fluorescein

INTRODUCTION

Resveratrol (*trans*-3,5,4′-trihydroxystilbene) is a natural polyphenol found in a variety of plants, for example in grapes and peanuts (*1*), where it is produced during environmental stress or pathogenic attack (*2*). It is found principally in the skins and seeds of grape berries and therefore usually occurs in greater concentrations in red than in white wines due to skin contact during fermentation.

This phytoalexin has been reported to have antiplatelet (*3*), anti-inflammatory (*4, 5*), and anticarcinogenic effects (*6*). The various activities of resveratrol have been attributed to its antioxidant properties (*7*), which contribute to controlling the intracellular redox balance by inhibiting the formation of reactive oxygen species (*6*). The phenolic nature of resveratrol explains its antioxidant activity.

The high hydrophobicity of resveratrol and its sensitivity to external agents such us air, light, and oxidative enzymes may constitute a serious problem for its bioavailability in the formulation and manipulation of functional foods.

In recent years, cyclodextrins (CDs) complexation has been successfully used to improve the solubility, chemical stability and bioavailability of a number of poorly soluble compounds. CDs are a group of naturally occurring cyclic oligosaccharides derived from starch with six α-cyclodextrins, seven β-cyclodextrins, or eight γ-cyclodextrins glucose residues, linked by $\alpha(1\rightarrow4)$ glycosidic bonds (*8*). In the pharmaceutical, cosmetics and food industries, cyclodextrins have been used as complexing agents to increase the water solubility of various compounds, such as drugs, vitamins, and food colorants, increasing the solubility, stability, and bioavailability of the guest molecule (*9–11*).

As has been recently described by our group, CDs can be used as resveratrol complexation agent, not only to increase the total resveratrol concentration in aqueous solution, while the free resveratrol concentration remains constant, but also to decrease the free resveratrol concentration in aqueous solution, while the total concentration remains constant (*12*). In both cases, CDs acts as substrate reservoir in a dosage-controlled manner.

The complexation of resveratrol by CDs delays resveratrol enzymatic oxidation (*12*), but it is not known whether entrapment in the internal cavity of CDs affects the antioxidant capacity of the polyphenol.

There are several methods available to determine antioxidant activity in vitro (*13*). However, the oxygen radical absorbance capacity (ORAC) method, adapted to use fluorescein (FL) as fluorescent probe, is increasingly used for this purpose in biological samples and foods. The ORAC method is based on inhibition of the peroxyl-radical-induced oxidation initiated by thermal decomposition of azo-compounds, like 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), and is the only

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method that combines both inhibition time and inhibition degree into a single quantity (*14*).

In the present paper, the effect of hydroxypropyl-β-cyclodextrins (HP-β-CDs) on the antioxidant capacity of resveratrol against reactive oxygen species (ROS) is studied by ORAC-fluorescein (ORAC-FL) method (*15*). The antioxidant capacity of resveratrol in aqueous solution in the absence and presence of CDs has been studied for the first time, using an ORAC-FL assay adapted to manual handling and a conventional fluorescence plate reader.

MATERIAL AND METHODS

Chemicals. FL, AAPH, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox C), and resveratrol were purchased from Sigma (Madrid, Spain). HP-β-CDs were from TCI (Europe). All other chemicals used were of analytical grade.

An FL stock solution (4 μM) was made in 75 mM sodium phosphate buffer (pH 7.4) and was stored at −20 °C for 4 weeks. An FL solution was prepared daily in 75 mM sodium phosphate buffer (pH 7.4) by diluting the stock FL to a final concentration of 6 nM. Solutions of 0.25 mM Trolox C and 15 μM resveratrol in 75 mM sodium phosphate buffer (pH 7.4) were prepared and aliquoted into small vials for storage at −80 °C until use. A 127 mM AAPH solution in 75 mM sodium phosphate buffer (pH 7.4) was prepared daily.

ORAC-FL Assay. The ORAC analyses were carried out on a Synergy HT multidetection microplate reader, from Bio-Tek Instruments, Inc. (Winooski, VT), using 96-well polystyrene microplates with black sides and clear bottom, purchased from Nalge Nunc International. Fluorescence was read through the clear bottom, with an excitation wavelength of 485/20 nm and an emission filter of 528/20 nm. The plate reader was controlled by KC4, version 3.4, software. The oxygen radical absorbance capacity was determined as described by Dávalos et al. (*16*) with slight modifications. The reaction was carried out in 75 mM sodium phosphate buffer (pH 7.4), and the final reaction mixture was 200 μL. FL (100 μL; 3 nM, final concentration) and resveratrol in the absence or presence of HP-β-CDs (70 μL) solutions were placed in the wells of the microplate. The mixture was preincubated for 30 min at 37 °C, before rapidly adding the AAPH solution (30 μL; 19 mM, final concentration) using a multichannel pipet. The microplate was immediately placed in the reader and the fluorescence recorded every 1.14 min for 120 min. The microplate was automatically shaken prior to each reading. A blank with FL and AAPH using sodium phosphate buffer instead of the antioxidant solution and eight calibration solutions using Trolox C (6.25, 12.5, 15, 18.75, 21.25, 25, 27.5, and 31.25 μM) as antioxidant were also used in each assay. All reaction mixtures were prepared in triplicate, and at least three independent assays were performed for each sample. In order to avoid a temperature effect, only the inner 60 wells were used for experimental purposes, while the outer wells were filled with 200 μL of distilled water.

The results were expressed as relative fluorescence with respect to the initial reading. The area under the fluorescence decay curve (AUC) was calculated by the equation

$$\mathrm{AUC} = 1 + \sum_{i=1.14}^{i=120} \frac{f_i}{f_0} \qquad (1)$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i. The net AUC corresponding to the sample was calculated by subtracting the AUC corresponding to the blank. Data processing was performed using Sigmaplot software package (Jandel Scientific, German).

Phase Solubility Diagram. A phase solubility diagram was constructed according to Higuchi and Connors (*17*). Excess amounts of resveratrol were added to aqueous solutions containing increasing concentrations HP-β-CDs (up to 100 mM) in 10 mL of 75 mM sodium phosphate buffer (pH 7.4) at 25 °C. The samples were placed in an ultrasonic bath for 60 min to reach equilibrium. The aqueous solutions were filtered through a 0.2 μm cellulose membrane filter and diluted in 80% ethanol–water. The resveratrol (ϵ_{312} = 28 993 M^{-1} cm^{-1}) concentration was spectrophotometrically determined in a Shimadzu model UV-1063 spectrophotometer.

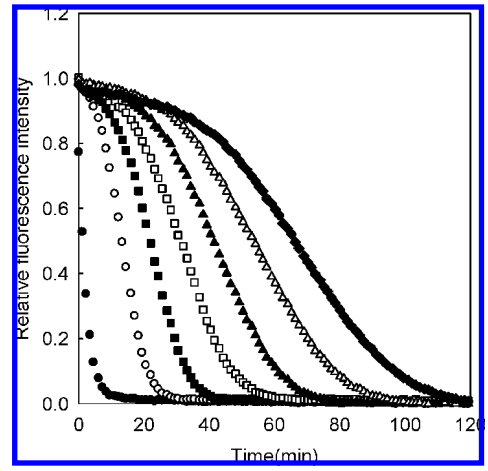

Figure 1. FL fluorescence decay curves induced by AAPH in the presence of resveratrol at different concentrations: blank (●), resveratrol: 0.3 μM (○), 0.5 μM (■), 0.75 μM (□), 1.12 μM (▲), 1.5 μM (△) and 2.02 μM (◆).

RESULTS AND DISCUSSION

The purpose of this paper is to demonstrate the effect of the inclusion of resveratrol in CDs on the antioxidant activity of resveratrol using the ORAC-FL method.

Method Validation. The fluorescence signal of FL was constant for at least 120 min when no AAPH was added, indicating that FL was photostable under the excitation conditions used (data not shown).

Linearity. Figure 1 shows the FL fluorescence decay curves in the presence of resveratrol and AAPH. The linear relationship between net area and antioxidant concentration was calculated using Trolox C (**Figure 2A**) and resveratrol (**Figure 2B**) at different concentrations. The regression analysis in **Figure 2B** points to the linear response between the resveratrol concentration and the net AUC, yielding the following equation:

$$y = 0.664 + 17.1097x \qquad (2)$$

with a correlation coefficient (r^2) of 0.998 (**Table 1**).

Limit of Quantitation (LOQ) and Limit of Detection (LOD). The limit of detection or least detectable dose (LOD) is the smallest concentration of the analyte that produces a signal significantly different from zero with a stated degree of confidence. There is a general consensus in favor of selecting the analyte dose providing 3 times the standard deviation (SD) from the mean measurement of the blank dose signal. The LOD was estimated by analysis of five sets of 12 replicates of the zero standards (*18*). The mean absorbance value plus 3-fold SD corresponded to an estimated limit of detection of 0.042 μM of resveratrol.

The limit of quantification (LOQ) is the smallest concentration of analyte that can be measured in samples so as to yield a predicted concentration with a stated relative precision and accuracy (*19*). Commonly, the selected LOQ is defined by the mean absorbance value plus 10-fold SD. The LOQ was calculated on the basis of the analysis of 20 samples with 0.141 μM of resveratrol (data not shown). The mean concentration determined for the 20 samples was 0.15 μM with a SD of 0.007 μM (**Table 1**).

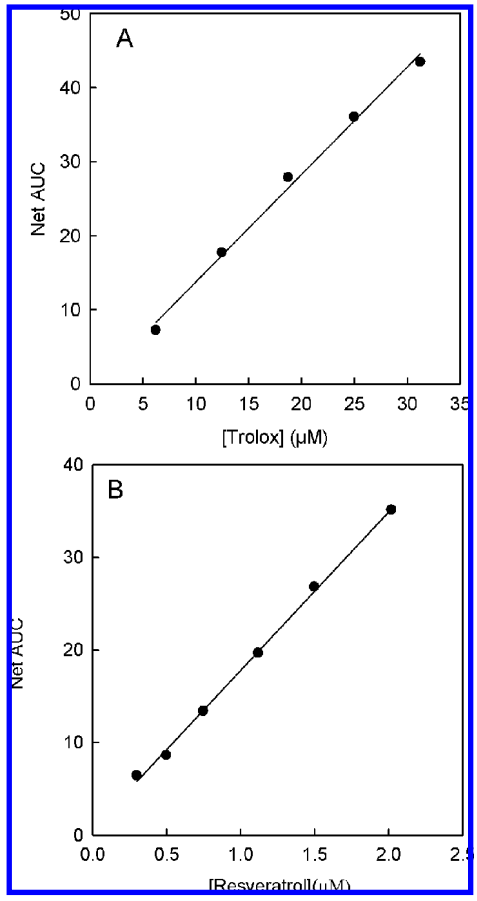

Figure 2. (**A**) Regression of net AUC of Trolox C on different concentrations of Trolox C. The net AUC = $AUC_{sample} - AUC_{blank}$. (**B**) Regression of net AUC of resveratrol on different concentrations of resveratrol. The net AUC = $AUC_{sample} - AUC_{blank}$.

Table 1. Values of More Representative Validation Parameters

linearity (μM)	LOD (μM)	LOQ (μM)	precision (%) interday	precision (%) intraday	ruggedness (%)
0.25–2.0	0.042 ± 0.004	0.15 ± 0.007	11	3	7.6

Precision. Assay precision was tested by repeatability (intraday variation) and reproducibility (interday variation) studies. Six samples were fortified at 0.5 μM, and the resveratrol concentration was measured six times (by triplicate), on the same day (repeatability) and of five different days (reproducibility). The SD and intra and interday coefficient of variation (% CV) of the ORAC values were calculated giving values of 3% and 11%, respectively (**Table 1**).

Ruggedness. To determine the ruggedness of the method, 1.5 μM of resveratrol was analyzed for 30 days, during which time the net AUC presented a CV of 7.6% (data not shown), confirming the reliability of the assay (**Table 1**).

Antioxidant Capacity of Resveratrol in the Presence of CDs. When the decay curves of FL in the presence of resveratrol alone were compared with those in the presence of HP-β-CDs 2 mM, an increase in the area under the curve was observed (**Figure 3A**). As can be seen in **Figure 3A**, the slope of the curves from resveratrol alone [0.75 μM (○) or 1.5 μM (□)] were higher than those for resveratrol–CDs complex [0.75 μM (●) or 1.5 μM (■)]. This fact originated an increase in the lasting time of its activity.

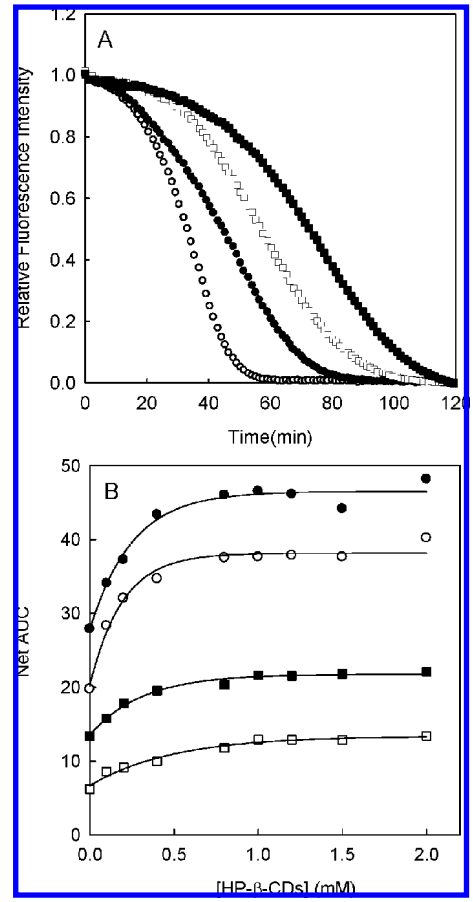

Figure 3. (**A**)FL fluorescence decay curves induced by AAPH in the presence of resveratrol alone, (0.75 μM (○) and, 1.5 μM ()) and with HP-β-CDs 2 mM (resveratrol 0.75 μM (●) and, resveratrol 1.5μM (■)). (**B**) Effect of HP-β-CDs concentration on the net AUC of resveratrol 1.5 μM (●), 1.12 μM (○), 0.75 μM (■) and 0.5 μM (□).

When increasing concentrations of HP-β-CDs were added to the reaction medium at different resveratrol concentrations (0.5, 0.75, 1.12, and 1.5 μM), a clear increase in the antioxidant activity was observed in all cases (**Figure 3B**). It was not possible to measure the net AUC of resveratrol concentrations higher than 1.5 μM in the presence of CDs because the measure times exceeded 2 h, established as optimum for the ORAC assay. As can be seen in **Figure 3B**, when HP-β-CDs increased, the net AUC of resveratrol also increased up to saturation at (0.4 mM) HP-β-CDs. At the saturation level, resveratrol showed almost double the antioxidant activity in the presence than in the absence of CDs in all cases studied (**Figure 3B**). This effect on antioxidant activity may be due to the formation of inclusion complexes between resveratrol and HP-β-CDs. Indeed, such complexation has recently been demonstrated for β-CDs and maltosyl-β-CDs (G_2-β-CDs) by our group (*12*). From these results, it was possible to deduce that the OH group of monophenolic ring of resveratrol is implied in the complexation process, because of the increase in antioxidant activity observed after complexation process and the decrease observed in the oxidation of resveratrol by lipoxygenase (*12*). Moreover, it has been described in the literature that phenolic groups are entrapped in the hydrophobic cavity of cyclodextrins.

In order to corroborate that resveratrol is complexed by HP-β-CDs in 75 mM sodium phosphate buffer (pH 7.4), and to calculate its complexation constant, K_c, a phase solubility diagram was carried out (*17*) (**Figure 4**). The linear relationship observed between HP-β-CDs and resveratrol concentration

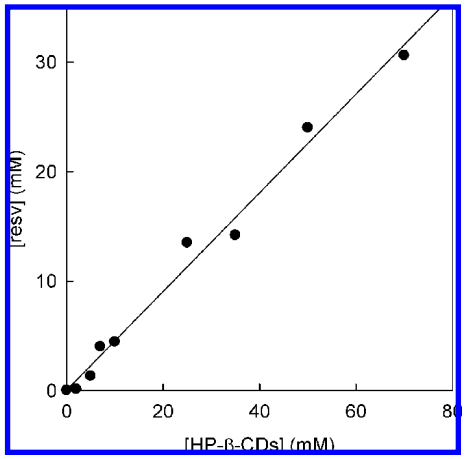

Figure 4. Phase solubility diagram of resveratrol with HP-β-CDs in sodium phosphate buffer (75 mM, pH 7.4) at 25 °C.

(**Figure 4**) corresponded to a 1:1 complex formation, so that the equilibrium between resveratrol and HP-β-CDs can be expressed as

$$[\text{resv}]_f + [CD]_f \overset{K_c}{\leftrightarrow} [\text{resv-CD}] \quad (3)$$

where the complexation constant, K_c, is defined as

$$K_c = \frac{[\text{resv-CD}]}{[\text{resv}]_f[\text{CD}]_f} \quad (4)$$

The K_c value was calculated using the equation described by Higuchi and Connors (*17*):

$$K_c = \frac{\text{slope}}{S_0(1 - \text{slope})} \quad (5)$$

where S_0 is the solubility of resveratrol in 75 mM sodium phosphate buffer (pH 7.4) ($S_0 = 45\ \mu$M). The K_c value obtained was 18 048 M^{-1}. This K_c value is much higher than that obtained for β-CDs or G_2-β-CDs (4317 and 5130 M^{-1}, respectively) (*12*).

At the concentrations studied in this paper (up to 2 μM), resveratrol is soluble in the reaction medium ($S_0 = 45\ \mu$M). So, the increase in net AUC observed in **Figure 3** with increasing CDs, can not be attributed to the solubilization of resveratrol, as has previously been described to the case of α-tocopherol (*20*) and lycopene (*21*). In the case of resveratrol, when it is complexed, HP-β-CDs acts as a reservoir of controlled substrate dosage that protects resveratrol against its rapid oxidation by AAPH radical. So, its antioxidant activity is prolonged and only reaches a maximum when all resveratrol is complexed (a minimum quantity of free resveratrol is always present because the complexation phenomenon is a dynamic equilibrium).

In order to clarify whether the increase in antioxidant activity of resveratrol is due to protection by HP-β-CDs, the free resveratrol concentration ($[\text{resv}]_f$) at each point of **Figure 3** was calculated using the K_c value previously obtained (18 048 M^{-1}) and the following equation (*12*):

$$[\text{resv}]_f = (-([\text{CD}]_t K_c - [\text{resv}]_t K_c + 1) + \sqrt{([\text{CD}]_t K_c - [\text{resv}]_t K_c + 1)^2 + 4K_c[\text{resv}]_t})/2K_c \quad (6)$$

replotting the data and data in **Figure 5**. This **Figure 5** showed that the points of different curves in **Figure 3** that reached the

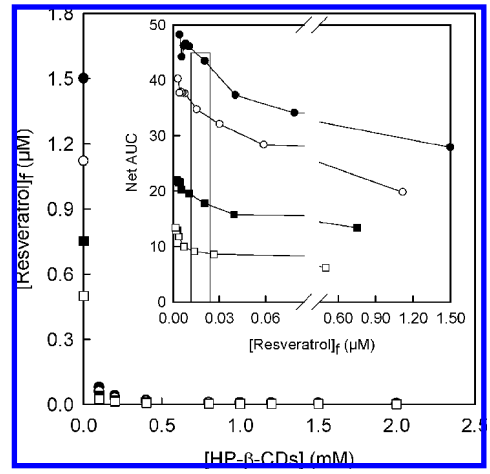

Figure 5. Effect of increasing concentrations of HP-β-CDs on free resveratrol concentration. Total resveratrol concentration: 1.5 μM (●), 1.12 μM (○), 0.75 μM (■), and 0.5 μM (□). Inset: Effect of free resveratrol concentration on net AUC. The free resveratrol concentrations were calculated from data shown in Figure 3, using eq 6 (see text for details). Total resveratrol concentration: 1.5 μM (●), 1.12 μM (○), 0.75 μM (■), and 0.5 μM (□).

maximum net AUC, independently of total resveratrol present (0.5, 0.75, 1.12, and 1.5 μM), have the same $[\text{resv}]_f$ (4 ± 0.8 nM, practically null). This result indicates that due to the high K_c value between resveratrol and HP-β-CDs (18 048 M^{-1}), when working at resveratrol concentrations below its S_0 (0.5–1.5 μM, in our case), the HP-β-CDs complexed all the resveratrol. When the $[\text{resv}]_f$ was plotted vs net AUC (**Figure 5**, inset), points with the same $[\text{resv}]_f$ did not have the same net AUC. Moreover, as free resveratrol increased, the net AUC decreased to reach the same net AUC value for resveratrol as in the absence of CDs, that is, when all the resveratrol is free. All these data point to the fact that the $[\text{resv}]_f$ is not the determinant factor of the increase in the antioxidant activity of resveratrol in the presence of CDs.

To determine whether the increase in antioxidant activity is a function of complexed resveratrol ([resv-CDs]), the concentration of [resv-CDs] was calculated at each point of **Figure 3**, using the equation

$$[\text{resv-CD}] = [\text{resv}]_t - [\text{resv}]_f \quad (7)$$

The results (data not shown), indicated that the net AUC increased as [resv-CDs] increased, until all the resveratrol was complexed. This result was obtained because the ORAC method combines both inhibition time and degree of inhibition into a single quantity.

To complete the study, an experiment was carried out in which increasing concentrations of resveratrol were added to the reaction medium at fixed HP-β-CDs concentrations (0, 0.05, 0.2, 0.4, and 2 mM), and the net AUC was measured (**Figure 6**). The results indicated that the net AUC increases with the resveratrol concentration. However, at each point of **Figure 6**, for the same resveratrol concentration, the net AUC increased as HP-β-CDs increased up to 0.4 mM, above which the HP-β-CDs concentration had no effect on the antioxidant activity of resveratrol because of all the resveratrol had already been complexed.

In order to discard any antioxidant effect of HP-β-CDs per se, the disappearance of fluorescence signal of FL by the attack of AAPH radical were measured in the presence of increasing concentrations of HP-β-CDs (in the absence of resveratrol or Trolox C) (**Figure 7**). In this case, no effect was observed as CDs

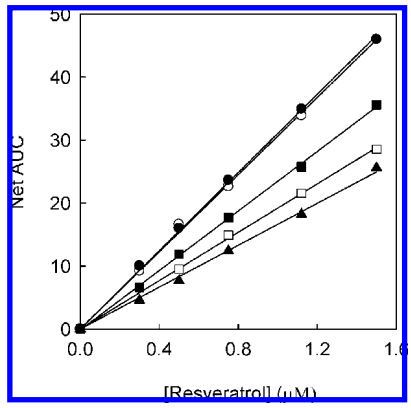

Figure 6. Effect of resveratrol concentration on the net AUC at fixed HP-β-CDs concentration: 2 mM (●), 0.4 mM (○), 0.2 mM (■), 0.05 mM (□), and 0 mM (▲).

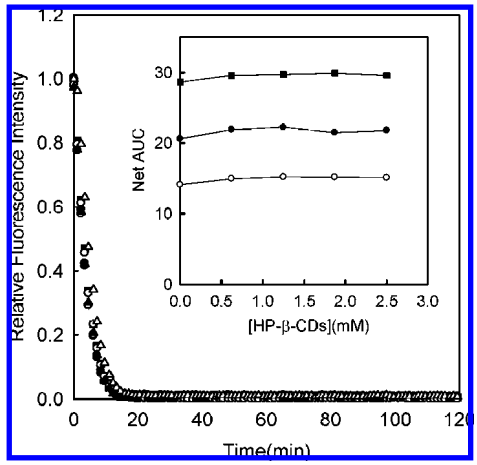

Figure 7. FL fluorescence decay curves induced by AAPH in the presence of HP-β-CDs at different concentrations: HP-β-CDS 0 mM (●), 0.2 mM (○), 0.4 mM (■), 1 mM (▲), 2 mM (△). (Inset) Effect of HP-β-CDs concentration on the net AUC of Trolox C 25 μM (■), 18.75 μM (●), and 12.5 μM (○).

concentration increased, indicating that HP-β-CDs, at the studied concentrations, do not present antioxidant effect per se.

Moreover, the antioxidant activity of Trolox C by ORAC-FL was measured in the presence of HP-β-CDs (**Figure 7**, inset). In this case, no effect was observed as CDs concentration increased, indicating that Trolox C was not complexed by HP-β-CDs and so the net AUC did not vary. This result indicates that CDs only increase the antioxidant activity of compounds that can be complexed in their hydrophobic cavity.

In conclusion, the complexation of resveratrol in HP-β-CDs increases its antioxidant activity, with CDs acting as a controlled dosage reservoir that protects resveratrol against its rapid oxidation by free radicals. In this way, its antioxidant activity is prolonged with time and reaches its maximum when all the resveratrol has been complexed.

ABBREVIATIONS USED

AAPH, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; CDs, cyclodextrins; CV, intra- and interday coefficient of variation; FL, fluorescein; G_2-β-CDs, maltosyl-β-cyclodextrins; HP-β-CDs, hydroxypropyl-β-cyclodextrins; K_c, complexation constant; LOD, limit of detection; LOQ, limit of quantitation; net AUC, net area; ORAC, oxygen radical absorbance capacity; ORAC-FL, oxygen radical absorbance capacity-fluorescein; resv, resveratrol; ROS, reactive oxygen species; SD, standard deviation.

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