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Comparative study of different methods to measure antioxidant activity of resveratrol in the presence of cyclodextrins

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ABSTRACT

The antioxidant activity of resveratrol in the absence and presence of increasing concentrations of HP-β-CDs was determined using three different methods: ORAC, ABTS and DPPH. The three methods were validated and compared for their linearity, precision and accuracy in measuring resveratrol antioxidant activity. The results indicated that the most sensitive method is the ORAC assay, which can measure the lowest resveratrol concentration (0.15–2 μM) with the highest precision. In the presence of increasing concentrations of HP-β-CDs, the antioxidant activity of resveratrol was seen to increase when it was measured by the ORAC and ABTS assays. However, no increase was observed when the DPPH assay was used. With the ORAC assay, the antioxidant activity increased until all the resveratrol had been included in HP-β-CDs (0.4 mM CDs), whereas in the case of ABTS assay the plateau in antioxidant activity was reached after 2 mM HP-β-CDs, suggesting that the CDs interferences in the measurement method. When the DPPH assay was used, no effect was observed when increasing concentrations of HP-β-CDs, indicating that in a methanolic medium resveratrol is free. Therefore, so this method cannot be used to measure the effect of resveratrol complexation with CDs on its antioxidant activity.

1. Introduction

Reactive oxygen species (ROS) are an entire class of highly reactive molecules derived from the metabolism of oxygen and, are often generated as by-products of biological reactions or by exogenous factors. *In vivo*, some of these ROS play a positive role in cell physiology; however, they may also do great damage to cell membranes and DNA, inducing the oxidation that causes membrane lipid peroxidation, decreased membrane fluidity and other DNA mutations, that may lead to cancer, degenerative diseases and others (Finkel and Holbrook, 2000).

Antioxidants are chemical substances that reduce or prevent oxidation. The growing interest in free radical biology and the lack of effective therapies for most chronic diseases have meant that the usefulness of antioxidants in protection against these diseases is increasingly studied.

Many studies have shown that phenolic compounds display antioxidant activity as a result of their capacity to scavenge free radicals (Seyoum et al., 2006). They can also act as antioxidants by chelating metal ions, preventing radical formation and improving the antioxidant endogenous system (Al-Azzawie and Alhamdani, 2006). These compounds are known to act as antioxidants not only because of their ability to donate hydrogen or electrons, but also because they are stable radical intermediates.

trans-Resveratrol (trans-3,5,4′-trihydroxystilbene) is a triphenolic phytoalexin found in a variety of plant species such as grapevines, mulberries, peanuts and the dried roots and stems of *Polygonium cuspidatum* (Japanese knotweed). Its synthesis is triggered by plant stress conditions such as fungal infection, UV irradiation, and exposure to ozone or heavy metal ions (Frémont, 2000; Sanders et al., 2000). Resveratrol has attracted considerable attention due to its cardioprotective and cancer chemopreventive activities (Jang et al., 1997), which explain the great interest shown in consuming grapes, wines and dietary products containing this phytoalexin. However, its therapeutic usefulness is limited because of its high hydrophobicity and sensitivity to external agents such as air, light and oxidative enzymes. In order to avoid this limitation, our group has recently suggested including resveratrol in cyclodextrins (CDs) (Lucas-Abellán et al., 2008b).

The growing interest in the physiological benefits of natural antioxidants has been matched by acceleration in the development of analytical and biological methodologies for measurement of both the levels and antioxidant potential of these compounds. Numerous *in vitro* studies have been conducted to evaluate the total antioxidant capacity of food products. So far, however, there is no official standardised method, and therefore it is recommended that

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each evaluation should be made with various oxidation conditions and different methods of measurement (Frankel and Meyer, 2000).

The methods most commonly used to determine the total antioxidant capacity differ in terms of their assay principles and experimental conditions, and fall into two major groups: assays based on a single electron transfer reaction, monitored through a change in colour as the oxidant is reduced, and assays based on a hydrogen atom transfer reaction, where the antioxidant and the substrate (probe) compete for free radicals (Huang et al., 2005). Electron transfer reaction assays include the Trolox equivalent antioxidant capacity (TEAC or ABTS) assay, the ferric reducing ability of plasma (FRAP) assay, the copper reduction (CUPRAC) assay, and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay. Hydrogen atom transfer reaction assays include the crocin bleaching assay, the total peroxyl radical-trapping antioxidant parameter (TRAP) assay, and the oxygen radical absorbance capacity (ORAC) assay (Huang et al., 2005; Prior et al., 2005). Of all these methods, ABTS, DPPH and ORAC are among the most widely used.

$ABTS^{\cdot+}$ and $DPPH^{\cdot}$ radicals are foreign to biological systems. The ABTS assay measures the relative ability of antioxidants to scavenge the $ABTS^{\cdot+}$ generated in an aqueous phase, compared with the Trolox standard. The method is usually expressed as Trolox equivalent antioxidant capacity (TEAC). The method is rapid and can be used over a wide range of pH values, in both aqueous and organic solvent systems (Arnao et al., 1999; Lemanska et al., 2001).

The $DPPH^{\cdot}$ is a stable free radical with an absorption band at 515 nm. This assay measures the losses in absorption when $DPPH^{\cdot}$ radical is reduced by an antioxidant or free radical species. This method is widely used to determine the antioxidant activity of purified phenolic compounds as well as natural plant extracts (Brand-Williams et al., 1995; Sripriya et al., 1996; Bondet et al., 1997; Mahinda and Shahidi, 2000; Peyrat-Maillard et al., 2000; Fukumoto and Mazza, 2000). Using this method, the antioxidant activity can be evaluated over time, because most phenolic antioxidant reacts slowly with $DPPH^{\cdot}$, reaching the steady state in 1–6 h (Bondet et al., 1997). However, like ABTS, it has limited relevance to biological systems.

The ORAC-FL assay is said to be more relevant because it uses a biologically relevant radical source (Prior et al., 2003). This method measures the ability of antioxidants to protect fluorescein (FL) from damage by free radicals. It consists of measuring the decrease in the fluorescence of FL when it suffers oxidative damage caused by a source of peroxyl radical ($ROO^{\cdot}$) such as 2,2′-azobis(2-amidino-propane) dihydrochloride (AAPH). A major advantage of ORAC is that the method is automated and largely standardised; hence, values can be easily compared across laboratories. Also, the ORAC method is reported to mimic antioxidant activity of phenols in biological systems better than other methods since it uses biologically relevant free radicals and integrates both time and the degree of activity of antioxidants (Cao et al., 1996; Cao and Prior, 2001; Ou et al., 2002).

As been recently described by our group, CDs can be used as phenolic compounds complexation agent, not only to increase the total phenolic compounds concentration in aqueous solution, while the free phenolic compounds concentration remains constant, but also to decrease the free phenolic compounds concentration in aqueous solution, while the total concentration remains constant (Lucas-Abellán et al., 2008a). In all cases, CDs acts as substrate reservoir in a dosage-controlled manner. In the case of resveratrol, its complexation with hydroxypropyl-β-cyclodextrins (HP-β-CDs) led to an increase not only in its aqueous solubility but also in its antioxidant activity (Lucas-Abellán et al., 2008b).

The aim of this paper is to compare the efficiency of ORAC ABTS and DPPH, assays for the *in vitro* evaluation of the antioxidant activity of resveratrol in the presence of CDs.

2. Materials and methods

2.1. Reagents and chemicals

Methanol (MeOH) of analytical grade was supplied by Merck (Darmstad, Germany). Mili-Q system (Millipore Corp., Bedford, MA) ultrapure was used throughout this research. Resveratrol was purchased from Sigma (Madrid, Spain). Hydroxypropyl-β-cyclodextrins (HP-β-CDs) were from TCI (Europe). Trolox (6-hidroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), 2,2′-azinobis (3-ethylbenzothialozinesulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) and fluorescein (FL) were purchased from Sigma (Madrid, Spain). All other chemicals used were of analytical grade.

2.2. DPPH assay

Samples were analysed according to the technique reported by Brand-Williams et al. (1995). The stock solution was prepared by dissolving 24 mg of DPPH with 100 mL in MeOH, and then stored at −20 °C in the dark until needed. The working solution was obtained by diluting 10 mL of stock solution with 45 mL MeOH, to obtain an absorbance of 1.1 ± 0.1 units at 515 nm, using a Shimadzu UV-1063 spectrophotometer. Briefly, a volume of 10 μL of different resveratrol concentrations, alone or in the presence of HP-β-CDs, was added to 990 μL of 0.094 mM $DPPH^{\cdot}$ in MeOH, to reach 1 mL. To determine the reaction kinetics, the assays were continuously monitored at 515 nm over a 1 h period at 25 °C. Most of the samples tested showed residual reactivity even after 1 h. However, changes in absorbance were minimal for all samples after 20 min.

The antioxidant abilities were expressed as μM Trolox equivalents by using a Trolox calibration curve prepared for a concentration range of 0–5 μM. Each sample was analysed in triplicate.

2.3. ABTS method

The antioxidant activity of resveratrol alone or in the presence of CDs, was determined by using $ABTS^{\cdot+}$ radical cation (Miller et al., 1993). The method is based on the ability of an antioxidant to reduce $ABTS^{\cdot+}$ into its colourless form. For $ABTS^{\cdot+}$ generation from ABTS salt, 3 mM of $K_2S_2O_8$ was reacted with 8 mM ABTS salt in sodium phosphate buffer (75 mM, pH 7.4), for 16 h at room temperature in the dark. The $ABTS^{\cdot+}$ solution was then diluted with sodium phosphate (buffer 75 mM, pH 7.4) to obtain an initial absorbance of 1.1 ± 0.1 at 414 nm, using a Shimadzu UV-1063 spectrophotometer. Fresh $ABTS^{\cdot+}$ solution was prepared everyday. The reaction kinetics was determined at 414 nm over a 1 h period, by continuously monitoring, at 25 °C. Most of the samples tested showed residual reactivity even after 1 h. However, changes in absorbance were minimal for all samples after 30 min.

The antioxidant abilities were expressed as μM Trolox equivalents by using a Trolox calibration curve prepared for a concentration range of 0–3 μM. Each sample was analysed in triplicate.

2.4. ORAC-FL assay

The oxygen radical absorbance capacity was determined as described by Dávalos et al. (2004) with slight modifications. The ORAC analyses were carried out on a Synergy HT multi-detection microplate reader, from Bio-Tek Instruments, Inc. (Winooski, VT, USA), 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 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 pipette. 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 (from 6.25 to 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 antioxidant abilities were expressed as μM Trolox equivalents.

3. Results and discussion

The antioxidant activity of resveratrol in the absence and presence of increasing concentrations of HP-β-CDs was determined using three different methods: ORAC, ABTS and DPPH. In order to compare the results obtained by these three methods, Trolox was

used as a standard in all cases, and the antioxidant capacity was expressed as μM Trolox equivalent.

3.1. Methods validation

The three methods were validated to quantify the antioxidant activity of resveratrol. ABTS and DPPH were stable when no antioxidant compound was added for at least 60 min under the reaction conditions used. FL was also stable at least 120 min when no AAPH was added to the reaction medium under the excitation conditions used.

3.2. Linearity

The linear relationship between the antioxidant activity and antioxidant concentration was calculated using Trolox (Fig. 1) and resveratrol (Fig. 2) at different concentrations in the ORAC, ABTS and DPPH assays. The regression analysis in Fig. 2 points to the linear response between the resveratrol concentration and the μM Trolox equivalents, yielding the following equations:

ORAC method: $y = 0.45 + 12.05x \quad r^2 = 0.998$

ABTS method: $y = 6.8 \times 10^{-4} + 0.33x \quad r^2 = 0.998$

DPPH method: $y = 0.095 + 0.044x \quad r^2 = 0.996$

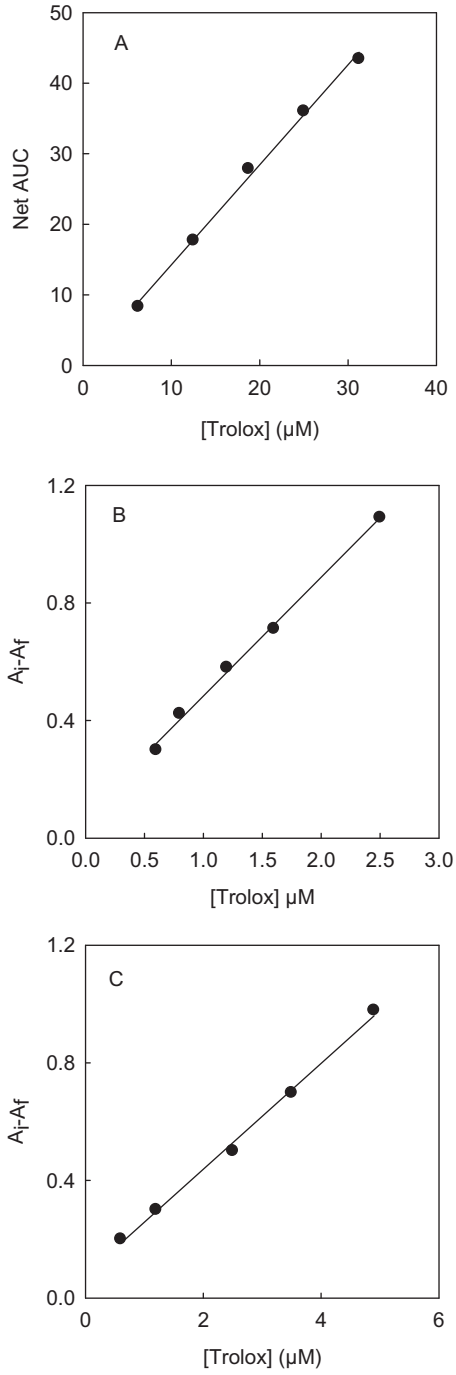

Fig. 1. Regression of Trolox C on different concentrations of Trolox C in ORAC (A), ABTS (B) and DPPH (C) assays.

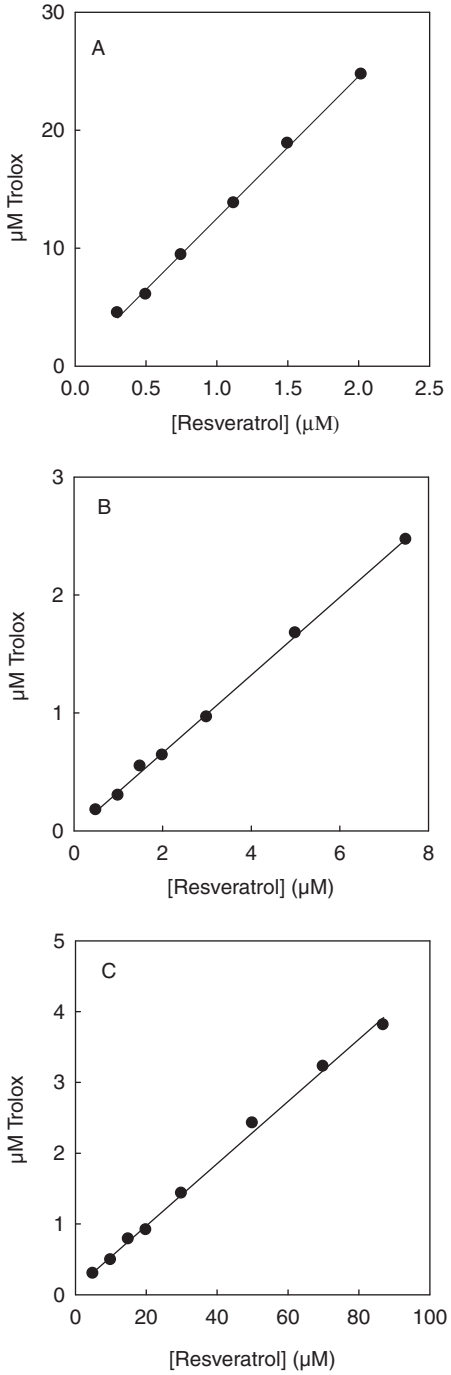

Fig. 2. Regression of μM Trolox equivalents of resveratrol at different concentrations in ORAC (A), ABTS (B) and DPPH (C) assays.

Table 1
Values of more representative validation parameters.

Assay method	Linearity	LOD (μM)	LOQ (μM)	Precision (%)		Ruggedness (%)
				Intraday	Interday	
ORAC	0.15–2	0.042 ± 0.003	0.15 ± 0.007	3	11	7.6
ABTS	0.5–7.5	0.3 ± 0.01	0.5 ± 0.03	5	13	9.3
DPPH	5–90	1.3 ± 0.06	5 ± 0.17	8	15	10.5

3.3. Limit of quantification (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 favour of selecting the analyte dose providing three 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 (Gabaldón et al., 2007). The mean value plus 3-fold SD corresponded to an estimated limit of detection of 0.042, 0.3 and 1.3 μM of resveratrol for ORAC, ABTS and DPPH assays, respectively (Table 1).

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 (Michaliak et al., 1995). Commonly, the selected LOQ is defined by the mean value plus 10-fold SD. The LOQ was calculated on the basis of the analysis of 20 samples with 0.15, 0.5 and 5 μM of resveratrol for ORAC, ABTS and DPPH assays, respectively (data not shown). The mean concentration determined for the 20 samples was 0.15 ± 0.007, 0.5 ± 0.03 and 5.02 ± 0.17 μM for ORAC, ABTS and DPPH assays, respectively (Table 1).

3.4. Precision

Assay precision was tested by repeatability (intra-day variation) and reproducibility (inter-day variation) studies. Six samples of resveratrol (1.5 μM) were analysed six times (in triplicate), on the same day (repeatability) and of five different days (reproducibility) by using the ORAC and ABTS assays. As can be seen in Table 1, the SD, intra-day and inter-day coefficient of variation (%CV) of the three methods were calculated, giving values of 3% and 11%, respectively, for the ORAC assay; close to those obtained by ABTS method (5% for repeatability and 13% for reproducibility). In the case of the DPPH assay, six samples of 15 μM resveratrol were analysed, obtaining values of 8% for repeatability and 15% for reproducibility.

3.5. Ruggedness

To determine the ruggedness of three methods, 1.5 μM of resveratrol was analyzed using the ORAC and ABTS assay; and 15 μM using the DPPH assay. During a 30 days period the μM Trolox equivalent presented a CV of 7.6%, 9.3% and 10.5% for ORAC, ABTS and DPPH assays, respectively (data not shown), confirming the reliability of the assays (Table 1).

The data obtained with these three methods showed that the highest antioxidant activity was observed using the ORAC method, whereas ABTS and DPPH assays reported lower values (Table 2). These results agree with those described in the literature (Awika et al., 2003; Zulueta et al., 2009). The ORAC assay as used in this paper permitted us to measure the antioxidant activity of resveratrol in the absence of CDs in a range from 0.15 to 2 μM. In the case of the ABTS assay, the range of resveratrol that could be measured was from 0.5 to 7.5 μM and in the case of DPPH method from 5 to 90 μM (Table 1). These results indicated that the most sensitive method for measuring resveratrol antioxidant activity was the ORAC assay, which can measured the lowest resveratrol concentration (0.15–2 μM) with the highest precision (Table 1). The least sensitive method to measure the antioxidant activity of resveratrol was the DPPH assay, which only serves to measure high resveratrol concentrations (5–90 μM), but is not capable of quantifying the antioxidant activity of resveratrol concentrations below 5 μM. It is important to note that resveratrol concentration in foods is very low, so, the ORAC method must be considered best for quantifying the antioxidant activity of resveratrol in such cases.

Table 2
Antioxidant activity values obtained with ORAC, ABTS and DPPH assays.

[Resveratrol] (μM)	μM Trolox		
	ORAC	ABTS	DPPH
0.5	6	0.17	–
1.5	18	0.49	–
5	–	1.65	0.315
15	–	–	0.755

3.6. Antioxidant capacity of resveratrol in the presence of CDs

To compare the efficiency of each method for measuring the antioxidant activity of phenolic compounds in the presence of cyclodextrin, the antioxidant activity of resveratrol was evaluated in the presence of increasing concentrations of HP-β-CDs using the ORAC, ABTS and DPPH assays.

When increasing concentrations of HP-β-CDs were added to the reaction medium at different resveratrol concentrations, a clear increase in the antioxidant activity was observed when the ORAC and ABTS assays were used (Fig. 3A and B), but no effect was observed when the DPPH assay was used (Fig. 3C). As can be seen in Fig. 3A and B, when HP-β-CDs increased the antioxidant activity of resveratrol also increased up to saturation, although this effect was only evident when measured by the ORAC or ABTS assays. When the ORAC assay was used at the saturation level (0.4 mM CDs), resveratrol showed almost double the antioxidant activity in the presence of CDs than in its absence of CDs for resveratrol at 0.5 and 1.5 μM (Fig. 3A).

This effect on the antioxidant activity of resveratrol may be due to the formation of inclusion complexes between resveratrol and HP-β-CDs as has been previously described in the literature (Lucas-Abellán et al., 2008a,b; Lu et al., 2009).

It is important to note that when the ORAC assay was used, the plateau was reached at 0.4 mM HP-β-CDs, indicating that all the resveratrol had been complexed, and so, the antioxidant activity of resveratrol remained constant (Lucas-Abellán et al., 2008a,b).

However, in the case of the ABTS assay, the plateau in the antioxidant activity curve was reached at 2 mM HP-β-CDs (Fig. 3B). The higher CDs concentration needed to reaches the plateau than into the ORAC assay may be because CDs interfere with the measurement method, complexing the $ABTS^{\cdot+}$. This fact was demonstrated when the ABTS assay was carried out in absence of resveratrol, obtaining a decay of $ABTS^{\cdot+}$ absorbance, with increasing concentration of HP-β-CDs, indicating that the radical was complexed by

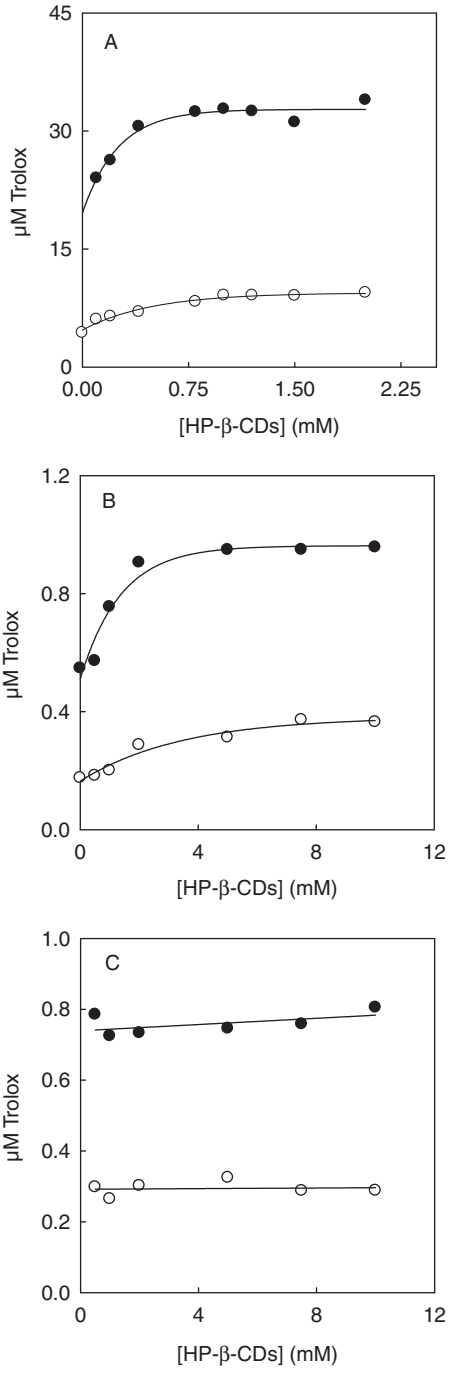

Fig. 3. Effect of HP-β-CDs concentration on the μM Trolox equivalents of resveratrol: (A) 0.5 μM (○), 1.5 μM (●) by using ORAC assay; (B) 0.5 μM (○), 1.5 μM (●) by using ABTS assay; (C) 5 μM (○), 15 μM (●) by using DPPH assay.

CDs. As a result, resveratrol and $ABTS^{\cdot+}$ compete for the hydrophobic cavity of HP-β-CDs. Using the ORAC assay it was not possible to measure the antioxidant activity of resveratrol at concentrations higher than 1.5 μM in the presence of CDs because the measurement times exceeded the 2 h established as optimum for this assay. In the case of the ABTS assay, the highest resveratrol concentration that could be measured in the presence of HP-β-CDs was 3.5 μM above which the decrease in absorbance was so great that in less that 2 min the absorbance was less than 0.1, and the method could not be considered valid.

When the DPPH assay was used to measure the antioxidant activity of resveratrol in the presence of HP-β-CDs, no effect was observed as the CDs concentration increased (Fig. 3C), suggesting that the methanolic medium of the DPPH assay prevents resveratrol complexation in the hydrophobic cavity of CDs. In such conditions, resveratrol is free in the assay medium, so CDs have no effect on its antioxidant activity. Therefore, the DPPH assay cannot be used to measure the effect of resveratrol complexation in CDs on its antioxidant activity.

4. Conclusion

The ORAC-FL approach is the best method for quantifying the antioxidant activity of phenolic compounds complexed in CDs, because of its sensitivity and the fact that the FL and AAPH do not interference with CDs complexation.

In the case of the ABTS assay, the $ABTS^{\cdot+}$ competes with phenolic compounds to enter into the hydrophobic cavity of CDs, so CDs interferes with the measurement method.

With respect to the DPPH assay, the hydrophobic nature of the medium prevents the complexation of phenolic compounds in the hydrophobic cavity of CDs, so it cannot be used to measure this effect.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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