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Analytical Methods

Complexation of resveratrol by native and modified cyclodextrins: Determination of complexation constant by enzymatic, solubility and fluorimetric assays

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ABSTRACT

The complexation of resveratrol with native α-, β- and γ-cyclodextrins (CDs) and modified CDs (hydroxypropyl-β-(HP-β-CDs), maltosyl-β-(G_2-β-CDs), methyl-β-, carboxymethyl-β- and acetyl-β-cyclodextrins) was studied, and the complexation constants (K_c) were compared. The complexation constant between resveratrol and each type of CD was calculated using three different methods: enzymatic, solubility and fluorimetric. The K_c values obtained showed that HP-β-CDs with their very high K_c of 18,048 ± 625 M^{-1}, were the most effective for complexing resveratrol. Moreover, comparison of the results obtained by the three methods revealed that the fluorimetric method undervalued the K_c between resveratrol and all cyclodextrins, while the enzymatic and solubility methods were more precise for calculating the K_c between resveratrol and CDs, as demonstrated by the cyclodextrin-assay.

1. Introduction

Resveratrol (trans-3,5,4′-trihydroxystilbene), a phytoalexin belonging to the stilbene class of polyphenolic compounds, is found both in the free and conjugated forms in high concentrations in a number of plant species (Soleas, Diamandis, & Goldberg, 1997), including grapevine (*Vitis vinifera*) (Langcake & Pryce, 1976; Lamuela-Raventos, Romero-Pérez, Waterhouse, & De la Torre-Boronat, 1995), peanut, mulberry and pistachios (Tokusoglu, Ünal, & Yemis, 2005). It is produced by plants in response to stress conditions, including infection by microorganisms, the application of chemicals, injury from light exposure and change in temperature (Agrios, 1998; Bailey & Mansfield, 1982).

Resveratrol has been shown to provide health-promoting activities such as lowering the incidence of coronary heat disease (Frankel, Waterhouse, & Kinsella, 1993) and cancer chemopreventive activity (Jang et al., 1997).

The high hydrophobicity and sensitivity of resveratrol to external agents such as air, light and oxidative enzymes constitutes a serious problem for its bioavailability, formulation and manipulation in the manufacture of functional foods. In order to avoid this limitation, inclusion of resveratrol in cyclodextrins has recently been suggested by our group (Lucas-Abellán, Fortea, López-Nicolas, & Núñez-Delicado, 2007).

Cyclodextrins (CDs) are a group of naturally occurring cyclic oligosaccharides derived from starch with six, seven or eight glucose residues linked by α (1 → 4) glycosidic bonds in a cylinder-shaped structure, and named as α-, β- and γ-CDs, respectively. The central cavity of these molecules is hydrophobic, while the rims of the surrounding walls are hydrophilic (Martín Del Valle, 2004). This hydrophobic cavity forms inclusion complexes with a wide range of organic and inorganic guest molecules (Cai et al., 1990). It has been demonstrated that complexation can considerably increase the solubility, stability and bioavailability of the guest molecule (Martín Del Valle, 2004).

To improve the complex-forming abilities of CDs, a wide variety of chemically modified CDs have been designed and prepared by introducing various functional groups into the macrocyclic ring (Kahn, Forgo, Stine, & ÓSouza, 1988). The inclusion complexes of some chemically modified CDs are even more soluble than those from natural CDs (Luong, Male, & Zhao, 1993).

There are many studies in the literature about the calculation of K_c between CD and different compounds, using different methods, such as fluorescence, nuclear magnetic resonance (RMN), enzymatic, solubility, equilibrium dialysis, conductometry and affinity capillary electrophoresis (Álvarez-Parrilla, De la Rosa, Torres-Rivas, Rodrigo-García, & González-Aguilar, 2005; Bertacche, Lorenzi, Nava, Pini, & Cínico, 2006; Lucas-Abellán et al., 2007; Platzer, Schward, & Neubert, 1999; Polyakov, Leshina, Konovalova, Hand, & Kispert, 2004; Sheehy & Ramstad, 2005; Ugwu, Alcala, Bhardwaj, & Blanchard, 1996). However, this variance in the calculation methods complicates the comparation of K_c values obtained for

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different compounds. There is not a reference method to calculate the K_c.

The complexation of resveratrol in CDs has been described previously not only by our group (Lucas-Abellán et al., 2007) but also by Bertacche, Lorenzi, Nava, Pini, and Cínico (2006). However, no comparative studies exist in which a wide range of natural and modified CDs are used to complex resveratrol and the respective K_c values calculated by different methods are compared.

In this paper, the complexation of resveratrol with native CDs (α-, β- and γ-CDs) and modified CDs (hydroxypropyl-β-(HP-β-CDs), maltosyl-β-(G_2-β-CDs), methyl-β-, carboxymethyl-β- and acetyl-β-CDs) was studied, in order to establish which type of CDs would produce the most stable complex.

Moreover, in this contribution comparison of three different methods to calculate K_c, namely enzymatic, solubility and fluorimetric were compared using the model system of resveratrol-CD to determine which of them offers the most precise results.

2. Materials and methods

Electrophoretically pure (Cook & Kulkarni, 1988) soybean lipoxygenase (LOX) (EC 1.13.11.12) Type V (701,000 Sigma units/mg protein) (one unit will cause an increase in A_{234} of 0.001per min at pH 9.0 at 25 °C when linoleic acid is the substrate in 3.0 mL volume) prepared by affinity chromatography and *trans*-resveratrol, were obtained from Sigma (Madrid, Spain). Hydrogen peroxide was supplied from Aldrich (Madrid, Spain). HP-β-, α- and γ-CDs were purchased from TCI Europe N.V. (Zwijndrecht, Belgium). Carboxymethyl-, acetyl- and methyl-β-CDs were supplied for Sigma (Madrid, Spain). G_2-β and β-CDs were kindly supplied by Amaizo, American Maize-Products Company, Hammond, IN. All other chemicals used were of analytical grade.

The hydrogen peroxide, LOX and resveratrol solutions were freshly prepared each day, and their concentrations calculated using $\varepsilon_{240} = 39.4\ M^{-1}\ cm^{-1}$ (Nelson & Kiesow, 1972), $\varepsilon_{280} = 1600{,}000\ M^{-1}\ cm^{-1}$ (Spaapen, Veldink, Liefkens, Vliegenthart, & Kay, 1979) and $\varepsilon_{312} = 33{,}400\ M^{-1}\ cm^{-1}$ (Pinto, García-Barrado, & Macías, 1999), respectively.

The calculations of K_c by each method were made in triplicate and SD was calculated in each case.

2.1. Enzymatic method

The enzymatic oxidation of resveratrol was followed spectrophotometrically using a Shimadzu (Kyoto, Japan) model UV-1063 spectrophotometer at the absorption maximum of the resveratrol oxidation product (250 nm), using $\Delta\varepsilon_{250} = 29{,}600\ M^{-1}\ cm^{-1}$ (Lucas-Abellán et al., 2007). Unless otherwise stated, the standard reaction medium at 25 °C contained 100 mM sodium borate buffer (pH 9.0), 400 μM H_2O_2, 30 μM resveratrol, 50 nM of LOX and increasing concentrations of CDs in a final volume of 1 mL. The steady state was calculated from the linear zone of the product accumulation curve. A reference cuvette containing all the components of the reaction medium except the enzyme served as the control.

Free resveratrol is the only form of substrate which LOX can oxidise. So, the formation of 1:1 inclusion complexes by the addition of increasing concentrations of CDs leads to an inhibitory effect in the oxidation of resveratrol by LOX (Fig. 1A). The complexation constant (K_c) can be calculated using the equations previously described by our group, in which a Michaelis–Menten

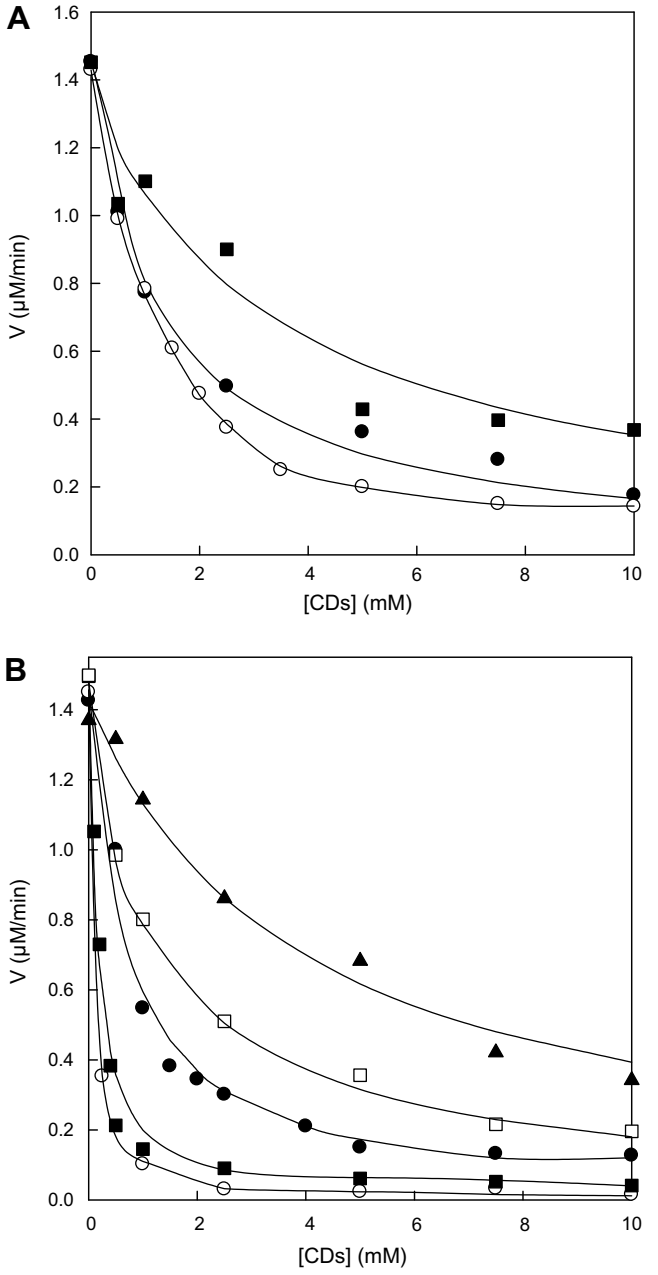


Fig. 1. Effect of native CDs on the oxidation of resveratrol by lipoxygenase in the presence of H_2O_2 The reaction medium at 25 °C contained 100 mM sodium borate buffer, pH 9.0, 30 μM resveratrol, 400 μM H_2O_2, 50 nM lipoxygenase and increasing concentrations of CDs. (A) native CDs: (●) α-CDs, (○) β-CDs, (■) γ-CDs. (B) Modified CDs: (●) G_2-β-CDs, (○) HP-β-CDs, (■) methyl-β-CDs (□), acethyl-β-CDs (▲), carboxymethyl-β-CDs.

modified equation for free substrate was used (Lucas-Abellán et al., 2007):

$$[\text{resv}]_f + [\text{CD}]_f \overset{K_c}{\leftrightarrow} [\text{resv} - \text{CD}] \tag{1}$$

$$K_c = \frac{[\text{resv} - \text{CD}]}{[\text{resv}]_f [\text{CD}]_f} \tag{2}$$

$$[\text{resv}]_f = (-([\text{CD}]_t K_c - [\text{resv}]_t K_c + 1) + \sqrt{([\text{CD}]_t K_c - [\text{resv}]_t K_c + 1)^2 + 4K_c[\text{resv}]_t})/2K_c \tag{3}$$

$$v = \frac{V_m\left[(-([CD]_tK_c - [resv]_tK_c + 1) + \sqrt{([CD]_tK_c - [resv]_tK_c + 1)^2 + 4K_c[resv]_t})/2K_c\right]}{K_M + \left[(-([CD]_tK_c - [resv]_tK_c + 1) + \sqrt{([CD]_tK_c - [resv]_tK_c + 1)^2 + 4K_c[resv]_t})/2K_c\right]} \quad (4)$$

where $[resv]_f$ is free resveratrol $[CDs]_f$ is free cyclodextrin, $[resv]_t$ is total resveratrol $[CDs]_t$ is total cyclodextrin [resv-CDs] is complex resveratrol-cyclodextrin, K_c is complexation constant, V_m and K_M are Michaelis–Menten constants and v is the oxidation rate of free resveratrol.

2.2. Solubility method

Phase solubility diagrams were constructed according to Higuchi and Connors (1965). Excess amounts of resveratrol were added to aqueous solutions of increasing concentrations of β-CDs, G_2-β-Ds and HP-β-CDs up to 100 mM (15 mM in the case of β-CDs, its solubility limit), in 10 mL of water at 25 °C. The samples were maintained in an ultrasonic bath at 25 °C for 60 min to reach equilibrium. The aqueous solutions were filtered through 0.2 μm membrane filter cellulose and diluted in 80% ethanol. The resveratrol (ε_{312} = 28,994 M^{-1} cm^{-1}) concentration was spectrophotometrically determined.

The K_c between resveratrol and CDs was calculated by using the equation below (Higuchi & Connors, 1965):

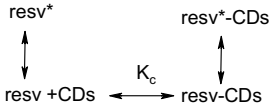
$$K_c = \frac{\text{slope}}{S_0(1 - \text{slope})} \quad (5)$$

were S_0 is the water solubility of resveratrol and "slope" the slope of the phase solubility diagram.

2.3. Fluorimetric method

Steady-state fluorescence measurements were performed using a Shimadzu RF 5301 PC spectrofluorimeter. Emission fluorescence spectra were acquired in the 330–600 nm range (1 nm step), at a fixed excitation wavelength of 330 nm. The reaction medium contained 30 μM resveratrol and increasing concentrations of β-, G_2-β- and HP-β-CDs prepared in water to reach a final volume of 3 mL at 25 °C.

Assuming that the increase in the resveratrol fluorescence intensity observed in the presence of increasing concentrations of CDs is due to the formation of the inclusion complexes between resveratrol and CDs, the following equilibrium can be described:



here resv* and resv*-CDs are resveratrol and the complex in the excited state. They are the only fluorescent species and there is no association or dissociation process in the excited state. K_c is the complexation constant as defined in the enzymatic method.

The fluorescence intensity at any wavelength (F) can be related to the CDs concentration by the equation (Connors, 1987):

$$F = F_0 + \frac{(F_\infty - F_0)K_c[CDs]_t}{1 + K_c[CDs]_t} \quad (6)$$

where F_∞ is the fluorescence intensity when total resveratrol has been complexed in CDs and F_0 is the fluorescence of resveratrol in the absence of CDs. Experimental data of F as a function of [CDs] can be fitted to Eq. (5), using as initial parameters (K_c and F_∞) those obtained from the analysis of the experimental data using the Benesi-Hildebrand equation for 1:1 complexes (double reciprocal plot) (Benesi & Hildebrand, 1949):

$$\frac{1}{F - F_0} = \frac{1}{F_\infty - F_0} + \frac{1}{(F_\infty - F_0)K_c[CDs]_t} \quad (7)$$

The K_c value can also be calculated by using Scatchard's equation for the 1:1 complex formation, which relates the enhanced fluorescence intensity with CD concentration (Scatchard, 1949):

$$\frac{F - F_0}{[CDs]_t} = (F_\infty - F_0)K_c - (F - F_0)K_c \quad (8)$$

3. Results and discussion

In this paper, the K_c between resveratrol and each type of native CDs was calculated using the enzymatic method described by Lucas-Abellán et al. (2007). For that, the oxidation of resveratrol by the hydroperoxidase activity of LOX was achieved in the presence of increasing concentrations of α-, β- or γ-CDs (Fig. 1A). As can be seen in Fig. 1A, LOX activity decreased as the CDs concentration increased, the degree depending on the type of cyclodextrin used, inhibition being strongest in the case of β-CDs.

To calculate the K_c between resveratrol and each type of native CDs, increasing concentrations of each type of CDs were added to the reaction medium at different resveratrol concentrations, a clear decrease in LOX activity being observed in all cases (data not shown). Assuming that this inhibitory effect was due to the formation of 1:1 inclusion complexes and that free resveratrol is the only form of substrate which LOX can use, Eq. (4) was used to obtain the K_c values for α-, β- and γ-CDs shown in Table 1. As can be seen from the Table 1, β-CDs were the native type showing the highest K_c for the complexation of resveratrol (4220 ± 387 M^{-1}), similar to that described previously by Lucas-Abellán et al. (2007), while α- and γ-CDs presented lower values of 805 ± 75 and 226 ± 32 M^{-1}, respectively. This result indicated that the size of the hydrophobic cavity of β-CDs permits stronger interactions with resveratrol, while the hydrophobic cavity of α- and γ-CDs, is too small and too large, respectively, thus reducing their interaction with resveratrol.

As β-CDs were the most effective for complexing resveratrol, different types of modified β-CDs were studied, adding different functional groups to the macrocyclic ring (HP-β-, G_2-β-, methyl-β-, carboxymethyl-β- and acetyl-β-CDs). Their K_c for resveratrol complexation was calculated using the enzymatic method in all cases. The inhibition curves for the oxidation of 30 μM resveratrol by LOX in the presence of increasing concentrations of each type of modified β-CDs are presented in Fig. 1B: HP-β-CDs were the most effective (Fig. 1B, open circles). The oxidation of different concentrations of resveratrol by LOX in the presence of the different types of modified CDs was also studied (data not show), and their K_c was calculated. As can be seen in Table 1, the K_c values for modified β-CDs ranged from 1518 ± 205 for carboxymethyl-β-CDs to 18,048 ± 652 M^{-1} for HP-β-CDs, according to the functional group added. HP-β-CDs was the most effective in resveratrol complexation (K_c = 18,048 ± 652 M^{-1}), followed by methyl-β-CDs (K_c = 10,089 ± 675 M^{-1}). Moreover some modified CDs has lower K_c values than native β-CDs, e.g. carboxymethyl-β-CDs and acetyl-β-CDs,

Table 1
Complexation constant (K_c) for complexes formed between native and modified cyclodextrins and resveratrol calculated by the enzymatic, solubility and fluorimetric methods

	Cyclodextrin type	Enzymatic method	Solubility method	Fluorimetric method		
		K_c (M^{-1})	K_c (M^{-1})	K_c (M^{-1}) non-linear regression	K_c (M^{-1}) Benesi-Hildebrad	K_c (M^{-1}) Scatchard
Native CDs	α-CDs	805 ± 75				
	β-CDs	4220 ± 387	4438 ± 401	2205 ± 364	2145 ± 265	2382 ± 285
	γ-CDs	226 ± 32				
Modified CDs	Hydroxypropyl-β-CDs	18,048 ± 625	18,018 ± 723	11,035 ± 897	10,314 ± 889	10,229 ± 845
	Methyl-β-CDs	10,089 ± 675				
	Maltosyl-β-CDs	5098 ± 495	5425 ± 503	3011 ± 435	3314 ± 298	3224 ± 301
	Carboxymethyl-β-CDs	1518 ± 205				
	Acetyl-β-CDs	1789 ± 187				

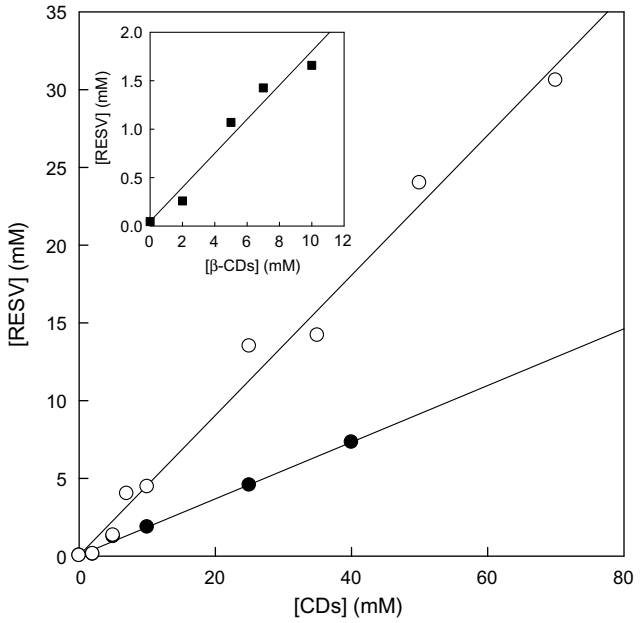


Fig. 2. Phase of solubility diagram of resveratrol 100 mM sodium borate buffer (pH 9.0) with HP-β-CDs (○) and G_2-β-CDS (●). Inset: Phase of solubility diagram of resveratrol with β-CDs (■).

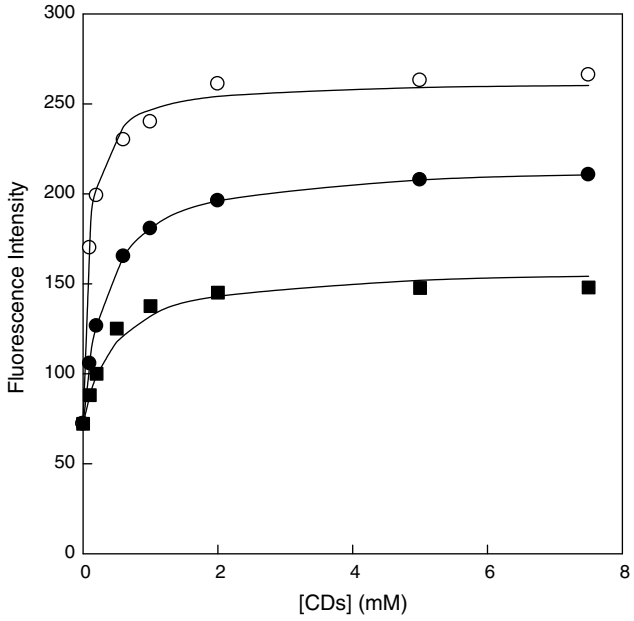


Fig. 3. Fluorescence intensities of resveratrol at 380 nm versus different concentrations of CDs 100 mM sodium borate buffer (pH 9.0). (■) β-CDs, (●) G_2-β-CDs, (○) HP-β-CDs. The lines show the best fits to Eq. (5).

indicating that the presence of carboxymethyl or acetyl as functional group hinders the complexation process.

For comparison purposes, the physical method described by Higuchi and Connors (1965) was used to calculate the K_c values for β- and two modified β-CDs. The phase solubility diagrams made for resveratrol using β-, HP-β- and G_2-β-CDs (Fig. 2) were all of the A_L type, indicating the formation of 1:1 complexes in both the native and modified CDs studied. Using Eq. (5), the K_c values were calculated (Table 1). As can be seen in Table 1, the K_c values obtained using the physical method were similar to those obtained with the enzymatic method (Table 1), indicating the validity of both methods.

A fluorimetric method was also used to calculate the K_c value for the complexation of resveratrol with β-, HP-β- and G_2-β-CDs. In this method, the increase in fluorescence intensity as a function of resveratrol complexation by CDs was used to calculate K_c.

When increasing concentrations of CDs were added to the reaction medium, the fluorescence intensity increased until a maximum was reached (Fig. 3). An increase due to the formation of inclusion complexes between resveratrol and CDs. This effect has been previously described for other compounds (Álvarez-Parrilla et al., 2005; Mahedero, Muñoz de la Peña, Bautista, & Aaron, 2002; Muñoz de la Peña, Mora-Diez, Mahedero-García, Bohoyo-Gil, & Cañada-Cañada, 2007; Wagner & Fitzpatrick, 2000).

The representation of $1/(F - F_0)$ vs. $1/[CDs]$ (double reciprocal plot), known as a Benesi–Hildebrand plot (Fig. 4A), leads to a straight line for the three type of CDs used, indicating the 1:1 stoechiometry of the complexes. This linear relation agrees with to that described by the Benesi–Hildebrand Eq. (7). The linear plots of Fig. 4A can be used to determine K_c values by simply dividing the intercepts by the slopes (Table 1). But since Benesi–Hildebrand plots tend to place more emphasis on low CD concentrations than on higher values, the slope of the line is more sensitive to the ordinate values of the points for the lowest concentrations. In order to avoid this effect, a non-linear regression analysis of the plots in Fig. 3 was carried out by applying the Eq. (6) and using as the initial parameters those estimated by the Benesi–Hildebrand plot. The K_c values obtained by the non-linear regression (Table 1) were similar to those calculated by the Benesi–Hildebrand plot.

The fluorescence data (Fig. 3) were also used to the calculate K_c values between resveratrol and CDs by Scatchard's method (Scatchard, 1949). In this case, when 1:1 complexes are formed, the relationship between the enhancement in fluorescence intensity ($F - F_0$) and CD concentration is given by the Eq. (8). Reploting the data of Fig. 3 as $(F - F_0)/[CDs]$ vs. $(F - F_0)$ (Fig. 4B), straight lines were obtained confirming the 1:1 complex formation between resveratrol and the studied CDs. The K_c values give by the slope of the plots (Table 1) were similar to those obtained by the Benesi–Hildebrand plot and non-linear regression.

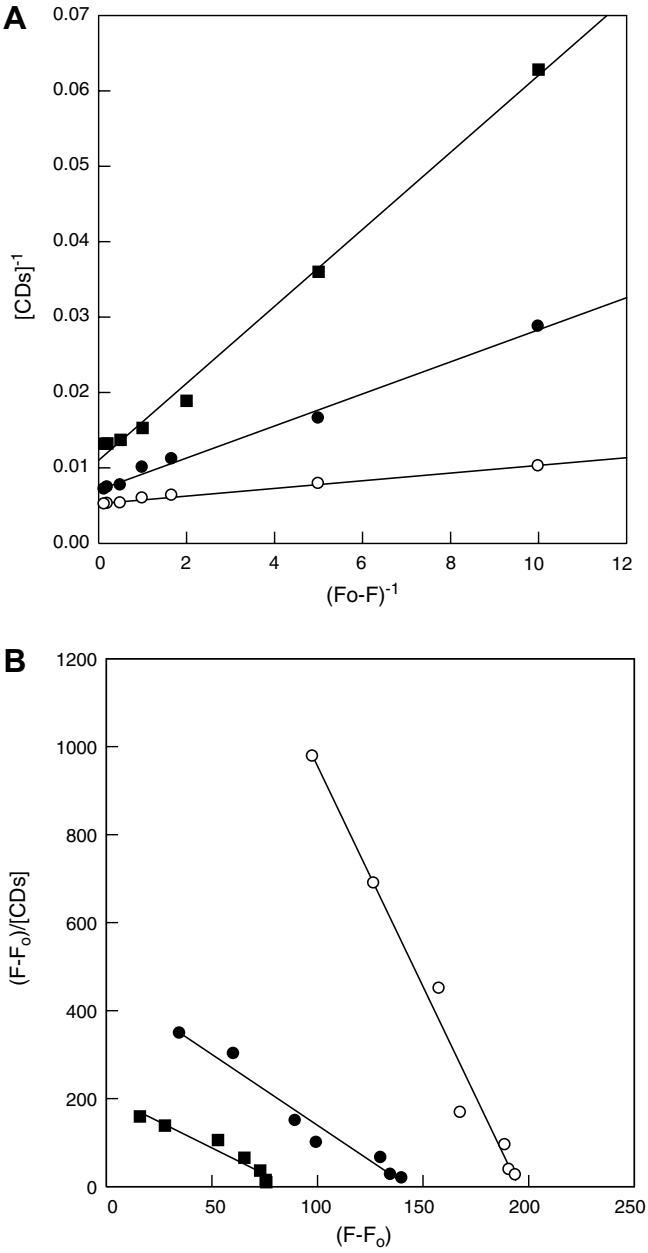


Fig. 4. (A) Benesi-Hildebrand plot for resveratrol-CDs (Eq. (7)). (■) β-CDs, (●) G_2-β-CDs, (○) HP-β-CDs. (B) Scatchard plot for resveratrol-CDs (Eq. (8)). (■) β-CDs, (●) G_2-β-CDs, (○) HP-β-CDs.

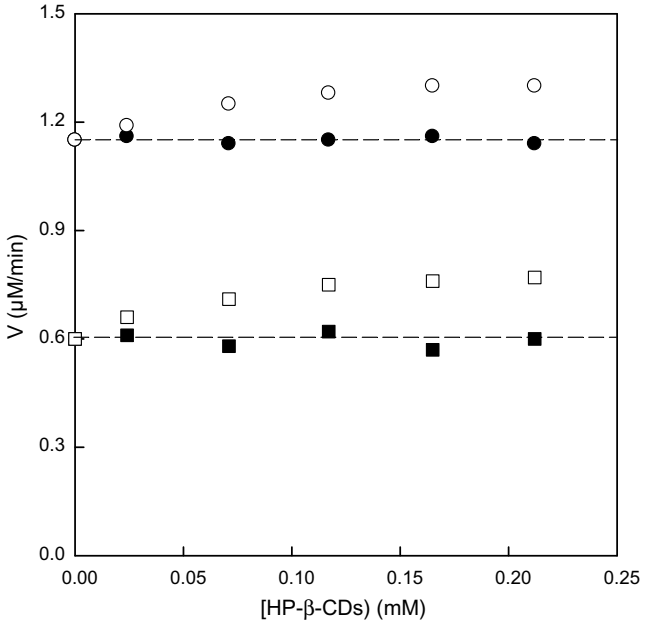


Fig. 5. Cyclodextrin assay. The HP-β-CDs and resveratrol total concentrations used in each case, were calculated to obtain two different free resveratrol concentrations, by using the K_c values obtained by the enzymatic method and Eq. (3) ((●) 15 μM free resveratrol, (■) 5 μM free resveratrol) and the fluorimetric method ((○) 15 μM free resveratrol, (□) 5 μM free resveratrol). The oxidation of free resveratrol was followed in 100 mM sodium borate buffer (pH 9.0) using 50 nM of LOX.

Comparing the K_c values obtained for the complexation of resveratrol by β-, HP-β- and G_2-β-CDs using the different methods (Table 1), shows that the enzymatic and solubility methods provide similar values, while the fluorimetric method gives lower values for all three CDs studied.

To determine which method is more accurate (enzymatic or fluorimetric), the so-called CD assay (López-Nicolás, Bru, & García-Carmona, 1997; Lucas-Abellán et al., 2007), in which the total CD and resveratrol concentrations are set so that the free resveratrol concentration remains constant, was carried out for the HP-β-CDs. By using the K_c values determined enzymatically or fluorimetrically and Eq. (3), we have performed the corresponding CD assay. It is expected that when the concentration of effective free resveratrol is constant, the rate of resveratrol oxidation by LOX (μM/min) is constant too, independent of the total resveratrol concentration. As shown in Fig. 5, the results obtained using enzymatically determined K_c (18,048 ± 625 M^{-1}) were as expected (the LOX activity was independent of total CDs and resveratrol concentrations), but those obtained with fluorimetrically determined K_c (11,035 ± 897 M^{-1}) deviated from expectations (the LOX activity did not remain constant), indicating that the free resveratrol concentration is not the same in all cases, so, the value of K_c used is not correct. Thus, the differences observed between the enzymatically and fluorimetrically obtained constants (Table 1) are very important as regards the real effective resveratrol concentration. It can be concluded that fluorimetric determination undervalues the K_c, the free resveratrol concentration being higher than expected and explaining the increase in LOX activity observed in Fig. 5.

In conclusion, HP-β-CDs are the most effective agents for complexing resveratrol regardless of the K_c calculation method used. Fluorimetric method undervalues the K_c, in the resveratrol case, so the enzymatic and solubility methods are most accurate for calculating the K_c between resveratrol and CDs. Moreover, in the case of resveratrol and other compounds that could be oxidized by different enzymes the optimum method to calculate its K_c is the enzymatic one, because of the high specificity of the enzymes acting only on free substrate.

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