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Use of reversed phase high pressure liquid cromatography for the physicochemical and thermodynamic characterization of oxyresveratrol/β-cyclodextrin complexes

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ABSTRACT

Knowledge of the complexation process of oxyresveratrol with β-cyclodextrin (β-CD) under different physicochemical conditions is essential if this potent antioxidant compound is to be used successfully in both food and pharmaceutical industries as ingredient of functional foods or nutraceuticals, despite its poor stability and bioavailability. In this paper, the complexation of oxyresveratrol with natural CDs was investigated for first time using RP-HPLC and mobile phases to which α-, β-, and γ-CD were added. Among natural CDs, the interaction of oxyresveratrol with β-CD was more efficient than with α- and γ-CD. The decrease in the retention times with increasing concentrations of β-CD (0–4 mM) showed that the formation constants (K_F) of the oxyresveratrol/β-CD complexes were strongly dependent on both the water–methanol proportion and the temperature of the mobile phase employed. However, oxyresveratrol formed complexes with β-CD with a 1:1 stoichiometry in all the physicochemical conditions tested. Moreover, to obtain information about the mechanism of the oxyresveratrol affinity for β-CD, the thermodynamic parameters ΔG°, ΔH° and ΔS° were obtained. Finally, to gain information on the effect of the structure of different compounds belonging to the stilbenoids family on the K_F values, the complexation of other molecules, resveratrol, pterostilbene and pinosylvin, was studied and compared with the results obtained for the oxyresveratrol/β-CD complexes.

1. Introduction

Cyclodextrins (CDs) are a group of structurally related natural products formed during the bacterial digestion of starch [1]. These cyclic oligosaccharides consist of α-(1–4) linked α-D-glucopyranose units and contain a somewhat lipophilic central cavity and a hydrophilic outer surface. Natural α-, β-, and γ-CDs consist of six, seven and eight glucopyranose units, respectively. The most important functional property of CDs is their ability to form inclusion complexes with a wide range of organic guest molecules [2,3]. Because CDs are able to increase the bioavailability of different compounds with proven health properties, their use in food and pharmaceutical industries is increasing [4,5].

Among the guest molecules which have been complexed by CDs, several works have studied the inclusion of different antioxidant molecules to facilitate their use as ingredients of functional foods or nutraceuticals [6]. In recent years, different stilbenoids with high antioxidant activity such as resveratrol, pterostilbene and pinosylvin have been complexed by natural and modified CDs [7–11]. However, to date, no research about the complexation of oxyresveratrol, one of the most potent antioxidants known, with CDs has been published. Oxyresveratrol (trans-2,3′,4,5′-tetrahydroxystilbene) belongs to a group of phenolic compounds known as stilbenes, and is found in different sources such as mulberry (*Morus alba* L.) fruits and twigs [12]. Its pharmacological properties include a wide range of biological activities: antioxidant [12], antiviral [13], hepatoprotective [14], and cyclooxygenase and tyrosinase-inhibitory [15,16] activities.

However, problems concerning the physicochemical properties of oxyresveratrol have meant that no "*novel food*" has been fortified with this antioxidant. Indeed, oxyresveratrol possesses low bioavailability and is easily oxidized by prooxidant agents. For these reasons, the complexation of oxyresveratrol with types of molecules which can increases its bioavailability and stability in the face of prooxidant agents is strongly desirable, as it would be the case of CDs. If the complexes between oxyresveratrol and natural CDs are to be used in the food industry, the first step is to characterize the molecular nature of the inclusion process, and to determine the stoichiometric coefficients and formation constants of the complexes (K_F). This has been done in this work for first time.

To characterize inclusion complexes several methods have been used including UV spectroscopy [17], fluorescence measurements

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[18], circular dichroism [19], potentiometry [20], mass spectrometry [21], nuclear magnetic resonance (NMR) [22], among others. In recent years, HPLC has been increasingly used for observing and characterizing CD-guest inclusion complexes [23]. Modifications of the retention properties of molecules with different CD concentrations in mobile phase were found to be related to the stoichiometry and stability of the inclusion complexes thus formed, as described by Fujimura et al. [24]. Recently, data on the retention behaviour in RP-HPLC, with and without CD in the mobile phase, have been published for monoaromatic compounds, aromatic amines, polyaromatic hydrocarbons, nitrogen heterocycles or aromatic hydroxyl compounds [25]. Several authors have reported the effect on the retention behaviour of the type and concentration of CD added to the mobile phase, and the interaction between the organic solvent and the CD used or the temperature and pH used in the measurements [26–28].

Bearing the above in mind, the five main objectives of this work were to: (i) study the retention mechanism of oxyresveratrol in a reversed-phase system involving the formation of different natural CD inclusion complexes in the mobile phase; (ii) calculate the stoichiometry and the apparent formation constants of β-CD inclusion complexes with oxyresveratrol from the relationship between the capacity factor and the β-CD concentration in the mobile phase; (iii) study the effect of temperature and organic solvent concentration on the apparent formation constants of oxyresveratrol/β-CD inclusion complexes; (iv) obtain the thermodynamic parameters (ΔG°, ΔH° and ΔS°) of the complexation of oxyresveratrol by β-CD; (v) study the effect of the structure of different stilbenoids such as resveratrol, pterostilbene, pinosylvin and oxyresveratrol on the inclusion mechanism.

2. Experimental

2.1. Chemicals and reagents

Oxyresveratrol was kindly supplied by Dr. José Luis Cenis Anadón. Resveratrol, α-CD, β-CD and γ-CD was purchased to Sigma (Madrid, Spain). Pterostilbene and pinosylvin were from Sequoia Research Products Limited (Pangbourne, United Kingdom). Copper sulphate and anhydrous D-glucose were supplied by Prolabo (Fontenay-Sous-Bois, France). The methanol and water used in this study were of HPLC grade purchased from Scharlau Chemie S.A. (Barcelona, Spain) and J.T. Baker (Deventer, Netherlands), respectively. Binary mixtures of water:methanol, with methanol percentages of 20–50%, were used without further purification.

2.2. Equipment and experimental procedures

Twenty microliters of stilbenes (prepared at a concentration of 0.05 mg/ml in methanol) were injected for HPLC analysis on equipment using a Merck-Hitachi pump L-6200 (Merck-Hitachi, Darmstadt, Germany) and a diode array detector Shimadzu SPD-M6A UV (Shimadzu, Kyoto, Japan). A commercially available reversed-phase column LiChrospher RP18 (Agilent, Waldbronn, Germany) (150 mm × 4 mm I.D. 5 μm particle size) was used.

For all experiments the mobile-phase flow-rate was set and systematically controlled at 1.00 ± 0.01 mL/min and the UV detector was operated at 328 nm (for oxyresveratrol) and 306 nm (for resveratrol, pterostilbene and pinosylvin).

Mobile phases were prepared according to the following procedure. After obtaining of the desired methanol–water mixture, an accurately weighed amount of CD was added to 250 mL of this binary mixture in a 500 mL volumetric flask. When total dissolution at ambient temperature was observed, the remaining amount of solvent was added to reach a final mobile-phase volume of 500 mL. The maximum quantity of β-CD that can be dissolved in such binary mixtures has been reported elsewhere [29,30]. The concentrations of β-CD employed were 0, 0.5, 1, 2, 3 and 4 mM. Whenever the mobile-phase solution was changed, the column was first conditioned for at least 1 h with the new solution mixture at a flow-rate of 1.0 mL/min.

The column void volume, t_0, was determined using reagent grade copper sulfate solution (0.01 mg/mL) as described by Clarot et al. [32].

2.3. Temperature studies

To study the effect of the temperature on the complexation process of oxyresveratrol by β-CD, increasing temperatures from 15 to 30 °C were selected. The thermodynamic relationship shown in Eq. (1) was used to determine the thermodynamic parameters standard enthalpy and entropy of transfer of the oxyresveratrol from the mobile phase to the CD:

$$\ln K_F = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \quad (1)$$

where K_F is the apparent formation constant of the inclusion complex, T is the temperature in Kelvin degrees, R is the gas constant, ΔH° and ΔS° are standard enthalpy and entropy changes of complexes formed in the mobile phase. For a linear plot of $\ln K_F$ *versus* $1/T$, the slope and intercept are respectively—$\Delta H^\circ/R$ and $\Delta S^\circ/R$. To determine the Gibbs free energy change for the interactions that take place during the inclusion process may be found, we used Eq. (2):

$$\Delta G^\circ = \Delta H^\circ - T\,\Delta S^\circ \quad (2)$$

3. Results and discussion

3.1. Selection of the optimum organic modifier to characterize oxyresveratrol/CDs complexes in RP-HPLC systems

Several studies have demonstrated that the use of CDs as additives in the mobile phases in reversed-phase high performance liquid chromatography (RP-HPLC) decreases the retention time of the guest as a result of host–guest interactions [25]. However, changes in retention behaviour, which are closely related to the stability constants of the complexes formed, are strongly dependent on factors such as the type of organic modifier. For this reason, the first step in our investigation was to select the most suitable composition of the mobile phase for the analysis.

The formation of CD inclusion complexes in the liquid phase proceeds more easily in an aqueous solution. However, an aqueous–organic solvent was used as a mobile phase in the present system (use of the non-polar stationary phase) because when water alone was used as mobile phase, very long retention times, with the associated experimental error, were required for the analysis.

To select the most appropriate organic solvent to be used in this work, two parameters—the affinity of the organic modifier for the CD cavity and the solubility of CDs in the organic solvent—were borne in mind due their influence on the retention value, the resolution of the sample solute and the binding constant of inclusion complexes of the solute. Although some types of organic solvents such as ethanol, acetonitrile or methanol have been used to identify different stilbenes using RP-HPLC [33], we selected methanol to be introduced in the correspondent mobile phases. The reasons of this selection were: (i) the very weak association of methanol with β-CD represented by the low value of K_m, the constant which describes the affinity of the organic modifier for the CD cavity, reported by Matsui and Mochida [31]. Indeed the K_m value described for the interaction between methanol and β-CD ($K_m = 0.32\ M^{-1}$) or α-CD ($K_m = 0.93\ M^{-1}$) makes it a more favourable

Table 1
Effect of additives in mobile phase on retention time of oxyresveratrol (flow-rate, 1.00 ± 0.01 mL/min, temperature, 25.0 ± 0.1 °C).

Retention time					
Mobile phase (% H_2O)	No addition	0.5 mM β-CD	1 mM β-CD	3.5 mM D-glucose	7 mM D-glucose
70%	13.6 ± 0.1	12.1 ± 0.1	10.3 ± 0.1	13.6 ± 0.1	13.6 ± 0.1

medium for oxyresveratrol–CD complexation than other alcohols such as ethanol (K_m for β-CD = 0.93 M^{-1}; K_m for α-CD = 5.62 M^{-1}) or 1-propanol (K_m for β-CD = 3.71 M^{-1}; K_m for α-CD = 23.44 M^{-1}); (ii) the fact that the solubility of β-CD in methanol is greater than in acetonitrile and THF permits the concentration of the β-CD in the mobile phase to be increased, thus improving characterization of the oxyresveratrol/β-CD complexes. For these reasons, binary mixtures of methanol:water were used as the optimum composition of the mobile phase in RP-HPLC to study the complexation of oxyresveratrol by β-CD.

3.2. Complexant behaviour of CDs in RP-HPLC systems

As mentioned above, the characterization of oxyresveratrol complexes in this study is based on the addition of CDs to different RP-HPLC mobile phases that may produce changes in the main chromatographic parameters. However, before using of CDs as components of mobile phases, several considerations must be taken into account. Because glucose is a constituent of the CD molecule, in order to confirm that the potential effect of CDs on the retention time of the guest molecule is due to it complexation ability and not to the glucidic nature of CDs, the effect of adding glucose and different CDs to the mobile phase on oxyresveratrol retention was studied, as shown in Table 1.

As can be observed, two amounts of D-glucose (3.5 and 7 mM), corresponding to 0.5 and 1 mM of β-CD, respectively, in the number of glucose units, were added to a binary mixture of methanol:water (30:70%). The results show that the addition of both 0.5 mM and 1 mM β-CD decreased the retention time, *Rt*, of oxyresveratrol, whereas the presence of D-glucose did not alter the *Rt* values even though the concentration of D-glucose was the same as that of β-CD as regards the number of glucose units.

Two conclusions can be deduced from these data. Firstly, the reduction in T_r values caused by the addition of β-CD to the mobile phase is due to the formation of an inclusion complex because no glucose/oxyresveratrol complexes were formed. Secondly, RP-HPLC appears to be a satisfactory method for observing and characterizing oxyresveratrol–β-CD inclusion complexes.

3.3. Effect of the cyclodextrin structure on the complexation of oxyresveratrol

To characterize the interaction between oxyresveratrol and the natural CDs at molecular level, the next step was to study the interaction between oxyresveratrol and several types of CD with differing structure, size and glucose number of glucose units. Three types of natural CD with GRAS status, all approved recently for use as additives in the European Union (α-, β- and γ-CD), were used to this end. The corresponding E-numbers assigned are E-457, E-459 and E-458, respectively. Fig. 1 depicts the effect of adding increasing α-, β- and γ-CD concentrations on the retention time of oxyresveratrol. As can be observed, the lowest retention time was obtained with β-CD, followed by α-CD and γ-CD.

At the molecular level, our data show that the inner diameter of the CD formed by seven units of glucose (β-CD: 6.0–6.4 Å) fitted oxyresveratrol better than the inner diameter of six units (α-CD: 4.7–5.2 Å) or eight units (γ-CD: 7.5–8.3 Å) of glucose.

The fact that β-CD was the optimum natural CD for complexing oxyresveratrol is in good agreement with the results of most of papers which compare the complexation of several stilbenoids compounds with natural CDs [7–11].

Since β-CD was the most effective CD for complexing oxyresveratrol, this natural CD was chosen to continue the investigation.

3.4. Mechanism of complexation of oxyresveratrol by β-CD

Based on different studies that have reported the competing equilibria in the column of an HPLC system upon introduction of the solute into a mobile-phase mixture consisting of β-CD and a primary organic modifier [7,32], the presence of oxyresveratrol in the column in the presence of β-CD in the mobile phase was studied, obtaining the equilibria presented in Fig. 2A and B for a 1:1 and 1:2 stoichiometry, respectively. As can be observed, when β-CD is added to the mobile phase, oxyresveratrol retention is governed by its partition between the mobile and stationary phases, and the oxyresveratrol complexation with β-CD.

To determine the K_F values for the oxyresveratrol/β-CD complexes, Eq. (3), which relates the capacity factor, *k*, and the β-CD mobile-phase concentration, [CD], is proposed [25,32,34]. In this equation we have assumed two conditions: (1) the complex presents a 1:1 stoichiometry and (2) interaction of the oxyresveratrol/β-CD complex with the stationary phase is negligible.

$$\frac{1}{k} = \frac{1}{k_0} + \frac{K_F}{k_0}[\mathrm{CD}] \quad (3)$$

where *k* is the capacity factor of the solute, k_0 the solute capacity factor in the absence of CD, K_F is the apparent formation constant of the inclusion complex and [CD] is the β-CD mobile-phase concentration.

Although several authors have claimed that stilbenes can not form CD complexes with a 1:2 stoichiometry, López-Nicolás et al. [10] demonstrated that trans-stilbene can able to be complexed by two molecules of HP-β-CD. For this reason, we studied the possible formation of a 1:2 oxyresveratrol/β-CD complex via a pre-

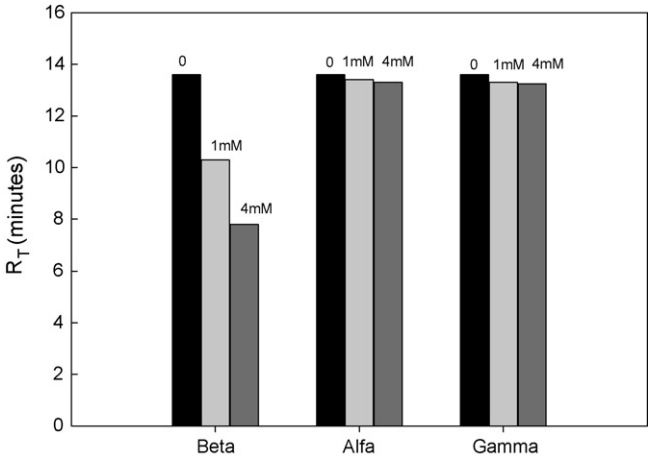

Fig. 1. Effect of different concentrations (0, 1 and 4 mM) of natural CDs on the retention time of oxyresveratrol at 25 °C and pH 7.0 with a methanol–water (30–70%) mobile phase (flow-rate, 1.00 ± 0.01 mL/min).

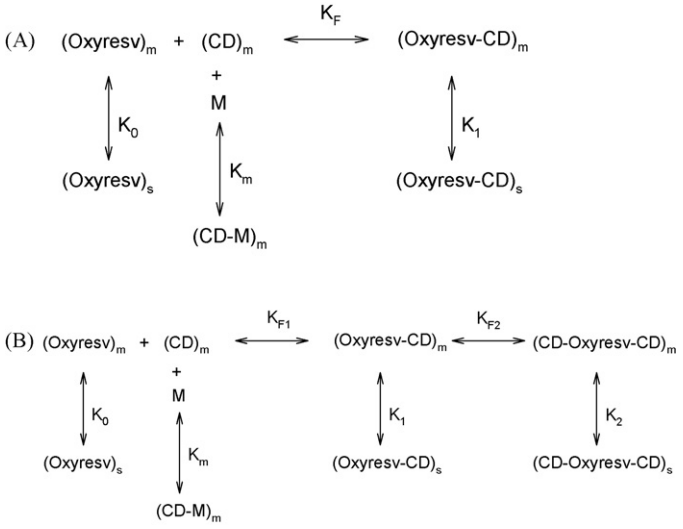

Fig. 2. (A) Equilibria proposed for a 1:1 oxyresveratrol–β-CD inclusion complex. (B) Equilibria proposed for a 1:2 oxyresveratrol–β-CD inclusion complex. Abreviatures: Oxyresv: oxyresveratrol; CD: β-cyclodextrin; Oxyresv–CD: oxyresveratrol–β-CD complex; CD–oxyresv–(CD): oxyresveratrol–$(\beta\text{-CD})_2$ complex; m: mobile phase; s: stationary phases; M: organic modifier; K_m: affinity constant of the modifier for the β-CD cavity; K_F: formation constant for the oxyresveratrol–β-CD complex in a 1:1 model; K_{F_1} : formation constant for the oxyresveratrol–β-CD complex in a 1:2 model; K_{F_2} : formation constant for the oxyresveratrol–$(\beta\text{-CD})_2$ complex in a 1:2 model; K_0: equilibrium constant of oxyresveratrol between mobile and stationary phase; K_1: equilibrium constant of oxyresveratrol–β-CD complex between mobile and stationary phase; K_2: equilibrium constant of oxyresveratrol–$(\beta\text{-CD})_2$ complex between mobile and stationary phase; CD-M: β-CD–organic modifier interaction.

cursor 1:1 complex (Fig. 2B). Eq. (4) is an extension of Eq. (3) and includes a second-order term that accounts for the possibility of 1:2 oxyresveratrol–β-CD complex formation:

$$\frac{1}{k} = \frac{1}{k_o} + \frac{K_{F_1}}{k_o}[\mathrm{CD}] + \frac{K_{F_1}K_{F_2}}{k_o}[\mathrm{CD}]^2 \quad (4)$$

where k_0 is the capacity factor of oxyresveratrol in the absence of β-CD modifier, K_{F_1} is the apparent formation constant for the 1:1 oxyresveratrol–β-CD complex and K_{F_2} is the apparent formation constant for the 1:2 oxyresveratrol–β-CD complex.

Using Eq. (3), a plot of the reciprocal of *k versus* [CD] should give a straight line, indicating the formation of 1:1 oxyresveratrol–β-CD complex. However, in the case of a 1:2 oxyresveratrol–β-CD complex formation, a plot of reciprocal of *k versus* $[\mathrm{CD}]^2$ should give a parabolic curve that fits Eq. (4).

3.5. Effect of organic solvent concentration on oxyresveratrol retention

Once methanol was selected as the best organic solvent and demonstrated the ability of CDs to complex oxyresveratrol in RP-HPLC systems, the next step in this research was to calculate the optimum methanol concentration for studying the inclusion process. For this, two factors were considered: the inclusion of methanol in the CD cavity and the analysis time. There is competition between methanol and oxyresveratrol for access to the CD hydrophobic capacity since the association constant of methanol with β-CD is $0.32\,\mathrm{M}^{-1}$ [31]. Therefore, a substantial amount of methanol can interact with β-CD when a significant percentage of methanol is present in the mobile phase, leading to competition with oxyresveratrol complexation. Several authors have reported that methanol concentrations higher than 30% in binary methanol:water mixtures reduce dramatically the inclusion process of guest molecules into β-CD [7]. On the other hand, as is shown in Fig. 3, the content of the organic solvent in the mobile

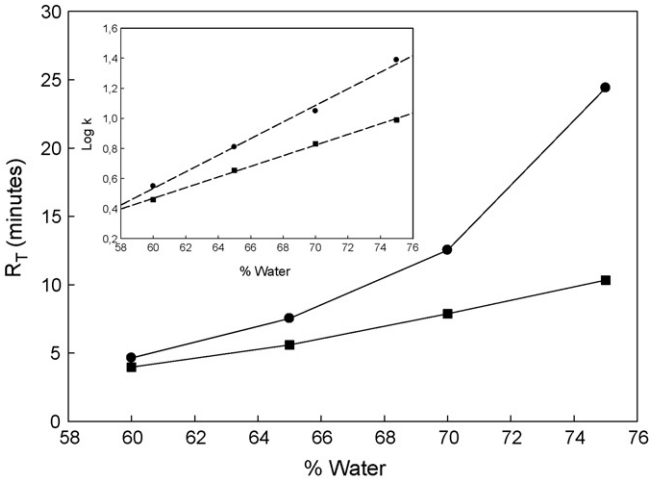

Fig. 3. Effect of water percentage on the retention time of oxyresveratrol in different methanol–water mobile phases. (Inset) Correlation of the log of the capacity factor (log *k*) of oxyresveratrol with volumetric fraction of water. (●) Without β-CD in the mobile phase, (■) with 4 mM β-CD in the mobile phase (flow-rate, 1.00 ± 0.01 mL/min; temperature, $25 \pm 1\,^{\circ}\mathrm{C}$). Each data point is the mean of 3 replicates.

phase also influences the retention of the guest molecule. Indeed, in the presence of β-CD, oxyresveratrol retention increased with increasing water percentage. Because water concentrations higher than 70% produced very long retention times (higher than 25 min), with the associated experimental error, and water concentrations lower than 70% increased the competitive effect of methanol for the CD cavity, a 30:70% methanol:water mobile phase was selected.

In addition, is necessary to verify that the presence of CDs in the mobile phase does not modify the classical "reversed-phase" elution mechanism. To this end, a representation of logarithm of *k* of oxyresveratrol as a function of water percentage in the mobile phase was plotted, in the absence or presence of β-CD (Fig. 3, inset). In the solvent composition range studied (60–75% of water in water–methanol mixtures), linear relationships (correlation coefficient >0.99) between the retention factor (*k*) logarithm of oxyresveratrol and the water percentage in the mobile phase were observed in both the absence and presence of different concentrations of 4 mM β-CD (Fig. 3, inset), confirming that the presence of β-CD does not affect the reversed-phase elution mechanism.

3.6. Effect of β-CD concentration on oxyresveratrol retention

In a previous section, we demonstrated that the presence of β-CD in the mobile phase reduced the oxyresveratrol retention time. However, is necessary to verify whether this behaviour is dependent on the β-CD concentration. Fig. 4 shows the capacity factors of oxyresveratrol in both the absence and presence of increasing β-CD total concentrations based on the solute retention time and the void time in different water–methanol proportions in the mobile phase.

As observed in Fig. 4, the formation of oxyresveratrol/β-CD complexes enhanced oxyresveratrol solubility in the mobile phase and reduced its residency time in the column, leading to a significant decrease in the retention time of the guest molecule. However, although the capacity factor of oxyresveratrol decreased regardless of the methanol percentage used when β-CD was added to the mobile phase (0–4 mM), such a decrease was always most pronounced at the lowest mobile-phase methanol concentration.

Several factors might explain this behaviour, including the polarity of the mobile phase or the interaction between CD and the organic solvent present in the mobile phase. For example, the sharp

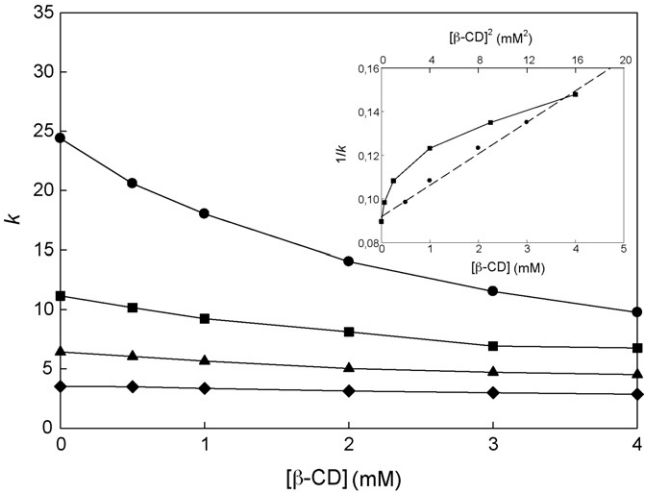

Fig. 4. Effect of total β-CD concentration on the retention factor *k* of oxyresveratrol in different methanol–water mobile phases. Methanol percentages: (●) 25%; (■) 30%; (▲) 35%; (♦) 40% (flow-rate, 1.00 ± 0.01 mL/min; temperature, 25 ± 1 °C). Each data point is the mean of 3 replicates. (Inset) Reciprocal plot of oxyresveratrol complexed to β-CD with a methanol–water (30–70%) mobile phase (flow-rate, 1.00 ± 0.01 mL/min) for determining the stoichiometry of oxyresveratrol–β-CD complexes: 1/*k versus* [β-CD] (assumption of 1:1 complex) (filled circles); (b) 1/(*k*) *versus* [β-CD]² (hypothesis of 1:2 complex) (filled squares).

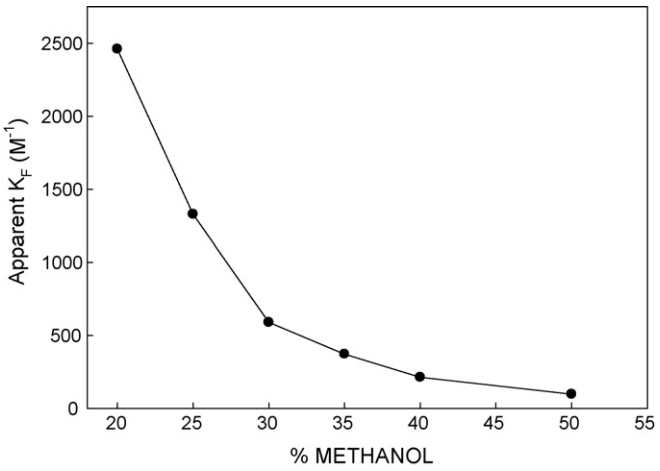

Fig. 5. Apparent formation constants (K_F) of oxyresveratrol–β-CD complexes as a function of methanol percentage in methanol–water mobile phases (flow-rate, 1.00 ± 0.01 mL/min; temperature, 2 ± 1 °C). Each data point is the mean of 3 replicates.

decrease in the oxyresveratrol capacity factor observed in Fig. 4 for different binary mixtures of methanol–water used as mobile phase could be explained by CD complexation: the higher the β-CD concentration, the faster the elution. As the eluent methanol concentration increases from 25 to 40%, the decrease in the mobile-phase polarity provokes a decrease in complexation. The solute affinity for the hydrophobic cavity of β-CD diminishes and part of the driving force for inclusion is removed because the amount of methanol present provides a less polar mobile phase, in which the non-polar solutes become more soluble. Similar data were presented by Clarot et al. [32] for the complexation of other guest molecules with native and modified CDs.

Another factor that would explain the behaviour presented in Fig. 4 is the interaction between methanol and β-CD described above. So, a substantial amount of methanol can interact with β-CD when a significant methanol percentage is present in the mobile phase, leading to competition with oxyresveratrol complexation.

3.7. Stochiometry of the oxyresveratrol–β-CD complexes

Several papers have reported the stoichiometry for complexes of some stilbenoids with natural and modified CDs, showing different results. Although CDs form complexes with resveratrol [7–9], pterostilbene [10] and pinosylvin [11] with a 1:1 stoichiometry, López-Nicolás et al. [10] showed that trans-stilbene forms 1:2 complexes with HP-β-CD. For this reason, and because this is the first study where the interaction of oxyresveratrol with β-CD is studied, it was necessary to investigate the stoichiometry for β-CD complexes of oxyresveratrol. Using Eqs. (3) and (4), the reciprocal of *k* for oxyresveratrol was plotted as a function of [β-CD] to determine the stoichiometric ratios for oxyresveratrol/β-CD complexes. In our study, a plot of 1/*k versus* [β-CD] gave a straight line with a linear correlation higher than 0.99, indicating that the presumed stoichiometry of the oxyresveratrol/β-CD complexes formed was 1:1 (Fig. 4, inset, filled circles). On the other hand, when 1/*k* was plotted against $([\beta\text{-CD}])^2$, a non-linear relationship was obtained (linear correlation of 0.82) (Fig. 4, inset, filled squares), which indicates that the stoichiometry of the inclusion complex is not 1:2.

3.8. Effect of methanol percentage on both apparent and effective K_F values

The K_F values for β-CD complexes indicate the stability of the complex and the conditions that can modify the inclusion process such as temperature, pH, presence of modifiers, etc. Moreover, these K_F values have an important role for the development of food, pharmaceutical, analytical and other uses for β-CD. For these reasons and since the K_F values between oxyresveratrol and β-CD will reveal the amount of oxyresveratrol complexed in equilibrium with free oxyresveratrol, the next step of this investigation was to calculate these constants.

In the previous section we demonstrated that, in all the conditions tested, oxyresveratrol forms complexes of 1:1 stoichiometry with β-CD. For this reason the equilibrium 1:1 with β-CD was used to calculate the K_F values for oxyresveratrol complexes with β-CD at different methanol concentrations.

The values of apparent K_F for mobile phases containing different water percentage were obtained from linear graphs of 1/*k versus* [CD] using the data shown in Fig. 4 and linear regression slopes and intercepts. The strong dependence of apparent K_F values on the methanol concentration employed in the mobile phase is shown in Fig. 5, where a similar behaviour to that obtained for the complexes between resveratrol and this type of natural CD can be observed [7]. The existence of a strong competition on the part of methanol and solute for the β-CD cavity reported by different authors [32,34] explains the dramatic decrease in K_F values observed between 25 and 40% methanol content. Moreover, the decrease in K_F values can be interpreted by reference to hydrophobic interactions, which are known to play a key role in the inclusion process. The transfer of a solute containing a hydrophobic moiety from a polar solvent to the hydrophobic β-CD cavity, leads to a large decrease in solute free energy and favours complexation. As the mobile phase increases in polarity, the polarity difference between the β-CD cavity and the eluent will become more intense. Consequently, complex formation will be even more strongly favoured [30].

3.9. Effect of temperature on the complexation of oxyresveratrol by β-CD

One of the most important parameters that must be studied concerning the use of oxyresveratrol/β-CD complexes as ingredients in food industry is the effect of temperature on the complexation mechanism. Indeed, several researchers have studied the changes

that occur in the equilibrium between CD and different compounds when the temperature of the medium varies. While some authors found that an increase in the temperature of the system leads to an increase in the K_F values, as is the case of the fatty acids-CD complexes [18], others found that a decrease in the system's temperature causes a dissociation of these complexes independently of the temperature used [1].

In order to clarify the role of temperature on the complexation of oxyresveratrol with β-CD, the effect of temperature on the effective K_F was studied for the oxyresveratrol–β-CD interaction between 15 and 30 °C (Fig. 6). For all the temperatures tested the stoichiometry of the oxyresveratrol/β-CD complexes was 1:1, the reciprocal of k for oxyresveratrol *versus* [CD] showing a correlation coefficient higher than 0.99 (data not shown) for a binary mixture of methanol–water (30–70%) as mobile phase. From these data, different K_F values were obtained for different temperatures (Fig. 6), it being found that an increase in temperature leads to a lower degree of complexation of oxyresveratrol by β-CD.

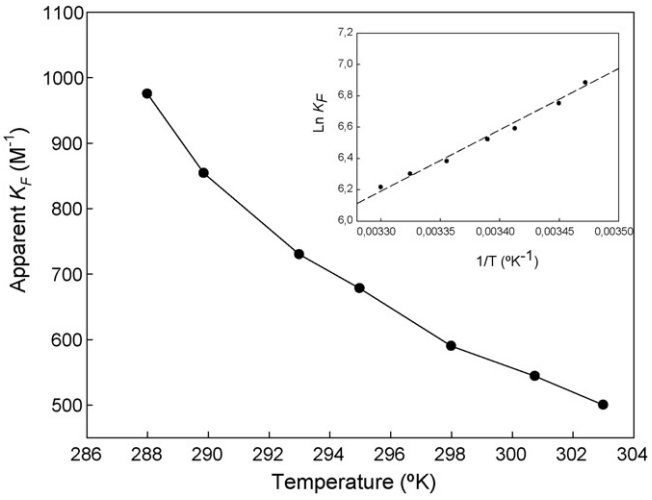

Fig. 6. Apparent formation constant (K_F) of oxyresveratrol–β-CD complexes as a function of temperature. (Inset) Van't Hoff plot ($\ln K_F$ *versus* $1/T$) for oxyresveratrol–β-CD complexes. Mobile phase: methanol–water (30–70%) (flow-rate, 1.00 ± 0.01 mL/min). Each data point is the mean of 3 replicates.

3.10. Thermodynamic parameters for the oxyresveratrol–β-CD complexes

The next step of our investigation was to study the main thermodynamic parameters of the complexation process (ΔH°, ΔS° and ΔG° at 25 ± 0.2 °C) in order to study mechanistic aspects of the affinity of oxyresveratrol for β-CD. For this, a van't Hoff plot (Eq. (1)) was used and the $\ln K_F$ was plotted *versus* $1/T$. The data showed a lineal representation, with correlation coefficient higher than 0.99 (Fig. 6, inset).

The results obtained to three main conclusions being drawn concerning the nature of the complexation of oxyresveratrol by β-CD: (i) The process is *exothermic*: the negative values obtained for enthalpy changes (-32.61 ± 1 kJ mol^{-1}) indicate the exothermic nature of the interaction processes of oxyresveratrol with β-CD. This behaviour is typical of hydrophobic interactions, van der Waals interactions, the displacement of water molecules from the cavity of β-CD or the formation of hydrogen bonds; (ii) The process presents a negative value for entropy changes (-56.13 ± 2 J mol^{-1} K^{-1}) due to a decrease in the translational and rotational degrees of freedom of the complexed oxyresveratrol compared with the free ones; (iii) The process is *spontaneous*, as seen for the negative value obtained for the Gibbs free energy change (-15.88 ± 1 kJ mol^{-1}) for the interactions that take place during the inclusion process at 25 ± 0.2 °C.

3.11. Effect of the structure of different stilbenes on the apparent formation constants of the stilbene/HP-β-CD complexes

To obtain information on the mechanism involved in the complexation of different compounds of the stilbenoid family by β-CD, the last step of this work was to compare both the effective K_F values and the stoichiometry for the complexation of four stilbenoids (oxyresveratrol, resveratrol, pterostilbene and pinosylvin) that differ in the number of the hydroxyl groups and in the type of substituents of the aromatics ring (Fig. 7) by β-CD.

Firstly, the stoichiometry calculated for all the stilbenoid/β-CD complexes studied was 1:1 (Table 2), indicating that only one molecule of this type of oxyresveratrol, resveratrol, pterostilbene or pinosylvin can be complexed by a molecule of β-CD. However, several differences were evident from the comparison of the K_F values of the different complexes. As Table 2 shows, the highest K_F value was obtained for the resveratrol/β-CD complexes, followed by the oxyresveratrol/β-CD complexes, pterostilbene/β-CD complexes and finally the pinosylvin/β-CD complexes.

This behaviour can be attributed to a variety of reasons, such as the resonance structure of the guest molecules or the hydropho-

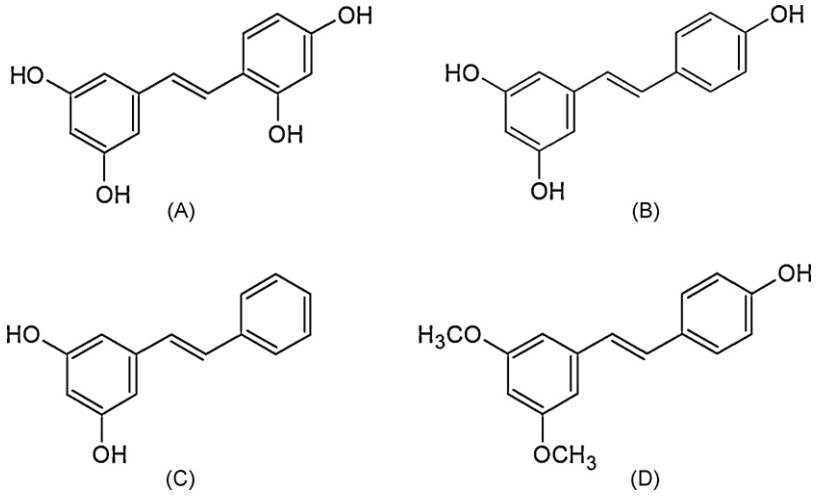

Fig. 7. Structure of oxyresveratrol (A); resveratrol (B); pinosylvin (C) and pterostilbene (D).

Table 2
Apparent K_F values and correlation coefficients arising from Eqs. (3) and (4) for different stilbenes β-*CD* complexes at 25 °C at pH 7.0 0 in a methanol:water (30:70%) medium.

Complex	K_F (M^{-1})	Correlation coefficient	
		1:1 using Eq. (3)	1:2 using Eq. (4)
Resveratrol–β-CD	1805 ± 35	0.99	0.91
Oxyresveratrol–β-CD	590 ± 23	0.99	0.86
Pterostilbene–β-CD	213 ± 11	0.99	0.92
Pinosilvin–β-CD	145 ± 10	0.99	0.89

bicity of the stilbenoids studied. So, the resonance structure of resveratrol and oxyresveratrol produces a high stability in this type of stilbenes, which is not shown by pterostilbene or pinosylvin and which facilities the inclusion mechanism, as was recently reported by López-Nicolás et al. [10]. Moreover, the hydrophobicity of the guest molecules also influences the complexation behaviour of the different stilbenes. The major hydrophilic structure of resveratrol and oxyresveratrol permits a more effective interaction with β-CD complexes than for the pterostilbene or pinosylvin complexes. Indeed, oxyresveratrol and resveratrol have four and three hydroxyl groups in their structure, respectively, while pinosylvin has two hydroxyl groups and pterostilbene has only one. Moreover, resveratrol and oxyresveratrol do not contain any methyl groups in their structure, while pterostilbene has two methyl groups, which may diminish the efficiency of the complexation by CDs.

4. Conclusion

Recent years have seen increased research into the health properties of oxyresveratrol. However, several problems associated with its low stability and bioavailability and the ease with which it is oxidized by prooxidant agents have limited its use in the food and pharmaceutical industries. To resolve these problems in this work, we propose, for first time, the use of cyclodextrins, a type of molecule which facilitates the "solubilization" of this stilbene and protects it against prooxidants agents. Moreover, the use of oxyresveratrol–β-CD complexes could slow down the rapid metabolism and elimination of oxyresveratrol, improving its bioavailability, as has been demonstrated for other β-CD complexes. Our results show that, although the stoichiometry of the complex is 1:1 for all the conditions used, the K_F values for the oxyresveratrol–CD complexes are strongly dependent on several factors, such as temperature, pH, presence of organic solvents, type of CD and structure of the guest molecule.

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