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Host–Guest Interaction Study of Resveratrol With Natural and Modified Cyclodextrins

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Abstract

The aim of this work is to increase the stability and water solubility of resveratrol by complexation with different cyclodextrins. Furthermore, physical–chemical properties of each inclusion compound were investigated. Complexes of resveratrol with cyclodextrins both native (α, β, γ) and modified (2-hydroxypropyl-β-cyclodextrin, dimethyl-β-cyclodextrin) were obtained by using the suspension method. An inclusion complex with β-cyclodextrin was also prepared by using the microwave. Solid state characterization of the products was carried out using Fourier transform infrared spectroscopy (FT-IR), differential scanning calorimetry (DSC), X-ray diffraction (DRX); solution studies were performed by UV–Vis spectrophotometry and ^{1}H-NMR spectroscopy. Phase solubility profiles with all cyclodextrins employed were classified as A_N type, indicating the formation of 1:1 stoichiometric inclusion complexes. Stability constants (K_c) from the phase solubility diagrams were calculated. Stability studies in the solid state and in solution were performed; the photodegradation by UV–Vis spectrophotometry was monitored. The isomerization rate *trans* to *cis*, in ethanol solution, decreased with inclusion. The dissolution studies revealed that resveratrol dissolution rate was improved by the formation of inclusion complexes.

Introduction

Resveratrol (*trans* 3, 5, 4′-trihydroxystilbene) (Figure 1) is a polyphenolic phytoalexin that is found, free and conjugated, in high concentrations in grapes juice, peanuts, mulberries and in other plant extracts [1, 2]. Phytoalexines are low molecular weight secondary metabolites made by plants as a defence response to microbial infections [3].

Extracts containing resveratrol have been already used in traditional Chinese and Japanese medicine for treating inflammation and cardiovascular diseases, dermatitis, gonorrhea, fever and hyperlipidemia [4]. Recent studies have shown that resveratrol is responsible for the cardiovascular benefits associated with moderate red wine consumption [5], that used to explain the so-called "French paradox" (a very low mortality rate due to coronary heart disease despite a high-fat diet and partially smoking habits) [6].

Some studies have provided evidence that resveratrol modulates lipid metabolism [7], inhibits platelet activation and aggregation [8], protects the lipoproteins against oxidative and free radical damage [9–11], and it has also chemopreventing activity by inhibition of cellular events associated with tumour initiation, promotion and progression [12–14]. It has also been found that resveratrol has activity against the bacterial and fungal species that are major etiological agents in human skin infections [15].

However, due to its low water solubility, resveratrol cannot be used for oral administration; this limitation could be overcome by the formation of inclusion complexes with cyclodextrins.

Cyclodextrins have been used extensively as pharmaceutical excipients to increase the solubility of poorly water-soluble compounds by the formation of an inclusion complex between the host cyclodextrin molecule and the guest drug molecule. Formation of the inclusion complex involves molecular encapsulation of the guest molecule by cyclodextrin and this results in modification of the physical–chemical properties such as solubility and stability of the guest molecule [16–18].

The present work has involved the preparation and characterization of inclusion complexes of resveratrol with native (α, β, γ) and modified cyclodextrins (2-hydroxypropyl-β-cyclodextrin, dimethyl-β-cyclodextrin);

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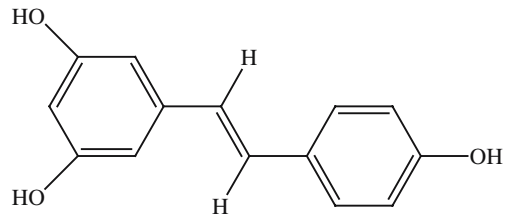


Figure 1. Chemical structure of *trans* resveratrol.

moreover, in this work it was investigated whether the low water solubility problem could be overcome by the complex formation. In addition the stability of resveratrol, pure and complexed, under different storage conditions was evaluated. As a matter of fact, from the literature [19] it is known that *trans* resveratrol exposed to sunlight at room temperature isomerizes in *cis* resveratrol within about 2 h.

The complex formation was confirmed by Fourier transform infrared spectroscopy (FT-IR), differential scanning calorimetry (DSC), powder X-ray diffraction (DRX) and ^{1}H-NMR spectroscopy. The combined use of different characterization techniques, according to the physical state considered, gave by far the best results in terms of reliability of the models.

Phase solubility diagrams and dissolution profiles of the complexes were plotted and comparatively evaluated.

Experimental

Materials

Resveratrol ($C_{14}H_{12}O_3$; molecular weight = 228,25 Dalton) was purchased from Polichimica (purity >98%, Bologna, Italy); cyclodextrins (α-CD, γ-CD), 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) and dimethyl-β-cyclodextrin (DM-β-CD) were purchased from Aldrich, while β-cyclodextrin (β-CD) was furnished by Bayer (Milan, Italy).

Preparation of the inclusion complex

The inclusion complexes between resveratrol and cyclodextrins were prepared using wet technologies and without the use of rotary evaporation because of resveratrol instability to this process [20]. Each cyclodextrin (4.38×10^{-5} mol) was finely suspended in water (2 ml) at room temperature with vigorous stirring and an equimolar amount of resveratrol (4.38×10^{-5} mol) was directly added to the suspension. After stirring for 120 h, the solvent was removed by evaporation in vacuum. An inclusion complex of β-cyclodextrin with resveratrol was also prepared by using microwave irradiation [21]: a mixture with molar ratio 1:1 of β-CD and resveratrol was grounded in a glass container with the minimum amount of solvents (ethanol:water = 1:1, v/v). The mixture was reacted for 90 s at 60 °C in a microwave oven (Microwave multimode milestone MycroSynth labstation) and than the solvents were evaporated under vacuum.

Preparation of the physical mixture

Each physical mixture (1:1) of resveratrol and cyclodextrin was prepared by mixing of pulverized powder in equimolar amounts.

Characterization of the inclusion complexes

The DSC curves of different sample were recorded on a Perkin Elmer DSC 6 differential scanning calorimeter calibrated with indium at the heating rates of 10 °C/min. The thermal behaviour was studied by heating 2 mg samples in aluminium crimped pans under nitrogen gas flow within the temperature range 50–350 °C.

The powder X-ray diffraction patterns of resveratrol, cyclodextrins, physical mixtures and inclusion compounds were recorded with a Rigaku Miniflex diffractometer with a Ni-filtered CuKα radiation detector ($\lambda = 1.5405$ Å) operating at a voltage of 30 kV and a current of 35 mA. The samples were analysed in the 2θ range from 3° to 60° with a scan angular speed of 2°/min and a scan step time of 2.00 s.

FT-IR spectra of resveratrol, cyclodextrins and corresponding inclusion compounds were collected using a Perkin Elmer FT-IR Spectrometer Spectrum One in a spectral region between 4000 and 450 cm^{-1}. Samples were mixed in a mortar with KBr (1:100) and pressed in a hydraulic press (14 tons) to small tablets, which were then placed in the infrared beam.

An optical Microscope Multiscope Perkin Elmer examined the surface morphology of resveratrol, cyclodextrins, their physical mixture and their complexes. All photographs were taken using WINTV32 and a magnification of 560×.

Proton NMR spectra of inclusion complexes, cyclodextrins and resveratrol were taken on a Bruker Avance 500 operating at 500 MHz using TMS as internal standard. Chemical shifts were expressed as ppm (δ). Samples of about 5 mg were dissolved in 0.7 ml DMSO-d_6 or D_2O.

Phase solubility studies were carried out according to the method described by Higuchi and Connors [22]. An excess of resveratrol (10 mg) was added to a series of aqueous solutions (10 ml) containing increasing concentrations of cyclodextrins (0 to 4.5×10^{-3} mmol/l) and it was shaken at 25 °C over 7 days in an orbital shaker (Instruments s.r.l., Type M201-OR). After equilibrium was reached suspensions were centrifuged (microCentrifugette 4214 ALC, 14,000 rpm). Aliquots of supernatant were taken and suitably diluted with water and resveratrol concentration was spectrophotometrically determinate (UV–Vis spectrophotometer Jasco V-530) at $\lambda = 307$ nm. Final values of resveratrol concentration are the mean of three replicate determinations. The drug standard followed Beer's law and the cyclodextrins did not interfere with the assay; the apparent stability constants (K_c) and stoichiometry of the complexes were

determined from the initial straight-line portion of the phase solubility diagrams according to the following equation: $K_c = \text{slope}/S_0(1\text{-slope})$ where S_0 is the solubility of resveratrol in the absence of cyclodextrin.

Calibration curve of resveratrol was performed in ethanol using known concentrations ranging from 0 to 20 μg/ml. Sample preparation and analyses were carried out at room temperature. Calibration graphs were plotted according to the linear regression analyses, which gave a correlation coefficient value (R^2) of 0.9986.

In vitro dissolution studies

A comparison between the dissolution behaviour of the complexes with β-CD and HP-β-CD and of pure resveratrol was carried out using transparent gelatine capsule containing an amount of formulation equivalent to 5 mg of resveratrol.

The dissolution profiles were collected using a Erweka apparatus according to the USP rotating basket method. The dissolution media consisted of 500 ml of distilled water; the experiments were performed at 37 ± 0.3 °C at a rotation speed of 100 ± 2 rpm.

At pre-selected time, 2 ml samples were withdrawn, filtered through polycarbonate membrane (Millipore 0.45 μm) and replaced with 2 ml of prethermostated fresh dissolution medium. Quantitative determination of resveratrol was carried out by an HPLC system consisting of a liquid chromatograph Alliance 2690 (Waters) equipped with a photodiode array detector and a computer integrating apparatus (Millenium 32). Data represent the mean value of two separate determinations. Analyses were performed at 306 nm with a Nova Pack C_{18} column. The mobile phase was a mixture of 75% water, 20% methanol and 5% acetic acid, delivered with a flow rate of 1.2 ml/min.

Stability studies

The stability studies of resveratrol and the corresponding inclusion compounds were assessed both in the solid state and in ethanol solution at room temperature in the dark, at 4 °C and under light exposure.

Photostability tests were performed using a UV set lamp at 365 and 254 nm (UV CM24/F, intensity 300/310 $\mu W/cm^2$) and natural sunlight in selected sunny and clear sky days (considering the few hours required for the experiment).

At specified time intervals, samples were taken and the concentration of *trans* resveratrol was determined by UV–Vis spectrophotometry; the results were expressed as percentage of the remaining *trans* resveratrol. Each test was carried out in triplicate.

Each sample of pure resveratrol powder and corresponding complexes with cyclodextrins was placed and spread uniformly as a thin film on open glass plate (Ø 48 mm) according to the ICH guidelines [23]. The fine powders on the plates were discrete enough to allow for uniform irradiation.

The solutions for stability studies were prepared dissolving 2 mg of each sample in 20 ml of ethanol. The clear solutions obtained were stored in the same conditions described above.

Result and discussion

DSC

Thermal analysis has been reported as a method to characterize cyclodextrin complexes [24–26].

In Figure 2, DSC profiles of resveratrol, α-CD, physical mixture and the corresponding inclusion complex are shown.

In the DSC curves of all complexes the absence of the melting endothermic peak of resveratrol at 267 °C can be noticed. Furthermore, the loss of crystal water at around 100 °C, as endothermic peak, was the result of displacement of bound water in the cavity by the guest [27]. On the other hand, in the thermograms of all physical mixtures the endothermic peak of resveratrol was present.

These results confirmed that the real inclusion complexes were formed between resveratrol and all tested cyclodextrins.

DRX

The DRX patterns of pure resveratrol, cyclodextrins, physical mixture and corresponding inclusion compounds, confirmed the results of DSC analyses.

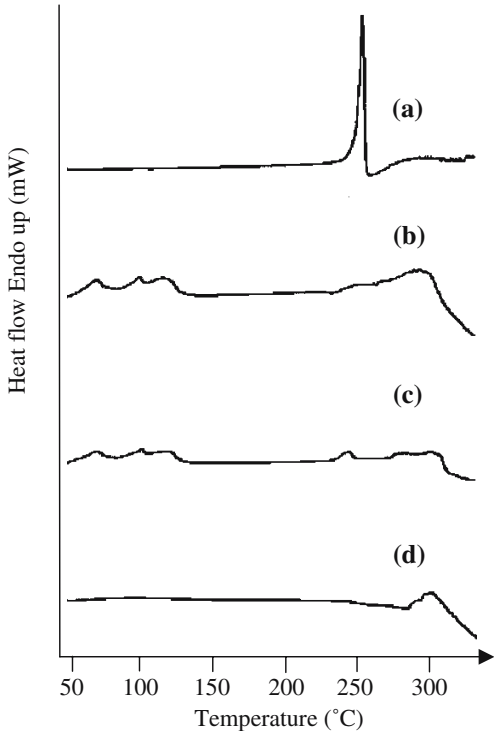


Figure 2. DSC thermograms of resveratrol (a), α-CD (b), physical mixture (c) and inclusion complex (d).

The diffraction peaks relating to crystalline resveratrol were no longer detectable in all cyclodextrin systems, and the reduction of the crystalline degree may be considered as an indirect proof of complexation [28].

In the complexes a reduced number of peaks were noticed, and remarkably lowered intensity were noticed too, thus meaning a greater amorphousness of the inclusion compounds compared to the free molecule.

In Figure 3 are reported, as example, the X-ray powder diffraction patterns of pure resveratrol, β-CD, the corresponding physical mixture and inclusion compounds obtained using the microwave and the suspension method. The physical mixture pattern is approximately the superposition of the raw materials. The intensity of the characteristic resveratrol profile ($2\theta = 6.640$) decreases extending the raw materials mixing time; this behaviour could be related with a starting complexation.

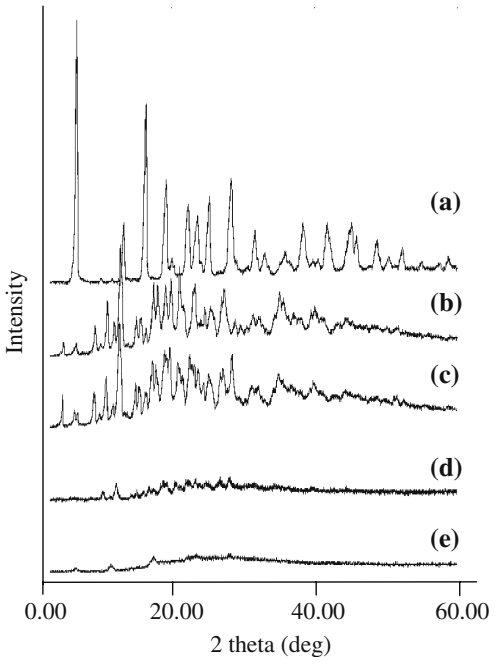


Figure 3. X-Ray diffractograms of resveratrol (a), β-CD (b), physical mixture (c), inclusion complex using suspension method (d) and inclusion complex using microwave (e).

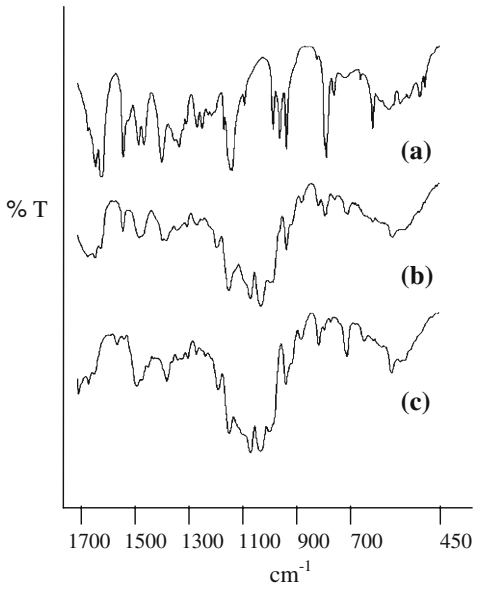


Figure 4. IR spectra of resveratrol (a), inclusion complex (b) and DM-β-CD (c).

FT-IR

Figure 4 shows infrared spectra of resveratrol, inclusion complex and DM-β-CD.

FT-IR absorption in the range of 1700–450 cm^{-1} of resveratrol showed three characteristic intense bands at 1606, 1587, 1384 cm^{-1} corresponding to C–C aromatic double bond stretching, C–C olefinic stretching and C–O stretching. The typical *trans* olefinic band is at 965 cm^{-1}.

The FT-IR spectra of the inclusion compounds showed changes in the spectral features of the guest molecule; in fact the band intensities at 1384 and 965 cm^{-1} decreased while the bands at 1606 and 1587 cm^{-1} disappeared. These evidences could be due to the formation of inclusion complexes [29].

Optical microscopy

The photographs (Figure 5) were collected for resveratrol, cyclodextrins, corresponding physical mixture and inclusion compounds in different molar ratios (1:1, 1:2, 2:1).

These analyses confirmed the real formation of new compounds. In fact, resveratrol (Figure 5a) appeared as small agglomerate crystals, DM-β-CD had prismatic like crystallites (Figure 5b) and the physical mixture showed the presence of both raw compounds (Figure 5c). The complexes obtained (Figure 5d) with equimolar amounts of host and guest showed a morphological and dimensional modification of the crystallites.

The photographs of 2:1 (Figure 5e) and 1:2 (Figure 5f) guest:host complexes exhibit the crystallites of the complex and agglomerate of resveratrol or DM-β-CD in excess, respectively.

All the cyclodextrins investigated showed similar morphological characteristics.

^{1}H-NMR

The NMR-techniques are commonly used to study inclusion complexes of cyclodextrins. In Table 1 the ^{1}H-NMR chemical shift displacements of each cyclodextrin are summarized. The shifts of H_3 and H_5 protons located inside the torus were by far the most affected [30], while the externals protons (H_1, H_2 and H_4) were shifted much less by the inclusion process, indicating a lower probability of interaction with resveratrol. H_6 proton, located on the cavity rim, was also shifted, but to a smaller extent. Unfortunately, the HP-

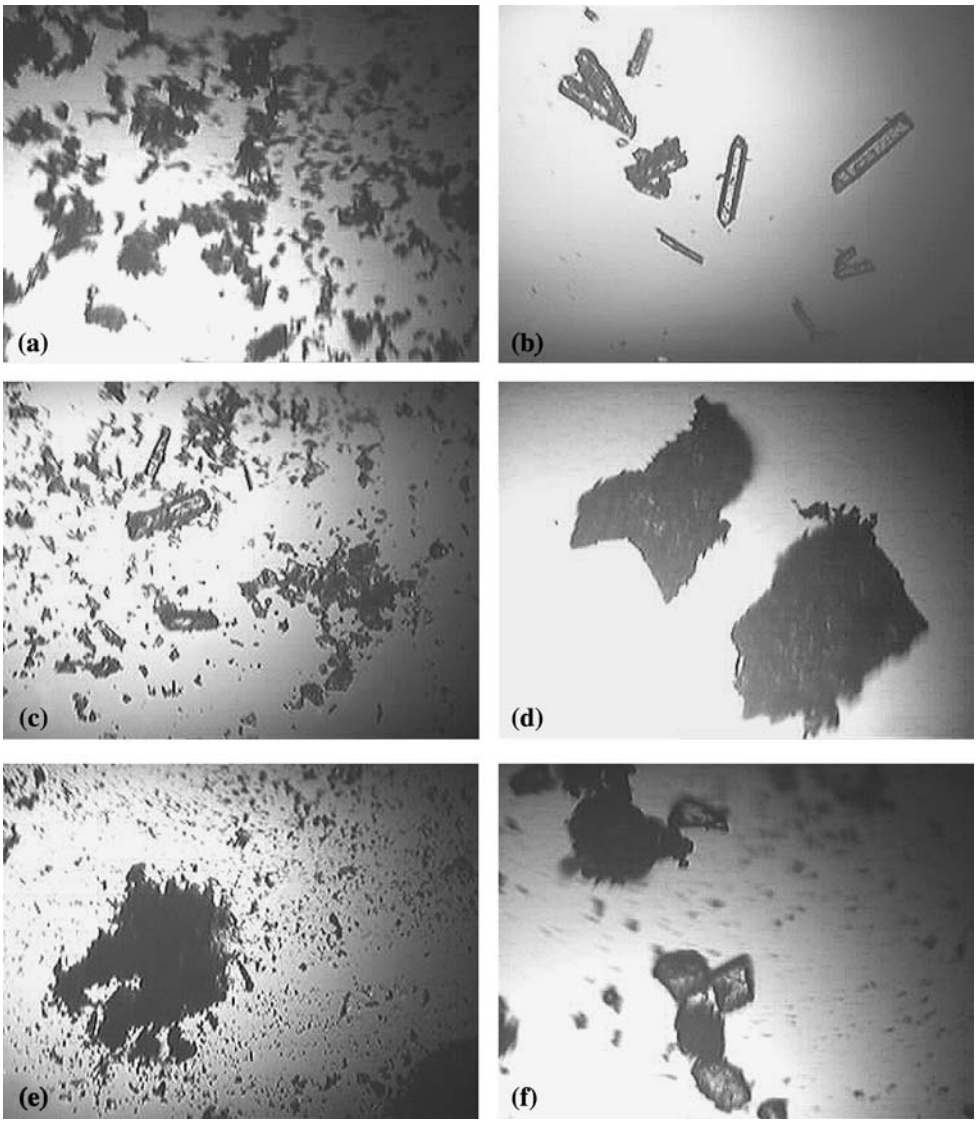


Figure 5. Photographs collected with an optical miscroscope: resveratrol (a), DM-β-CD (b), physical mixture (c), 1:1 (d), 2:1 (e), 1:2 (f) guest:host inclusion compounds.

β-CD system cannot be quantitatively determined because of the overlapping with other signals due to its multicomponent mixture. The shift of most cyclodextrins signals to higher field suggested that the driving forces for the formation of the inclusion complexes were predominantly hydrophobic interactions [31, 32]. The larger upfield shift observed for the internals protons was undoubtedly due to the ring current effects generated by the circulating π electrons of the aromatic guest [33].

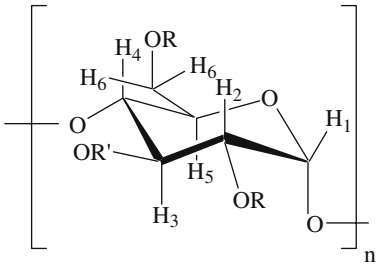
In Figure 6 and 7 are shown as an example the ^{1}H-NMR spectra of resveratrol and β-CD free and complexed. The signal of H_5 was not detectable in the free β-CD, since it was hidden by H_6 signal, whereas it became evident in the complex, as a consequence of the upfield shift.

As shown in Table 2 the proton signals of resveratrol presented up or downfield shifts between the free and complexed state indicating they are all affected as a result of complexation. Since only low shift changes of signal took place, it followed that the complexation was a dynamic process in which a fast exchange existed between the free and bounded states [34, 35].

Phase solubility studies

The phase solubility diagrams were obtained by plotting the means solubilities against cyclodextrins concentrations (linear region were fitted by least-squares regression) (Figure 8). For all cyclodextrins an A_N type diagram was obtained, according to the Higuchi and Connors classification [22], where the guest solubility increased linearly with cyclodextrin concentration and showed a downward curvature. From these curves, it can be seen that the apparent solubility of resveratrol increases due to the formation of a soluble inclusion complex. Apparent stability constants (K_c) for complexes with each cyclodextrin were calculated from the ascending segments of the solubility curves by assuming that all the complexes had 1:1 stoichiometry. These values are listed in Table 3. The highest value of K_c

Table 1. ^{1}H-NMR chemical shift displacement ($\Delta\delta$, ppm) of cyclodextrins by complexation with resveratrol in D_2O. $\Delta\delta$ were expressed as $\Delta\delta = \delta_{complex} - \delta_{free\ CD}$



α-CD (n=6), β-CD (n=7), γ-CD (n=8) ; R=H

HP-β-CD (n=7); R, R'= H or $CH_2CH(OH)CH_3$

DM-β-CD (n=7); R=CH_3

Proton	with α-CD	with β-CD	with γ-CD	with HP-β-CD	with DM-β-CD
H_1	−0.0050	−0.0133	−0.0011	−0.0350	−0.0410
H_2	+0.0035	−0.0145	+0.0001	*	−0.0250
H_3	−0.1960	−0.0328	−0.0023	−0.0299	−0.0632
H_4	+0.0015	−0.0051	−0.0011	*	−0.0400
H_5	*	−0.0800	*	*	−0.1120
H_6	*	−0.0337	*	*	−0.0700

*Could not be determined due to the overlapping with other signals.

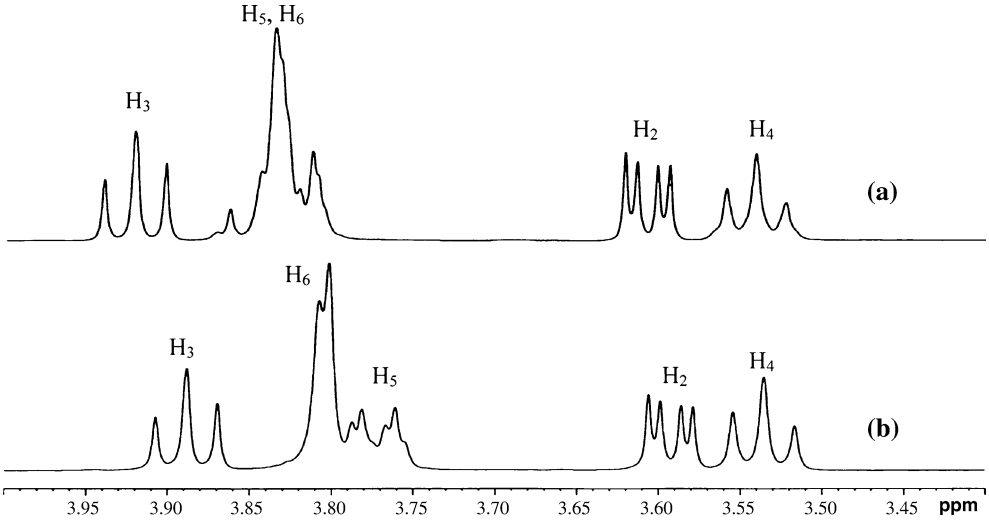


Figure 6. ^{1}H-NMR spectra: free β-CD (a) and inclusion complex (b).

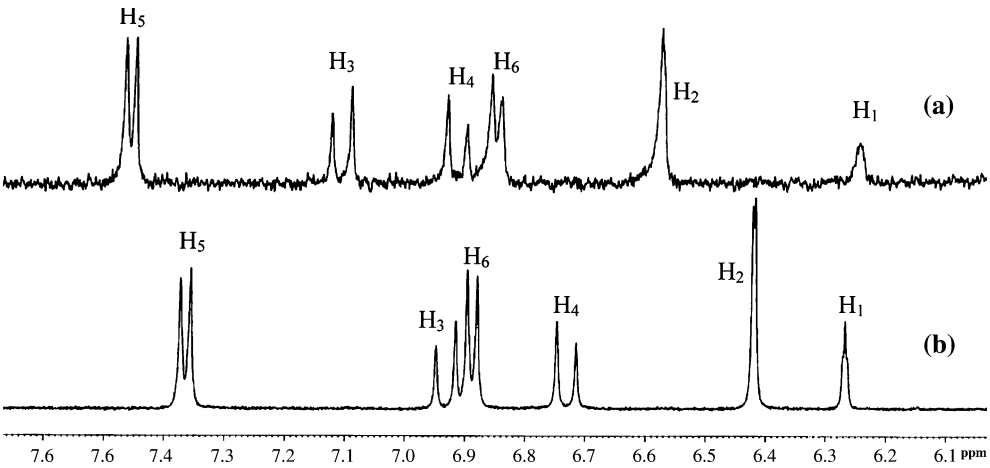
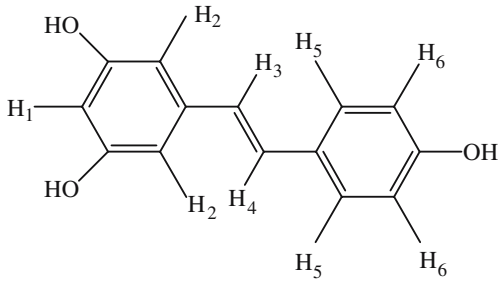


Figure 7. ^{1}H-NMR spectra: free resveratrol (a) and inclusion complex (b).

Table 2. ^{1}H-NMR chemical shift displacement ($\Delta\delta$, ppm) of resveratrol by complexation with different cyclodextrins in D_2O. $\Delta\delta$ were expressed as $\Delta\delta = \delta_{complex} - \delta_{free\ resveratrol}$



Proton	with α-CD	with β-CD	with γ-CD	with HP-β-CD	with DM-β-CD
H_1	+0.0738	+0.0225	−0.0514	+0.0057	−0.0300
H_2	−0.1517	−0.1542	−0.1156	−0.1256	−0.2032
H_3	−0.1317	−0.1763	−0.1540	−0.1286	−0.2447
H_4	−0.1988	−0.0265	−0.1656	−0.1276	−0.2300
H_5	+0.0450	−0.0949	−0.1186	−0.0629	−0.1465
H_6	+0.1040	−0.1182	−0.0440	+0.0162	−0.0278

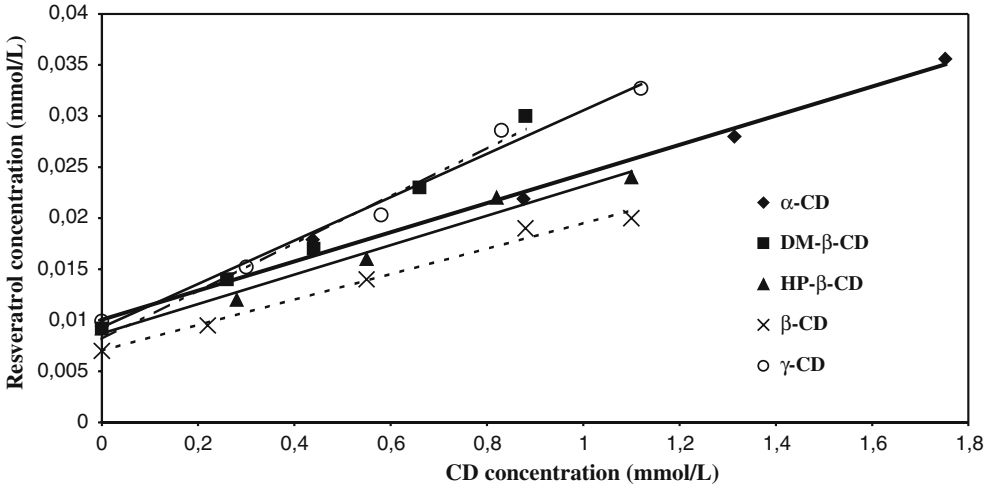


Figure 8. Initial straight line portion of the phase solubility diagrams for resveratrol with different cyclodextrins at 25 °C.

($K_c = 2604\ M^{-1}$) was found for DM-β-CD, indicating that resveratrol interacts more strongly with this cyclodextrin; this was probably due to the deeper cavity of methylated cyclodextrins than β-CD (11 against 8 Å) [36] and also to the hydrophobicity [37]. For all cyclodextrins, the slope of the straight line was less than 1, thus it was assumed that the increase in solubility observed was due to the formation of a 1:1 complex.

In vitro dissolution studies

The preliminary dissolution tests were performed on β-CD and HP-β-CD complexes because of their better water solubility.

In Table 4 dissolution percentages are reported.

The increasing of resveratrol dissolution upon combination with cyclodextrins has been noticed. The best dissolution behaviour of HP-β-CD complex can be explained by the great water solubility, high amorphizing, wetting, solubilizing and complexing power of this cyclodextrin.

Stability studies

The stability of resveratrol and the corresponding cyclodextrins complexes in ethanol solution was investi-

Table 3. Summary of the findings from phase solubility studies

CDs	R^2	S_0 (10^{-3} M)	K_c (M^{-1})
α-CD	0.989	0.0094	1014
β-CD	0.990	0.0092	2057
γ-CD	0.985	0.0093	2187
HP-β-CD	0.980	0.0092	1588
DM-β-CD	0.980	0.0092	2604

Table 4. Percentage of drug dissolved at different times

	Resveratrol	with β-CD	with HP-β-CD
10 min	n.d*	n.d*	n.d*
15 min	n.d*	n.d*	5
20 min	n.d*	5	11
30 min	3	9	45
40 min	7	14	77
50 min	10	20	76
60 min	11	21	81

*n.d, not detected.

Table 5. Stability of resveratrol and its inclusion complexes under different storage conditions: the values reported are expressed as percentage of resveratrol remained after 120 h (4 °C, room temperature in the dark and room temperature under sunlight) and after 24 h (254 and 365 nm)

		After 120 h		After 24 h	
	4 °C	Dark	Sunlight	254 nm	365 nm
resveratrol	98	88	41	37	35
resveratrol/α-CD	98	96	83	68	45
resveratrol/β-CD	98	93	37	57	50
resveratrol/γ-CD	98	93	38	50	58
resveratrol/DM-β-CD	97	94	49	61	53
resveratrol/HP-β-CD	98	99	43	44	37

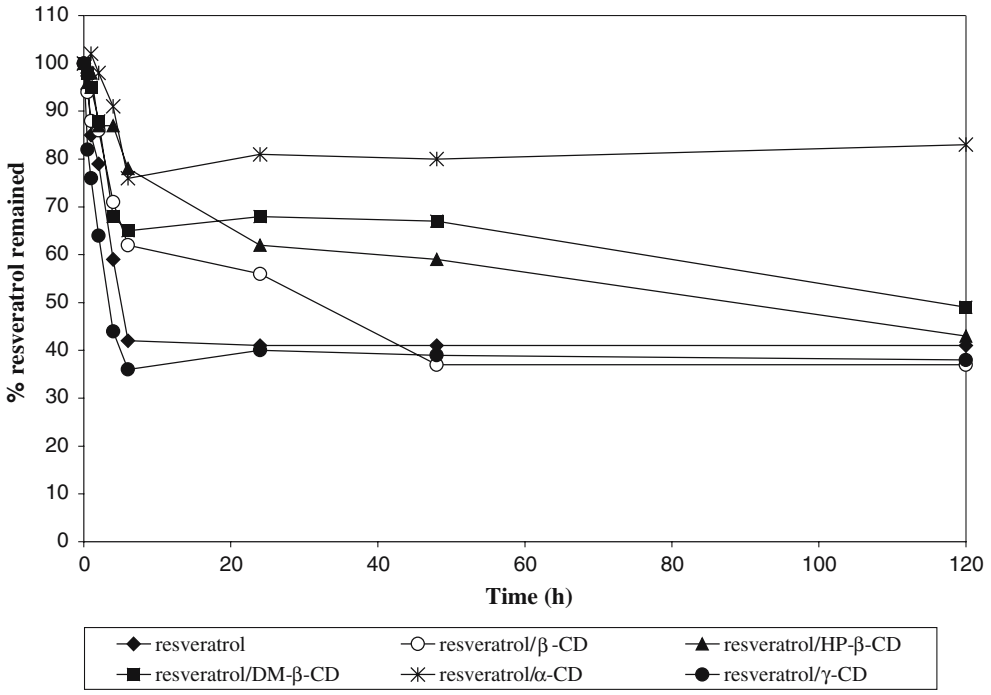


Figure 9. Stability profiles of resveratrol and inclusion complexes under sunlight exposure.

gated under different storage conditions including 4 °C, room temperature in the dark and under light exposure ($\lambda = 254$ nm, $\lambda = 365$ nm and sunlight).

As shown in Table 5, resveratrol and corresponding inclusion compounds were stable at 4 °C for up to 5 days. When samples were stored at room temperature in the dark resveratrol concentration decreased to 88% of reference value, but resveratrol concentration in the complexes remains about 95%. After 5 days of sunlight exposure the pure resveratrol level decreased to 41%; after irradiation at 254 nm and 365 nm. Only the inclusion complex with α-CD presented 83% of resveratrol. In Figure 9 the sunlight stability profile of resveratrol and corresponding inclusion compounds is reported. As shown the resveratrol percentage level strongly decreases within 6 h.

In the solid state in the same storage conditions both resveratrol and inclusion compounds were stable.

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