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DIRECCIÓN DE NUEVAS CREACIONES

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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Patente de Invención	☒	Patente de Modelo de Utilidad	☐
Vía Nacional	☐		
Vía PCT (Fase Nacional)	☒	Capítulo I	☒
		Capítulo II	☐
No. Solicitud Internacional	PCT/BR2008/000216		
Fecha de Presentación Internacional	23/07/2008		

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 9 a 29	23/02/2010
Reivindicaciones:	Folios 30 a 34	23/02/2010
Prioridad:	BR PI0705319-3	23/07/2007

2. Análisis de la Prioridad

Se acepta la reivindicación de Prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486), y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

Título de la solicitud: “Proceso de preparación del complejo soluble en agua conteniendo el compuesto resveratrol, productos formando dicho complejo y usos de los mismos”.

El problema planteado en esta solicitud consiste en suministrar un sistema que mejore la solubilidad acuosa del trans-resveratrol para mejorar su biodisponibilidad.

Para resolver este problema técnico la solicitud en estudio proporciona un proceso para la obtención del complejo soluble de resveratrol y el compuesto ciclodextrina, el complejo y una composición que lo contiene.

Expediente 10-21163 1er Art 45

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K31/05; A61K47/40

4. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

El uso de las reivindicaciones 24 a 26 no es patentable, de acuerdo con el Art 14.

5. De la Solicitud de Patente

Título

El título no corresponde con el objeto de la solicitud porque se refiere a los usos los cuales no son patentables, por lo cual se sugiere al solicitante hacer la corrección necesaria.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

La expresión "mas baja o igual a 60mM" de la reivindicación 1 carece de claridad, debido a que implica que la concentración de ciclodextrina puede ser 0mM, se recomienda establecer un rango aceptable.

La expresión "un solvente orgánico miscible en agua" carece de concisión ya que no limita el alcance de la solicitud y abarca todos los compuestos conocidos y que puedan desarrollarse que cumplan con dicha condición.

Las reivindicaciones 1, 17, 22 y 23 indican la presencia de cualquier ciclodextrina y cualquier resveratrol, sin embargo los ejemplos 1 a 3 presentados en la descripción (folio 26 y siguientes) y los estudios de solubilidad se refieren específicamente a β -ciclodextrinas y trans-resveratrol, por lo cual no es posible establecer a partir de la información proporcionada que los resultados sean extrapolables a cualquier tipo de ciclodextrina o resveratrol, sin que ello implique múltiples desarrollos experimentales por parte de la persona versada en la materia tanto para generar los complejos de los diversos tipos de resveratrol con las diferentes ciclodextrinas, como para establecer el efecto en la solubilidad de cada complejo. Se recomienda al solicitante realizar una restricción de los procesos, complejos y composiciones del capítulo reivindicatorio basado en una generalización razonable de los ejemplos definidos en la descripción.

La expresión "vehículo nutracéuticamente aceptable" de la reivindicación 22 carece de concisión y debe ser especificada, igualmente sucede con las definiciones funcionales "diluentes, lubricantes, aglutinantes, desagregantes, mezclas efervescentes, colorantes, agentes humectante; y sustancias nutracéuticamente inactivas y atóxicas", se recomienda especificar el alcance de la solicitud definiendo químicamente los componentes.

6. Determinación del Estado de la Técnica

El Estado de la Técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 23 de julio de 2007, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Item	Documento	Título	Fecha de Publicación
D1	Journal of inclusion Phenomena and Macrocyclic Chemistry (2006) 55:279-	Host-Guest Interaction Study Reveratrol with Natural and	2006

	287	Modified Cyclodextrins	
D2	US2006/0292099	Treatmen of eye disorders with sirtuin modulators	28/12/2006

D1 enseña un método para incrementar la estabilidad y solubilidad del resveratrol mediante la formación de complejo con diferentes ciclodextrinas tanto naturales (α , β , γ), como modificadas (2-hidroxipropil- β -ciclodextrina, dimetil- β -ciclodextrina). Adicionalmente, las propiedades fisicoquímicas de cada complejo de inclusión fueron investigadas, los cuales fueron obtenidos utilizando el método de suspensión.

D2http://worldwide.espacenet.com/publicationDetails/biblio?DB=EPODOC&II=2&ND=3&adjacent=true&locale=en_EP&FT=D&date=20061228&CC=US&NR=2006292099A1&KC=A1 divulga la complejación del trans-resveratrol con β -ciclodextrina, la cual es estudiada mediante la técnica de cromatografía líquida en fase reversa

7. Examen de Patentabilidad (Art 14 D486)

Nivel Inventivo (Art18 D486)

La reivindicación 1 consiste en un proceso para la obtención del complejo soluble de resveratrol y el compuesto ciclodextrina.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
proceso para la obtención del complejo soluble de resveratrol y el compuesto ciclodextrina caracterizado por:	Proceso para la obtención del complejo soluble de resveratrol y una ciclodextrina.	Proceso para la manufactura de un complejo amorfo de ciclodextrina-resveratrol. (Párrafo 1239)
a. Ciclodextrina completamente homogenizada en agua, con calentamiento de 50 a 80°C, hasta que una concentración de ciclodextrina más baja o igual a 60mM.	Suspender la ciclodextrina en agua a temperatura ambiente	Solucion salina que contiene 0.9% de hydroxypropyl- β -ciclodextrina (Párrafo 1239)
b. Agregar a la solución resultante, un solvente orgánico fisiológicamente aceptable y miscible en agua, hasta una proporción de solvente orgánico-agua que oscile de 1:1 a 1:5	No lo divulga	No lo divulga
c. Agregar a la mezcla resultante del paso (b), una solución del compuesto resveratrol diluido en un solvente orgánico fisiológicamente aceptable y miscible en agua, y caliente la mezcla resultante a una temperatura que oscile entre 50 a 80°C.	Adicionar una cantidad equimolar de resveratrol	Adicionar el resveratrol a la solución en una relación 1:1 y 1:100. (párrafo 1240)
d. Enfriar lentamente la solución resultante del paso (c) hasta precipitación de cristales del complejo de dicho compuesto resveratrol/ciclodextrina; y	No lo divulga	No lo divulga
e. Separar los cristales de dicho complejo del compuesto resveratrol/ciclodextrina.	Remover el solvente por evaporación al vacío	Separar el producto.

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención

definida en la reivindicación 1 porque el documento D1 divulga un proceso para preparar complejos de resveratrol con ciclodextrinas, los cuales mejoran la estabilidad y solubilidad de dicho compuesto (resumen)

La diferencia entre la invención, definida en la reivindicación 1 y el procedimiento de D1 consiste en que no se especifica la adición de un solvente orgánico.

El documento D2 igualmente divulga complejos de ciclodextrina con resveratrol y procesos para obtenerlos (párrafos 1239 y 1240).

El efecto que logra el incluir el solvente orgánico en este proceso no se puede determinar ya que la solicitud carece de información que permita establecer el efecto técnico sorprendente o superior del proceso reclamado.

Por lo tanto, el Problema Técnico Objetivo que pretende resolver esta invención se puede formular así: cómo modificar el procedimiento para la preparación de complejos de ciclodextrina y resveratrol conocido en D1 para conseguir un proceso alternativo para mejorar la solubilidad del resveratrol?.

Sin embargo, el desarrollo de un proceso alternativo para la preparación de complejo de ciclodextrinas y resveratrol para aumentar la solubilidad acuosa, ya está divulgado en el documento D5 que establece la adición de resveratrol a una solución que contiene 0.9% de hydroxypropyl-β-ciclodextrina y formar complejos (Párrafo 1239).

En consecuencia, la persona del oficio normalmente versada en la materia, modificaría el procedimiento de D1 para lograr una metodología alternativa como lo enseña D2 y llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

Conclusión:

La reivindicación 1 y sus dependientes cumplen con el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

Las reivindicaciones 17 y 22 reclaman un complejo compuesto de resveratrol y ciclodextrina y composiciones que lo contienen .

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
Complejo compuesto de resveratrol y ciclodextrina caracterizado por ser sólido en su forma cristalina	Complejo de resveratrol con ciclodextrina (resumen)	Composiciones que comprenden un complejo de ciclodextrina con resveratrol (párrafos 1239 a 1243)

Los documentos D1 y D2 se consideran el estado de la técnica cercano porque D1 divulga complejos de resveratrol con ciclodextrinas (resumen y pág. 280).

Los complejos reivindicados difieren de los de D1 en la anterioridad no especifica que el sólido obtenido sea cristalino.

El documento D2 divulga complejos de hydroxypropyl-β-ciclodextrina y resveratrol y composiciones que los contienen (párrafos 1239 a 1243).

La diferencia entre los complejos divulgados en D2 y la presente solicitud, consiste en que

la anterioridad no establece que los compuestos correspondan a una forma cristalina.

El efecto logrado por el hecho de desarrollar una forma cristalina del complejo resveratrol-ciclodextrina no se puede determinar debido a que la solicitud carece de información que permita establecer una ventaja o efecto sorprendente de los complejos y composiciones reclamadas con relación a la solución propuesta en el estado de la técnica.

Por lo tanto, el Problema técnico objetivo a resolver consiste en cómo desarrollar complejos de resveratrol con ciclodextrinas alternativos a los divulgados en D1 que provean una adecuada solubilidad del resveratrol en agua.

Sin embargo, D2 enseña complejos alternativos de resveratrol con hydroxypropyl-β-ciclodextrina que igualmente proveen adecuada solubilidad en agua a este compuesto.

De manera que el estado de la técnica sugiere a la persona versada en la materia la forma en que se resuelve el problema de mejorar la solubilidad en agua del resveratrol mediante la misma metodología propuesta en esta solicitud. Por lo cual se considera obvia.

Conclusión:
Las reivindicaciones 17 y 22 y sus dependientes cumplen el requisito de novedad (Art. 16), pero no poseen nivel inventivo (Art. 18).

8. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Journal of inclusion Phenomena and Macrocyclic Chemistry (2006) 55:279-287	1-23	Nivel Inventivo
D2	US2006/0292099	1-23	Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.


JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

1978 SET. 2002

El anterior requerimiento fue notificado por fijación en lista, de fecha _____

Elaborado por : Emilce Cano Urrego. 
Revisado por: Jacqueline Alfonso Rozo.

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

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Key words: cyclodextrin inclusion complex, dissolution tests, phase solubility studies, resveratrol, stability

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 ¹H-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]. Phytoalexins 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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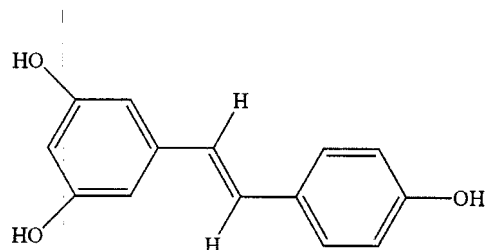


Figure 1. Chemical structure of *trans* resveratrol.

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 ^1H -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 ($\text{C}_{14}\text{H}_{12}\text{O}_3$; 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 then 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 $\text{CuK}\alpha$ radiation detector ($\lambda = 1.5405 \text{ \AA}$) 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 $\times$.

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 determined (UV-Vis spectrophotometer Jasco V-530) at $\lambda = 307 \text{ nm}$. 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 $\mu\text{g/ml}$. 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 $37 \pm 0.3^\circ\text{C}$ 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 C₁₈ 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 $\mu\text{W/cm}^2$) 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 ($\varnothing$ 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.

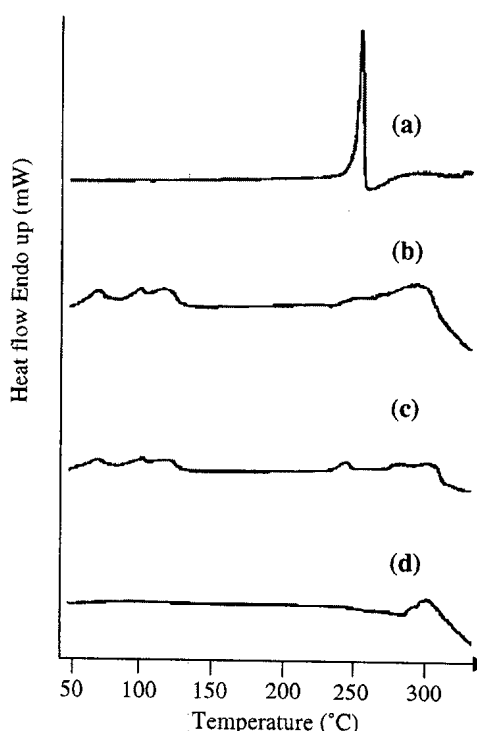


Figure 2. DSC thermograms of resveratrol (a), α -CD (b), physical mixture (c) and inclusion complex (d).

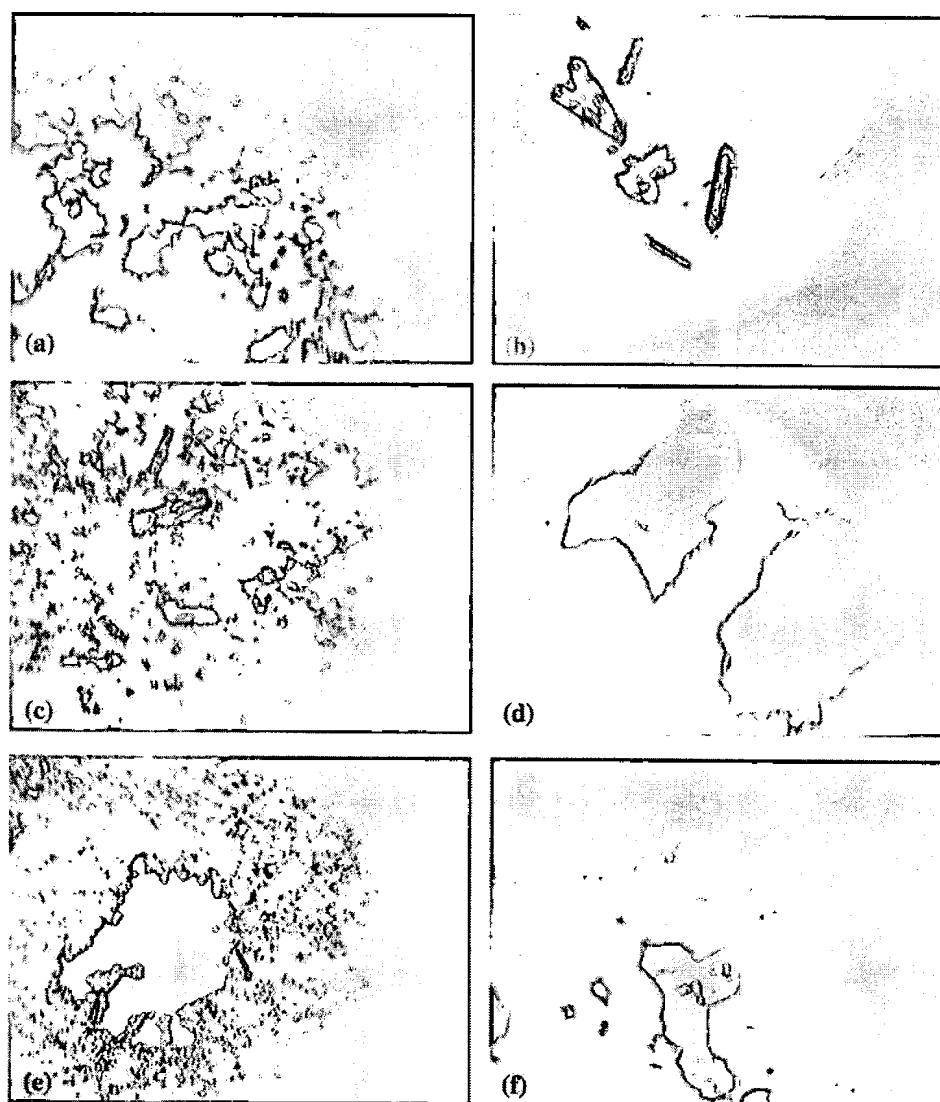


Figure 5. Photographs collected with an optical microscope: resveratrol (a), DM- β -CD (b), physical mixture (c), 1:1 (d), 2:1 (e), 1:2 (f) guest:host inclusion compounds.

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 internal 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 ^1H -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

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.

FT-IR

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

FT-IR absorption in the range of $1700\text{--}450\text{ cm}^{-1}$ 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

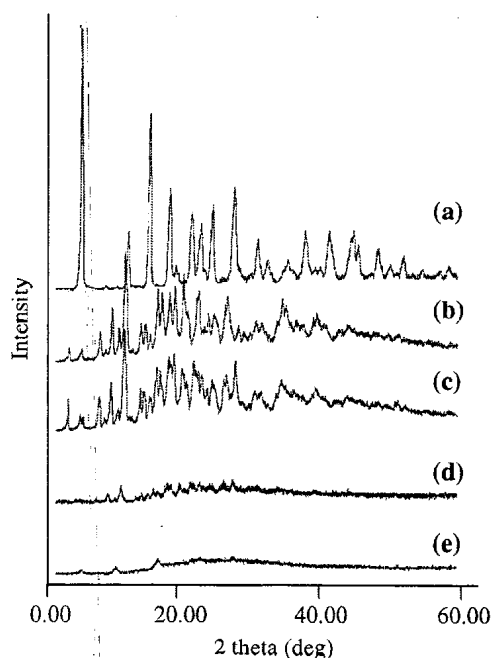


Figure 3. X-Ray diffractograms of resveratrol (a), β -CD (b), physical mixture (c), inclusion complex using suspension method (d) and inclusion complex using microwave (e).

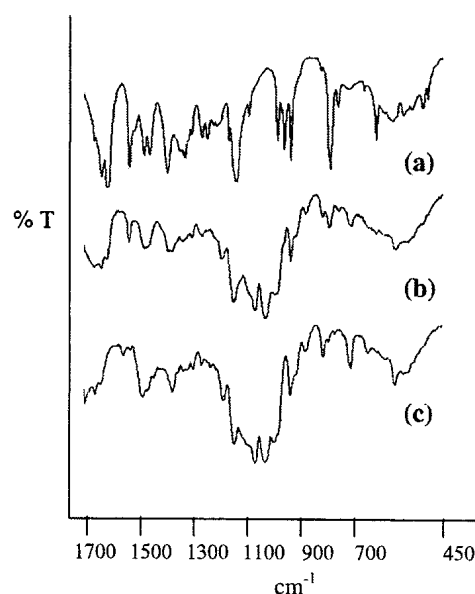


Figure 4. IR spectra of resveratrol (a), inclusion complex (b) and DM- β -CD (c).

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.

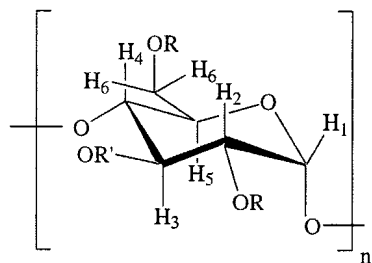
The photographs of 2:1 (Figure 5e) and 1:2 (Figure 5f) guest:host complexes exhibit the crystallites of the complex and agglomerate of resveratrol or DM- β -CD in excess, respectively.

All the cyclodextrins investigated showed similar morphological characteristics.

$^1\text{H-NMR}$

The NMR-techniques are commonly used to study inclusion complexes of cyclodextrins. In Table 1 the $^1\text{H-NMR}$ 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 external 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-

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



α -CD ($n=6$), β -CD ($n=7$), γ -CD ($n=8$); $\text{R}=\text{H}$

HP- β -CD ($n=7$); $\text{R}, \text{R}' = \text{H}$ or $\text{CH}_2\text{CH}(\text{OH})\text{CH}_3$

DM- β -CD ($n=7$); $\text{R}=\text{CH}_3$

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.

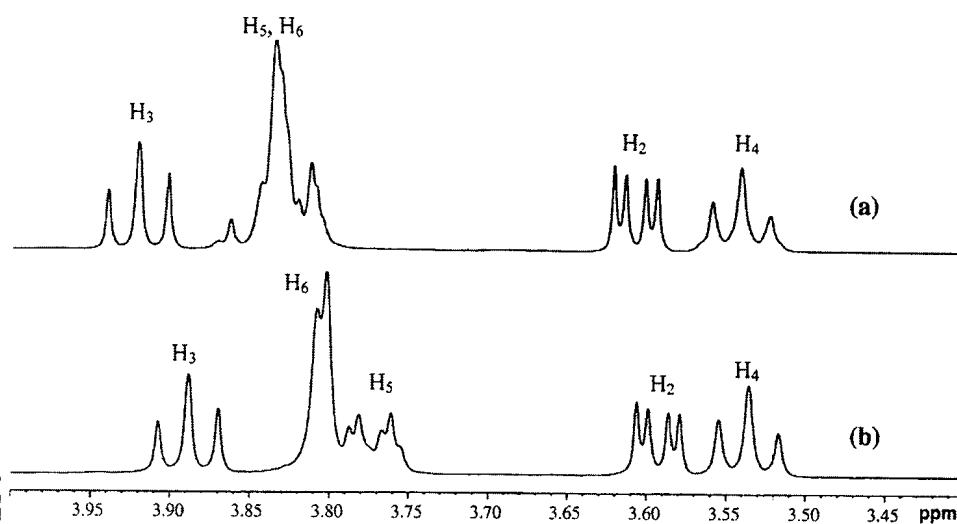


Figure 6. ^1H -NMR spectra: free β -CD (a) and inclusion complex (b).

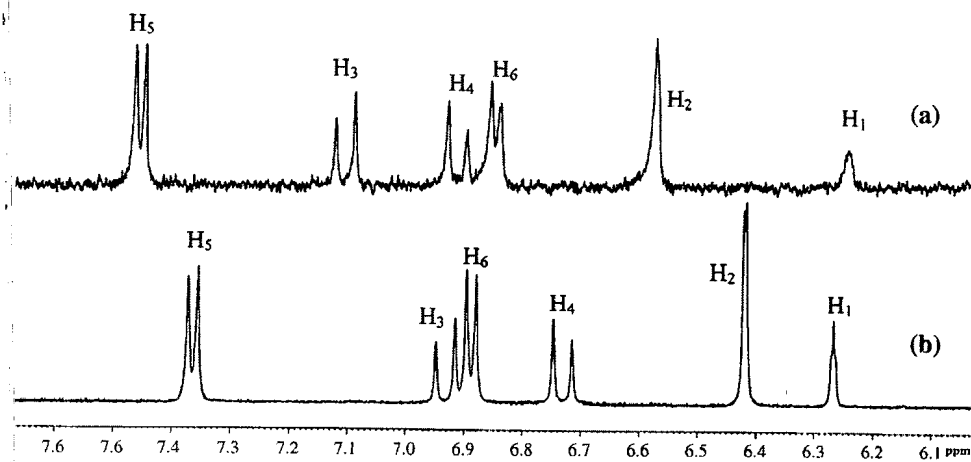
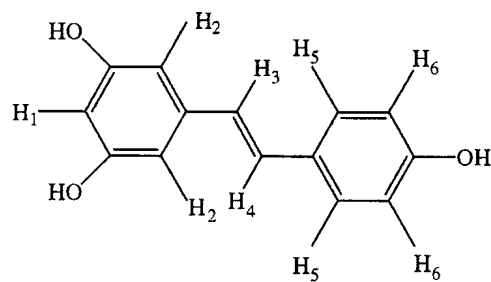


Figure 7. ^1H -NMR spectra: free resveratrol (a) and inclusion complex (b).

Table 2. ¹H-NMR chemical shift displacement (Δδ, ppm) of resveratrol by complexation with different cyclodextrins in D₂O. Δδ were expressed as Δδ = δ_{complex} - δ_{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

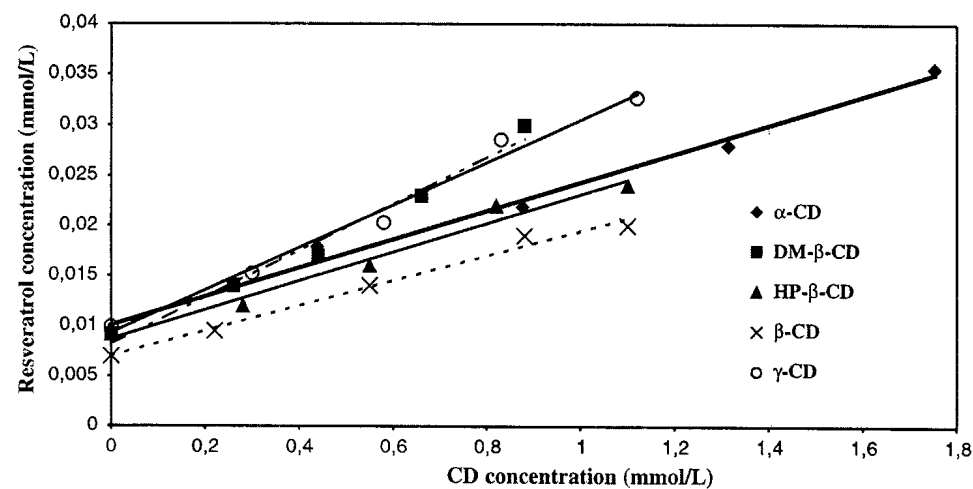


Figure 8. Initial straight line portion of the phase solubility diagrams for resveratrol with different cyclodextrins at 25 °C.

($K_c = 2604\text{ M}^{-1}$) 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)

	4 °C	After 120 h		After 24 h	
		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

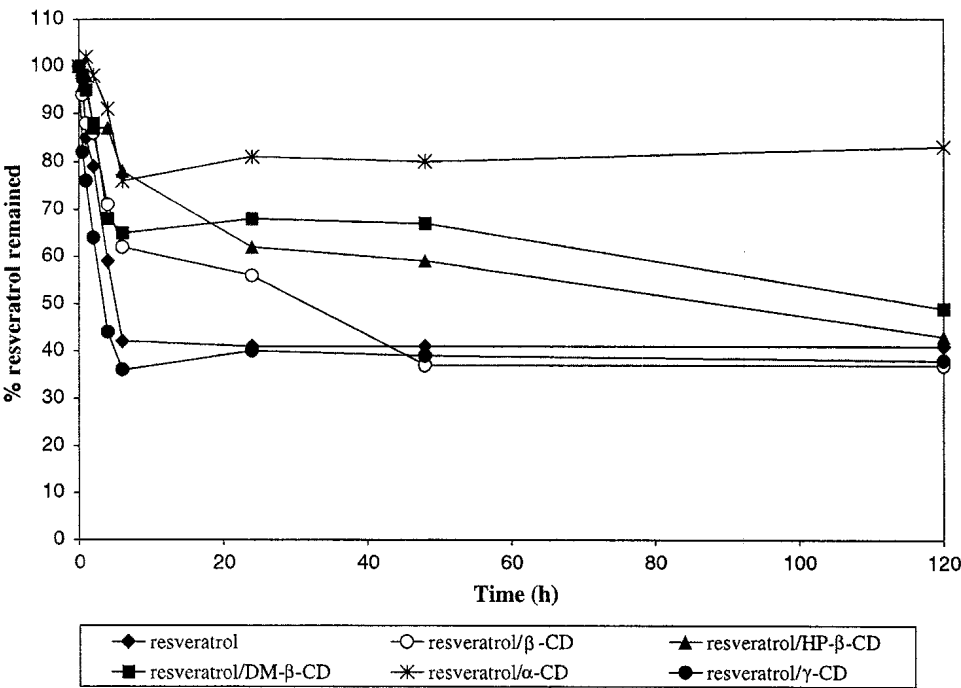


Figure 9. Stability profiles of resveratrol and inclusion complexes under sunlight exposure.

gated under different storage conditions including 4 °C, room temperature in the dark and under light exposure (λ = 254 nm, λ = 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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