UNIVERSIDAD DE MURCIA

FACULTAD DE BIOLOGÍA

Molecular Nanoencapsulation of Stilbenes by Cyclodextrins. Study of Biochemical, Chromatographic and Antioxidant Applications

Nanoencapsulación Molecular de Estilbenos por Ciclodextrinas. Apllicaciones Bioquímicas, Cromatográficas y Antioxidantes

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NANOENCAPSULACIÓN MOLECULAR DE ESTILBENOS POR CICLODEXTRINAS. APLICACIONES BIOQUÍMICAS, CROMATOGRÁFICAS Y ANTIOXIDANTES

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- Pilar Rodríguez-Bonilla, José Manuel López-Nicolás, Lorena Méndez-Cazorla, Francisco García-Carmona. Kinetic mechanism and product characterization of the enzymatic peroxidation of pterostilbene as model of the detoxification process of stilbene-type phytoalexins. *Phytochemistry* (2011) 72: 100-108.

- Pilar Rodríguez-Bonilla, José Manuel López-Nicolás, Francisco García-Carmona. Use a reversed phase high pressure liquid cromatography for the physicochemical and thermodynamic characterization of oxyresveratrol/β-cyclodextrin complexes. *Journal of Chromatography B* (2010) 878: 1569-1575.

- José Manuel López-Nicolás, Pilar Rodríguez-Bonilla, Lorena Méndez-Cazorla, Francisco García-Carmona. Physicochemical Study of the Compelxation of Pterostilbene by Natural and Modified Cyclodextrins. *Journal of Agricultural and Food Chemistry* (2009) 57: 5294-5300.

- José Manuel López-Nicolás, Pilar Rodríguez-Bonilla, Francisco García-Carmona. Complexation of Pinosylvin, an Analogue of Resveratrol with High Antifungal and Antimicrobial Activitiy, by Different Types of Cyclodextrins. *Journal of Agricultural and Food Chemistry* (2009) 57:10175-10180.

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En Murcia, a 10 de Octubre de 2015

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A mi madre

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"Recordarás algo de lo que leas,

bastante de lo que oigas,

mucho de lo que veas,

y todo lo que hagas".

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CAPÍTULO I

Introducción

En los últimos años ha cobrado gran fuerza en el sector de alimentos y bebidas la aparición de los denominados alimentos funcionales, entendiéndose como tales aquellos que le confieren al consumidor un beneficio para la salud, más allá de sus propiedades puramente nutritivas. Más concretamente, pero siempre dentro del campo de este tipo de nuevos productos, uno de los grupos de alimentos funcionales que más cuota de mercado está obteniendo es el de los enriquecidos en sustancias bioactivas con un alto valor biológico. Debido a ello, en esta tesis doctoral se propone la investigación necesaria para el diseño de alimentos funcionales enriquecidos en diversos tipos de estilbenos, moléculas que han demostrado poseer grandes propiedades beneficiosas para la salud. El estudio de los estilbenos favorecerá además, su uso en las industria farmacéutica y cosmética.

1. ESTILBENOS

Los estilbenos son compuestos de bajo peso molecular encontrados de forma natural en gran variedad de fuentes, tales como plantas, productos de aromaterapia y suplementos dietéticos. Además, los compuestos estilbenoides pueden ser constitutivos de la savia de los árboles o inducirse en respuesta a estrés medioambiental *(Roupe et al., 2006)*.

La inducción de la síntesis y secreción de estilbeno se produce en el fruto y/o hojas donde los estilbenos actúan como fitoalexinas debido a su acción protectora tras su secreción. Esta acción protectora se refiere a la defensa de la planta frente a ataques víricos y microbiológicos, excesiva exposición ultravioleta y enfermedades. Frente a la amenaza ambiental, la planta activa el itinerario fenilpropanoide y los estilbenos se producen y

secretan como consecuencia de ello. Por tanto, el tipo de estilbeno que se produce depende mucho de la región de origen y de los estímulos medioambientales *(Chong et al., 2009).*

En base a las propiedades que presentan algunos de ellos, estudiaremos a lo largo de esta tesis los principales estilbenos: oxyresveratrol, resveratrol, pinosilvina y pterostilbeno.

1.1. Oxyresveratrol

1.1.1. Características generales y estructura

Oxyresveratrol (trans-2,3´,4,5´-tetrahydroxystilbene) es un potente antioxidante y captor de radicales libres, encontrándose principalmente en la morera (*Morus alba L.*). Su estructura corresponde a la **Figura 1**:

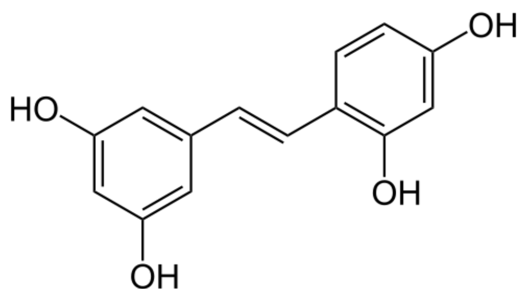

Figura 1. *Estructura de oxyresveratrol*

1.1.2. Función en la naturaleza y propiedades

Entre sus principales beneficios se encuentran: efectos neuroprotectivos (Webber et al*.,* 2012), fuerte inhibidor de la tirosinasa (Likhitwitayawuid et al., 2006; Song et al., 2012), antivírico (P. Sasivimolphan, et al., 2009) y hepatoprotectivo (Zhang et al., 2012).

1.2. Resveratrol

1.2.1. Características generales y estructura

El *trans*-resveratrol (*trans*-3,4´,5-trihidroxyestilbeno) es un estilbeno presente en altas concentraciones en una amplia variedad de plantas y frutos, incluyendo uvas, cacahuetes, arándanos, pinos y plantas utilizadas en la medicina oriental (Burns et al., *2002).* Además*,* en plantas el *trans*-resveratrol es sintetizado como respuesta a condiciones de estrés, tales como golpes, infecciones fúngicas, rayos UV, metales pesados, etc. La estructura presentada por este estilbeno se muestra en la **Figura 2**.

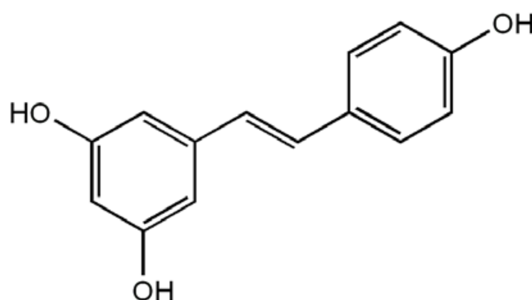

Figura 2*.* *Estructura de resveratrol*

En los últimos años este compuesto está cobrando especial relevancia gracias a la llamada "Paradoja Francesa", la cual implica una disminución de la mortalidad por enfermedades coronarias en el sur de Francia, debido al consumo regular de vino tinto.

1.2.2. Funciones en la naturaleza y propiedades

Debido a la similitud estructural entre el trans-resveratrol y el agente estrogénico dietilestilbestrol, este estilbeno es considerado como fitoestrógeno, atribuyéndole por tanto, los efectos beneficiosos que aportan estas moléculas (Gambini et al., 2013).

También se le han atribuido efectos anti-carcinogénicos y actividades cardioprotectoras basándose en sus propiedades antioxidantes y anticoagulantes (Huang et al., 2014). Además de esos efectos, el trans-resveratrol inhibe la agregación plaquetaria y la peroxidación lipídia, alterando la síntesis de eicosanoides y, por tanto, modulando el metabolismo lipoproteínico (Yashiro et al., 2012;Konstantinos M et al., 2013).

Finalmente, el trans-resveratrol posee actividad anticancerígena debida a su capacidad para reducir la incidencia de carcinógenos sobre animales de experimentación (Frisdelli et al., 2009).

1.3. Pinosilvina

1.3.1. Características generales y estructura

La pinosilvina (*trans*-3´,5´dihidroxiestilbeno) es un estilbeno natural encontrado en extractos de la madera de diversas especies de pino y en eucaliptos. Su estructura se presenta en la **Figura 3**.

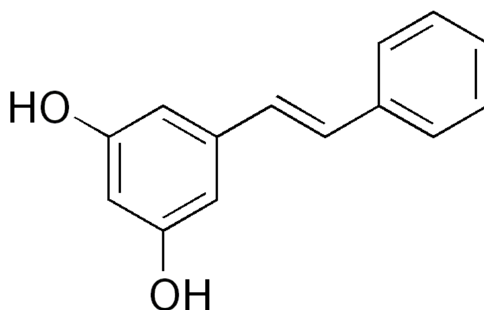

Figura 3. *Estructura de Pinosilvina*

La formación de pinosilvina se produce en las acículas de los pinos, inducida por infección o estrés medioambiental *(Antikainen et al., 2012)*. Por ello, muchos estudios sobre la pinosilvina se han centrado en la capacidad de proteger a varias especies de pinos contra enfermedades y podredumbre (*Plumed-Ferrer et al., 2013*).

1.3.2. Funciones en la naturaleza y propiedades

A la pinosilvina se le ha adjudicado un papel importante como anticancerígeno. Esta afirmación se basa en que la pinosilvina tiene efectos estrogénicos ya que es un modulador de receptores de estrógenos, al igual que el piceatannol y el resveratrol *(Laavola et al., 2015).*

Diversos estudios han comparado la capacidad antioxidante del resveratrol y la pinosilvina usando radicales lipídicos y la conclusión obtenida de dichos estudios es que según aumenta el número de hidroxilos en las moléculas de los estilbenos, la capacidad antioxidante aumenta; por tanto, la estructura de pinosilvina es menos eficiente que el

resveratrol en la captación de radicales libres *(Stojanovic et al., 2001; Perecko et al., 2008).* Sin embargo, existe controversia con los resultados obtenidos por algunos autores.

1.4. Pterostilbeno

1.4.1. Características generales y estructura

El pterostilbeno (*trans*-3,5-dimetoxy-4´-hidroxiestilbeno) es un estilbeno natural, obtenido por dimetilación del resveratrol en las posiciones 3 y 5, tal y como se muestra en la **Figura 4**:

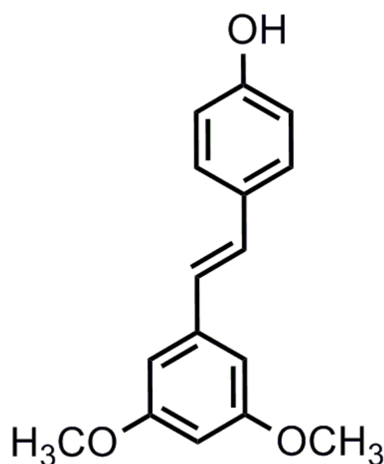

Figura 4. *Estructura de Pterostilbeno*

Este compuesto se encuentra en frutos rojos, arándanos azules, en gran variedad de uvas inmaduras o infectadas con *Botritis vinifera* y en árboles de las especies *Pterocarpus marsupium* (utilizado en el tratamiento para la diabetes) *(Chakraborty et al., 2010).*

Por último, se han encontrado altas concentraciones de pterostilbeno en darakchasava, una bebida medicinal India compuesta principalmente de uvas desecadas, usada en el tratamiento de enfermedades cardiovasculares *(Paul et al., 1999).*

1.4.2. Funciones en la naturaleza y propiedades

Las principales propiedades que se atribuyen al pterostilbeno son las siguientes: antioxidante, anticancerígena, antidiabética, hipolipidémica, antifúngica, etc. *(Robb, E.L. & Stuart J.A., 2014; Nikhil et al., 2015; Perecko et al., 2015).*

La actividad antioxidante y antiinflamatoria *in vitro* del pterostilbeno es comparable a la del resveratrol *(Rimando et al., 2002; Choo et al., 2014),* aunque existe controversia con los resultados. Además, al pterostilbeno se le atribuyen propiedades anticancerígenas tanto en estudios *in vitro* como *in vivo (Paul et al., 2010;McCormack & McFadden, 2012)*. En esos estudios, el pterostilbeno mostraba una inhibición del crecimiento, adhesión, inhibición de la expansión de la metástasis y ser un agente apoptótico sobre ciertas células cancerígenas *(Ferrer et al., 2005; Schneider et al., 2010; Li et al., 2013).*

En cuanto a la actividad antidiabética del pterostilbeno, extractos de *Pterocarpus marsupium* con alto contenido en pterostilbeno han demostrado ser capaces de reducir los niveles de glucosa en sangre *(Grover et al., 2002; Chakraborty et al., 2010).*

El pterostilbeno también posee propiedades hipolipidémicas, ya que disminuye los niveles de lipoproteínas de baja densidad y aumenta los niveles de lipoproteínas de alta

densidad en el plasma de hámsteres suplementados con altas dosis de pterostilbeno *(Zhang et al., 2013).*

Por otra parte, se han encontrado propiedades antifúngicas en el pterostilbeno, debido a que inhibe la germinación de conidios de *Botrytis cinerea in vitro*. Estudios realizados para evaluar los efectos sobre conidios, muestran que el pterostilbeno destruye los ribosomas, el retículo endoplásmico y las membranas del núcleo y mitocondria, y desorganiza la membrana plasmática *(Caruso et al., 2011*).

Respecto a su catálisis enzimática, el pterostilbeno es oxidado por la enzima lacasa de uva contaminada por *Botrytis cinerea*, dando lugar a un producto de estructura dimérica insoluble en agua *(Breuil et al., 1999).*

1.5. Problemas derivados del uso de los estilbenos

El hecho de que, por una parte, los miembros de esta familia de compuestos muestren un alto grado de inestabilidad y que, por otra, la gran mayoría de ellos presenten un carácter poco hidrofílico, dificulta el enriquecimiento de alimentos con estas moléculas, por lo que se hace necesario el diseño de nuevas estrategias para que puedan ser utilizados en alimentos funcionales, como es el caso de la encapsulación molecular. A continuación se enumeran los principales problemas que presentan los estilbenos y que dificultan su uso en la industria alimentaria, farmacéutica y cosmética:

- Presentan baja solubilidad
- Son fácilmente oxidables

- Presentan baja biodisponibilidad
- Son fácilmente alterables por agentes físico-químicos (pH, temperatura, luz)

2. ENCAPSULACIÓN MOLECULAR

La encapsulación es un proceso mediante el cual ciertas sustancias bioactivas (sabores, vitaminas o aceites esenciales) son introducidas en una matriz o sistema pared con el objetivo de impedir su pérdida, para protegerlos de la reacción con otros compuestos presentes en el alimento o para impedir que sufran reacciones de oxidación debido a la luz o al oxígeno. Una ventaja adicional es que un compuesto encapsulado se liberara gradualmente del compuesto que lo ha englobado o atrapado y se obtienen productos alimenticios con mejores características sensoriales y nutricionales. Se utiliza también el término nanoencapsulación en la industria alimentaria o farmacéutica cuando se encapsulan sustancias de bajo peso molecular o en pequeñas cantidades.

Los principales métodos de encapsulación pueden dividirse en tres grupos:

a) Procesos físicos: secado por aspersión, extrusión y recubrimiento por aspersión.
b) Procesos fisicoquímicos: coacervación simple o compleja y atrapamiento en liposomas.
c) Procesos químicos: polimerización interfacial e inclusión molecular.

El método de encapsulación a elegir dependerá del tamaño medio de la partícula requerida y las propiedades fisicoquímicas del agente encapsulante y la sustancia a

encapsular, de las aplicaciones deseadas para el material microencapsulado, el mecanismo de liberación deseado y el costo total del proceso.

A causa de la hidrofobicidad y fácil oxidación que presentan las moléculas principales de esta tesis, los estilbenos, numerosos trabajos han estudiado su inclusión en diferentes complejos para conseguir una mayor conservación de sus propiedades físico químicas.

El agente complejante más comúnmente utilizado para la formación de complejos de inclusión con estilbenos son las ciclodextrinas (CDs). Estas ciclodextrinas tienen un centro hidrofóbico mientras que la superficie exterior es hidrofílica, lo cual permite un equilibrio dinámico en el cual agua u otro compuesto, son reemplazados en la cavidad de la molécula de CD. La estabilidad de estos complejos depende de la estructura, tanto del estilbeno encapsulado como del tipo de CD utilizada, hidrofobicidad de la molécula huésped, pH, disolvente orgánico, temperatura y concentración de la CD.

Debido a que tanto la estructura como tipo de CD son esenciales para la formación de los complejos de inclusión, se van a describir a continuación las principales características de dicho agente encapsulante.

3. CICLODEXTRINAS

3.1. Definición y estructura

Las ciclodextrinas (CDs) son oligosacáridos cíclicos obtenidos de forma natural mediante la degradación enzimática del almidón de maíz o de patata, a través de la acción de la enzima ciclodextrin-glicosil-transferasa (CGT-asa) presente en diversos

microorganismos, entre los que destaca *Bacillus macerans*. Esta enzima es un tipo de amilasa que puede cortar un fragmento de la hélice del almidón uniendo a continuación los extremos finales de dicho fragmento para formar la CD *(Szetjli, 1998).*

Estas estructuras contienen de 6 a 12 unidades de glucopiranosa unidas por enlaces α-(1-4)-glucosídicos. El número de unidades de glucosa determina el nombre de cada CD, que es designado por una letra griega: α-CD (6 unidades de glucosa), β-CD (7 unidades de glucosa), γ-CD (8 unidades de glucosa), etc. Aunque han sido identificadas CDs de hasta 12 unidades de glucosa, solamente los tres primeros miembros de la serie (α-, β-, γ-) han sido estudiados detalladamente *(López-Nicolás et al., 2008).*

Las CDs tienen forma de anillo tronco cónico con los C2 y C3 de los residuos de glucosa hacia el interior del anillo y con el C6 hacia el exterior. A su vez, los –OH 6 de las CDs se encuentran situados en la cara ancha del tronco cónico, mientras que los –OH 2 y 3 lo hacen en la cara estrecha de dicho tronco **(Figura 5).**

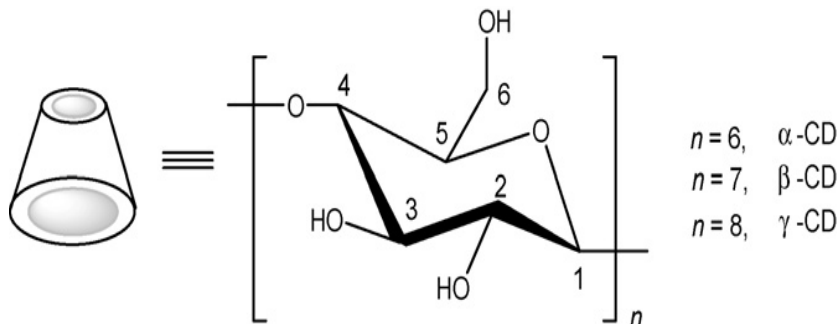

Figura 5. *Estructura molecular de las CDs*

Mediante modelos de relleno espacial y difracción de Rayos-X se ha demostrado que las CDs tienen un interior altamente apolar (grupos –CH) y una superficie exterior hidrofílica debido a los grupos hidroxilo **(Figura 6).**

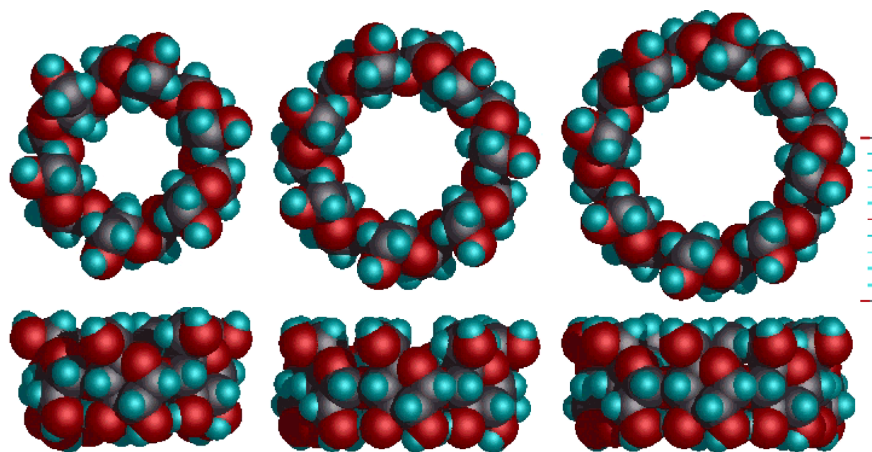

Figura 6. *Estructura de α-CD (izquierda), β-CD (centro) y γ-CD (derecha)*

Además, las CDs están sujetas a posibles adiciones, sustituciones o eliminaciones que den lugar a modificaciones en su estructura. Las CDs resultantes se denominan CDs modificadas y se obtienen principalmente mediante la sustitución de uno o más átomos de hidrógeno de los grupos hidroxilo primarios y/o secundarios por diferentes sustituyentes (grupos halógeno, amino, etc.) *(López-Nicolás et al., 2008).*

3.2. Propiedades

La principal propiedad de las CDs es la de incluir una amplia variedad de moléculas orgánicas e inorgánicas, comúnmente denominadas moléculas huésped, dentro de su cavidad hidrofóbica, formando los llamados complejos de inclusión.

La formación de estos complejos de inclusión ha dotado a las CDs de la capacidad de aumentar la biodisponibilidad de diferentes compuestos y proteger a gran variedad de moléculas contra la acción de agentes externos, por lo cual su aplicación en diferentes industrias se ha visto incrementada en los últimos años.

3.3. Aplicaciones

Las ventajas mencionadas anteriormente de los compuestos de inclusión formados entre las CDs y distintas moléculas huésped han sido aprovechadas en procesos de microencapsulación, lo que ha llevado al uso de las CDs en distintos campos de aplicación como son:

3.3.1. Industria farmacéutica

Aunque las ciclodextrinas se han estudiado durante más de 100 años y pueden encontrarse en por lo menos 35 productos farmacéuticos, aún se consideran como nuevos excipientes farmacéuticos (Kurvovs & Loftsson; 2013).

Existen numerosas aplicaciones para las CDs en la industria farmacéutica. Las CDs se han usado principalmente como agentes complejantes para aumentar la solubilidad acuosa de fármacos poco solubles en agua y mejorar su biodisponibilidad y estabilidad, aumentando el efecto farmacológico y consiguiendo, por tanto, una reducción en la dosis del fármaco administrado (Frömming et al., 1994; Kurvovs & Loftsson; 2013).

Además, las CDs han sido utilizadas para reducir o prevenir la irritación gastrointestinal u ocular, reducir o eliminar olores o sabores desagradables, prevenir o oeliminar interacciones entre sustancias y fármacos, o incluso para convertir aceites y medicamentos líquidos en compuestos de estructura microcristalina o polvos amorfos.

Las principales ventajas que presentan los complejos de inclusión de CDs con fármacos y que han dado lugar a su extendido uso, son las siguientes:

- Mejora de la solubilidad: las CD aumentan la solubilidad acuosa de muchos fármacos poco solubles mediante la formación de complejos de inclusión con moléculas apolares o sus grupos funcionales. En el complejo resultante se oculta la mayor parte de la estructura hidrofóbica en la cavidad interior de la CD, mientras que los grupos hidroxilo hidrófilos permanecen expuestos al medio ambiente en la superficie externa. El efecto neto es que se forma un complejo fármaco CD soluble en agua (Archontaki et al., 2002).
- Mejora de la biodisponibilidad: Cuando la biodisponibilidad es baja debido a la escasa solubilidad, las CDs pueden aportar un valor añadido. El principal condicionante para la absorción de un fármaco administrado por vía oral es la liberación de la formulación en forma disuelta. Cuando el fármaco está complejado con CDs, la velocidad de disolución y, en consecuencia, su absorción se ve mejorada. La reducción de la hidrofobicidad de los fármacos por la formación de complejos con CDs también mejora su absorción percutánea o rectal. (Arima et al., 2001)
- Mejora de la estabilidad: la complejación con CDs es de inmensa aplicación en la mejora de la estabilidad química, física y térmica de los fármacos. Para una molécula activa que se degrada cuando se expone al oxígeno, el agua, la radiación o el calor,

estar atrapada dentro de la cavidad de la CD, dificulta la reacción de los reactivos con la molécula protegida. (Arima et al., 2001)

- Reducción de la irritación: Los fármacos que contienen sustancias que irritan el estómago, la piel o los ojos pueden ser encapsulados dentro de una cavidad de CDs para reducir su irritación. Al formar complejos de inclusión con CDs se reduce la concentración local del fármaco libre, por debajo del umbral de irritación. A medida que el complejo se disocia gradualmente y el fármaco libre se libera, es absorbido en el cuerpo, manteniéndose siempre la concentración libre local por debajo de los niveles que podrían ser irritantes para la mucosa (Arias et al., 2000; Figueiras et al., 2010).
- Prevención de la incompatibilidad: Algunos medicamentos pueden ser incompatibles entre sí o con otros ingredientes inactivos presentes en una formulación. La encapsulación de los ingredientes incompatibles dentro de una molécula de CD estabiliza la formulación por la separación física de los componentes con el fin de prevenir la interacción fármaco-aditivo o fármaco-fármaco (Stroger et al., 2005)
- Olor y sabor enmascarado: El olor desagradable y el sabor amargo de muchos fármacos pueden ser enmascarados por la formación de complejos con las CDs. Las moléculas o grupos funcionales que causan sabores u olores desagradables pueden ocultarse de los receptores sensoriales mediante el encapsulado dentro de la cavidad de las CDs. Los complejos resultantes tienen poco o ningún sabor u olor y son mucho más aceptables para el paciente.
- Manipulación de sustancias: Las sustancias que son aceites/líquidos a temperatura ambiente pueden ser difíciles de manipular y formular en formas de dosificación sólidas estables. Al ser complejadas con CDs se pueden convertir dichas sustancias en

microcristalinas o polvos amorfos que pueden ser utilizados en formas de dosificación sólidas permitiendo un formato alternativo de administración de medicamentos, como puede ser en forma de comprimidos (Nakagawa et al., 2000;Stella & He, 2008).

3.3.2. Industria alimentaria

Desde hace algunos años, en la industria alimentaria se están usando las CDs para prolongar la vida útil de los alimentos, ya que los complejos de inclusión impiden que las moléculas huésped reaccionen con otras especies químicas (Szente et al., 2004; Kfoury et al., 2015; Martínez-Alonso et al., 2015).

El uso de las CDs en este sector está dirigido principalmente a la prevención de la degradación del color de determinadas frutas y vegetales debido al pardeamiento enzimático. En las últimas décadas, los tratamientos térmicos se han empleado para la inactivación de la enzima polifenoloxidasa, principal agente responsable del pardeamiento enzimático. Sin embargo, las propiedades nutricionales y sensoriales de los alimentos suelen ser alteradas por los abusos térmicos. Para evitar la pérdida de calidad derivada de estos tratamientos se utilizan las CDs, que al complejar los principales fenoles presentes en la estructura de frutas y vegetales, predominantemente de naturaleza hidrofóbica, impiden que la polifenoloxidasa oxide dichos compuestos y, por tanto, se ralentiza el pardeamiento enzimático (López-Nicolás et al., 2008). Diferentes trabajos publicados por nuestro grupo de investigación muestran como las CDs ralentizan el pardeamiento enzimático de distintos zumos de frutas como los de manzana, pera, melocotón o uva (López-Nicolás et al., 2007a; López-Nicolás et al., 2007b; López-Nicolás et al., 2007 c; Núñez-Delicado et al., 2007).

Por otra parte, diversos trabajos muestran cómo las CDs pueden actuar como antioxidantes secundarios, protegiendo a otros agentes reductores comúnmente empleados, como es el ácido ascórbico, de posibles oxidaciones externas (López-Nicolás et al., 2007b; López-Nicolás et al., 2007e). La acción sinérgica de las CDs junto con el ácido ascórbico ofrece un alto poder antioxidante que retrasa de forma significativa el pardeamiento de diversos zumos de frutas (López-Nicolás et al., 2008).

3.3.3. Cosmética

Las aplicaciones de las CDs en este sector incluyen el uso en pasta de dientes, cremas para la piel, suavizantes líquidos y sólidos, pañuelos, etc.

El área de la cosmética demanda el uso de CDs principalmente para evitar la volatilización de los perfumes, ambientadores y detergentes, mediante el control de la pérdida de fragancias de los complejos de inclusión. La interacción entre la molécula huésped y las CDs necesita una energía mayor para volatilizar, por tanto se producen fragancias de mayor duración (Ries et al., 2013; Pérez-Fernández et al., 2013).

3.3.4. Agricultura y Medio Ambiente

Las CDs forman complejos con una amplia variedad de compuestos agrícolas, incluyendo herbicidas, insecticidas, fungicidas, repelentes, feromonas y reguladores del crecimiento. Por ello, en la agricultura el uso de CDs puede ir orientado a retrasar la germinación de las semillas (Stepniak et al., 2015).

Las CDs también se usan en tratamientos de aguas para aumentar la estabilización, encapsulación y adsorción de contaminantes. Utilizando CDs, la alta toxicidad de dichas sustancias puede ser eliminada a través de efluentes industriales mediante la formación de complejos de inclusión (Sikder et al., 2014; Chauke et al., 2015; Cui et al., 2015).

Otro uso de las CDs sobre aguas es su utilización para la remediación del derrame de petróleo y la eliminación de contaminantes orgánicos de las aguas residuales (Kumar et al., 2015), así como para la eliminación de los contaminantes orgánicos en aguas de descarga industriales, ayudando a una reducción de la contaminación (Charles et al., 2014) .

Además, las CDs pueden tener una gran importancia sobre el medio ambiente ya que contribuyen a la solubilización de contaminantes orgánicos favoreciendo su eliminación del suelo y atmósfera (Morin-Crini & Crini, 2013). Esta mejora de la solubilidad permite el uso de CDs en determinadas pruebas sobre la recuperación del suelo.

Otra aplicación adicional de las complejación con CDs es la producción de combustible biodiesel a partir de aceite de cocina usado (Zou et al., 2013).

3.3.5. Cromatografía

En los últimos años, se ha producido un incremento en el número de publicaciones que estudian el uso de CDs en el análisis cromatográficos de diversos compuestos (López-Nicolás et al., 2008; Pàpay et al., 2016). Concretamente, las CDs son ampliamente usadas en cromatografía líquida de alta resolución (HPLC) para separar isómeros y enantiómeros

de diversos compuestos, bien sea por su uso en la fase móvil o en la fase estacionaria (López-Nicolás et al., 2008; Zhou et al., 2016).

Por último, el uso de CDs como aditivos añadidos a la fase móvil también ha sido empleado para la determinación de las constantes de complejación entre diferentes CDs y varias moléculas huésped siguiendo diversos métodos (López-Nicolás et al., 2009).

Una vez expuestos los beneficios de la formación de complejos de inclusión con CDs, se plantean los siguientes objetivos para esta Tesis Doctoral.

CAPÍTULO II

Objetivos

El objetivo general de la presente tesis es la nanoencapsulación de diferentes estilbenos mediante ciclodextrinas con el fin de incrementar, por un lado, su estabilidad frente a diversos agentes físico-químicos y, por otro, la solubilidad de los derivados más hidrofóbicos; de esta forma se facilita su uso en la industria alimentaria y farmacéutica. Con este fin, los *objetivos concretos* que se plantean son los siguientes:

a) Caracterizar mediante métodos fluorimétricos, cromatográficos la formación de distintos complejos de inclusión de los estilbenos (oxyresveratrol, resveratrol, pinosylvin y pterostilbeno) con diferentes tipos de ciclodextrinas. Las ciclodextrinas empleadas serán:

   1. Ciclodextrinas naturales

   - α-CD (compuesto con estatus GRAS y aditivo E-457)
   - β-CD (compuesto con estatus GRAS y aditivo E-459)
   - γ-CD (compuesto con estatus GRAS y aditivo E-458)

   2. Ciclodextrinas modificadas

   - Etil-β-CD
   - Metil- β-CD
   - Hidroxipropil-β-CD

b) Estudiar el efecto de diferentes agentes físico-químicos sobre las constantes de formación de dichos complejos. Los agentes estudiados que pueden modificar la estabilidad de los complejos de formación serán:

   - pH

- Temperatura
- Presencia de disolventes orgánicos

c) Evaluar el comportamiento de estilbenos en presencia de ciclodextrinas frente a agentes enzimáticos, proponiendo el papel como moduladores biológicos.

d) Determinar las propiedades antioxidantes de los complejos formados comparándolas con el potencial antioxidante de los estilbenos en ausencia de ciclodextrinas. ¿Esto no era cuando el artículo iba a comparar el uso y no uso de CDs con ORAC, ABTS Y FRAP?

e) Proponer el uso de ciclodextrinas para desarrollar nuevos métodos cromatográficos de análisis de compuestos alimentarios y farmacéuticos.

f) Determinar la capacidad antioxidante de estilbenos mediante diferentes técnicas de medida: ORAC, ABTS y FRAP.

g) Evaluar el efecto que produce la adición de CDs sobre la capacidad antioxidante de los estilbenos, medido con diferentes métodos: ORAC, ABTS y FRAP.

CAPÍTULO III

Ciclodextrinas y antioxidantes

ABSTRACT

In recent years the growth of the functional foods industry has increased research into new compounds with high added value for use in the fortification of traditional products. One of the most promising functional food groups are those enriched in antioxidant compounds of a lipophilic nature. In spite of the numerous advantages reported for such antioxidant molecules, they may also have disadvantages that impede their use in functional foods, although these problems may well avoided by the use of encapsulant agents such as cyclodextrins. This explains the recent increase in the number of research papers dealing with the complexation of different guest molecules possesing important antioxidant properties using natural and modified cyclodextrins. This paper presents a review of the most recent studies on the complexes formed between several important types of antioxidant compounds and cyclodextrins, focusing on the contradictory data reported in the literature concerning to the antioxidant activity of the host/guest molecule complexes, the different complexation constants reported for identical complexes, the bioavailability of the antioxidant compound in the presence of cyclodextrins and recommendation concerning the use of natural or modified cyclodextrins. Moreover, the use of cyclodextrins as antibrowning agents to prevent enzymatic browning in different foods is revised. Finally, we look at studies which suggest that cyclodextrins act as ‘‘secondary antioxidants”, enhancing the ability of traditional antioxidants to prevent enzymatic browning.

INTRODUCTION

One of the priorities of the food industry is the development of functional foods with high added value such as its the case of antioxidant compounds of lipophilic nature used to fortify hydrophobic solvents. Although the benefits of these compounds have been demonstrated, their use as functional ingredients in aqueous media has several disadvantages. First, the use of these molecules as fortifiers and nutraceuticals is limited by their poor solubility in water. Secondly, the presence of structures in these compounds means that they are easily oxidised by prooxidant agents. Finally, although these compounds are well absorbed by humans when taken orally, their bioavailability is quite low as a result of their rapid metabolism and elimination.

In recent years several novel foods supplemented with a variety of hydrophilic antioxidant compounds have appeared on the market, although the above mentioned problem has meant that very few of these foods can be enriched with hydrophobic antioxidants molecules. It is for this reason that the complexation of these antioxidant compounds in aqueous medium with molecules such as cyclodextrins (CDs) has been tried in order to increase their stability, bioavailability and solubility.

CDs are are naturally occurring cyclic oligosaccharides derived from starch with six, seven, or eight glucose residues linked by α(1-4) glycosidic bonds (Szente and Szejtli, 2004) (Fig. 1). The steric arrangement of these glucose units in the CD molecule results in a hollow truncated cone with a hydrophilic outside surface, which makes CDs water soluble, and a hydrophobic internal cavity, which enables CDs to form inclusion complexes with various hydrophobic guest molecules (Li et al., 2007). The advantageous changes in guest

molecule properties after the formation of inclusions complexes with CDs have led to many applications of CDs in industries related with food, pharmaceuticals, cosmetics, chemicals, agriculture, etc. (Martin Del Valle, 2004; Szente and Szejtli, 2004).

CDs are produced from starch or starch derivates by using cyclodextrins glycosyltransferase (CGTase, EC 2.4.1.19). The resulting enzymatic product is usually a mixture of CDs, mainly α-, β-, and γ-CD consisting of six, seven, or eight glucose units, respectively (Fig. 2), and trace amounts of large-ring CDs with more than nine glucose units (Endo et al. 2002). Although a few interesting large-ring CDs (modified CDs) showing novel structural features have been isolated and characterized during the past decade (Zheng et al., 2002; Qi et al., 2007), the most extensively studied and utilized products remain α-, β-, and γ- CDs (Szejtli, 1998). Moreover α-, β- and γ-CD enjoy GRAS status and have been approved recently as additives in the European Union. Indeed, all three natural CDs have recently been included in the European lists of additives approved for alimentary use with the corresponding E-numbers E-457, E-459 and E-458 assigned to α-, β- and γ-CD, respectively.

After the first fundamental review on CDs in 1957 (French, 1957), several other excellent reviews and monographs have been published, providing readers with a compilation of CD-related literature (Szejtli, 1990; Singh et al., 2002; Martin Del Valle, 2004). As regards the food field, most previous reviews focused on the aspects of CDs related with their impact on the sensory characteristics of foods, food packaging, nutrient sequestering properties, on food preservation or on the nutritional properties of different food products (Szente and Szejtli, 2004; Cravotto et al., 2006; Astray et al., 2009). However, in this review we focuse on the literature published about the effect of the

encapsulation of different lipophilic antioxidants used in the design of functional foods on some aspects such as their solubility, bioavaibility, application or antioxidant activity.

In recent years, several compounds with antioxidant properties have been complexed with CDs. However, the contradictory data published concerning: (i) the complexation constant (K_F) values, (ii) the nature of the best type of CD for the complexation process, (iii) the effect of the complexation on the antioxidant capacity of the guest molecules and (iv) the influence of encapsulation on the bioavaibility of the antioxidant compound, means that an exhaustive revision of the complexation process between important groups of antioxidants (carotenoids, stilbenes, fatty acids, vitamins, phenols and Coenzyme Q_{10}) and both natural and modified CDs is overdue.

Moreover, one of the main factors which must be controlled to prevent the browning of several foods is the enzymatic activity of polyphenoloxidase (PPO) and much research has focused on the use of postharvest chemical treatments to avoid such browning (Sánchez-Ferrer et al., 1995). However, many of these treatments present serious disadvantages for use in the food industry. Although synthetic antioxidants have a higher antibrowning effect in foods than those from natural sources, there is growing consumer interest in natural antioxidants. For this reason CDs have been recently used to prevent the enzymatic browning of different foods by encapsulating the phenols (natural substrates of PPO) present in the same.

Finally, in this review we revise the capacity of CDs as *"secondary antioxidants"*. Another strategy to avoid the browning of foods would be to look for "*preservers*" of the natural antioxidant capacity of a particular food. In this review, we show how CDs can

enhance the ability of other antioxidants to prevent the enzymatic browning due to the protective effect they offer against the oxidation of these antioxidants. In this respect, CDs seem to act as a *‘‘secondary antioxidants’’*, reducing food browning and enhancing the naturally occurring antioxidant capacity of the food itself.

CYCLODEXTRINS AND STILBENES

Stilbenes are a small family of plant secondary metabolites derived from the phenylpropanoid pathway, and produced in a number of unrelated plant species (Chong et al., 2009). These compounds are involved in various ways in plant disease resistance and human health. One of the most important properties of stilbenes is their antioxidant activity. In recent years, several works on the encapsulation of these molecules with CDs have been published. However, contradictory results have been reported concerning the effect of the complexation of stilbenes by natural and modified CDs on the physico-chemical and functional properties of these natural molecules. For this reason, we hope to throw light on this problem by revising the data published on the encapsulation of four important stilbenes (resveratrol, pterostilbene, pinosylvin and oxyresveratrol, Fig. 3) by CDs.

Resveratrol

Resveratrol (3,5,40-trihydroxy-stilbene) (Fig. 3B) is a phytoalexin found in at least 72 species of plant distributed among 31 genera and 12 families (Jang et al., 1997). In recent years, research into resveratrol has uncovered several beneficial biological effects of this compound on human health (Latruffe et al., 2002). However, the limited number of foods with high levels of resveratrol has led to the search for new strategies to incorporate this in new foods. Recently, transgenic fruits, including lettuce (Liu et al., 2006) and apple (Ruhmann et al., 2006), with high levels of free resveratrol or its derivates, have been developed. Another alternative is to use resveratrol as an ingredient in the functional food industry as a fortifier and nutraceutical compound.

Although the benefits of this stilbene have been demonstrated, its use has several disadvantages as a functional ingredient in aqueous medium. Although resveratrol is well absorbed by humans when taken orally, its bioavailability is quite low as a result of its rapid metabolism and elimination (Walle et al., 2004). Furthermore, the use of this stilbene as fortifier and nutraceutical is limited by its poor solubility in water (less than 0.05 mg/ml). Finally, the above mentioned presence of conjugated double bounds on the structure of these compounds means that they are easily oxidised by different prooxidant agents (Fan and Mattheis, 2001). However, the above mentioned problems which prevent resveratrol from being used as a fortifier of foods in an aqueous medium have meant that no novel food has been enriched in this important antioxidant compound. Due to these problems, the complexation of resveratrol in aqueous medium using compounds to increase its stability, bioavailability and solubility is considered a worthwhile goal. The use of CDs is promising in this respect and its has been reported in several recent papers.

In these works, different methods have been used to determine the K_F values (e.g. liquid chromatography, enzymology, solubility, RMN, Fourier transform infrared spectroscopy, differential scanning calorimetry or Xray diffraction). However, most works published have limited their investigation to the determination of K_F values using techniques based on solubility (Bertacche et al., 2006), enzymology (Lucas-Abellán et al., 2007) or liquid chromatography, (López-Nicolás and García-Carmona, 2008; López-Nicolás et al., 2006). However, results concerning the K_F values of resveratrol complexed by CDs under different reaction conditions are contradictory. While some papers have claimed that natural CDs are the most suitable for the inclusion process (Bertacche et al., 2006), others have reported that modified CDs are more appropriate for complexing

resveratrol (Lucas-Abellán et al., 2007). Moreover, papers have reported very different K_F values for the same type of complexes. Finally, contradictory data have been published about the effect of the encapsulation with CDs on the antioxidant activity of resveratrol.

Our study may help to resolve this problem by taking a look at several important aspects of the complexation mechanism, such as the aggregation state of resveratrol, the pH values of the reaction medium, the pKa values of the guest molecule and the UV–vis or the fluorospectrometric properties of resveratrol in the presence of CDs.

HPLC is an appropriate technique for determining the stoichiometry and K_F values of resveratrol/CD complexes (López-Nicolás et al., 2006; López-Nicolás et al., 2008). In organic medium, the complexation of resveratrol with β-CD was investigated using reversed-phase liquid chromatography and mobile phases to which β-CD was added. López-Nicolás et al. (2006) reported a decrease in retention times with increasing concentrations of β-CD (0–2.5 mM), and showed that resveratrol forms a 1:1 complex with β-CD, while the apparent K_F values were strongly dependent on the water–methanol proportion of the mobile phase used. Moreover, the K_F values for the resveratrol–β-CD interaction decreased when the temperature was raised from 20 to 37 ºC In order to gain information about the mechanism of resveratrol affinity for β-CD, the thermodynamic parameters of the complexation were obtained by López-Nicolás et al. (2006). Their results showed that the complex formation of resveratrol with β-CD (AG^o =−17.01 kJ/mol) is largely driven by enthalpy (AH^o =−30.62 kJ/mol) and slight entropy (AS^o =−45.68 J/mol K) changes.

As regards complexation in aqueous medium using HPLC, López-Nicolás and García-Carmona (2008) studied the complexation of resveratrol with natural CDs in aqueous medium under different physicochemical conditions (pH or temperature) which are essential if this antioxidant compound is to be used successfully in the food industry as an ingredient of functional foods due to its poor stability, bioavailability and solubility. In the above paper, a rapid, simple and sensitive way of determining of the apparent K_F values of resveratrol/CD complexes by HPLC in aqueous medium was investigated for the first time. It was observed that resveratrol forms a 1:1 complex with α-, β-, γ-CD. The highest value of the apparent K_F values ($K_F = 1922 \pm 89 M^{-1}$) was found for β-CD and a strong dependence of K_F on pH was seen in the region where resveratrol begins the deprotonation of its hydroxyl groups. Moreover, López-Nicolás and García-Carmona (2008) reported that an increase in the system's temperature produced a decrease in the values of K_F.

A complete study of the host–guest interaction of resveratrol with natural and modified CDs was published by Bertacche et al. (2006). The main objective of that paper was to increase the stability and water solubility of resveratrol by complexation with different CDs. Furthermore, the physical–chemical properties of each inclusion compound were investigated by these authors. Complexes of resveratrol with CDs, both native (α-, β-, γ-CD) and modified (HP-CD, dimethyl-β-CD), were obtained by using the suspension method. The authors also prepared an inclusion complex with β-CD by microwave and characterized the solid state of the products using Fourier transform infrared spectroscopy, differential scanning calorimetry and Xray diffraction. The solution studies were performed by UV–Vis spectrophotometry and H-NMR spectroscopy. In the same paper, phase solubility profiles with all the CDs used indicated the formation of 1:1 stoichiometric

inclusion complexes and the K_F values were calculated for each case from the phase solubility diagrams, values in the same order as those shown by other author being reported. Moreover, stability studies in the solid state and in solution were performed, while the photodegradation by UV–Vis spectrophotometry showed that the isomerisation rate *trans* to *cis*, in ethanol solution, decreased with inclusion.

In another paper, Lucas-Abellán et al. (2007) studied the formation of resveratrol/CD inclusion complexes in aqueous solutions using the hydroperoxidase activity of lipoxygenase as the enzymatic system. The addition of CDs to the reaction medium had an inhibitory effect on resveratrol oxidation by lipoxygenase due to the complexation of phytoalexin into the CD cavity, which is in equilibrium with free CDs and free resveratrol, the only effective substrate for lipoxygenase. This inhibitory effect, which has also been described in the case of the oxidation of other substrates, such as fatty acids by lipoxygenase (Bru et al., 1995; López-Nicolás et al., 1997), depends on the K_F values between resveratrol and the type of CD used. In the work of Lucas-Abellán et al. (2007), β-CD and maltosyl-β-CD were used and their K_F values were calculated by nonlinear regression of the inhibition curves obtained in the presence of CDs. The values obtained were 4317 and 5130 M^{-1} for β-CD and maltosyl-β-CD, respectively.

In addition, different methods were used by Lucas-Abellán et al. (2008 b) to study the complexation of resveratrol with native CDs (α-CD; β-CD and γ-CD) and modified CDs (HP-β-CD; maltosyl β-CD; methyl- β-CD; carboxymethyl- β-CD and acetyl-β-CDs) and the K_F were compared. The K_F values between resveratrol and each type of CD were calculated using three different methods: enzymatic, solubility and fluorimetric. The K_F values obtained by Lucas-Abellán et al. (2008 b) showed that HP-β-CD with their very high

K_F of 18048 ± 625 M^{-1} were the most effective type of CD for complexing resveratrol. Moreover, comparison of the results obtained by the three methods revealed that the fluorimetric method undervalued the K_F values between resveratrol and all the CDs, while the enzymatic and solubility methods were more precise for calculating the K_F values between resveratrol and CDs. However, these undervaluated K_F values obtained by the fluorimetric method have been discussed recently in a paper published by López-Nicolás and García-Carmona (2010).

In that paper, the authors demonstrated that, at pH values higher than the pK_{a1} of resveratrol, the coexistence of different protonated/deprotonated forms of this antioxidant does not permit the fluorimetric determination of the K_F value of resveratrol with HP-β-CD. However, when the Hildebrand–Benesi equation was used to calculate this constant at physiological pH, the problem was resolved, a K_F value of 14490 ± 723 M^{-1} and a 1:1 stoichiometry of the complexation process being found for all the cases tested. The results obtained in this paper resolve the contradictory data published by Lucas-Abellán et al. (2008b) about the complexation process of resveratrol by CDs. Moreover, López-Nicolás and García-Carmona (2010) studied the aggregation state of resveratrol in the presence and absence of HP-β-CD using absorption and steady-state fluorescence at different pH values. The results revealed that this potent antioxidant shows a monomer/aggregate equilibrium which is dependent on the protonation state of resveratrol. This equilibrium can be modified by the presence of HP-β-CD, which reduces the aggregation of the resveratrol molecules, producing individual molecules of the solute and preventing side effects due to aggregation phenomena.

As a second part of this work, our research group recently investigated the influence of the presence of CDs on the hydroperoxidation of resveratrol by lipoxygenase, bearing in mind the protonation and aggregation state of this potent antioxidant (López-Nicolás et al., 2009c). When the enzyme uses monomers of resveratrol as substrate, LOX shows a Michaelian behavior but when the resveratrol concentration is increased to values higher than critical it shows strong inhibition. These results can be interpreted as a previously unreported aggregate-induced enzyme inhibition, which can be modified by the use of modulators of the aggregation state of resveratrol such as CDs. Indeed, the addition of increasing concentrations of HP-β-CD produced a change in the LOX enzymatic activity due to the ability of CDs to sequester part of the resveratrol to form soluble inclusion complexes, thereby reducing the concentration of the free resveratrol. Thus, free resveratrol is the only effective substrate and the oxidation of the complexed substrate requires the previous dissociation of the complex. When HP-β-CD is added to the reaction medium, the observed inhibition by substrate aggregation is produced at a higher resveratrol concentration than observed in the absence of any agent. Thus, the concentration of resveratrol at which the LOX activity was inhibited increased in the presence of HP-β-CD, reflecting the formation of inclusion complexes and resulting in the extension of the range of monomeric resveratrol. This result is due to the ability of CDs to increase the critical concentration at which the aggregation of different substrates is produced, thus widening the apparent monomer concentration range (Bru et al., 1995; López-Nicolás et al., 1995). Indeed, as deduced from the fluorescence experiments and our previous studies (López-Nicolás et al., 1995), free and complexed monomers of resveratrol exist in the premicellar region, whereas in the postmicellar region there is the additional presence of resveratrol

aggregates. In both regions, the complexed resveratrol simply constitutes a pool of substrate to which the enzyme might or might not have direct access.

In a recent work, the photostability and biological properties of the resveratrol/ HP-β-CD complex were studied by Sapino et al. (2009). When the rate of resveratrol degradation was followed in different systems under UVA irradiation, the results showed the photoprotective effect of the complex.

An important paper on the application of the complexes formed between resveratrol and CDs in cellular biology was published by Bru et al. (2006), who investigated in detail the properties of CDs as elicitors of the defense responses in grapevine cell cultures. This group showed that certain modified CDs are able to elicit some of these responses, as evidenced by the high levels of accumulated resveratrol and stilbene-like compounds, changes in extracellular cell wall-like peroxidase and their isoenzyme expression pattern, as well as the effect on *B. cinerea* growth. In grapevine (*Vitis vinifera* L.), defense responses after microbial infection or treatment with elicitors involve the accumulation of phytoalexins, oxidative burst, and the synthesis of pathogenesis-related proteins. Oligosaccharide fractions from fungal or algal cell walls efficiently induced the defense responses, but a detailed analysis of the elicitor-plant cell surface interaction at the molecular level was precluded due to the lack of chemically pure oligosaccharide elicitors. The above work presented by Bru et al. (2006) was a continuation of the report by the same research group (Morales, et al., 1998) in which they investigated the effect of DIMEB, the doubly methylated α-CD in hydroxyls 2 and 6, on resveratrol metabolism in grapevine cell cultures inoculated with the grapevine pathogenic bacteria *Xylophilus ampelinus*. The inoculated cell cultures accumulated significant levels of *trans*-piceid in the cells, whereas

no resveratrol was observed in the extracellular medium. When the culture medium was supplemented with 5 mM DIMEB, both infected and non-infected cell cultures accumulated *trans*-piceid in the cells and secreted resveratrol in the spent medium. It was suggested that DIMEB might be acting as an elicitor independent of the presence of the bacterial pathogen.

In order to obtain information about the effect of the complexes between resveratrol and CDs on the bioavailability of the lipophilic antioxidant, Das et al. (2008) studied the impact of aqueous solubility and dose manipulation on the pharmacokinetics of resveratrol, using water-soluble intravenous and oral formulations of resveratrol prepared with HP-β-CD and randomly methylated-β-CD (RM-β-CD), respectively. Sodium salt and a suspension of resveratrol in carboxymethyl cellulose (CMC) were used as the reference intravenous and oral formulations, respectively. The pharmacokinetics of resveratrol was assessed in Sprague–Dawley rats and the plasma resveratrol concentrations were measured by HPLC. The results showed that both HP-β-CD and RM-β-CD enhanced the aqueous solubility of resveratrol. However, after intravenous administration, rapid elimination of resveratrol was observed at all the tested doses (5, 10, and 25 mg kg^{-1}) regardless of formulation type, with non-linear elimination occurring at 25 mg kg^{-1}. RM-β-CD significantly increased the maximal plasma concentration of orally administered resveratrol, but did not increase the oral bioavailability in comparison with the CMC suspension. Furthermore, in the same paper the oral bioavailability remained unchanged at all tested doses (15, 25, and 50 mg kg^{-1}). In conclusion, these authors suggested that the aqueous solubility barrier might affect the speed but not the extent of resveratrol absorption. Further dose manipulation (up to 50 mg kg^{-1}) did not have a significant impact

on the oral bioavailability of resveratrol. Thse data rebat the hyphotesis that the use of CDs always increases the bioavailability of the guest molecule.

In another interesting paper (Lu et al., 2009), the molecular modelling of the complexation of resveratrol with two kinds of CD, β-CD and HP-β-CD, was carried out. The molecular modelling showed that part of the A-ring and the B-ring of resveratrol are placed in the cavity of β-CD, and the hydroxyl groups are projected outside. As regards resveratrol in HP-β-CD, the B-ring of resveratrol is included in the cavity of HP-β-CD, and part of the A-ring is points outwards. ^{1}H-NMR spectroscopy showed that H2, H3, H4 and H5 protons of resveratrol are more affected by the complexation, indicating that they are located inside the torus of CDs, which is in agreement with the results of the molecular modeling.

In a recent work, Li et al. (2010) studied the thermal effects of inclusion processes of α, β-, γ- and Mβ-CD with resveratrol in aqueous solutions by isothermal titration calorimetry with nanowatt sensitivity at the temperature of 298.15 K, and the standard enthalpy changes, stoichiometry and K_F of the inclusion complexes were derived from the direct calorimetric data by nonlinear simulation. Moreover, the thermodynamic parameters were discussed in the light of weak interactions between the host and the guest molecules combining with the UV spectral message. The results indicate that, although all the complexes formed in the aqueous solutions are in 1:1 stoichiometry, the binding processes of α-, β-, and Mβ-CD with the guest are mainly driven by enthalpy, while that of γ-CD with the drug is driven by both enthalpy and entropy.

As indicated previously, resveratrol is a potent lipophilic antioxidant. For this reason, the effect of complexation with CDs on its antioxidant capacity has been described in different papers. For example, Lucas-Abellán et al. (2008a) reported the effect of the complexation of resveratrol with HP-β-CD on the antioxidant capacity of this antioxidant using the oxygen radical absorbance capacity (ORAC) method, with fluorescein (FL) as the fluorescent probe. The method was validated through its linearity, precision and accuracy for measuring the ORAC of resveratrol in the absence or presence of CDs. Lucas-Abellán et al. (2008a) reported that the complexation of resveratrol in CDs increased the net area under the FL decay curve of resveratrol up to its saturation level, at which the stilbene showed almost double the antioxidant activity it shows in the absence of CDs. For these authors, the antioxidant activity of resveratrol was dependent on the complexed resveratrol because CDs acts as a controlled dosage reservoir that protects resveratrol against rapid oxidation by free radicals. In this way, its antioxidant activity is prolonged and only reaches its maximum when all the resveratrol is complexed.

However, results contrary to those published by Lucas-Abellán et al. (2008a) were reported in another paper concerning the effect on the antioxidant activity published by Lu et al. (2009). These authors compared the scavenging capacity towards the DPPH of free resveratrol and complexed resveratrol at the same concentration. The concentration of resveratrol in resveratrol/6 mM β-CD and resveratrol/6 mM HP-β-CD complexes were 0.68 and 2.72 mM, respectively, as determined by the molar absorption coefficients of 40,800 M^{-1} cm^{-1} at 306 nm and 34300 M^{-1} cm^{-1} at 307 nm. These authors reported that the differences in scavenging capacity between free and complexed resveratrol are slight, which suggests that the inclusion process had little influence on the antioxidant activity.

Finally, and also concerning the effect of CDs on the antioxidant activity of resveratrol, in the previously mentioned study by Sapino et al. (2009) the radical scavenging activity, the metal-chelating efficiency and the anti-lipoperoxidative potential of resveratrol were assessed: the data showed that the inclusion phenomenon did not significatively interfere with these biological properties. Finally, in vitro experiments revealed that the skin accumulation of resveratrol was higher when released from the complex then when deposited alone.

Pterostilbene

Pterostilbene (*trans*-3,5-dimethoxy-4′-hydroxystilbene) (Fig. 3D) is a stilbenoid present in different sources such as the berries of some *Vacciunium* species, the leaves of *Vitis vinifera*, the heartwood of sandalwood (*Pterocarpus santalinus*) and *Pterocarpus marsupium* or different medicinal products (Chong et al., 2009). This compound presents a variety of healthy properties, acting, for example, as antihyperglycemic, antioxidative, anticancer, antiinflammatory, anticholesterol, antifungus, hypolipidemic or analgesic (Remsberg,et al., 2008). In spite of these advantages, pterostilbene shows very poor solubility in water, possesses low bioavailability and is easily oxidized by several enzymes (Breuil, et al., 1999). For these reasons, the complexation of pterostilbene with molecules that can increase its bioavailability, solubility and stability in the face of prooxidant agents is strongly desirable, as it when CDs are used.

Recently, the complexation of pterostilbene with CDs was described by López-Nicolás et al. (2009a) using steady state fluorescence. Pterostilbene forms a 1:1 complex with different natural and modified CDs. Among natural CDs, the interaction of pterostilbene

with β-CD was the most efficient. However, all the modified CDs showed higher K_F than β-CD. The highest K_F was found for HP-β-CD (17520 ± 981 M^{-1}), in which its value showed a strong dependence on pH in the region where the pterostilbene begins the deprotonation of its hydroxyl group. Moreover, the values of K_F decreased as the system temperature increased. Furthermore, to obtain information on the mechanism of pterostilbene affinity for CD, the thermodynamic parameters of the complexation (ΔH^o, ΔS^o and ΔG^o) were studied. Finally, a comparison of the K_F values obtained for three types of stilbenes revealed that both the stoichiometry and the K_F values of the complex are dependent on the structure of the guest molecule. While the resveratrol/HP-β-CD and pterostilbene/HP-β-CD complexes showed a 1:1 stoichiometry (with a higher K_F value for the resveratrol-HP-β-CD complexes), *trans*-stilbene showed a 1:2 stoichiometry.

Moreover, Rodríguez-Bonilla et al. (2011) recently reported on a kinetic mechanism and provided a product characterization of the enzymatic peroxidation of pterostilbene as a model of the detoxification process of stilbene-type phytoalexins. In this work, the addition of increasing concentrations of HP-β-CD produced a change in the peroxidase enzymatic activity that depended on the pterostilbene concentration used. This typical behaviour is due to the ability of both natural and modified CDs to sequester part of the pterostilbene to form soluble inclusion complexes, thereby reducing the concentration of the free pterostilbene. Thus, CDs act as substrate reservoir in a dose-dependent manner. Indeed, free pterostilbene is the only effective substrate and the oxidation of the complexed substrate requires the prior dissociation of the complex. Moreover, although CDs have usually been used for complexing the substrates of enzymatic reactions, due to the importance of the pterostilbene oxidation products in the detoxification of phytoalexins, the authors studied

the potential effect of the addition of CDs on the three main products of the oxidation of pterostilbene by peroxidase. Indeed, the presence of increasing concentrations of HP-β-CD had different effects on the concentration of the three reaction products determined by HPLC.

Finally, in a recent work (Rodríguez-Bonilla et al. doi:10.1016/j.jchromb.2011.03.025), a RP-HPLC method was developed for the determination of pterostilbene in food samples. The novel method is based on the addition of CDs to the mobile phase where the complexation of pterostilbene by CDs is carried out. In order to select the most suitable conditions for the RP-HPLC method, the effect of several physico-chemical parameters on the complexation of pterostilbene by CDs was studied. The results show that the addition of 12 mM HP-β-CD to a 50:50 (v/v) methanol:water mobile phase at 25ºC and pH 7.0 significantly improves the main analytical parameters. In addition, it was seen that pterostilbene forms a 1:1 complex with HP-β-CD, showing an apparent complexation constant of 251 ± 13 M^{-1}. Finally, in order to study the validity of the proposed method, blueberries were analysed and the concentration of pterostilbene was been determined.

Pinosylvin

Pinosylvin (*trans*-3,5-dihydroxystilbene) $C_{14}H_{12}O_2$, (Fig. 3C) is a stilbenoid with several pharmacological (antimicrobial, antifungal, anticancer, antiinflammatory, antioxidative and antibacterial) properties (Lee et al., 2005). This compound is present in the wood pulp of pine and eucalyptus trees, and is present in tea oils and herbal remedies (Roupe et al., 2005).As has indicated previously, the disadvantages of stilbenes related with

their poor solubility in water, the ease with which they are oxidized by different agents or their tendency to be photodegraded have meant that pinosylvin has not been used as an ingredient in food products. Recently, López-Nicolás et al. (2009) studied the formation of inclusion complexes between both natural and modified CDs and pinosylvin. Using steady state fluorescence, these authors demonstrated that natural and modified CDs are able to complex pinosylvin following a 1:1 stoichiometry. Their results show that the K_F values were higher for all the modified CDs than for natural CDs, the highest K_F value being that determined for HP-β-CD/pinosylvin complexes (12112 ± 761 M^{-1}). The effect of pH and temperature on the K_F values was reported in this work and a thermodynamic study of the inclusion process was carried out to determine the three thermodynamic parameters, ΔH^o, ΔS^o and ΔG^o. The results show that the complexation of pinosylvin by HP-β-CD is a spontaneous and exothermic process.

Oxyresveratrol

Oxyresveratrol (trans-2,3´,4,5´) (Fig. 3A) is found in different sources such as mulberry (Morus alba L.) fruits and twigs (Lorentz et al., 2003). Its pharmacological properties include a wide range of biological (antioxidant, antiviral, hepatoprotective and cyclooxygenase and tyrosinase-inhibitory) activities (Lorentz et al., 2003). Recently, Rodriguez-Bonilla et al. (2010) studied the complexation of oxyresveratrol with natural CDs 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-CD and γ-CD. The decrease in the retention times with increasing concentrations of β-CD (0–4mM) showed that the K_F values 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 oxyresveratrol affinity for β-CD, the thermodynamic parameters ΔG^o, ΔH^o and ΔS^o were obtained. Finally, to better understand on the effect of the structure of different compounds belonging to the stilbenes 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. The authors show that the stoichiometry for all the stilbenoid β-CD complexes studied was 1:1, indicating that only one molecule of this type of oxyresveratrol (resveratrol, pterostilbene or pinosylvin) can be complexed by a molecule of β-CD. However, differences were evident in a comparison of the K_F values of the different complexes. 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.

CYCLODEXTRINS AND VITAMINS

Since Pitha (1981) reported the use of substituted cycloamyloses for enhancing the water solubility of vitamins A, D, E, and K, the use of CDs as complexant agents of vitamins with high antioxidant capacity has been described in numerous works. Due to the capacity of CDs to form inclusion complex with compounds of a hydrophobic nature, in Pitha´s work a revision of the main articles published about the interaction between different CDs and the lipophilics vitamins A, D, E and K has been resumed. However, as will be seen in forthcoming sections, very few papers have studied the effect of complexation of vitamins by CDs on the antioxidant activity of these fat-soluble compounds. For this reason, further research is needed into the antioxidant capacity of the CD/lipophilic vitamin complexes.

Vitamin A

One of the most important groups of hydrocarbons with important nutritional roles in humans is the so called “Vitamin A” (Loveday and Singh, 2008). The group of compounds which forms “Vitamin A” is made up of retinoids (chemical derivatives of retinol) and provitamin A carotenoids (which are partially converted to retinoids). Supplementation with large, pharmaceutically administered doses of vitamin A can substantially reduce the incidence and severity of some infectious diseases (Villamor and Fawzi, 2005). The fortification of foods is another strategy for combating vitamin A deficiency, although such fortification is not straightforward for several reasons (Loveday and Singh, 2008). Vitamin A is poorly dispersible in aqueous systems such as beverages and high moisture foods, and is highly labile under ambient conditions, a problem that

affects food supplementation route (Dary and Mora, 2002). Technologies that enhance the stability of vitamin A in foods are required for ensuring the safety and efficacy of vitamin A fortification of foods. In this review we discuss the factors affecting vitamin A encapsulation by CDs.

The complexation of provitamin A carotenoids by several CDs will be studied in future sections. For this reason, we shall confine this part of the review to the main papers published on the interaction between CDs and retinoids. Several methods have been reported to prepare retinoid/CD complexes. Semenova et al. (2002) and Guo et al. (1995) prepared them by mixing solutions at room temperature in aqueous alcoholic solutions of ethanol or methanol, respectively. Others authors (McCormack and Gregoriadis, 1998; Muñoz-Botella et al., 2002) reported another method based on the formation of a film of retinoid on the surface of a flask, to which aqueous CD solution is added and stirred for several days. The complexation of all-trans-retinoic acid with β-CD increased the aqueous solubility of all-trans retinoic acid by more than 100 fold (Qi and Shieh, 2002). However, this aqueous solubility increased more than 10 000 times after complexation with HP-β-CD (Lin et al., 2000). This solubility is strongly dependent on several factors such as pH (the β-CD/retinoic acid complex is better at neutral pH than at acidic pH) (Lin et al., 2000; Yap et al., 2005) or the presence in the medium of organic salts Qi and Shieh (2002) reported a 26-fold increase in the β-CD/retinoic acid complex after the addition of 1.5% sodium acetate.

On the other hand, cis-retinoids have also been complexed by several CDs to inhibit their photoisomerization (Munoz-Botella et al., 2002) and photodegradation (Yap et al., 2005). The molar ratio of CD to the retinoid molecule in the inclusion complex is usually 1:1 or 2:1 (Guo et al., 1995; Munoz-Botella et al., 2002), but ratios as high as 4.5:1 have

been reported (McCormack and Gregoriadis, 1998). However, different factors such as CD concentration can modify the stoichiometry of the inclusion complexes, e.g. HP-β-CD forms complexes with all-trans retinoic acid in a molar ratio of 1:1 at low CD concentration and in a molar ratio of 2:1 at higher CD concentration (Lin et al., 2000).

Recently, Weisser et al. (2009) investigated a topically applied vitamin A loaded amphiphilic CD nanocapsules. In this work, a new cholesteryl-CD derivative, obtained by grafting a single cholesterol on a CD, proved suitable for the manufacture of nanocapsules. These nanocapsules were loaded with a lipophilic drug, e.g., vitamin A propionate which is a highly unstable and poorly water soluble molecule of therapeutic interest. The oily nature of vitamin A propionate leads to the formation of nanocapsules with a reproducible size distribution and long term stability. The colloidal suspension can be used to form a gel which permits the encapsulated vitamin A propionate to penetrate the skin.

In another work, Lin et al. (2007) reported the biopharmaceutics of 13-cis-retinoic acid (isotretinoin), a molecule commonly used in the management of severe acne, formulated with modified β-CDs, studying the influence of the presence of CDs on the kinetic profile and bioavailability of isotretinoin administered to rats.

Vitamin K

Vitamin K is a necessary participant in the synthesis of several proteins that mediate both coagulation and anticoagulation (Wallin et al., 2008). Vitamin K deficiency is manifested as a tendency to bleed excessively. Indeed, many commercially-available rodent poisons are compounds that interfere with vitamin K and kill by inducing lethal hemorrhage. Vitamin K serves as an essential cofactor for a carboxylase that catalyzes the

carboxylation of glutamic acid residues in vitamin K-dependent proteins. The key vitamin K-dependent proteins include coagulation proteins (factors II (prothrombin), VII, IX and X), anticoagulation proteins (proteins C, S and Z) and others such as bone proteins, osteocalcin and matrix-Gla protein, and certain ribosomal proteins (Yamauchi et al., 2010).

Although the antioxidant activity of vitamin K has been reported in numerous studies (Vervoort et al., 1997), very few papers have reported the complexation between this type of vitamin and CDs. In a study about the solubilization of lipid-soluble vitamins by complexation with CDs, Okada et al. (1990) reported the inclusion complex formation of eight kinds of lipid-soluble vitamins with 6-O-α-D-glucopyranosyl-β-CD (G-β-CD) in aqueous solution and in solid phase, which were assessed by the solubility method and thermal analysis. All lipid-soluble vitamins were highly solubilized in water by complexation with G-β-CD. As regards the complexation of vitamin K with G-β-CD, analysis of the phase solubility diagrams showed, the stoichimetric ratio of the main complex in water to be 1:3 for Vitamin K_1/G-β-CD, 1:3 for Vitamin K_2/G-β-CD, and 1:1 for Vitamin K_3/G-β-CD. Later, Zhenming et al. (2003) studied the interaction of the Vitamin K_3-CD inclusion complex and its analytical application. The inclusion interaction of the complexes between Vitamin K_3 and β-CD, HP-β-CD and sulfobutylether-β-CD (SBE-β-CD) were studied using steady-state fluorescence measurements. The K_F values were calculated and a 1:1 inclusion stoichiometry for Vitamin K_3/CDs was determined. The results showed that the inclusion ability of β-CD and its derivatives was in the order: SBE-β-CD>HP-β-CD>β-CD. Finally, the results obtained were applied to determining Vitamin K_3 in pharmaceutical preparations. In another work, Berzas et al. (2000) published a similar spectrofluorimetric study of the β-CD/Vitamin K_3 complex and reported a different K_F

value although the 1:1 stoichiometric ratio was confirmed. This vitamin not naturally fluorescent but yields fluorescence when it is reduced. However, it is possible to yield a fluorescent derivative in aqueous medium when complexed to β-CD and the procedure was applied to pharmaceutical formulations. The procedure was also applied to pharmaceutical formulations. Furthermore, Lengyel and Szejtli (1985) prepared a stable, nonsublimable γ-CD inclusion complex of menadione (Vitamin K_3) in solution, in suspension, and by kneading. The biological activity of the complex was tested on baby chickens by prothrombin time determination. Reduced prothrombin times showed an enhanced bioavailability of menadione in complexed form. The stability of Vitamin K_3 is greatly improved in pharmaceutical and veterinary products prepared with a menadione- γ-CD -CD inclusion complex.

Vitamin D

Vitamin D is a group of fat-soluble secosteroids, the two major physiologically relevant forms of which are vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). Vitamin D without a subscript refers to either D_2 or D_3 or both. Vitamin D_3 is produced in the skin of vertebrates after exposure to ultraviolet B light from the sun or artificial sources, and occurs naturally in a small range of foods. In some countries, staple foods such as milk, flour and margarine are artificially fortified with vitamin D, and it is also available as a supplement in pill form. Food sources such as fatty fish, mushrooms, eggs, and meat are rich in vitamin D and are often recommended for consumption by those suffering vitamin D deficiency (Holick, 2005).

The complexation of Vitamin D by different type of CDs has been reported in several studies. Palmieri, et al. (1993) evaluated different complexation methods (phase solubility diagram, HPLC, DSC, X-RAY diffractometry and NMR) to form inclusion complexex between β-CD and Vitamin D_2 in aqueous solution and solid state. The solid inclusion complexes were prepared by spray-drying, kneading and solid dispersion. The dissolution profiles of the complex either in powder or in tablets were studied in order to select the best inclusion process. Their results show that while kneading provided a good yield, spray drying led to complete complexation and best dissolution.

Studies on the stability and structure of the β-CD/Vitamin D_2 inclusion complex by Peng et al. (1999) showed that the Vitamin D_2 content in the inclusion complex was still 85%-95% after two years, while that in the β-CD and Vitamin D_2 mixture was just 17%-22%. This indicates that Vitamin D_2 in the inclusion complex was much more stable than pure Vitamin D_2. Therefore, adding the inclusion complex β-CD-Vitamin D_2 into "Longmu" Zhuanggu Chongji, a medicine for children to prevent and cure rickets, may not only decrease the expense of Vitamin D_2, but also enhance the stability of the products.

One of the most interesting applications of the complexes between Vitamin D and CDs was reported in the paper by Takeda et al. (1994). These authors studied the application of CDs to the microbial transformation of VD_3 to 25-hydroxy Vitamin D_3 and 1α,25-dihydroxy Vitamin D_3. In this microbial hydroxylation, it was found that CD had the ability to enhance the hydroxylation of Vitamin D_3.

In another paper, Comini et al. (1994) studied the interaction of β-CD with bile acids (cholic, taurocholic, chenodeoxycholic and lithocholic) and their competition with

vitamins A and D_3. These vitamins were seen to compete with cholic and taurocholic acids but not with lithocholic and chenodeoxycholic acids. The affinity of vitamins A and D_3 for β-CD was lower than that of the bile acids. The results of this study suggest that depletion of lipophilic vitamins will not occur it of β-CD, thus providing further support for the safety and suitability of β-CD as an ingredient in foods and orally administered drugs.

In another study, Tian and Holick (1995) studied the catalyzed thermal isomerisation between previtamin D_3 and vitamin D_3 via β-CD complexation, observing a strong increase in the velocity of isomerisation when CDs are present in the reaction medium. This conformation-controlled process may play an important role in the modulation of the previtamin D_3/vitamin D_3 endocrine system *in vivo,* as it has been seen to do in the sea urchin.

One of the main applications of the use of CDs in the analytical chemistry field is to reduce of the retention time and the separation of antioxidant compounds analyzed by different analytical techniques. In 1997, Spencer and Purdy studied the separation of fat-soluble vitamins using several types of CDs in high-performance liquid chromatography and micellar electrokinetic chromatography (MECK). The use of MEKC rather than HPLC provided comparable or even improved resolution of these hydrophobic solutes. Both techniques proved successful in the separation of vitamins D_2 and D_3. Dimethyl-13-CD as mobile phase and buffer additive led to the best separation of these two compounds. Moreover, the addition of CD to the running buffer in MEKC provided little improvement in the resolution of these two compounds.

Vitamin E

Tocopherols and tocotrienols share common structures both having a chromanol head and a phytyl tail. They are collectively named vitamin E, which has been shown to be the most effective lipid-soluble antioxidant in nature, interfering with one or more propagation steps of the lipid peroxidation process**.** Due to their medical, biological, and physiochemical significance, tocopherols have been extensively studied. In contrast with other lipohilic vitamins, the complexation of vitamin E by CDs has been reported in many papers along with the effect of encapsulation on the antioxidant activity.

In a recent report on CD inclusion complex formation and solid-state characterization of the natural antioxidants α-tocopherol and quercetins, Koontz et al. (2009) suggested that, natural antioxidant/CD inclusion complexes may serve as novel additives in controlled-release active packaging to extend the oxidative stability of foods. In this work, variations of the coprecipitation from aqueous solution technique were optimized for the CD complexation of α-tocopherol and quercetin.

The use of CDs to separate different tocopherols has been reported by several authors. For example, Adibi and Mounts (1994) separated α-, β-, γ-, and δ-tocopherols and 5,7-dimethyltocol by normal-phase HPLC on β- or γ-CD-bonded silica (CDS) with fluorescence detection. Generally, the k′ values obtained with hexane mobile phases or with the β-CDS phase were greater than those observed in HPLC using cyclohexane mobile phases or with the γ-CDS phase. With a similar objective, Chang et al. (2006) published a study about CD-modified microemulsion electrokinetic chromatography for the separation of α-, γ-, δ-tocopherol and γ-tocopherol acetate. Microemulsion electrokinetic chromatography, claimed to attain high peak efficiency with great solubilization power, has

not previously been applied to the separation of tocopherols. The results obtained by these authors were applied to vitamin E preparations with optimum results.

Siró et al. (2006) studied the influence of complexation in β-CD on the release of alpha-tocopherol from antioxidative low-density polyethylene film into fatty food simulants. The release of alpha-tocopherol from two formulations (with and without complexation with β-CD) of low-density polyethylene film was examined and it was concluded that complexation with β-CD had a significant effect on the antioxidant release rate. A decrease in the diffusion coefficient of one order of magnitude was calculated in the case of complexed alpha-tocopherol compared with the free form and total migration of alpha-tocopherol from both films was observed, meaning that the partition coefficient of tocopherol was not influenced by incorporation with CD. Thus, complexation might be the key to a long-lasting antioxidative effect of this kind of active packaging.

Several works have been published concerning the antioxidant activity of vitamin E complexed with different types of natural and modified CDs. Iaconinoto et al. (2004) studied the influence of complexation with β-CD (β-CD), HP-β-CD or HP-γ-CD on the antioxidant activity during the light-induced decomposition of vitamin E (alpha-tocopherol). The photodegradation of alpha-tocopherol was examined in emulsion vehicles and was not significantly influenced by complexation with β-CD, whereas HP-β-CD and HP-γ-CD enhanced the light-induced decomposition of alpha-tocopherol. On the other hand, accelerated stability studies indicated that the degradation of non-irradiated alpha-tocopherol was reduced by complexation with HP-β-CD or HP-γ-CD. The radical scavenging activity of alpha-tocopherol was evaluated *in vitro* using the xanthine/xanthine oxidase enzymatic system. No significant differences were observed between the free form

of the vitamin and its complexes with β-CD, HP-β-CD or HP-γ-CD. Therefore, complexation of alpha-tocopherol with these CDs does not interfere with the antioxidant activity of this vitamin.

One of the applications of the encapsulation of vitamin E by CDs is in the development of new assays to measure the antioxidant activity of different compounds. Thus, in an interesting work, Huang e al. (2002) developed an oxygen radical absorbance capacity assay for lipophilic antioxidants including the vitamin E family, using RM-β-CD as the solubility enhancer. RM-β-CD was introduced as the water solubility enhancer for lipophilic antioxidants. For the first time, by using 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid as a standard (1.0), the ORAC values of α-tocopherol, (+)-γ-tocopherol, (+)-δ-tocopherol, α-tocopherol acetate, tocotrienols, 2,6-di-tert-butyl-4-methylphenol, and γ-oryzanol were determined: 0.5 ± 0.02, 0.74 ± 0.03, 1.36 ± 0.14, 0.00, 0.91 ± 0.04, 0.16 ± 0.01, and 3.00 ± 0.26, respectively.

On the other hand, Celik et al. (2007) reported a simple, low-cost, and widely applicable total antioxidant capacity assay for different molecules named the CUPRAC method. This is an improvement over classical CUPRAC methodology because reports the assay for both lipophilic and hydrophilic antioxidants simultaneously, by making use of their 'host–guest' complexes with methyl-β-CD in aqueous medium. The turbidity limit for α-tocopherol was 1:3 (v/v) alcohol–water solutions, but when these suspensions were mixed with equal volumes of 7% methyl-β-CD aqueous solution, clear solutions were obtained in which the CUPRAC assay could be directly performed.

Moreover, Oyurek et al. (2008) reported another method to determine the antioxidant capacity of several molecules using an assay of lipophilic and hydrophilic antioxidants in the same acetone–water solution containing 2% methyl-β-CD and also using the cupric reducing antioxidant capacity (CUPRAC) method. In this way the order of antioxidant potency of various compounds irrespective of their lipophilicity could be established in the same solvent medium. Methyl-β-CD was introduced as the water solubility enhancer for lipophilic antioxidants. Two percent methyl-β-CD (w/v) in an acetone–H_2O (9:1, v/v) mixture was found to sufficiently solubilize carotene, lycopene, vitamin E, vitamin C, synthetic antioxidants and other phenolic antioxidants. This assay was validated through linearity, additivity, precision, and recovery assays, which demonstrated that the CUPRAC assay is reliable and robust.

Finally, one of the most important applications of the encapsulation of vitamin E is in the cosmetic industry. Thus, the formation of an inclusion complex between γ-CD and a broad variety of organic compounds increases the stability and solubility of active cosmetic ingredients and provides a better control over the release of fragrances. Lipophilic vitamin E is essential in skin care because of its nature as a free radical scavenger; however, it is sensitive to light and oxidation-induced degradation. For this reasons, Regiert (2005) studied the light stability of vitamin E by inclusion in γ-CD for its use in the cosmetic industry.

CYCLODEXTRINS AND CAROTENOIDS

The presence of carotenoids in higher plants, algae, fungi and bacteria has been widely discussed in recent years (Boussiba et al., 1992; Castenmiller and West, 1998; Scotter et al., 2003). Moreover, recently papers have investigated their presence in animals, such as birds and crustaceans. Because carotenoids can only be biosynthesized by plants and microorganisms, their presence in animals is attributed to their ingestion in the food and their accumulation in certain tissues, e.g. the feathers of flamingos, egg yolk, and the exoskeleton of invertebrates. Furthermore, over 600 compounds constitute the carotenoid family, among which the best known are α-carotene, β-carotene, lutein, lycopene, astaxanthin and zeaxanthin. With respect to it structure, carotenoids predominantly occur in their *all-trans* configuration, which is the thermodynamically more stable isomer. However there is ample proof supporting the presence of *cis* isomers in plants, especially in chlorophyll-containing tissues, but also in a number of fruits (Cortes et al., 2004). Low levels of *cis*-isomers of β-carotene were also detected in hydroponic leafy vegetables, although below the limit of quantification.

Several healthy effects have been attributed to the consumption of a diet rich in carotenoids, such as the decreased risk of certain types of cancer, atherosclerosis and age-related degeneration. Moreover, a key property of carotenoids is their capacity to quench singlet oxygen and free radicals; since this capacity depends on the number of conjugated double bonds, carotenoids inevitably show high antioxidant capacity (Nelson et al., 2003).

In spite of the numerous advantages reported for different carotenoids, these molecules present two main disadvantages that impede their use as added antioxidant

agents in functional foods: i) the ease with which they are oxidized by different agents due to the system of double bonds and ii) their poor solubility in aqueous solvents. In an attempt to avoid these problems, several papers on the complexation of carotenes with CDs have been published. Below, we review the most recent works on the complexation of the main types of carotenoid with different CDs.

Astaxanthin

Astaxanthin, generally known as all-*trans* astaxanthin, is a red-orange carotenoid pigment, naturally found in many aquatic animals, such as shrimp, crab and salmon. Because it belongs to the xanthopyll class of carotenoids, astaxanthin is closely related to β-carotene, lutein and zeaxanthin, sharing with them many of the general metabolic and physiological activities attributed to carotenoids (Ciapara et al., 2004).

The use of astaxanthin in the aquaculture industry is increasing since it provides the typical muscle colour of salmon a property that is widely accepted by consumers. Different biological functions have been reported for astaxanthin in fish. For example, this type of carotene is associated with protecting cells against oxidative damage and also with reproduction and embryo development (Parajo et al., 1996). Moreover, the use of astaxanthin as nutraceutical agent in human nutrition has increased because of the numerous investigations which justify its antioxidant, anticancer and antiinflamatory properties (Wei and Yan, 2001). Several astaxanthin products derived from microalgae are available for consumers.

The antioxidant properties of astaxanthin are strongly related with the eleven conjugated carbon–carbon double bonds which are present in its structure. Several authors

have shown that the antioxidant property of astaxanthin is ten times that of β-carotene, and up to 500 times stronger than that of vitamin E (Shimidzu et al., 1996).

However, several difficulties limit the use of astaxanthin as an antioxidant agent to produce novel lipophilic foods is limited by including its sensitivity to heat or light and its poor aqueous solubility.

For this reason, Chen et al. (2007) recently published a paper describing the preparation and stability of an inclusion complex of astaxanthin with β-CD. Using HPLC, IR and absorbance methods, the complexes investigated by these authors showed a stoichiometry of 1:4 for astaxanthin: β-CD. Moreover, their results showed that the water solubility of the inclusion complex was <0.5 mg/ml, which is better than that of astaxanthin itself. Moreover, the heat stability of the inclusion complex and its stability in light were greatly enhanced. The aqueous solubility of the inclusion complex was also slightly enhanced (<0.5 mg/ml).

In another paper and in order to distinguish between the complexation of this carotene with natural and modified CDs, Yuan et al. (2008) reported the inclusion complex of astaxanthin with HP-β-CD, using IR spectroscopy. The water solubility of the resulting inclusion complex was >1.0 mg/ml, which is much better than that of astaxanthin and that published by Chen et al. (2007) for the complexes of this type of carotenoid with β-CD. Moreover, when the solid state thermal behaviour of the inclusion complex was investigated by thermogravimetric/differential thermal analysis, the temperature at which astaxanthin began to decompose was enhanced to about 290 ºC. The stability of the inclusion complex in solution was also tested by Chen et al (2007), who found that the

complex greatly improved the stability of astaxanthin against light and oxygen. The release of astaxanthin from the inclusion complex was also reported in that paper.

Lycopene

Lycopene is one of the major carotenoids in the diet of North Americans and Europeans, and is therefore a nutraceutical for which is great demand. The most important source of lycopene is tomato and its processed food products, in which lycopene may constitute more than 60% of the carotenoids present. Lycopene is also present in watermelon, apricot, papaya, passion fruit, guava, pink grapefruit, carrots, pepper, persimmon, balsam pear and a large number or red berries (e.g., cranberries) and the fruits of autumn olive (Charoensiria et al., 2009).

Several epidemiological studies have suggested that the high consumption of tomatoes and tomato products containing lycopene may protect against CVD and reduce the risk of several types of cancer, most notably those of the prostate, breast, lung and digestive tract. Serum and tissue levels of lycopene have also been inversely related with chronic disease risk (Ramandeep and Savage, 2006). Such epidemiological leads have stimulated a number of animal models and cell culture studies designed to test this hypothesis and to establish the beneficial effects of lycopene. The evidence gained in from these studies suggests that lycopene has anti-carcinogenic and anti-atherogenic effects both *in vitro* and *in vivo*.

A key property of carotenoids is their capacity for quenching singlet oxygen and free radicals, a capacity that depends on the number of conjugated double bonds, and makes

lycopene exceptionally effective compared with other carotenoids. The *in vitro* quenching constant of lycopene has been found to be more than twice that of β-carotene and 100 times that of α-tocopherol (Di Mascio et al., 1989). Lycopene may also interact with reactive oxygen species, such as hydrogen peroxide and nitrogen dioxide. Despite the many disputes in the literature about lycopene behaviour, lycopene presents the same problems for use in functional foods as those mentioned above for astaxanthin. For this reason, its complexation with CDs has been reported in several papers, which we will now look at.

Blanch et al. (2007) reported the stabilization of all-*trans*-lycopene from tomato by encapsulation using α, β and γ-CDs. To that end, two different encapsulation methods were studied: a conventional method and a supercritical fluid extraction process. An optimization procedure taking into consideration the distinct molar ratios of CD/lycopene (1/0.0026, 1/0.005 and 1/0.05) as well as the type of CD to be used was accomplished. Encapsulation was determined by using micro-Raman spectroscopy. As a result, a CD/lycopene molar ratio of 1/0.0026 was selected as it provided the best complexation yields (93.8%), whilst β-CD seemed to be the most favourable for stabilizing the lycopene. A comparison of the two methods studied showed higher encapsulation yields with the conventional method. The results reported by the above authors (Blanch et al., 2007) showed that the supercritical fluid approach offers numerous advantages, such as the possibility of carrying out the extraction, fractionation and encapsulation of lycopene from tomato in one step, notably shortening the overall procedure time and minimizing sample handling.

In another paper, Quaglia et al. (2007) produced lycopene-containing powders from tomato products by a solvent-free method making use of β-CD. The powders were prepared by spray-drying a tomato concentrate, one of the most bioavailable forms of lycopene after

mechanical treatment with β-CD in different weight ratios. The obtained product was centrifuged to partly eliminate the food matrix and characterized by the amount of lycopene hydrodispersed/hydrosolubilized in the aqueous fraction. The chemical antioxidant activity of sera was also evaluated by these authors and a progressive increase of this antioxidant capacity when CDs were presented in the reaction medium was observed. The powders obtained by spray-drying sera exhibited good flow properties, a lycopene content of between 0.4 and 1.09 mg/g and excellent water dispersability. The process developed, which makes use of β-CD, is of great interest for obtaining nutraceuticals with better bioavailability than that of lycopene alone.

In their study, Nunes and Mercadante (2007) obtained encapsulated lycopene in a powder form, using either spray-drying or molecular inclusion with β-CD followed by freeze-drying. The encapsulation efficiency using spray-drying ranged from 94 to 96%, with an average yield of 51%, with microcapsules showing superficial indentations and an absence of cracks and breakages. Lycopene/β-CD complexes were only formed at a molar ratio of 1:4, and irregular structures of different sizes that eventually formed aggregates, similar to those of β-CD, were observed after freezedrying. About 50% of the initial lycopene did not form complexes with β-CD. Lycopene purity increased from 96.4 to 98.1% after spray-drying, whereas lycopene purity decreased from 97.7 to 91.3% after complex formation and freeze-drying. Both the drying processes yielded pale-pink, dry, free-flowing powders.

Recently, Fernández-García et al. (2010) analyzed the assimilation efficiency of carotenoids when they are delivered as inclusion complexes with β-CD in water and showed that the *in vitro* intestinal absorption of several carotenoids (lycopene, lutein, and

β-carotene) delivered as molecular inclusion complexes with β-CD led to a significant increase in carotenoid assimilation compared with the corresponding carotenoid suspensions in Tween and assimilation was not inhibited by high-density lipoproteins.

In 2006, Vertzoni and co-workers described the solubilization and quantification of lycopene in aqueous media in the form of binary CD systems. The optimized kneading method for preparing the lycopene–CD binary systems improved the solubility of lycopene in water and 5% (w/v) dextrose solution. The lycopene in the binary systems was quantified by a spectrometric method that followed single-step extraction with dichloromethane. The storage stability characteristics of the binary systems were studied at 4 ºC in solution and at −20 ºC in the lyophilized products, monitoring the lycopene content at λ_{max} = 482 nm. The results obtained by these authors with the spectrometric method were confirmed by HPLC. In the presence of CDs, the lycopene concentration in water was 8.0 ± 1.0, 27.1 ± 3.2 and 16.0 ± 2.2 μg/ml for β-CD, HP-β-CD and Me-β-CD, respectively. In 5% (w/v) aqueous dextrose solutions the corresponding values were 16.0 ± 1.8, 48.0 ± 5.1 and 4.0 ± 0.5 μg/ml, respectively. At 4 ºC, the storage stability of the lycopene–CD binary systems in water or 5% (w/v) aqueous dextrose solutions was limited ($t_{1/2}$ = 1–4 days), although the addition of the antioxidant sodium metabisulfite increased the stability of the lycopene–HP-β-CD binary system in water. At −20 ºC, the lyophilized lycopene–CD binary systems were stable for at least two weeks.

As regards the effect of the addition of CD on the antioxidant capacity of lycopene, Bangalore et al., (2005) reported the effect that β-CD had on the relation between lycopene concentration and ORAC (oxygen radical absorbance capacity), which is used as an index of antioxidant activity for many hydrophilic antioxidants but not for lycopene. This study

served to validate the ORAC assay for different concentrations of lycopene in the presence of β-CD, a water-solubility enhancer. The lycopene concentration correlated poorly with ORAC in the absence of β-CD. However, these correlations improved with increasing levels of β-CD. The results demonstrated that the inclusion of β-CD in the ORAC assay improves the correlation between ORAC and lycopene concentration, thus expanding the scope of the assay to include fat-soluble antioxidants. These findings open up new avenues for using the ORAC assay as a tool for evaluating lycopene antioxidant activity, enabling us to better understand the relationship between lycopene concentration and antioxidant activity in various food systems.

β-carotene

β-carotene is the major carotenoid present in the human diet as well as in the human organism, where displays pro-vitamin A activity, among its other positive effects on human health. β-carotene is endogenously present in several isomers; all-*trans*-β-β´-carotene is followed, in decreasing order, by lower concentrations of the 15-*cis*, 13-*cis*- and 9-*cis*-isomers (Johnson el at., 1997). In addition to the β-carotene structure, the form α-carotene also exists, as a result of a shift of one of the conjugated double bonds in the head region of the β-carotene structure. β-carotene is mainly present in the vegetables that form part of the human diet and small amounts in the animal-derived diet. It is mainly green, yellow, orange and red vegetables like broccoli, Brussels sprouts, tomatoes, spinach, carrots and paprika as well as colored fruits like apricot, grapefruit, cherry and papaya, are rich in β-carotene (Biesalski et al., 2001). Additional nutritional sources of β-carotene are foods supplemented with β-carotene as a food colorant, like margarine, butter and many soft drinks. The bioavailability of β-carotene in the human organism varies greatly and depends on various

factors (Van het Hof et al., 2000) such as interaction between individual carotenoids, the amount of fat and individual fatty acids present in the diet and the matrix in which the carotenoids are located. The absorption rate is also modified by diseases of the gastrointestinal tract mainly due to the malabsorption of lipids.

The complexation of β-carotene with several types of CDs has been reported in recent years. For example, Mele et al. (2002) used the formation of complexes between CDs and *trans*-β-carotene in water to evidence the formation of large aggregates using light scattering and NMR spectroscopy. These authors also showed that the NMR spectra of CD/β-carotene in a D_2O solution pointed to a chemical shift of the CD protons upon complexation, indicating that a host-guest interaction had taken place in solution. The pattern of the chemical shift was, however, different from that expected in the case of classical inclusion complexes of defined stoichiometry (e.g., a 1:1 complex), indicating that the formation of a true inclusion compound between CD and β-carotene may be only one of the possible interaction mechanisms. Any possible CDs/β-carotene host-guest associations can be reasonably expected to have a neatly amphiphilic character due to the hydrophilic hosts and the hydrophobic guest. The study of Mele et al. (2002) confirmed that the hydrophobic moiety (β-carotene) of such inclusion complexes in water tends to self-associate, forming larger supramolecular micelles like aggregates, with the hydrophilic CD molecules arranged outside and in fast exchange (on the NMR timescale) with the free CD molecules in solution. Besides, the LS data presented by these authors showed that pure β-CD and γ-CD do not form large self-aggregates in water, at least in the experimental conditions used. This observation is in agreement with recent NMR studies showing that

pure β-CD is a monomer in a water solution (Azaroval-Bellanger and Perly, 1994), ruling out previous contrary conclusions obtained from LS experiments.

Another field of application for β-carotene complexation by CDs is in food science and technology. Szente et al. (1998) studied the stabilization and solubilization of natural lipophilic colorants with CDs and provided data on the practical application of the benefits of the molecular encapsulation of natural colorants by CDs. The experimental results confirmed the increased stability of CD-complexed curcumin, curcuma oleoresin, β-carotene and carotenoid oleoresins in the face of light, heat and oxygen. The parent β-CD stabilized carotenoides and, especially, curcumines. Methylated β-CD was found to be the most potent solubilizing agent for both carotenoids and curcuminoids.

The complexation of this potent antioxidant with CDs has also been used in cellular biology, where the use of CDs to complex β-carotene was reported to improve a novel method for the supplementation of cultured cells (Pfitzer et al., 2000). A physiological, water-soluble complex of carotenoids with methyl-β-CD was developed for the purpose of cell supplementation. The bioavailability, cytotoxicity and stability of the formulations were compared to carotenoid solutions in organic solvents and the stability of the different carotenoid solutions (0.5 μM) under cell culture conditions was determined by measuring absorbance 1 and 7 days after treatment. To determine the availability of β-carotene, human skin fibroblasts were incubated for up to 8 days with 5 μM β-carotene in methyl-β-CD or THF/DMSO and the cellular and medium β-carotene contents were determined by HPLC analysis. Depending on the solubilizer used, different orders of stability were found by Pfitzner et al (2000). Methyl-β-CD formulation: β-carotene>zeaxanthin>lutein>lycopene. Organic solvents: zeaxanthin>lutein>lycopene>β-carotene. Two days after supplementation

with 5 µM β-carotene in MLCD, cellular β-carotene levels reached a maximum of 140 ± 11 pmol/µg DNA, levelling off at 100 ± 15 pmol/ µg DNA until day 8. Incubation with β-CD dissolved in THF/DMSO resulted in a lower β-carotene uptake of 105 ± 14 pmol/ µg DNA and 64 ± 20 pmol/ µg DNA, respectively. No cytotoxic effects of these formulations were detected by Pfitzner and coworkers in their paper. The results show that the methyl-β-CD formulation is a better method for investigating carotenoids and other lipophilic compounds in *in vitro* test systems compared to methods using organic solvents.

In another paper, Lancrajan et al. (2001) used liposomes and β-CD as carriers for the incorporation of three dietary carotenoids (β-carotene and two other carotenoids lutein and canthaxanthin) into plasma and into the mitochondrial, microsomal and nuclear membrane fractions from pig liver cells or the retinal epithelial cell line D407. The uptake dynamics of the carotenoids from the carriers to the organelle membranes and their incorporation was followed by these authors by incubating at pH 7.4 for up to 3 h. The results showed the β-CD carrier to be more effective at incorporating lutein, while β-carotene was incorporated into natural membranes from liposomes or from CDs.

As regard the antioxidant capacity of β-carotene, Özyürek et al. (2008) reported the antioxidant capacity of both lipophilic and hydrophilic antioxidants simultaneously, by making use of their 'host–guest' complexes with methyl-β-CD in acetonated aqueous medium using the cupric reducing antioxidant capacity (CUPRAC) method. In this way, methyl-β-CD was introduced as the water solubility enhancer for lipophilic antioxidants. Two percent methyl-β-CD (w/v) in an acetone–H_2O (9:1, v/v) mixture was found to sufficiently solubilize β-carotene, lycopene, vitamin E, vitamin C, synthetic antioxidants and other phenolic antioxidants. This assay was validated through linearity, additivity,

precision, and recovery assays. The validation results demonstrated that the CUPRAC assay is reliable and robust. In an acetonated aqueous solution of methyl-β-CD, only CUPRAC and 2,2´-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays were capable of measuring carotenoids together with hydrophilic antioxidants. The CUPRAC antioxidant capacities of a wide range of polyphenol and flavonoids were reported in this work as trolox equivalent antioxidant capacity (TEAC) in the CUPRAC assay, and compared to those found by reference methods, ABTS/horseradish peroxidase (HRP)–H_2O_2 and ferric reducing antioxidant power (FRAP) assays. The TEAC coefficients of hydrophilic antioxidants did not differ significantly from those reported in the original CUPRAC method, while TEAC values of β-carotene and lycopene were reported for the first time in this modified CUPRAC assay. The authors demonstrated that synthetic mixtures composed of lipophilic and hydrophilic antioxidants provided the theoretically expected CUPRAC antioxidant capacities, indicating that there were no chemical deviations from Beer's law, and that the observed CUPRAC absorbances were additive.

Moreover, using different techniques, Polyakov et al. (2004) studied the formation of inclusion complexes between β-CD and carotene and carotene derivates, finding that although CD protects the carotenoids from reactive oxygen species, the complexation with CD results in considerable decrease in antioxidant ability of the carotenoids. Their results show that CDs does not prevent the reaction of carotenoids with Fe^{3+} ions, but reduces their scavenging rate toward OOH radicals. This means that different sites are responsible for the interaction of carotenoids with free radicals and Fe^{3+} ions. Because CDs are widely used as carriers and stabilizers of dietary carotenoids, the demonstration that CDs protect the

carotenoids from reactive oxygen species and provide their safe delivery to the cell membrane is of importance.

CYCLODEXTRINS AND COENZYME Q_{10}

Coenzyme Q_{10} (CoQ_{10}), also known as ubiquinone, is a lipid-soluble compound that has an important function in the mitochondrial electron transport chain as an electron carrier (Bhagavan and Chopra, 2006). Further, CoQ_{10} is an antioxidant whose activity is particularly important in regenerating vitamin E. Its ability to quench free-radicals also helps to maintain the structural integrity and stability of mitochondrial and cellular membranes, including intracellular membranes. A number of claims that are attributed to CoQ_{10} may be due to its antioxidant properties as well as to its enhancement of the cellular bioenergy capacity. In spite of these advantages, CoQ_{10} presents several inconveniences for use in the food industry as fortifier or nutraceutical. CoQ_{10} is sensitive to light and heat and will decompose when it exposed to light. Moreover, the bioavailability of CoQ_{10} is low and variable due to its poor solubility in water and high molecular weight (Bhagavan and Chopra, 2006). For these reasons, CoQ_{10} should be treated to protect it from light and heat and to increase its bioavailability. A number of strategies to improve the absorption of CoQ_{10} have been proposed, such as oily solutions, self-emulsified drug delivery systems, esterification, coadministration of pepper extract, binary solid dispersion and liposome. We shall now present, a review of the principal papers and patents on the complexation of this antioxidant by CDs.

In 2008, Hatanaka et al. reported the physicochemical and pharmacokinetic characterization of water-soluble CoQ_{10} formulations. In this work, a novel liquid (nano-emulsion, NE) and water-soluble powder formulations, including CD–CoQ_{10} complex (CoQ_{10}–CD) and dry-emulsion (DE), were prepared. The physicochemical properties of each formulation were characterized by dynamic light scattering, scanning electron

microscopy, powder X-ray diffractometry, and differential scanning calorimetry. In all the powder formulations prepared, CoQ_{10} existed mainly in an amorphous form, as determined by and each powder formulation exhibited high solubility and dispersibility in water, resulting in the formation of a nano-sized emulsions (NE; 60 nm) and micron sized particles (DEs and CoQ_{10}–CD; 0.77–2.4μm). The pharmacokinetic study of each dosage form, as opposed to a CoQ_{10} crystal suspension, was also carried out in rats after a single oral dose. Although similar kinetic values were seen with Tmax of 1.5 and 1.7 h, respectively, NE exhibited ca 1.7-fold higher AUC and Cmax than the crystalline CoQ_{10}. Considering the significant increase on both Cmax and AUC of CoQ_{10}, NE methodology could be the most effective of the formulations tested for improving of oral absorption of CoQ_{10}.

Recently, Miyamoto (2009) published a structural study of the CoQ_{10} inclusion complex with γ-CD, investigating the molecular composition and three-dimensional structure of the complex using chemical analyses and molecular modelling. Moreover, the molecular ratio of γ-CD and CoQ_{10} in the complex was investigated by NMR as well as by HPLC to determine the γ-CD/ CoQ_{10} ratio, which was found to be 2.5.

Increasing the bioavailability of CoQ_{10} is one of the main goals of the complexation of this potent antioxidant by CDs. An interesting study on the enhancement of the oral bioavailability of CoQ_{10} by complexation with γ-CD in healthy adults was published by Terao et al. (2006). The objective of this study was to compare the effect of the molecular encapsulation of CoQ_{10} by complexation with γ-CD (CoQ_{10}-γ-CD) with that of a mixture of CoQ_{10} and microcrystalline cellulose (CoQ_{10}-MCC) on absorption and bioavailability of CoQ_{10} in supplement form in healthy adults. After 6 and 8 hours mean CoQ_{10} plasma levels

in subjects given a single oral administration of the CoQ_{10} -γ-CD capsule were significantly than those found with the CoQ_{10}-MCC capsule. In addition, the mean plasma levels at 24 and 48 hours tended to be higher after CoQ_{10}-γ-CD administration. These results indicate that the oral absorption and bioavailability of CoQ_{10} in healthy adult volunteers could be significantly enhanced by complexation with γ-CD, and point to the potential use of γ-CD as formulation aid for orally administered CoQ_{10}.

Cuomo et al. (2000) also compared the bioavailability of CoQ_{10} in different formulations. The study was undertaken as part of a programme to develop a new CoQ_{10} formula providing high bioavailability of CoQuinone product, but without the synthetic solubilizers found in CoQuinone. Two new formulas were tested. The first, a dry tablet formula, contained CoQ_{10} complexed with CDs, while the second was an all-natural liquid formula based on lecithin, medium chain triglycerides and glycerine monooleate. The dry tablet formula with CDs did not provide high levels of bioavailability, but the new all-natural liquid formula did. For these reasons further studies are required into the bioavailability of CD/ CoQ_{10} complexes.

Recently, Higashi et al. (2009) prepared four kinds of complexes of CoQ_{10} with γ-CD using the kneading method and the solubility method with or without heating, and compared the resulting pharmaceutical properties. Differential scanning calorimetrical curves and powder X-ray diffraction patterns showed that the complexes formed pseudorotaxane-like supramolecular structures, although those included free γ-CD and CoQ_{10} when prepared without heating. Heating improved the complexation of CoQ_{10} with γ-CD in both methods. The dispersion rate of CoQ_{10} in water increased in the order of CoQ_{10} alone ~physical mixture with γ-CD < solubility/heating product < solubility product

< kneading/heating product < kneading product, possibly due to submicron-ordered particle formulation. Of the various ointments containing CoQ_{10} alone, the release of CoQ_{10} from the hydrophilic ointment was fastest, especially when heating was involved. The fast release of CoQ_{10} from hydrophilic ointment could be involved in propylene glycol in the ointment. These results suggest that supramolecular complexes of CoQ_{10} with γ-CD can be prepared by various methods, and among various complexes the pseudorotaxane-like CoQ_{10}0/γ-CD complexes prepared by the solubility method with heating show the best potential for preparing of ointments.

In 2008, Nishimura et al. confirmed that CoQ_{10} forms a pseudorotaxane-like supramolecular complex with γ-CD. The X-ray diffraction pattern of the CoQ_{10}/ γ-CD complex was different from that of the physical mixture, but almost the same as that of polypropylene glycol/γ-CD polypseudorotaxane. Also, a differential scanning calorimetric study and the FT-IR study confirmed the interaction between CoQ_{10} and γ-CD in the solid state. An 1H-NMR study and the yield study of the supramolecular complex of CoQ_{10} with γ-CD demonstrated that the stoichiometry was 5:1 (γ-CD : CoQ_{10}). The dispersion rate of CoQ_{10} was markedly increased by the formation of the supramolecular complex with γ-CD, possibly due to submicron-ordered particle formulation. In fact, CoQ_{10} was found to form submicron-sized supramolecular particles with γ-CD, when prepared by the solubility method. Consequently, the study showed that CoQ_{10} forms a pseudorotaxane-like supramolecular complex with γ-CD in water.

Milivojevic et al. (2009) recently reported the complexation of CoQ_{10} with β- and γ-CD in aqueous solutions in order to improve the water solubility, thermo- and photo-stability of CoQ_{10}. Complex formation resulted in an increase in water solubility at room

temperature and in the pH 6.5 by a factor at least 10^2. The solubility of CoQ_{10} in the presence of CDs linearly increased with temperature and pH. UV light (λ = 254) and temperature together had a great effect on CoQ_{10} stability. After 120 min of exposure at 80 ºC and UV light, about 72.3% of pure CoQ_{10} was degraded. Thermo- and photo-stability was strongly improved by complex formation; more than 64% of CoQ_{10} remaining unchanged.

A significant application of the CoQ_{10}/CD complexes is in the analysis field was described by Yang and Song (2006), who reported a novel polarographic method for the determination of CoQ_{10} in β-CD and iodinate system. These authors improved the stability of CoQ_{10} to light by forming an inclusion complex of CoQ_{10} with β-CD. In addition, the polarographic reductive wave of the inclusion complex was catalyzed by KIO_3 to produce an association/parallel catalytic wave, which was two orders of magnitude greater than the reductive wave of CoQ_{10} as regards analytical sensitivity. The proposed method is highly sensitive and allows CoQ_{10} to be determined under light, making it useful for the rapid analysis of CoQ_{10} in pharmaceutical preparation samples. In addition, it may meet the difficult requirements involved in biological samples. If complex biological samples are treated with the necessary separation and accumulation processes, the method could be applied to the determination of CoQ_{10} in more complex biological samples.

Finally, Uekaji et al. (2011) recently reported the enhancement of the stability and bioavailability of coenzyme CoQ_{10} oxidized form by γ-CD complexation. In a series of the studies, the authors investigated an easy and economical conversion of CoQ_{10} oxidized form to its reduced form in complex powder, using inexpensive vitamin C as the reductant.

CYCLODEXTRINS AND FATTY ACIDS

The complexation of fatty acids (both saturated and unsaturated by natural and modified CDs has been reported in numerous works. For example, the properties and applications of fatty acid/CDs were studied by Szente et al. (1993) in an interesting paper several years ago. Based on that paper, several researchers have focused their research on the encapsulation of these guest molecules. As regards the nature of encapsulation, the CD hydrophobic cavity can include, at least in part, fatty acid chains (Duchene et al., 2003). The exact nature of the CD and that of the fatty acid (chain length, double bonds) together, have a significant influence on the inclusion characteristics. Depending on the fatty acid chain length (C4–C18), one CD (or more) can interact and include the carboxylic chain. Duchene et al. (2003), in an excellent paper, that for both short (≤C8) and long (≥C12) chain fatty acids, the highest affinity is obtained with α-CD, which has the narrowest cavity (Gelb and Schwartz, 1989). In the case of intermediate chain fatty acids (≈C10), part of the chain is outside the β-CD cavity so these can better interact with α-CD than with β-CD at the same 1:1 molecular ratio (Szente et al., 1993). These results were confirmed by a study of the water solubility of fatty acids from C6 to C12 in the presence of α- or β-CD (Schlenk and Sand, 1961). As will be discussed below, the presence of double bonds has been investigated by different authors (Szente et al., 1993; Jyothirmayi et al., 1991; López-Nicolás et al. 1995). On the other hand the influence of fatty acid chain length was demonstrated by Schlenk and Sand (1961), Gelb and Schwartz (1989) and Shimada et al. (1992). The number of CDs capable of interacting with fatty acids increases as in the hydrocarbon chain increases in length. The formation of inclusion compounds occurs

through two types of interaction: (1) the creation of hydrogen bonds between the carboxyl of the fatty acid chain and the hydroxyls in position 6 on the CD; and (2) the creation of hydrophobic interactions between the fatty acid hydrocarbon chain and the CD cavity.

In a recent paper, Parker and Stalcup (2008) studied the complexation by β-CD of different fatty acids such as octanoate, 2-octenoate, decanoate, 9-decenoate and dodecanote, using affinity capillary electrophoresis and isothermal titration calorimetry.

As regards to the application of fatty acid CD complexes, Ajisaka et al. (2002) showed that the addition of medium-chain fatty acid (caprylic capric and lauric acid)/CD complexes to ruminant diets may be effective in reducing methane production.

Moreover, Greenberg-Ofrath et al. (1993) used CDs as carriers of cholesterol and fatty acids in the cultivation of mycoplasmas. The design of fully or partly defined media for mycoplasma cultivation needs the essential lipids, cholesterol and long-chain fatty acids to be provided in an assimilable and nontoxic form. Greenberg-Ofrath et al. (1993) introduced CDs as carriers of these lipids, thus providing alternatives to serum or bovine serum albumin. β-CD was found to inhibit the growth of the sterol-requiring *M. capricolum* in both serum and BSA media, but it stimulated the growth of the sterol-independent *A. laidlawii*. In sharp contrast to β-CD and DIMEB, HP-β-CD added at 5 and 10 mM to a basal medium supplemented with lipids permitted the growth of *M. capricolum.*

Linoleic acid and conjugated linoleic acid are the two polyunsaturated fatty acids most studied in connection with encapsulation with CDs.

Linoleic acid

Using nuclear magnetic resonance techniques, Jyothirmayi, et al. (1991) studied the complexation of linoleic and arachidonic acid by α- and β-CD. Moreover, the complexation of polyunsaturated fatty acids by several CDS was reported many years ago by our group. For example, López-Nicolás et al. (1995) and Bru et al. (1995) studied the equilibria of linoleic acid LA/CD complexes to investigate the behaviour of 'soluble lipids' in solution as a function of factors that typically affect biochemical processes, such as pH, temperature and CD structure. The above complexes were formed with a stoichiometry of 1:2 in solution. The first CD molecule interacts with linoléico acid and through hydrogen bonds when the pH is below the fatty acid pK_a; hydrophobic interactions may also play an important role at high pH. The second CD molecule makes only hydrophobic contact with the linoleic and hydrocarbon chain. The formation of hydrogen bonds was dependent on the inner diameter of the CD, whereas the strength of the hydrophobic interactions between CD and LA were related with the presence of hydrophobic groups in the CD. The first CD molecule interacts more strongly with linoleic and at increased temperatures. The quantitative description of the linoleic and-CD interaction allows absolute control of the effects of the lipid on biochemical processes.

Later, López-Nicolás et al. (1997) reported that the structural resemblance between CDs and starch in its helical conformation makes the former a suitable model system for studying the oxidation of polyunsaturated fatty acids such as linoleic acid, which naturally occur associated with amylose as inclusion complexes in several plant storage tissues. For the oxidation of linoleic and by lipoxygenase in the presence of β-CD, the authors proposed a model in which free linoleic is the only effective substrate; thus the oxidation of the complexed substrate required the previous dissociation of the complex. Consistently, β-CD

was shown to slow down the reaction rate of LOX oxidation, which was mainly due to the increases in K_m and V_{max} remaining unchanged. The apparent inhibition produced by β-CD (increased K_m) is due to the removal of effective substrate in the form of inclusion complexes. This "sequestered" substrate can, however, be converted since it is in equilibrium with the free form.

Moreover, the advantages of the presence of CDs in a reaction catalyzed by immobilized lipoxygenase were reported for the first time by Pérez-Gilabert and García-Carmona (2005). In this study the authors showed that the steady-state rate in the presence of β-CD was seven times higher than in control experiments using the same concentration of linoleic acid; furthermore, the percentage of substrate conversion (and product accumulation) in the presence of β-CD was higher than in the control assays. The operational stability of the immobilized enzyme increased in the presence of β-CD, while an increase in the percentage of the main reaction products was also observed.

Finally, Hadaruga et al. (2006) made a thermal stability study of linoleic acid/α- and β-CD complexes using bionanoparticles that were obtained by a solution method and were characterized by differential scanning calorimetry and transmission electron microscopy. The authors analyzed pure linoleic acid, the corresponding thermally (50–150 ºC) degraded raw linoleic acid samples and those recovered from the complexes by gas chromatography–mass spectrometry, after conversion to the methyl esters. The nanoparticles obtained in that work showed good yields of 88% and 74% for α- and β-CD complexes, respectively. The main degradation products (for the thermally degraded raw samples) were aldehydes, epoxy, dihydroxy derivatives, homologues, and isomers of linoleic acid. The same authors observed the good thermal stability of nanoparticles, especially for the linoleic acid/α-CD

complex, which contained a relative concentration of above 98% fatty acid in the case of temperature degradations of 50 and 100 ºC. However, a lower concentration of 92% was observed in the case of the linoleic acid/βCD complex while, for the temperature degradation of 150 ºC, the linoleic acid was partially converted to more stable geometrical isomers.

As regards the practical applications of the fatty acid/CD complexes, Regier (2007) showed that when in the form of a molecular inclusion compound with α-CD, linoleic acid is effectively protected against oxidation. Finally, investigations by this author into the storage and light stability, using olfactory tests and headspace analysis of the formulations, pointed to the stability of the suitable inclusion compound. Moreover, the reversible complexation makes it possible for the first time to use linoleic acid in various cosmetic formulations and personal-care products.

Conjugated linoleic acid

Conjugated linoleic acid (CLA) is a collective term describing a mixture of positional and geometrical isomers of linoleic acid including a conjugated double bond in various positions (Uehara et al., 2008). It is industrially manufactured by alkali-induced conjugation of linoleic acid-rich oils such as sunflower oil, in the presence of propylene glycol. After conjugation, a mixture consisting of almost equivalent amounts of the isomers, 9-*cis*, 11-*trans*-CLA (c9t11) and 10-*trans*, 12-*cis*-CLA (t10c12), which have two double bonds (*cis* and *trans*) at different positions of the aliphatic chains, is obtained. The two CLAs exhibit various biochemical properties, including a reduction of cancer incidence, beneficial effects in atherosclerosis, decreased body fat content and improved

immune functions. It has been reported that the biochemical properties of c9t11 and t10c12 differ in that the c9t11 isomer exhibits anti-tumor activity, whereas the t10c12 isomer decreases body fat, increases energy expenditure, and suppresses the development of hypertension (Uehara et al., 2008).

As indicated previously, free fatty acids and their derivatives are compounds which can be complexed in the hydrophobic inner cavity of CDs (Jyothirmayi et al., 1991). Thus, polyunsaturated fatty acids encapsulated in several types of CDs have been shown to be completely protected against oxidation even in pure oxygen (Reichenbach and Min, 1997). For this reason, Kim et al. (2000) expected that CLA could be protected against oxidation by microencapsulation in CDs and investigated for first time the oxidative stability of CLA microencapsulated in (α-, β-, γ)-CDs at various mole ratios, when reacted at 35 °C. These researchers studied the process of complexation by measuring headspace-oxygen depletion in air-tight serum bottles and by measuring the peroxide values. The rate of oxygen depletion determined by Kim et al. (2000) was reduced from 41.0 (control) to 21.5, 2.1, 1.2, and 1.1 μmol/L h^{-1} by CLA/ α-CD microencapsules at 1:1, 1:2, 1:4, and 1:6 mole ratios, respectively, indicating that CLA oxidation was completely protected by a 1:4 mole ratio of CLA/ α-CD. Such a protective effect by CLA/β-CD or CLA/γ-CD microencapsules was achieved at a 1:6 mole ratio, but the effect by CLA/β-CD was slightly greater than that of CLA/γ-CD. The protective effect of α-, β-, γ-CDs for CLA oxidation was confirmed by their POV-reducing abilities in CLA/CDs. The results suggested that α-CD was the most effective for the protection of CLA oxidation by microencapsulation, followed by β-CD and γ-CD.

However, In a later and similar work, Park et al. (2002) characterized the inclusion complex of CLA with natural CDs with opposite results.. These authors prepared the complexes to determine the mole ratio of CLA complexed with CDs and the oxidative stability of CLA in the CLA/CDs inclusion complexes. When measured by GC, NMR, and T_1 value analyses, 1 mole of CLA was complexed with 5 mol of α-CD, 4 mol of β-CD, and 2 mol of γ-CD. The results presented by these authors showed that the oxidation of CLA at 35 °C for 80 h was completely prevented by the formation of CLA/CDs inclusion complexes.

To date, the most important application of the complexes between CLA and CDs is that reported by Liu et al. (2005) in a study on the separation of CLA isomers by CD-modified micellar electrokinetic chromatography. Analytical methods for the determination of the composition of CLA isomers are important for both research and routine inspection purposes. Gas chromatography is one of the methods used to analyze CLA isomers, but it produces overlapping peaks of some CLA isomers, such as 9*cis*,*11trans* and *8trans*,*10cis* and their geometric isomers (*cis,cis* and *trans,trans*) and a retention time of more than 50 min is needed with this method. Furthermore, fatty acids needed to be methylated with the GC method. Another method, Ag^+-HPLC, allowed well-resolved separation of three groups of geometric isomers (*trans,trans, cis/trans*, and *cis,cis*) of a commercial CLA mixture, and each group could be further separated into positional isomers, such as 11,13-CLA; 10,12-CLA; 9,11-CLA; and 8,10-CLA. However, the four peaks of each group partially overlapped and were separated using three columns in series, while the retention time was prolonged up to 60 min. To resolve these problems, Liu et al. (2005) used the complexes between CLA and CDs. In this paper, a CD-modified micellar electrokinetic

chromatography (CD-MEKC) method was developed to separate the CLA isomers. All seven CLA isomers (9*cis*,11*cis*-CLA, 9*cis*,11*trans*-CLA, 9*trans*,11*trans*-CLA, 10*trans*,12*cis*-CLA, 11*cis*,13*cis*-CLA, 11*cis*,13*trans*-CLA and 11*trans*,13*trans*-CLA) were completely separated in the optimized conditions (4% (w/v) β-CD, 54mM sodium dodecyl sulphate (SDS), 80mMborate (pH 9.0), 8M urea, 4% (v/v) ethanol, 30 kV and 15 ºC). The CD-MEKC method reported was better than to the gas chromatographic and silver-ion high-performance liquid chromatographic methods generally used in analyzing CLA isomers.

Recently, Yang et al. (2009 and 2010) compared the formation of inclusion complexes between β-CD/CLA and amylose/CLA in different papers. In the first work (2009) a delivery system for bioactive CLA through a self-assembled amylase/CLA complex was investigated are compared with a β-CD/CLA complex. The results show the amylose-CLA complex offers better antioxidative protection against on CLA than β-CD/CLA complex, supporting the strong complexing interaction between CLA and amylose demonstrated by thermogravimetric analysis. Moreover, in a second paper (Yang et al., 2010), these authors confirmed the formation of an amylase/CLA/βCD three-component complex. Therefore, β-CD can be used to manipulate the crystallization process of amylose to modulate food product quality, and the amylose-β-CD complex could also be applied to improve the delivery efficiency of CLA and other bioactive compounds.

CYCLODEXTRINS AS ANTIOXIDANT MOLECULES

CDs as inhibitors of food browning

Color is a sensory property with a strong influence on food acceptance as it contributes decisively to the initial perception of a food's condition, ripeness, degree of processing, and other characteristics**.** One of the main factors that can alter the color of food and so limit its commercial shelf life is browning since the organoleptic and nutritional properties of foods may be strongly altered if this undesirable reaction is not controlled. Therefore, the control of the browning during the processing stages of food has always been a challenge for food researchers (Sapers et al., 2001; Walker, 1977; Sapers, 1993)**.** The degree of browning depends on the presence of oxygen, reducing substances, metallic ions, pH, temperature, and the activity of different oxidizing enzymes. One of the main factors that must be controlled is the enzymatic activity of PPO (monophenol dihydroxyphenylalanine: oxygen oxidoreductases, EC 1.14.18.1) (Sánchez-Ferrer et al., 1995). The presence of this enzyme in different fruits has been reported by several authors in the past decade, and much research has focused on the use of postharvest chemical treatments to avoid enzymatic browning (Sapers and Miller, 1988). However, many of these treatments present serious disadvantages for use in the food industry because they can have negative effects on the sensorial properties of the products (Abreu et al., 2003). Moreover, some chemical treatments have been associated with severe allergy-like reactions in certain populations, for which reason the Food and Drug Administration has restricted their use to only a few applications to inhibit the browning of foods (Sapers, 1993). Therefore, alternative methodologies are being investigated to extend the shelf life of foods, among them fresh juice fruit.

Recently, the use of CDs has been proposed by several authors for the control of enzymatic browning in different fruits, acting as *"primary antioxidants"*. To this end the effectiveness of CDs as browning inhibitors was determined as the difference between the colours observed in the CD-treated sample and the controls, using the colour space CIE-L*, a*, b* system. Different types of CDs (natural and modified) have been used to study the evolution of the color parameters of different fruit juices such as pear (López-Nicolás and García-Carmona, 2007), peach (López-Nicolás et al., 2007a), apple (López-Nicolás et al., 2007b) and grape (Nuñez-Delicado et al., 2005). In all cases, both the scalar (L*, a*, and b*) and the angular coordinates (H* and C*) were evaluated to define the color of fruits juice completely in the absence and presence of each type of CDs. To evaluate the behaviour of pear, peach, apple and grape juice enzymatic browning after the addition of CDs, increasing concentrations of different CDs were used. The evolution of the space CIE-L*, a*, b* parameters, reflecting inhibition of the darkening of the fruit juices with time shows that CDs are able of complexing PPO substrates, thereby preventing their oxidation to quinones and subsequent polymerization to brown pigments. Moreover, kinetic models to evaluate fruit juice enzymatic browning in the absence and presence of CDs have been proposed. The different K_F values between the mixtures of diphenols present in fruits juice and different CDs were calculated.

As mentioned above, the use of CDs as antibrowning agents in fruit juices has received growing attention. However, there has been no detailed study of the behaviour of these molecules as substances, which can also lead to the darkening of foods. For this reason the role of CDs as activators and inhibitors of latent banana pulp PPO was studied by Sojo et al. (1999). In this work, the effect of CDs on *o*-diphenol oxidation catalyzed by

banana PPO was studied. The oxidation of dopamine, the natural substrate of banana, in the presence of CDs was unaffected, because this hydrophilic phenol does not form inclusion complexes with CDs. However, when a hydrophobic phenol such as *tert*-butylcatechol (TBC) was used, a marked inhibition was observed with β-, HP-β-CD, and maltosyl-*β*-CDs. This inhibition was due to the complexation of TBC in the CD core, demonstrating that banana pulp PPO worked only toward free substrate and not toward the complex TBC/CDs. In addition, the effect of some inhibitors in the presence of CDs and dopamine as substrate was studied. Increasing concentrations of CDs, in the presence of two inhibitors (4-iodophenol and cinnamic acid) were able to activate the inhibited enzyme to reach the noninhibited level by complexing the inhibitors in the hydrophobic core of the CDs. This dual effect of CDs as activator and inhibitor was tested in crude banana pulp extracts, with surprising activation effects never before described being observed. To confirm these data, a kinetic study of the activation of banana juice enzymatic browning by the addition of maltosyl-β-CD was reported by López-Nicolás et al. (2007c). In this paper, when the colour of fresh banana juice was evaluated in the presence of different CDs, the evolution of several color parameters was the opposite of that observed in other fruit juices. Moreover, a kinetic model based on the complexation by CDs of the natural browning inhibitors present in banana was developed for the first time to clarify the enzymatic browning activation of banana juice. Finally, the apparent K_F values between the natural PPO inhibitors present in banana juice and maltosyl-β-CD were calculated. The results presented in this paper show that any antioxidant agent used to avoid enzymatic browning must be tested individually for each food because an opposite effect to that desired may be produced.

Knowledge of the kinetic models of the enzymatic processes occurring in foods is essential to understand their macroscopic behaviour, as in the case of organoleptic properties, such as color. The surprising observation that CDs chosen as natural antibrowning agents of fruit juice may behave as pro-browning agents, depending on the fruit source, is explained in this investigation through a biochemical model reflecting the interaction between natural compounds present in banana juice and CDs. Therefore, the presence of hydrophobic or hydrophilic phenols in the fruit structure and the inability of CDs to complex dopamine mean that a recognized antibrowning agent, such as CDs, can be converted into a browning agent, leading to changes in color parameters not previously suspected. In Fig. 4 we can observe that the negative values of the enzymatic browning percent variation of pear, peach, grape, and apple juices indicate that the addition of 90 mM maltosyl-β-CD as a browning inhibitor is effective to the extent calculated. However, the positive percent variation value obtained for banana juice indicates the activation of the enzymatic browning by the addition of maltosyl-β-CD.

To resume, the main phenolic compounds present in these fruit juices that can be oxidized by PPO are shown in Table 1. As can be seen in this table, in the case of apple, grape, pear, and peach juices the phenolic compounds are of a hydrophobic nature and have been reported as guest molecules for different CDs. However, the main natural phenol present in banana is dopamine, a hydrophilic compound that is not complex by CDs.

CDs as "secondary antioxidants"

In addition to their role as antibrowning agents in complexing the substrates of PPO and preventing the oxidation of the phenols present in fruit juices, other authors (López-Nicolás et al., 2007a; López-Nicolás and García-Carmona 2007) have reported the use of CDs as secondary antioxidants to improve the color of fresh juices. As is known, the synthetic antioxidants, metabisulfite and L-cysteine, have a higher antibrowning effect on fruits juice than those from natural sources, such as ascorbic acid (AA) or CDs. However, there is a growing interest in natural antioxidants for use in food, although another strategy would be to look for "preservers" of the natural antioxidant capacity of a particular food. In this approach, the development and use of new natural "*secondary antioxidants*" is a fresh and challenging task, as demonstrated by the oxidation of phenols by lipoxygenase (Nuñez-Delicado et al., 1997), in which CDs act as secondary antioxidants in synergism with AA, and seem to act as a *''secondary antioxidants"*, reducing fruits juice browning and enhancing the naturally occurring antioxidant capacity of the food. Thus, CDs can enhance the ability of AA to prevent enzymatic browning became of the protective effect provided by the CDs against AA oxidation.

This capacity of CDs to function as a secondary antioxidant in juices was evaluated and a kinetic model was proposed (López-Nicolás et al., 2007a; López-Nicolás and García-Carmona 2007). AA is the best known chemical agent for reducing the browning reaction. However, once the added AA has been completely oxidized to DHAA, *orto*-Quinones (*o*-Qs) accumulate and suffer browning. More stable forms of AA derivatives, such as erythorbic acid, 2- and 3-phosphate derivatives, phosphinate esters and ascorbyl- 6-fatty acid esters, have been developed to overcome these problems, but the results have not been very satisfactory. In the model proposed by us, CDs can enhance the ability of AA to

prevent enzymatic browning due to the protection that AA is offered against oxidation by *o*-Qs (Fig. 5). In the absence of CDs, the total concentration of PPO substrate is available to be oxidized to *o*-Qs by PPO in the presence of O_2. However, in the presence of CDs, AA is protected, due to the complexation of PPO substrates in the hydrophobic cavity of CDs. CDs slow down the production of *o*-Qs and, hence, the oxidation of AA, because only free substrates, in equilibrium with CD-bound phenol (CD-S), are oxidized by PPO in the presence of O_2 to *o*-Qs. In this way, the reaction is slowed down and the shelf life of the food is prolonged.

To confirm this hypothesis, we show an example of the evolution of pear juice color in the absence and presence of 2.28 mM AA and 90 mM maltosyl-*β*-CD, which was published in an interesting work by López-Nicolás and García-Carmona 2007 (Fig. 6A). Pear juice lightness (*L**) fell significantly when 90 mM maltosyl-*β*-CD was added to the medium. Moreover, when 2.28 mM AA was added, the rapid decay in *L** observed in the absence of any reagent was drastically reduced in the first 30 min. After which, *L** quickly decayed to reach the same values as observed in the absence of any agent. Furthermore, in Fig. 6A, we can see that when both 2.28 mM AA and 90 mM maltosyl-*β*-CD were added simultaneously to pear juice, the decrease in lightness (*L**) was less than in the absence of either of them individually. Moreover, in the presence of both agents, the pronounced decay observed in *L** values after the first 30 min was eliminated and the initial lightness of pear juice was almost totally maintained. This behavior is probably due to the preservation of the antioxidant capacity of AA by maltosyl-β-CD. To corroborate these results, the evolution of total color difference (A*E**) in the presence and absence of these enzymatic browning inhibitors was tested. As shown in Fig. 6B, the presence of AA in the medium

reduced the change in this parameter more effectively than maltosyl-*β*-CD but only during the first 30 min of the reaction time. After this time, the total color difference increased sharply to reach the maximum variation observed in the absence of any regent. However, in the presence of 2.28 mM AA plus 90 mM maltosyl-*β*-CD, the changes in A*E** were lower than when these reagents were added independently. Moreover, the inhibition rate of A*E** was maintained after the first 30 min. These results for *L** and A*E** confirm the hypothesis that the hydrophilic nature of AA precludes its inclusion in maltosyl-β-CD, and so the apparent CD-mediated protection of AA in pear juice is probably due to the complexation of the mixture of phenols present in pear juice into the hydrophobic cavity of the maltosyl-*β* -CD.

Although CDs are widely used as browning inhibitors in different fruit juices, the effect of the addition of CDs on others organoleptic properties, e.g. odor and aroma, has been reported in few works. Recently, some studies concerning the addition of CDs on the flavor profile of pear fruit juices have been published (Andreu-Sevilla et al. 2011, López-Nicolás et al., 2009d). Moreover, correlation of the results show in these papers, concerning the color and aroma of pear juice in the presence of CDs, with the consumer preferences has been also reported. Different descriptive sensory analysis of pear juices in both the presence and absence of CDs were carried out and odor/aroma attributes (fresh, fruity, pear-like, unnatural, etc.), plus global color, odor, aroma and quality, were quantified using a trained panel of judges. The addition of α-CD at 90 mM resulted in pear juices with the best color but with low aromatic intensity and low sensory quality. On the other hand, the addition of α-CD at 15 mM led to a pear juice also with an acceptable color but at the same

time with a high intensity of fruity and pear-like odors/aromas, making it the best appreciated juice by the panel.

CONCLUSIONS

In spite of the sharp increase in recent years of research into the encapsulation of lipophilic antioxidants by cyclodextrins, several problems still remain about the host/guest interaction and contradictory reports have been published. Although the positive effect of cyclodextrins on the solubility, stability and protection against prooxidant agents such as light, heat or oxidative enzymes have been demonstrated, the influence of encapsulation on such important factors as K_F values, the bioavailability of the guest molecule and the antioxidant capacity of the complexed compound is still not clearly defined and conflicting data have been reported by different authors for the same CD/antioxidant complex. This review shows that two principal factors are related with these problems. Firstly, several authors compare the K_F values determined under different physico-chemical conditions leading to erroneous conclusions. Indeed, factors such as pH of the medium or antioxidant pK_a play a fundamental role in encapsulation and are often not taken account. Secondly, although the analytical techniques to evaluate the complexes formed are well defined, the different assays used to measure the antioxidant capacity of the lipohilic antioxidant compounds may produce contradictory results. Thus, the antioxidant capacity of a host/guest complex cannot be determined indistinctly by techniques such as FRAP, ABTS, DPPH or ORAC because each of them measures different properties of the complex. This fact may explain why, for the same type of CD and lipohilic antioxidant, some researchers have reported an increase in the antioxidant capacity of the complex, while others have observed a decrease in the same property. Yet other authors have found that encapsulation of the antioxidant compound has no effect on its antioxidant activity. Finally, it should be clearly stated that while it is important to publish new studies that demonstrate the

interaction between others lipophilic antioxidant compounds and cyclodextrins, it is even more necessary to look for practical applications of the complexes that have already been reported.

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TABLES

Table 1. Effect of adding CDs on the browning of several fruit juices and the complexation of their main polyphenolic compounds (Adapted from López-Nicolás et al., 2007c).

FIGURE LEGENDS

Figure 1. Chemical structure of a cyclodextrin.

Figure 2. Structures of the natural cyclodextrins α, β and γ.

Figure 3. Structure of oxyresveratrol (A); resveratrol (B); pinosylvin (C) and pterostilbene (D).

Figure 4. Percent variation of enzymatic browning in several juices in the presence of 90 mM maltosyl-β-CD. Each data point is the mean of 3 replicates. (Adapted from López-Nicolás et al., 2007c).

Figure 5. Use of maltosyl-β-CD as secondary antioxidant on the browning of pear juice. PPO: polyphenol oxidase, CD: cyclodextrin, S: free PPO substrate, CD-S: complex between PPO substrates and CD, o-Q: orthoquinone, AA: ascorbic acid, DHAA: dehydroascorbic acid. (Adapted from López-Nicolás and García-Carmona, 2007).

Figure 6 Evolution of lightness (L*) (A), total color difference (ΔE*) (B) and Hue angle (H*) (C) in the absence of any agent (●) and in the presence of 2.28 mM AA (▲), 90 mM maltosyl-β-CD (■) and 90 mM maltosyl-β-CD plus 2.28 mM AA (♦). (Adapted from López-Nicolás and García-Carmona, 2007).

TABLE 1

Fruit juice	***Principal polyphenolic compounds***	***PPO substrates***	***Complexation by CDs***	***Effect of CD on browning***
Pear, Apple, Peach, Grape	Chlorogenic acid	+	+	Inhibitor
	Caffeic acid	+	+	
	Catechin	+	+	
	Epicatechin	+	+	
	Quercetin	+	+	
Banana	Dopamine	+	-	Activator

FIGURE 1

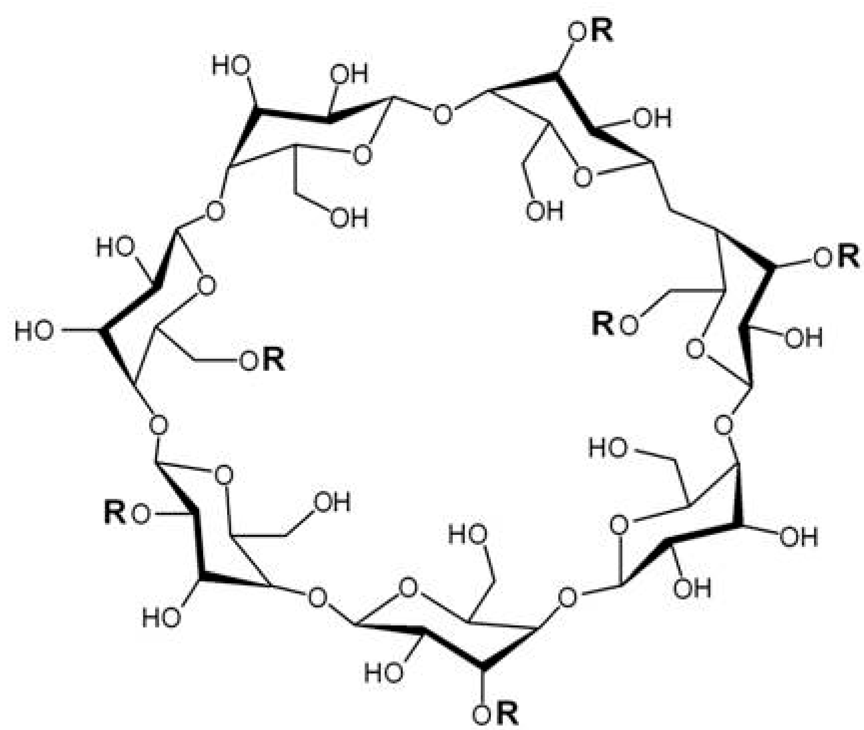


FIGURE 2

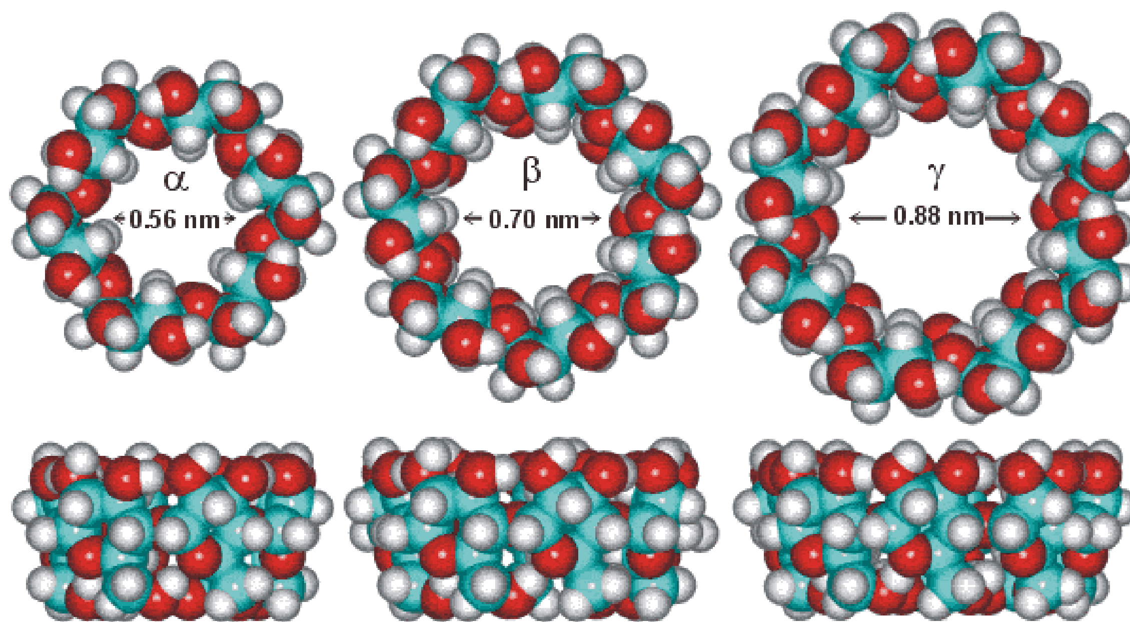


FIGURE 3

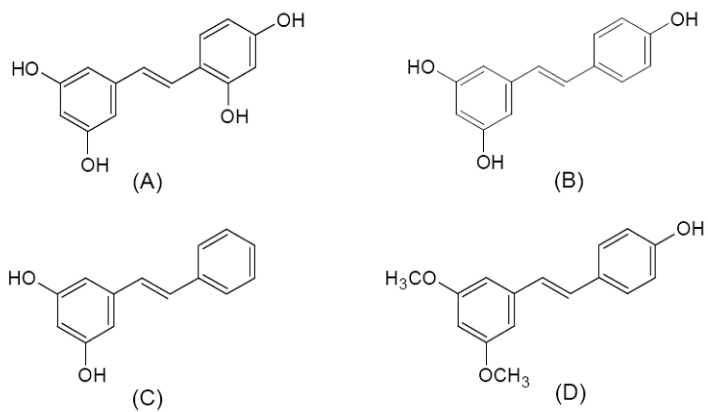


FIGURE 4

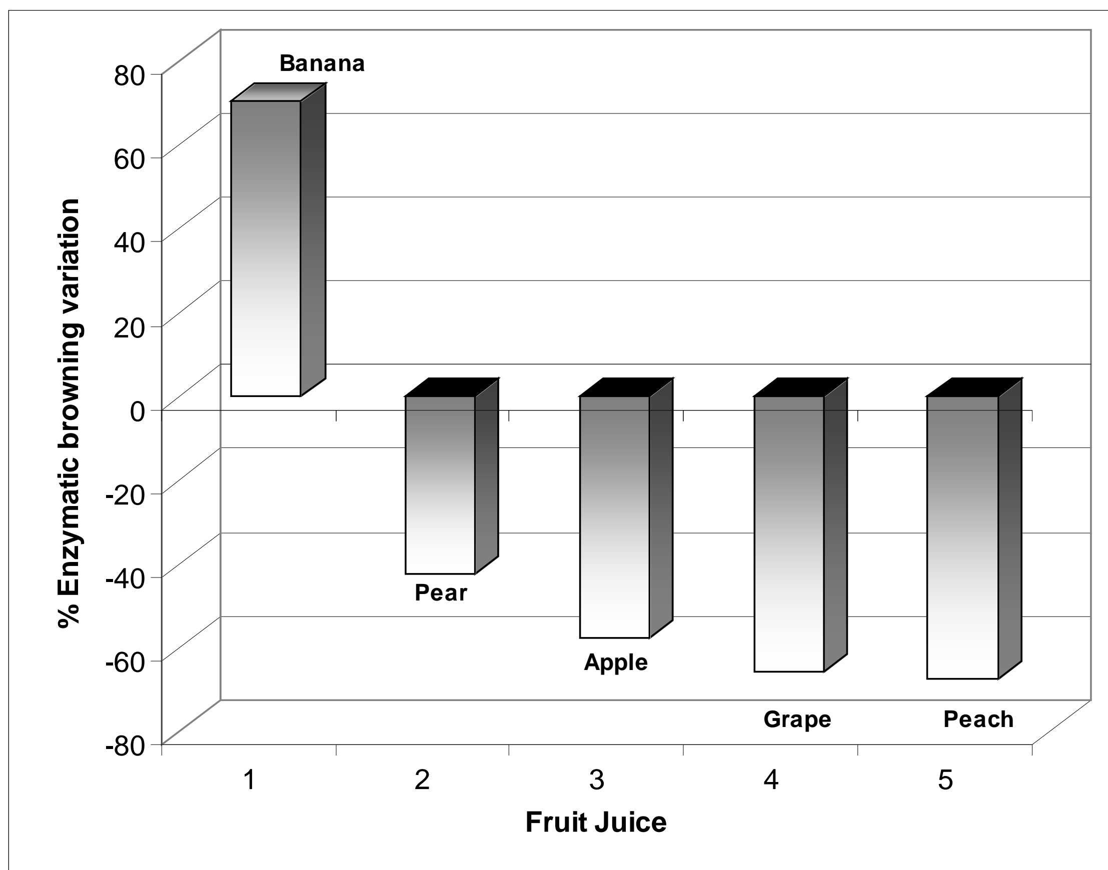


FIGURE 5

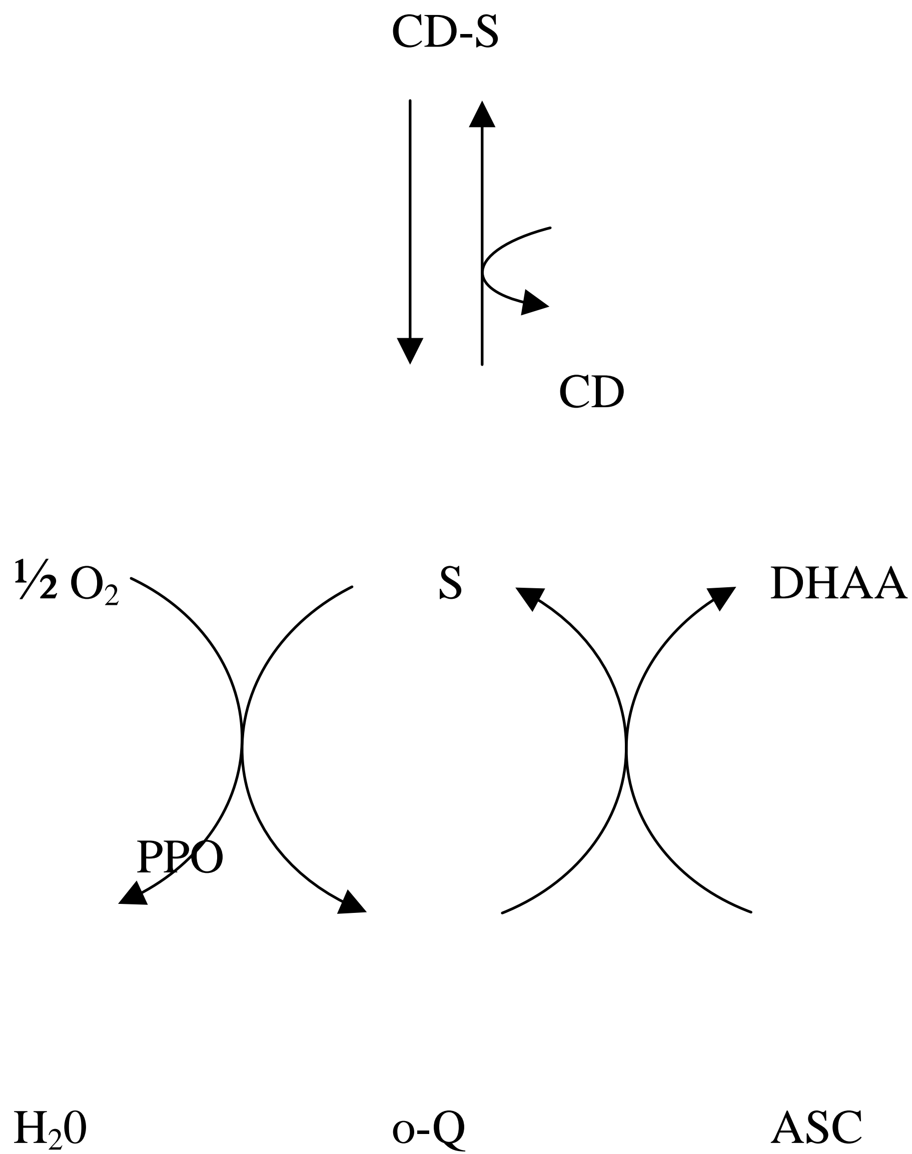
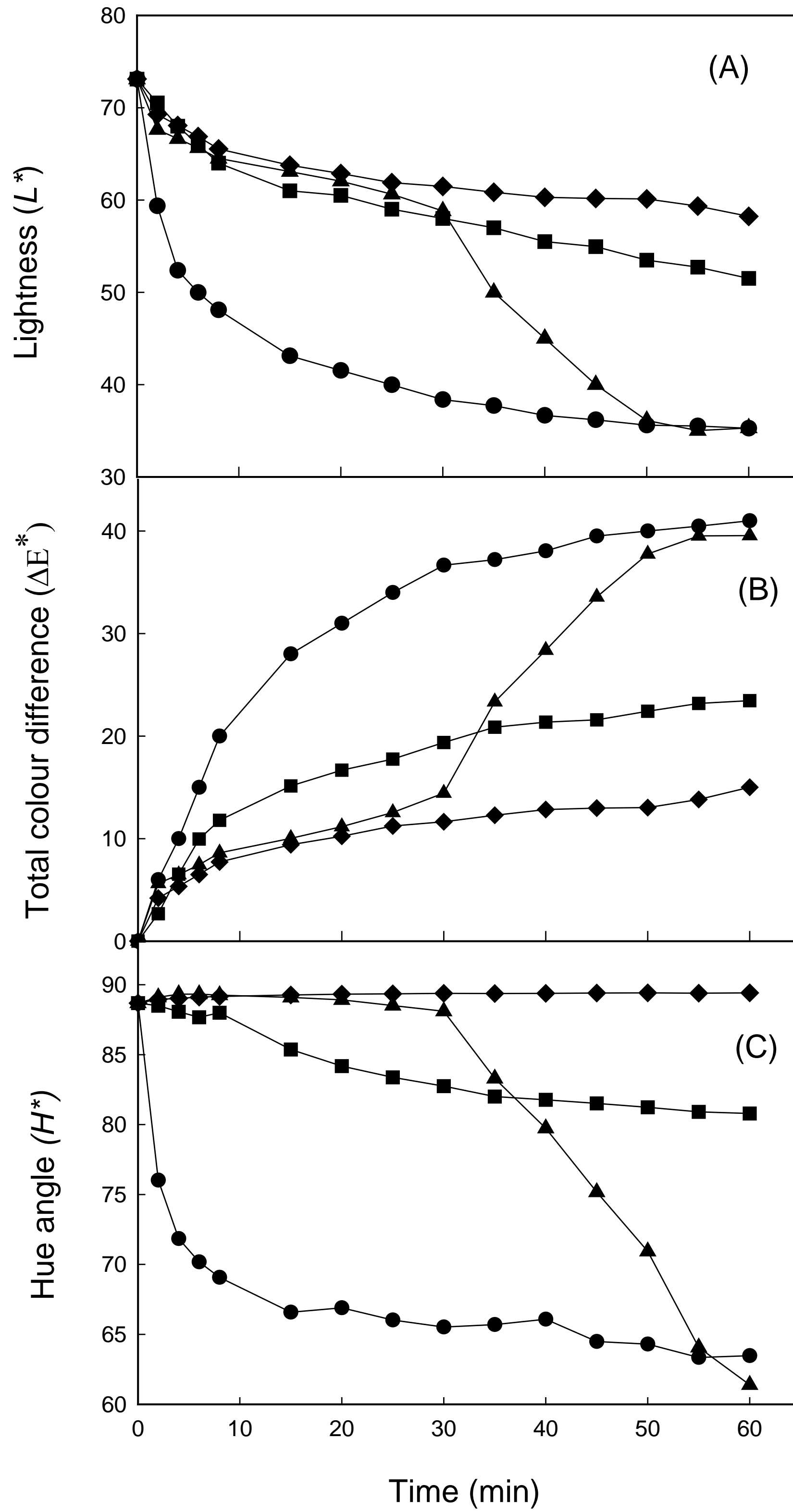


FIGURE 6



CAPÍTULO IV

Estudio físico-químico de la complejación de pterostilbeno con ciclodextrinas naturales y modificadas

ABSTRACT

In this paper, the interaction between pterostilbene and cyclodextrins (CDs) is described for the first time using steady state fluorescence. It was seen that pterostilbene forms a 1:1 complex with all the natural (α-,β- and γ-CD) and modified (HP-β-CD, methyl-β-CD and ethyl-β-CD) CDs tested. Among natural CDs, the interaction of pterostilbene with β-CD was the most efficient. However, all the modified CDs showed higher complexation constants (K_F) than β-CD. The highest K_F was found for HP-β-CD (17520 ± 981 M^{-1}), in which its value showed a strong dependence on pH in the region where the pterostilbene begins the deprotonation of its hydroxyl group. Moreover, the values of K_F decreased as the system temperature increased. To obtain information on the mechanism of pterostilbene affinity for CD, the thermodynamic parameters of the complexation (ΔH^o, ΔS^o and ΔG^o) were studied. Finally, a comparison of the K_F values obtained for three types of stilbenes revealed that both the stoichiometry and the K_F values of the complex are dependent on the structure of the guest molecule. While the *trans*-resveratrol-HP-β-CD and pterostilbene-HP-β-CD complexes showed a 1:1 (stoichiometry with a higher K_F value for the *trans*-resveratrol-HP-β-CD complexes), *trans*-stilbene showed a 1:2 stoichiometry.

INTRODUCTION

Pterostilbene (*trans*-3,5-dimethoxy-4′-hydroxystilbene) (**Scheme 1**) is a naturally occurring phytoalexin which has been identified in several plant species. It belongs to a group of phenolic compounds known as stilbenes, and is found in different sources such as the heartwood of sandalwood (*Pterocarpus santalinus*) *(1)* and *Pterocarpus marsupium (2)*. It was also identified in the leaves of *Vitis vinifera (3)*, in infected grape berries of *var*. Chardonnay and Gamay *(4)*, and in healthy and immature berries of *var*. Pinot Noir and Gamay *(5)*. Pterostilbene has also been found in the berries of some *Vacciunium* species *(5)*, while Paul et al. *(6)* reported high levels in darakchasava, a medicinal drink made primarily from dried grape berries used to treat cardiovascular and other ailments. Finally, pterostilbene also appears to be a constituent of the bark of *Guibourtia tessmanii*, a tree found in central Africa which is commonly used in folk medicine *(7)*.

In recent years, the perceived health benefits related with pterostilbene have led to a significant increase in its consumption in a variety of food products contains concentrations of this type of stilbene such as blueberries and it related products and some types of grapes and wines. It has also been included in medicaments. Among its pharmacological properties are a wide range of biological activities such as antihyperglycemic *(8)*, antioxidative *(9-11)*, anticancer *(9-14)*, antiinflammatory *(11),* anticholesterol *(15,16)*, antifungus *(17,18)*, hypolipidemic *(11)* or analgesic *(11)*. Moreover, Pan et al. *(12),* using transcript profiling, recently identified the cellular pathways targeted by pterostilbene. The observed response in lipid metabolism-related genes is consistent with its known hypolipidemic properties, and the induction of mitochondrial genes is consistent with its demonstrated role in

apoptosis in human cancer cell lines. Furthermore, their data show that pterostilbene has a significant effect on methionine metabolism.

However, problems concerning the physico-chemical properties of pterostilbene have meant that no "*novel food*" has been fortified with this antioxidant. Indeed, pterostilbene shows very poor solubility in water, (although it is more soluble in ethanol and other organic solvents), possesses low bioavailability and is easily oxidized by several enzymes such as laccase *(20,21)*. For these reasons, the complexation of pterostilbene with types of molecules which can increases its bioavailability, solubility and stability in the face of prooxidant agents is strongly desirable, as it is in the case of cyclodextrins (CDs).

CDs are torus-shaped oligosaccharides made up of α-(1,4) linked glucose units. The most common CDs are α, β and γ-CD, which contain six, seven and eight glucose units, respectively *(22,23)*. The cavity is carpeted by hydrogen atoms and so has a rather hydrophobic nature, unlike the outer surface of the molecule, in which the primary and secondary hydroxyl groups are exposed to the solvent, thus making the whole molecule highly water-soluble *(22,23)*. Poorly water-soluble compounds and hydrophobic moieties of amphiphilic molecules interact non-covalently with the CD cavity to form the so-called inclusion complexes, which are also highly water-soluble *(24-26)*. However, the solubility of these complexes depends of several factors such as the type of CD used *(24-26)*. Because CDs are able to increase the bioavailability of different compounds and to protect different molecules against the action of external agents, their use in both the pharmaceutical and food industries is increasing *(22,23)*.

To date, although CDs have been used to complex another type of stilbene such as *trans*-resveratrol *(27,28)*, the effect of CDs on pterostilbene has not been published in any paper. Indeed, this is the first work where the complexation between CD and this potent antioxidant is reported. Knowledge of the stoichiometric coefficients and of the complexation constants (K_F) of the CD complexes is essential if this stilbene is to be used in both the pharmaceutical and food industries.

Bearing the above in mind, the main objective of this work was to analyze the complexation mechanism of pterostilbene with different types of natural (α-, β- and γ-CD) and modified CDs (HP-β-CD, methyl-β-CD and ethyl-β-CD) under various experimental conditions of temperature and pH. The stoichiometry, K_F values and thermodynamic parameters for the pterostilbene-CD complexes are evaluated. Finally, the effect of the structure of the stilbene on both the stoichiometry and the K_F values are discussed using other molecules of this family such as *trans*-resveratrol and *trans*-stilbene.

To perform the study, a method which makes use of changes in fluorescence spectroscopic properties of pterostilbene in the presence of CDs, was used.

MATERIALS AND METHODS

Materials

Natural (α-, β- and γ-CD) and modified CDs (HP-β-CD, methyl-β-CD and ethyl-β-CD), *trans*-stilbene and *trans*-resveratrol were purchased from Sigma-Aldrich (Madrid, Spain) and used as received. Pterostilbene was from Sequoia Research Products Limited (Pangbourne, United Kingdom) and was used without further purification. Some stilbenes may be sensitive to the light and irradiation of solutions containing the analyte induces the formation of other molecules, which leads to the formation of a highly fluorescent compound. Because of this, the samples were stored in darkness.

Equipment and Experimental Procedure

Fluorescence studies. Fluorescence intensity was measured in a Kontron SFM-25 spectrofluorimeter equipped (Zurich, Switzerland) with thermostatically controlled cells and with a xenon lamp source and quartz cell, which were used to perform all fluorescence measurements. Excitation and emission bandwidths were both set at 2 nm. The excitation wavelengths for pterolstilbene, *trans*-resveratrol and *trans*-stilbene were 330nm, 334nm and 250nm, respectively. The emission wavelengths for pterostilbene, *trans*-resveratrol and *trans*-stilbene were 374nm, 385nm and 374nm, respectively. The relative fluorescence intensity values were recorded at 25 ºC. To avoid inner filter effects, 2-mm quartz cells were used.

Temperature studies. The K_F values were determined at the following temperatures: 15ºC, 25º C, 30º C and 37º C**,** using a Thermomixer Comfort (Eppendorf Ibérica, Madrid, Spain)

to control the temperature. The thermodynamic parameters ΔH^o, ΔS^o and ΔG^o can be calculated using the following thermodynamic relationship equation:

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

where K_F is the complexation constant of the inclusion complex, T is the temperature, R is the gas constant and ΔH^o and ΔS^o are standard enthalpy and entropy changes of complex formation in the mobile phase. For a linear plot of ln K_F versus 1/T, the slope and intercept are $-\Delta H^o/R$ and $\Delta S^o/R$ respectively. The Gibbs free energy change for the interactions that take place during the inclusion process may be found by the following equation:

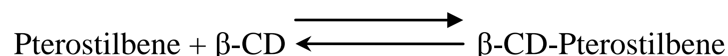
$$\Delta G^o = \Delta H^o - T\Delta S^o \quad \textit{(Equation 2)}$$

RESULTS AND DISCUSSION

Study of the complexation of pterostilbene by cyclodextrins. Stoichiometry of the complexes and determination of the complexation constants.

To date, no paper has reported the possible interaction between pterostilbene and any type of CD. For this reason we have selected β-CD, the most widely used natural CD, to evaluate the hypothetical interaction between this potent antioxidant and CDs. In order to quantify the interaction between pterostilbene and β-CD, the K_F was determined using, as analysis technique, the steady state fluorescence which takes into account the changes in the physico-chemical state of this antioxidant with the concentration and following the Benesi-Hildebrand method *(29)*.

Assuming that the composition of the complex was 1:1, the following expression can be written:



Pterostilbene + β-CD ⇄ β-CD-Pterostilbene

The complexation constant, K_F is given by:

$$K_F = \frac{[B\text{ - }CD/Pterostilbene]}{[Pterostilbene][B\text{ - }CD]} \qquad \textit{(Equation 3)}$$

where [β-CD], [Pterostilbene] and [β-CD/Pterolstilbene] are equilibrium concentrations.

From the experimental data of **Figure 1**, the difference in the intensity of the emission fluorescence of pterostilbene in the absence and presence of different amounts of

β-CD was plotted versus the β-CD concentration (**Figure 1** *inset*). A representative plot of the variation in fluorescence intensity at the wavelength band used as a function of β-CD concentration was analyzed by the Benesi-Hildebrand method.

The K_F value for the inclusion complex can be determined by typical double reciprocal (or Benesi–Hildebrand) plots:

$$\frac{1}{F - F_o} = \frac{1}{(F_\alpha - F_o)K_F[B - CD]} + \frac{1}{F_\alpha - F_o} \qquad \textit{(Equation 4)}$$

where [β-CD] denotes the β-CD concentration; F_0 the fluorescence intensity of pterostilbene in the absence of β-CD; F_∞ the fluorescence intensity when all of the pterostilbene molecules are essentially complexed with β-CD; and F the observed fluorescence intensity at each β-CD concentration tested.

In our study, a plot of $1/F - F_0$ vs. 1/[β-CD] gave a straight line with a linear correlation higher than 0.99, indicating that the presumed stoichiometry of the β-CD/pterostilbene complexes formed was 1:1 (**Figure 1** *inset*, filled circles).

The plot of $1/F\text{-}F_0$ as a function of $1/[\beta\text{-CD}]^2$ was also analyzed because it was thought it might provide information about the presence of higher order complexes, especially at higher β-CD concentrations. Assuming the stoichiometry of the inclusion complex to be 1:2, the following expression is obtained *(30)*:

$$\frac{1}{F - F_o} = \frac{1}{(F_\alpha - F_o)K_{F12}([\mathrm{B} - CD])^2} + \frac{1}{F_\alpha - F_o} \qquad \textit{(Equation 5)}$$

However, none of the experimental data provided a good linear fit in these plots, ruling out this possibility. When $1/F - F_0$ was plotted against $1/([\beta\text{-CD}])^2$, a nonlinear relationship was obtained (linear correlation of 0.86) (**Figure 1** *inset*, filled squares), which indicates that the stoichiometry of the inclusion complex is not 1:2.

Fitting the data obtained to equation 4, the K_F value for this physiological pH was calculated as 8120 ± 440 M^{-1}, demonstrating for first time the interaction between pterostilbene and CD. These results are in good agreement with those previously obtained for the 1:1 complexes between β-CD and several compounds with structures similar to pterostilbene *(27,28)*.

Effect of the cyclodextrin structure on complexation constants.

The K_F values between pterostilbene and CDs were determined with different types of CD in an attempt to characterize the interaction between pterostilbene and the host CD at a molecular level. Three types of natural CD with GRAS status and approved recently as additives in the European Union (α-, β- and γ-CD) were used to this end. Indeed, recently the three natural cyclodextrins have been included in the European lists of additives approved for alimentary use and the correspondent E-numbers have been assigned for α-, β-, and γ-cyclodextrins (E-457, E-458 and E-459 correspondently). Fitting the values of relative intensity to the equations previously described provides the corresponding K_F. As regards the different species, α-, β- and γ-CD, it can be observed that the highest K_F value (K_F =8120 ± 440 M^{-1}) was found for β-CD, followed by α-CD (K_F =4920 ± 340 M^{-1}) and, finally, γ-CD (K_F = 361 ± 25 M^{-1}). These results are in good agreement with those published for the most of the papers which compare the complexation of several

compounds and natural CDs *(22,23)*. In our study, the inner diameter of the CD formed by six units of glucose (β-CD: 6.0-6.4 Å) fitted pterostilbene better than the inner diameter of five units (α-: 4.7-5.2 Å) or seven units (γ-CD: 7.5-8.3 Å) of glucose. For this reason β-CD was considered the most suitable CD to continue the present investigation.

Effect of the chemical modification of β-CD on the complexation constants of the pterostilbene/CD complexes.

As β-CDs were the most effective for complexing pterostilbene, different types of modified β-CDs were studied, adding different functional groups to the macrocyclic ring. Qualitatively different results were obtained when HP-β-CD, methyl-β-CD and ethyl-β-CD were used instead of β-CD, the K_F values being higher for all the modified CDs than the natural CD. In **Figure 2A**, we can see the relative fluorescence values for all the modified CDs tested and β-CD. As is shown, the addition of increasing concentrations of modified CDs leads to greater increases in the relative intensity fluorescence values than when β-CD was used. As can be seen in **Figure 2B,** HP-β-CD showed the highest K_F value followed by ethyl-β-CD, methyl-β-CD and, finally, the natural CDs. As can be seen, the K_F for the complexation of pterostilbene by different modified CDs is dependent on the length of the aliphatic chain of the β-CD substituent, the greater the number of carbon atoms in the substituent, the higher K_F value for the resulting complex. Modification of β-CD occurs principally at position 2 of the sugar residues situated on one side of the torus at the edge and orientated inward *(32)*, thus increasing the hydrophobicity of the channel. The higher K_F observed for the pterostilbene/modified CDs complexes could be due to the

hydrophobic interactions with one side of the CD molecule (that bearing the methyl, ethyl or hydroxypropyl groups). Moreover, the dramatic changes occurring in the hydrophobicity of the CD torus provoked by the substitution of the internal –OH groups would also explain the behaviour of K_F. Moreover, our results are in good agreement with others that regard use of HP-β-CD as the most effective type of CD for complexing other stilbene compounds. For this reason, HP-β-CD was chosen as host CD for the following sections of the paper.

Effect of temperature on the complexation of pterostilbene by HP-β-CD.

One of the most important physico-chemical factors to be take into account when a compound is used as fortifier or nutraceutical in the food industry is the temperature. For this reason, we have studied in this paper the effect of the temperature in the K_F values of the pterostilbene/HP-β-CD complexes.

Although inclusion complexes usually dissociate when temperature is increased *(23,32)*, a previous paper published by our group showed that the K_F values between CDs and different polyunsaturated fatty acids increased with temperature *(31)*. For this reason, the next step in our investigation was to study the effect of temperature on the K_F values for the pterostilbene-HP-β-CD complex interactions at four different temperatures: 15ºC, 25º C, 30º C and 37º C. To prevent the results from being affected by changes in the buffer pH with temperature, the pH of the buffer was adjusted at the indicated temperature. The values of K_F for 15ºC, 25º C, 30º C and 37º C were 23800 ± 1120, 17520 ± 981, 14300 ± 720 and 872 ± 32 M^{-1} (**Figure 3**) , respectively. These results might be interpreted as a lower degree of interaction at higher temperatures possibly due to the fact that hydrogen

bonds are usually weakened by heating. Our results are in good agreement with those reported in recent years concerning the complexation of another type of stilbene, *trans*-resveratrol, by different types of CDs *(27,28)*.

Thermodynamic parameters for the pterostilbene-HP-β-CD complexes.

The thermodynamic parameters of the complexation of pterostilbene by HP-β-CD (ΔH^o, ΔS^o and ΔG^o at 25ºC) were obtained from a van´t Hoff plot (equation1) in order to gain information about mechanistic aspects of pterostilbene affinity for this type of CD. The ln K_F versus 1/T plot was obtained for HP-β-CD complexes and the van´t Hoff plot was linear, with correlation coefficient higher than 0.99 (**Figure 3** *inset*).

The exothermic nature of the interaction processes of pterostilbene with HP-β-CD is demonstrated by the negative values obtained for enthalpy changes. The enthalpy change is –29.20 kJ mol^{-1}, which is typical of: hydrophobic interactions, due to the displacement of water molecules from the cavity of HP-β-CD; increased van der Waals interactions between the molecules; the formation of hydrogen bonds and other interactions *(33)*. The changes of entropy are also negative in these processes (-17.64 J $mol^{-1}K^{-1}$). The fact that complexation decreases the translational and rotational degrees of freedom of the complexed pterostilbene compared with the free ones, leading to more ordered system, may justify this negative entropy value. Finally, our data show that the inclusion process is spontaneous due to the negative value obtained for the Gibbs free energy change (equation 2) for the interactions that take place during the inclusion process at 25ºC (–23.94 kJ mol^{-1}).

Effect of pH on the complexation of pterostilbene by HP-β-CD.

Another important factor to be considered when a CD complex is used in the food industry is its behaviour at different protonation states. To evaluate the effect of the pH medium on pterostilbene-HP-β-CD complexation, the K_F values for this type of complex were determined in the pH range 5.5-10.0. **Figure 4** shows the significant dependence of K_F on pH, passing from a stable value of around 17520 ± 981 M^{-1} (when the medium pH is between 5.5 and 7.5) to about 10050 ± 740 M^{-1} (when the medium pH is between 7.5 and 10.0), as happens during the titration of a weak ionizable group. This behaviour is similar to that reported by our group for the effect of pH on the K_F values of resveratrol-HP-β-CD complexes and other substances such as *trans*-resveratrol or polyunsaturated fatty acids *(28,32)*. As shown in **Figure 4**, the strong decrease in the K_F value coincides with the region where the stilbenoids begin the deprotonation of their hydroxyl groups. A possible cause for this dependence of K_F on pH is that the hypothetical formation of a hydrogen bond between the hydroxyl group of the pterostilbene and the hydrophilic groups of CD at pH values below the pK_a value, because hydrogen bonding is one of the most important types of interaction in the stabilization of inclusion complexes *(22,23*). The fact that the complexes between HP-β-CD and the protonated form of pterostilbene were more stable than the interaction with the deprotonated forms of this lipophilic antioxidant is of great interest for the food industry, because the protonated form of pterostilbene presents several beneficial biological effects on human health, as indicated above.

Effect of the structure of different stilbenes on the complexation constants of the

pterostilbene/ HP-β-CD complexes.

Finally, in order to obtain information on the mechanism involved in the complexation of different compounds of the stilbenoid family by HP-β-CD, both the K_F values and the stoichiometry for the complexation of three molecules (differing in the number of the hydroxyl groups and in the type of substituents of the aromatics ring) were determined. In **Scheme 1** the three type of stilbenoids studied (pterostilbene, *trans*-resveratrol and *trans*-stilbene) are presented.

As is shown in **Table 1**, the complexes formed between both pterostilbene and *trans*-resveratrol with HP-β-CD presented a 1:1 stoichiometry. Moreover, a comparison of the K_F values (**Table 1**) showed that the interaction was more effective for the *trans*-resveratrol-HP-β-CD complexes than for the pterostilbene-HP-β-CD complexes. Among causes of this behaviour may be the hydrophobicity or the resonance structure of the guest molecules can be the reasons of this behaviour. Indeed, *trans*-resveratrol has three hydroxyl groups in its structure while pterostilbene has only one. Moreover, *trans*-resveratrol does not contain any methyl groups in its structure, while pterostilbene has two methyl groups, which may diminish the efficiency of the complexation by CDs. On the other hand, the resonance structure of *trans*-resveratrol produces a high stability in this type of stilbene, which is not shown by pterostilbene, and may influence by its complexation by CDs.

However, the results obtained for the complexation of *trans*-stilbene by HP-β-CD showed important differences with respect to that shown by the pterostilbene and *trans*-resveratrol complexes. In first place, determination of the stoichiometry of the complexes provided different results from those obtained for the other stilbenoids. When a plot of 1/F

– F_0 vs. 1/[HP-β-CD] was constructed (**Figure 5**, filled circles), no straight line was obtained (and a linear correlation lower than 0.90), indicating that the presumed stoichiometry of the β-CD/pterostilbene complex formed was not 1:1 (**Table 1**). However, when a plot of 1/F – F_0 against $1/([HP\text{-}\beta\text{-}CD])^2$ (**Figure 5**, filled squares) pointed to a linear relationship (linear correlation higher than 0.99) (**Table 1**), which indicates that the stoichiometry of the inclusion complex was not 1:2.

These results can be explained by the symmetrical structure of *trans*-stilbene. As is shown in **Scheme 1**, this stilbenoid presents a symmetrical structure not present in the other guest molecules studied. This means that one molecule of *trans*-stilbene may be complexed by two molecules of HP-β-CD, each one of which complexed to *trans*-stilbene through one of the sides of its symmetrical structure. Finally, K_{F12} value determined for the *trans*-stilbene by HP-β-CD are shown in **Table 1**.

CONCLUSIONS

Although the number of works concerning the benefits of pterostilbene for human health has increased in recent years, its use as a functional ingredient (as a fortifier) or as nutraceutical compound has been limited because of problems associated with its low solubility and bioavailability and the ease with which it is oxidized by prooxidant agents. For this reason, we propose its complexation with cyclodextrins, a type of molecule which facilitates the "solubilization" of this stilbene and protects it against prooxidants agents. Our results show that the K_F values for the pterostilbene-CD complexes are strongly dependent on several factors, such as temperature, pH, type of CD and structure of the guest molecule. However, the stoichiometry of the complex is 1:1 for all the conditions used, except for the *trans*-stilbene-CD complexes where 1:2 complexes were formed. Potential applications of the resulting of pterostilbene-CD complexes can be found in the pharmaceutical and food ingredient industries as nutraceuticals due their high solubility and stability. Moreover, the use of pterostilbene-CD complexes could slow down the rapid metabolism and elimination of pterostilbene, improving its bioavailability, as has been demonstrated for other stilbenoid complexes.

ACKNOWLEDGMENT

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SCHEMES

Scheme 1. Structures of Pterostilbene, *Trans*-Resveratrol and *Trans*-stilbene.

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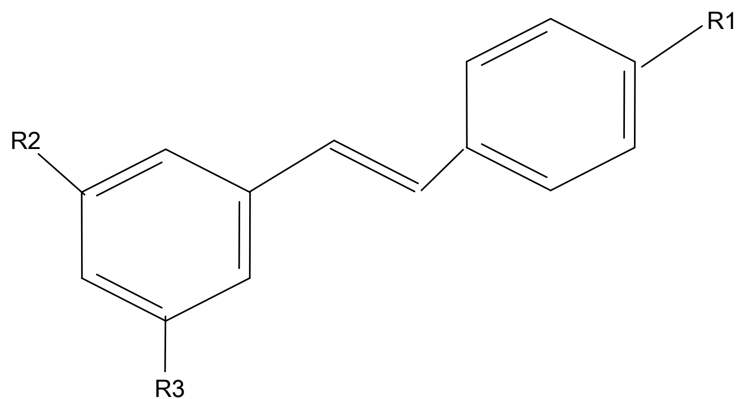


Pterostilbene: R1: OH; R2: OCH_3; R3: OCH_3

***Trans*-resveratrol**: R1: OH; R2: OH; R3: OH

***Trans*-stilbene**: R1: H; R2: H; R3: H

TABLES

Table 1. K_F values and correlation coefficients arising from equations (4) and (5) (for 1:1 and 1:2 *stilbenoids*-HP-β-CD complexes, respectively) at 25 ºC at pH 7.0.

Complex	**K_F (M^{-1})**	**K_{F12} (M^{-2})**	**Correlation coefficient**	
			1:1 using Eq.(4)	*1:2 using Eq.(5)*
Trans-resveratrol-HP-β-CD	24880 ± 1020	------------	0.99	0.91
Pterostilbene-HP-β-CD	17520 ± 981	------------	0.99	0.86
Trans-stilbene-HP-β-CD	----------------	$1.01 \times 10^{9} \pm 0.67 \times 10^{6}$	0.88	0.99

FIGURE LEGENDS

Figure 1. Dependence of emission fluorescence intensities of pterostilbene (30 μM) on HP-β-CD concentrations. *Inset*: Double reciprocal plot of pterostilbene complexed to β-CD for determining the stoichiometry of β-CD/pterostilbene complexes: $1/(F-F_0)$ versus 1/[β-CD] (assumption of 1:1 complex) (filled circles); (b) $1/(F-F_0)$ versus $1/[\beta\text{-CD}]^2$ (hypothesis of 1:2 complex) (filled squares).

Figure 2. (A) Dependence of emission fluorescence intensities of pterostilbene (30 μM) on modified β-CD concentrations. (B) Effect of the structure of modified and natural CDs on the complexation constant (K_F) of pterostilbene-CD complexes at 25 ºC in 0.1 M sodium phosphate buffer pH 7.0.

Figure 3. Effect of temperature on the complexation constant (K_F) of pterostilbene-HP-β-CD complexes at pH 7.0. *Inset:* Van´t Hoff plot (ln K_F vs. 1/T) for pterostilbene-HP-β-CD complexes in 0.1 M sodium phosphate buffer pH 7.0.

Figure 4. Effect of pH on the complexation constant (K_F) of pterostilbene-HP-β-CD complexes at 25 ºC.

Figure 5. Double reciprocal plot of *trans*-stilbene complexed to HP-β-CD for determining the stoichiometry of HP-β-CD/*trans*-stilbene complexes: $1/(F-F_0)$ versus 1/[HP-β-CD] (assumed of 1:1 complex) (filled circles); (b) $1/(F-F_0)$ versus $1/[HP\text{-}\beta\text{-}CD]^2$ (assumed of 1:2 complex) (filled squares). *Inset*: Dependence of emission fluorescence intensities of *trans*-stilbene (30 μM) on HP-β-CD concentrations.

FIGURE 1

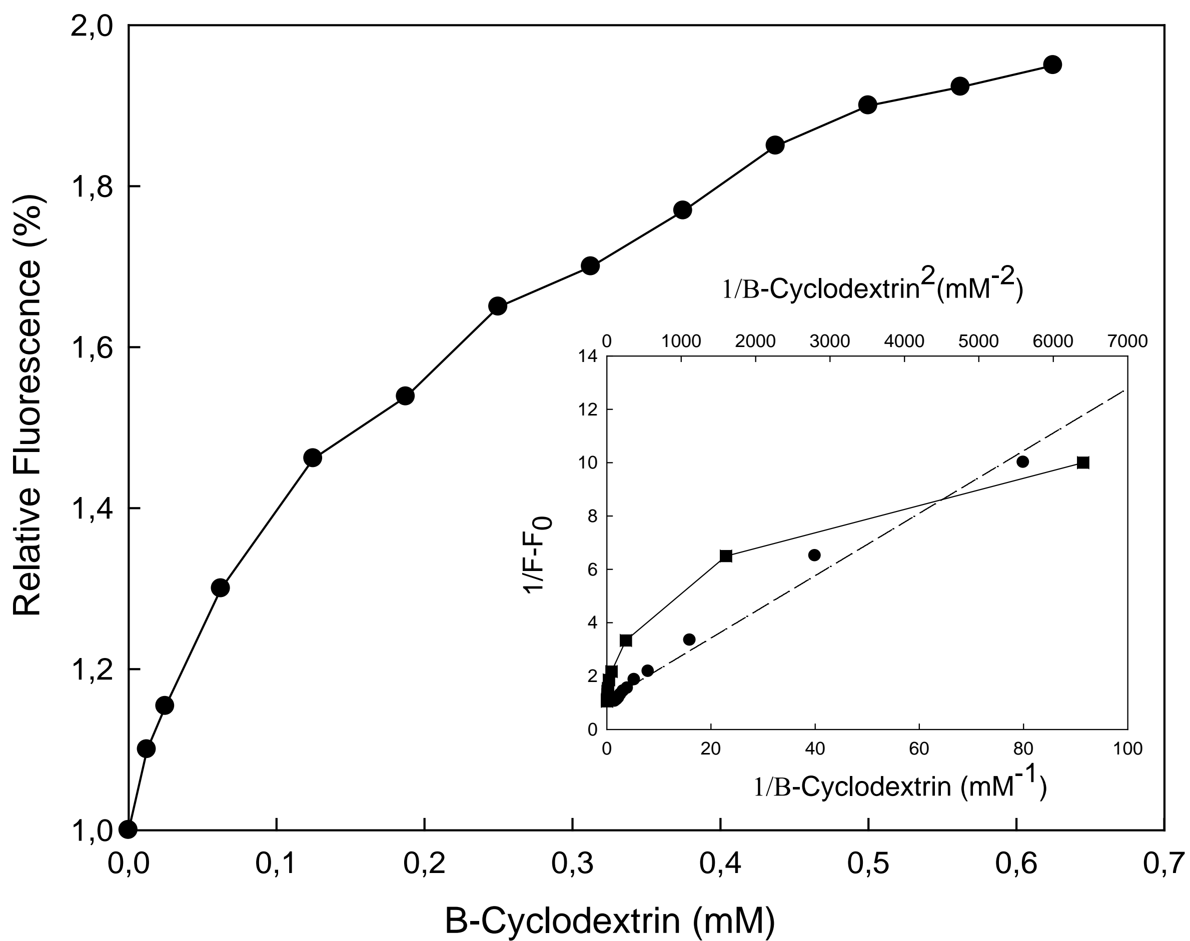
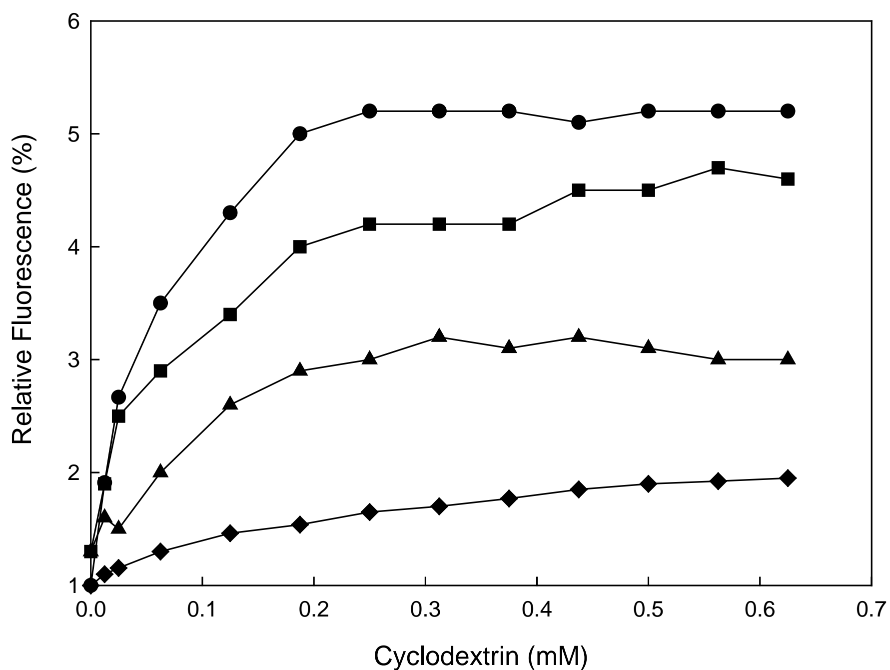


FIGURE 2



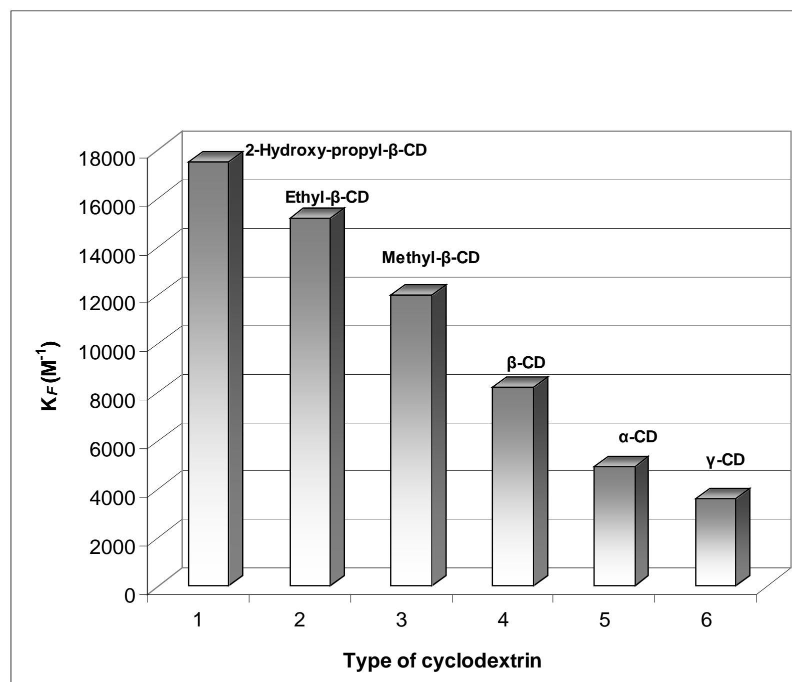


FIGURE 3.

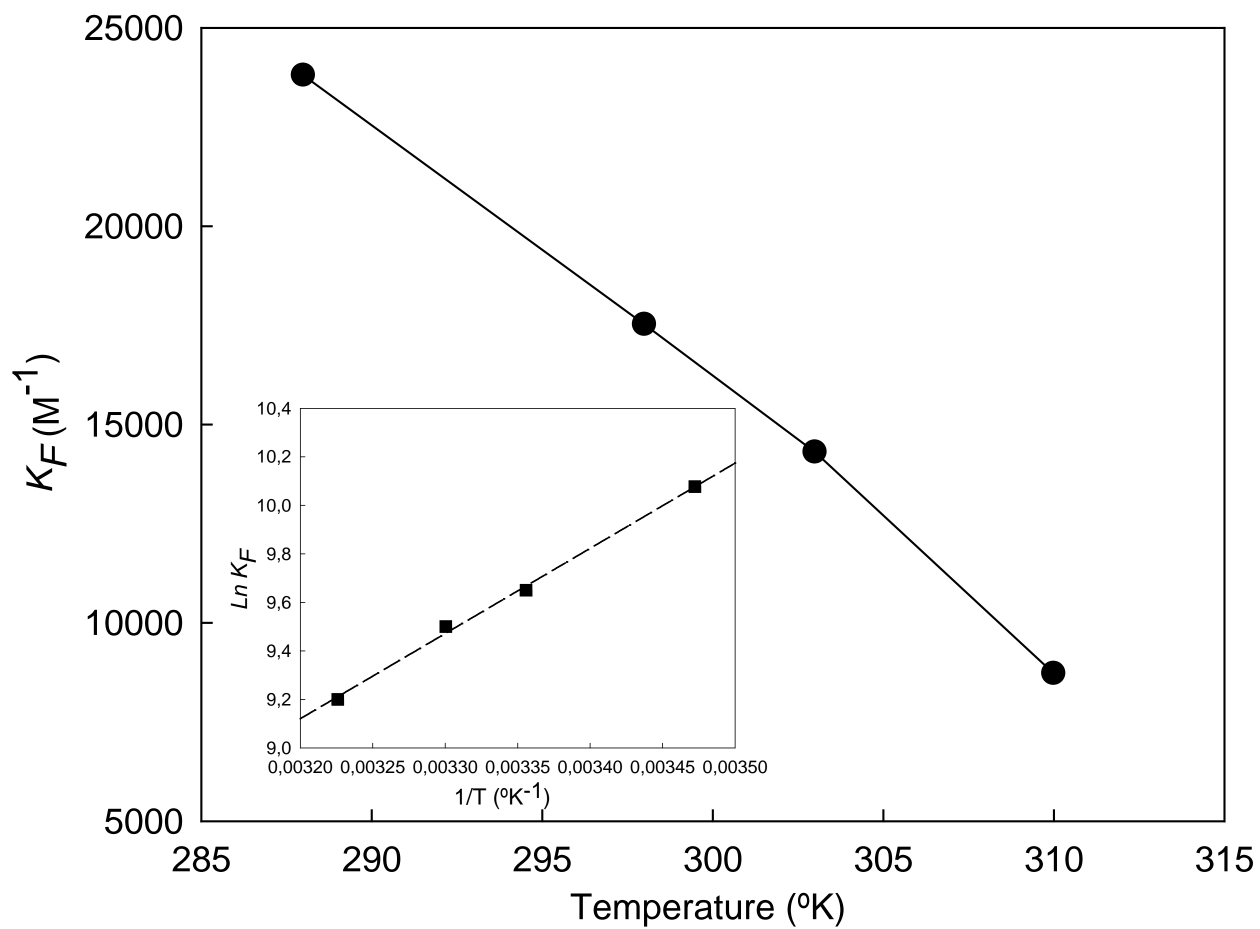


FIGURE 4

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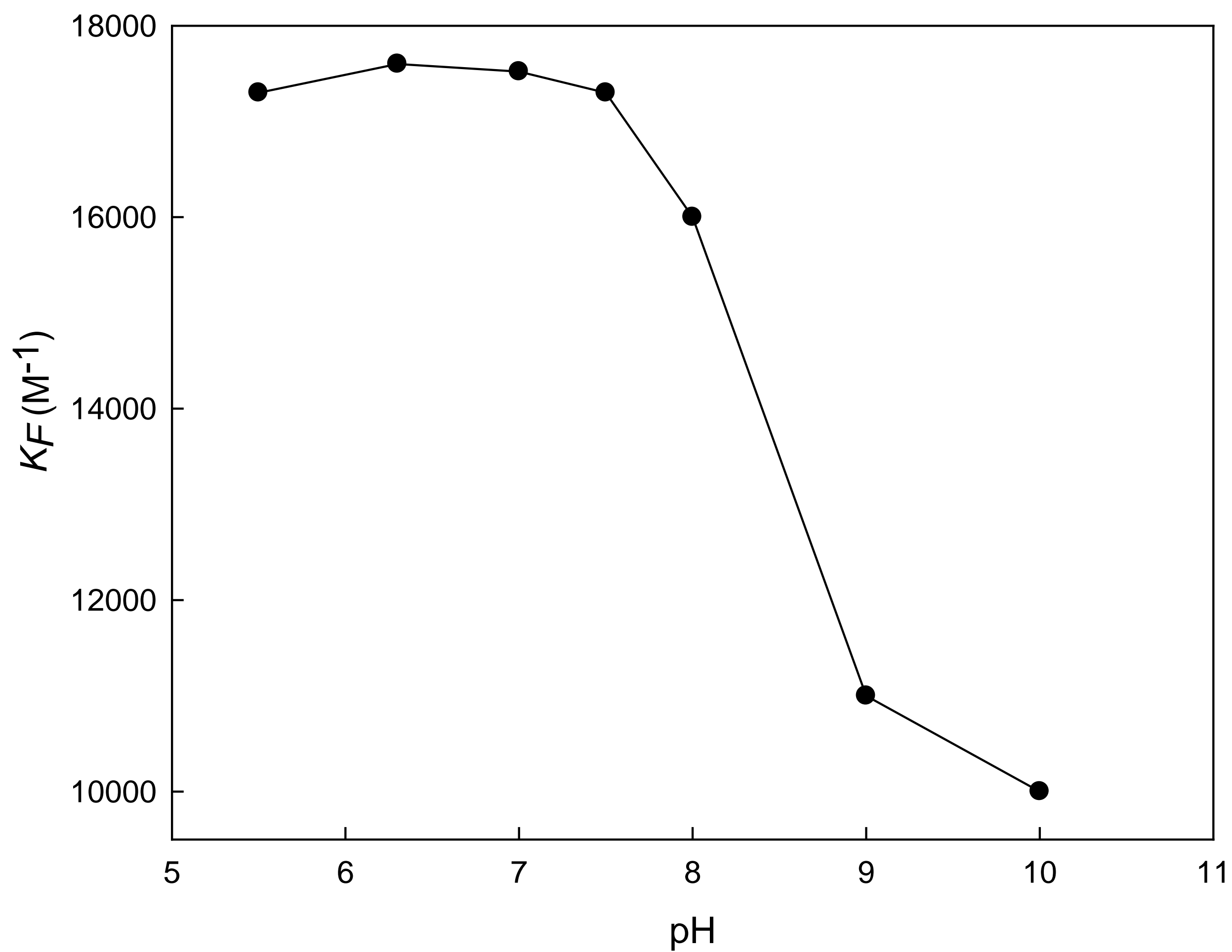
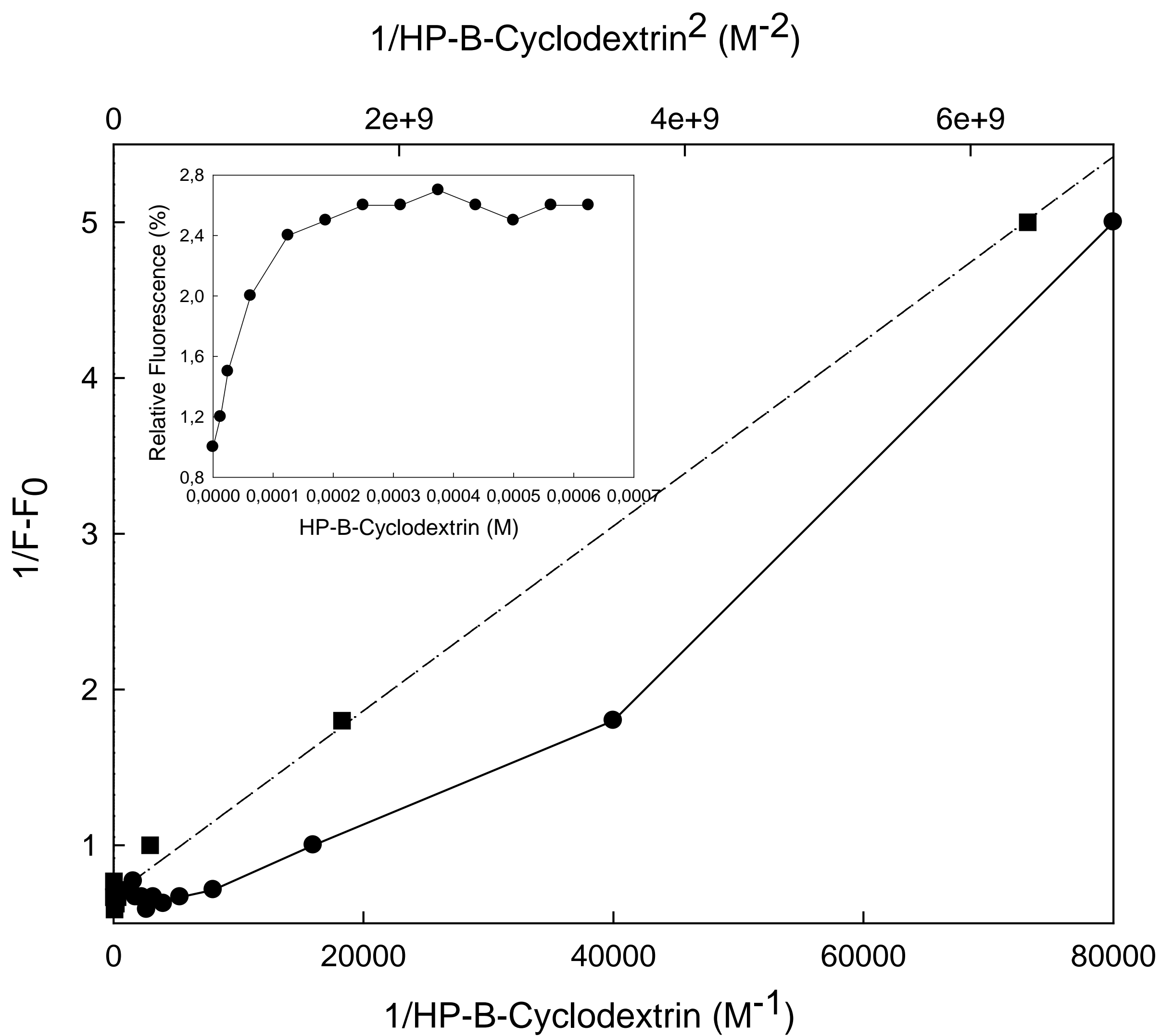


FIGURE 5



Capítulo V

Desarrollo de un nuevo método de cromatografía líquida de alta resolución basado en el uso de ciclodextrins para la determinación de pterostilbeno

ABSTRACT

In this work, a reversed phase high performance liquid chromatography (RP-HPLC) method was developed for the determination of pterostilbene in food samples. The novel method is based on the addition of cyclodextrins (CDs) to the mobile phase where the complexation of pterostilbene by CDs is carried out. In order to select the most suitable conditions for the RP-HPLC method, the effect of several physico-chemical parameters on the complexation of pterostilbene by CDs was studied. Our results show that the addition of 12 mM HP-β-CD to a 50:50 (v/v) methanol:water mobile phase at 25ºC and pH 7.0 significantly improves the main analytical parameters. In addition, it was seen that pterostilbene forms a 1:1 complex with HP-β-CD, showing an apparent complexation constant of 251 ± 13 M^{-1}. Finally, in order to study the validity of the proposed method, blueberries were analysed and the concentration of pterostilbene has been determined.

1. INTRODUCTION

In recent years, many works have been published on pterostilbene (*trans*-3,5-dimethoxy-4′-hydroxystilbene) (Fig. 1 *inset*), a stilbenoid compound with many beneficial health effects [1-5]. Pterostilbene is a naturally occurring phytoalexin which has been identified in some plant species and is found in different sources such as the leaves of *Vitis vinifera* [6], in infected grape berries [7] and in healthy and immature berries of *var*. Pinot Noir and Gamay [8]. Moreover, pterostilbene is present in the heartwood of sandalwood (*Pterocarpus santalinus*) [9] and *Pterocarpus marsupium* [10] or in the berries of some *Vacciunium* species [8]. Furthermore, this potent antioxidant also appears to be a constituent of the bark of *Guibourtia tessmanii* [11] and, finally, high levels of pterostilbene were found in darakchasava [12].

In spite of the beneficial properties for the health of pterostilbene, some problems concerning the identification of this potent antioxidant, such as the low concentrations present in different foods or the poor sensitivity of the methods used in its determination, have led fewer papers being published on the occurrence of this stilbenoid in other foods than those mentioned above. Pterostilbene has previously been quantified by gas chromatography after methylation and mass spectrometry [13-14]. However, few publications have reported the analysis of pterostilbene by HPLC [12, 3, 15-17]. Indeed, the works that have been published mention several disadvantages such as the long analysis times required, the high organic dissolvent concentration used in the mobile phase or the poor RP-HPLC analytical parameters obtained in the method, including poor linearity, precision and sensitivity or high detection and quantitation limits. For these reasons, we

have developed a new method for analyzing pterostilbene in blueberries using RP-HPLC based on the addition of cyclodextrins (CDs) in the mobile phase.

CDs are a group of structurally related natural products formed during the bacterial digestion of starch [18]. The most important functional property of CDs is their ability to form inclusion complexes with a wide range of organic guest molecules. Among the guest molecules which have been complexed by CDs, several works about the complexation of different stilbenoids with CDs have been recently published [19-23]. However, very few applications of these complexes have been reported. In this work, the use of the pterostilbene/CD complexes in the chromatographic analysis field is presented for first time.

Although the use of CDs as components of the HPLC mobile phases has been reported in several works, the principal aim of those papers was to determine the complexation constants of the guest/CD complexes under different physico-chemical conditions or to improve the separation of different enantiomers [24-25]. However, the use of CDs as additives to improve an HPLC analysis method that permits the rapid analysis of low levels of a compound has not previously been reported up date.

Bearing in mind the above, this work set five principal objectives: i) to study the complexation of pterostilbene by several type of CDs under various experimental conditions using HPLC method; ii) to select the optimum conditions of pH, temperature, organic dissolvent and type of CD for use in RP-HPLC; iii) to develop a new pterostilbene RP-HPLC method with high levels of precision and linearity, low detection and quantification limits and low percentage of organic solvent based on the addition of CDs to

the mobile phase using two types of detectors, UV-vis and fluorescence; iv) to calculate the apparent formation constant of the pterostilbene/HP-β-CD complexes and v) to apply the new RP-HPLC method for analyzing the pterostilbene concentration in blueberries variety *Duke*.

2. EXPERIMENTALS

2.1 Chemical and reagents

Pterostilbene was from Sequoia Research Products Limited (Pangbourne, United Kingdom) and was used without further purification (Purity of 98.1% determined by HPLC). Natural (α-CD, β-CD) and modified CDs (HP-β-CD and methyl-β-CD were purchased from Sigma-Aldrich (Madrid, Spain) The water and methanol used in this study, of HPLC grade, were purchased from Análisis vínicos, S.A. (Tomelloso, Spain). Anhydrous D-glucose was supplied by Prolabo (Fontenoy-Sous-Bois, France).

2.2 Equipment and Experimental Procedure

Twenty microlitres of pterostilbene prepared in methanol, at the concentration mentioned in the text, were injected for HPLC analysis using a Shimadzu LC-10A (Shimadzu Corporation, Kyoto, Japan) unit equipped with an RF-10AXL fluorescence detector (Shimadzu) and a SPD-M10A photodiode array detector (Shimadzu) was used. For the aqueous mobile phase studies, a commercially available C8 column Sunfire (Milford, USA) (150 x4.6 mm I.D. 5μm particle size) was used. For all experiments the mobile phase flow-rate was systematically controlled at 1.00 ± 0.01 mL/min, the UV-vis detector was operated at 306 nm and the fluorescence detector used an excitation wavelength of 330 nm and emission wavelength of 435 nm. Mobile phases were prepared according López Nicolás & García-Carmona [23].

2.3 Temperature and pH studies.

In the temperature studies, the retention factor was determined at the following temperatures: 15.0, 20.0, 25.0, 30.0 and 37.0 ± 0.1 ºC. To study the effect of mobile phase pH on the retention time of pterostilbene, buffers were used in the aqueous composition of

the mobile phase: 0.1 M sodium acetate buffer for pH 5.0, 0.1 M sodium phosphate for pH 5.5–8.5 and 0.1 M sodium borate for pH 9.0–11.0.

2.4 HPLC method validation

a) Determination of precision:

To evaluate precision, a 2 µg/ml pterostilbene solution was brought to 10 ml with 10% methanol in a volumetric flask, and injected at 20 µl for a total of 10 times. The precision of the method was expressed by the relative standard deviations (RSD) or coefficient of variation of the data set. The mean concentrations of pterostilbene were calculated while SD and RSD were calculated using equations described by Snyder et al. [26].

b) Determination of linearity:

The linearity of the method was evaluated by injecting 20 µl of the seven samples of pterostilbene standards, prepared at 0.2; 0.5; 1; 2; 4; 8 and 12 µg/ml. This set of standards was analyzed in triplicate. The correlation coefficient (r) between peak area and the concentration of pterostilbene was analyzed for each of the seven standards used.

c) Determination of LOD and LOQ:

The LOD and LOQ were determined by analyzing pterostilbene solutions that were sequentially diluted in a series with methanol to obtain the lowest level of analyte that gave a measurable response with a signal-to-noise ratio of 3 and 10, respectively.

2.5 Stilbene extraction and analysis

Blueberries were purchased from a local supermarket. The protocol used for stilbene extraction was adapted from the method previously described by Langcake and Pryce [27] and Jeandet et al. [28]. Blueberries analysis was performed using both the

photodiode array and fluorimetric detector. Concentrations of pterostilbene in samples were measured using the external standard method. Response factors (amount of standard/peak area) were calculated with data from the standard calibration curve.

3. Results and discussion

3.1.Selection of organic solvent of mobile phase

In this work, the use of the non-polar stationary phase did not allow determination of pterostilbene when water alone was used as mobile phase, as it involved very long retention times accompanied by the associated experimental error; it was therefore necessary to use an organic modifier in the mobile phase. In the selection of an organic solvent for a reversed-phase system, the retention value of the sample solute and the formation constant of inclusion complexes of the solute are important parameters since they depend on the type of organic solvent and its content in the mobile phase (López-Nicolás et al., [22].

Acetonitrile, ethanol, methanol and 1-propanol are the most widely used organic solvents in RP-HPLC. In this work, ethanol and 1-propanol were discarded for use as organic solvents because the strong interaction between these compounds and CDs can interfere in the complexation of pterostilbene by CDs [22]. Indeed, ethanol presented a constant Km (which describes the affinity of the organic modifier for the β-CD cavity) of $0.93M^{-1}$, and 1-propanol presented a Km = $3.71M^{-1}$ [29]. Several authors have selected acetonitrile [12, 3, 15, 17] or methanol [16] to identify pterostilbene in different samples. These two organic dissolvents have the lowest Km values of the above mentioned the organic solvents [29]. To choose between these two dissolvents for our investigations, pterostilbene was analysed using two different mobile phases in the presence of equal HP-β-CD concentrations. Theoretically, and as a result of host-guest interactions, the retention time of the guest will decrease when complexation occurs in the mobile phase and increase when it takes place in the stationary phase. These changes in the retention behaviour are closely related to the stability constants of the complexes formed. In our experiment, when

a MeOH:water:HP-β-CD (50:50:8mM) mobile phase was used, the retention time (*Rt*) of pterostilbene was reduced by about 70% compared with the same mobile phase in the absence of HP-β-CD. However, a reduction of only 10% was observed when ACN:water:HP-β-CD (50:50:8mM) mobile phase was used. Finally, several environmental problems have been associated with the use of acetonitrile. For these reasons, we selected methanol as the most suitable organic solvent to be used in the new RP-HPLC method described in this work.

3.2.Effect of mobile phase additives on pterostilbene retention

To validate the use of CDs as additives in the mobile phase for developing a new method of HPLC analysis it was first necessary confirm that the effect of CDs on the pterostilbene *Rt* is not due to the glucidic nature of the CDs but to their ability to complex hydrophobic compounds. To do this, we have studied the possible reduction of the pterostilbene *Rt* due to the presence of D-glucose in the reaction medium because glucose is a molecule included in the CD structure. Thus, various amounts of D-glucose (14 and 84 mM), corresponding to 2 and 12 mM of β-CD and HP-β-CD as regards the number of glucose units (each molecule of β-CD contains seven units of D-glucose in a ring), were added to the 50:50 (v/v) (methanol:water) mobile phase and the *Rt* of pterostilbene was checked. The *Rt* of pterostilbene in the absence of any additive was 44.62 min. Moreover, the *Rt* of pterostilbene decreased in the presence of β-CD at 2 mM (41.27 min.) and HP-β-CD at 12 mM (10.6 min.), whereas the addition of D-glucose did not alter the retention times (44.83 min.) even though the concentration of D-glucose was the same as that of β-CD and HP-β-CD as regards the number of glucose units. Several conclusions can be deduced from these results. Firstly, the addition of CD to the mobile phase reduces the

pterostilbene *Rt* due to its capacity to complex hydrophobic substances since no glucose/pterostilbene complexes exist. Moreover, the possible elution modifications observed in the presence of β-CD cannot therefore be attributed to modifications in solvent strength.

3.3.Effect of methanol concentration on pterostilbene retention in the presence and absence of cyclodextrin

The next step in our investigation was to analyze the effect of the concentration of the organic solvent in the mobile phase on the capacity factor. Fig. 1 shows, in both the absence and presence of 8mM HP-β-CD, pterostilbene retention increased exponentially with increasing percentages of water, in the mobile phase. As shown in Fig. 1, at methanol concentrations below 50% the pterostilbene analysis leads to very long *Rt* which can result in substantial experimental errors. On the other hand, at a high methanol percentage (more than 50% v/v in binary methanol–water mixtures), a substantial amount of methanol can interact with HP-β-CD, leading to competition with pterostilbene complexation. This high degree competition between the organic solvent and the guest molecule for the CD cavity means that the decrease in the *Rt* in the presence of HP-β-CD was not significant compared with that observed in the absence of this glucidic molecule. As can be seen in Fig. 1, at these high methanol concentrations the decrease in the mobile phase polarity provokes a decrease in both complexation and *Rt*. The amount of methanol present provides a less polar mobile phase, in which the non-polar solutes become more soluble; as a consequence, the solute affinity for the hydrophobic cavity of HP-β-CD diminishes and part of the driving force for inclusion is removed.

Furthermore, the use of only 50% methanol in the mobile phase rather than the high percentages of methanol or acetonitrile used by other researchers (up to 85%, see Table 1) provides environmental benefits that only serve to confirm the advantages of the method presented in this work with respect to those reported previously.

For this reason, we chose 50 % as the optimum methanol percentage to be used in our method.

3.4.Effect of pH on pterostilbene retention

Selection of the pH of the mobile phase is one of the most important steps in the development of a new HPLC method. For this reason, the pterostilbene *Rt* was studied in a wide range of pH (5.0–11.0). As shown in Fig. 2A the *Rt* of this potent antioxidant remains constant at pH values below pH 9.0. However, when the mobile phase pH is increased from pH 9.0 to 10.0, the *Rt* passes from a stable value of about 44.6 minutes to about 25 minutes in just one pH unit, as happens during the titration of a weak ionizable group. This strong decrease in the pterostilbene *Rt* may be due to the deprotonation of the hydroxyl group of this stilbenoid as has described for the complexation of *trans*-resveratrol by CDs [22, 23]. Indeed, the significant decrease in the *Rt* value between pH 9.0 and 10.0 coincides with the pH region where stibenoids usually suffer the deprotonation of their hydroxyl groups [23]. A likely explanation for the dependence of the pterostilbene *Rt* on the pH is that when the reaction medium pH is above the pK_a of the guest molecule, the number of hydrogen bonds between the CDs and several compounds are lower than when the guest molecules are protonated. For this reason, the guest/CDs complexation constants also decrease at pH values higher than the pK_a values of the complexed molecule. For this reason a pH of 7.0 was selected for our experiments.

3.5. Effect of temperature on pterostilbene retention

As is known, an increase in the system temperature generally leads to a decrease in the *Rt* of the analyte. However, also it is known that this increase in the temperature can weaken the interactions between CDs and the guest molecules. In this study the effect of temperature on the *Rt* was studied for pterostilbene at five different temperatures: 15, 20, 25, 30 and 37 ºC. As can be seen in Fig. 2B, an increase in the system temperature reduced the *Rt* from 65 minutes at 15ºC to 25 minutes at 37 ºC. According to these results, the optimum temperature to determine pterostilbene would be 37 ºC because the analysis times are lower than at high temperatures. However, according to the results of several researchers [23], temperature values higher than 25 ºC are not recommendable for analyzing guest molecules by HPLC in the presence of CDs because the complexes between CDs and guest molecules may be dissociated by the rupture of the interaction bonds. For this reason, a temperature of 25 ºC was selected for our experiments.

3.6. Effect of different types of CD on pterostilbene retention

The next step was to determine the best type of CD to be used in the new method. Four types of natural (α-CD and β-CD) and modified CDs (methyl-β-CD and hydroxypropyl-β-CD) were used to this end and the effect of the addition of 8 mM of α-CD, methyl-β-CD and HP-β-CD and 2 mM of β-CD (this low concentration is due to the poor solubility of β-CD) on the pterostilbene *Rt* was tested.

The two types of natural CD have GRAS status and have been approved as additives in the European for food use, and the corresponding E-numbers assigned are E-457 for α-CD and E-459 for β-CD.

As is shown, the *Rt* remained practically constant when β–CD was added to the mobile phase (43.2 min.) respect to the observed in the absence of CD (44.6 min.). Moreover, when a α-CD was used a slight decrease in *Rt* was observed (38.9 min.). As the new HPLC method proposed in this work is based in the use of increasing concentrations of CDs to reduce the *Rt* of pterostilbene, these data led us to two conclusions: (i) The interaction of pterostilbene with natural CDs is very weak at the concentration tested due to the poor formation of the pterostilbene/α-CD or pterostilbene/β-CD complexes and (ii) natural CDs cannot be recommended to be used as additives in the mobile phases to develop a rapid method for determining pterostilbene by RP-HPLC.

On the other hand, different studies have reported that modified CDs can complex stilbenoids better than natural CDs. For this reason, and due to these above results, we used modified CDs such as HP-β-CD and methyl-β-CD in this work. Our results show a strong decrease in the *Rt* of pterostilbene when 8 mM HP-β-CD or methyl-β-CD were added to the reaction medium. Thus, HP-β-CD reduced the *Rt* from 44.6 minutes to 14.1 minutes and methyl-β-CD to 17 minutes. Although similar data were obtained when both modified CDs were used, the fact that HP-β-CD presents the lowest *Rt* of all the CDs tested and because several studies indicate that this type of CD presents a high complexation constant for other stilbenoid/CD complexes led us to select HP-β-CD as the most suitable type of CD in our investigation.

3.7.Effect of HP-β-CD concentration on pterostilbene retention

After the RP-HPLC conditions were determined in the presence of CDs, validation tests were performed to check retention time, precision, linearity, range and limit of detection and quantitation.

The different pterostilbene chromatograms obtained at increasing concentrations of HP-β-CD (from 0 to 12 mM) in the mobile phase are presented in Fig. 3. As is shown in Fig. 3 *inset*, a strong reduction from 44.6 to 10.6 minutes (around 75%) in the pterostilbene Rt was observed when this antioxidant was determined in a C8 Sunfire column using 50:50 water–methanol proportions containing 12 mM HP-β-CD in mobile phase. These results are due to the ability of CD to reduce the *Rt* of pterostilbene because the inclusion complexes between this stilbenoid and HP-β-CD enhances guest solubility in the mobile phase and reduced its residency time in the column. Moreover, the *Rt* presented in this work (10.6 min. in the presence of 12 mM HP-β-CD) are the lowest times reported for the analysis of pterostilbene (up to 50 minutes) [12, 3, 15-17].

This reduction in the *Rt* of several compounds when different types of CDs are added to the mobile phases has been used by some researchers to determine the complexation constants of the guest/CD complexes. However, no work has suggested that this decrease in the *Rt* might be used to develop new method to analyse antioxidant compounds. With this in mind the effect of adding CDs on the main HPLC parameters, such as precision, linearity, reproducibility, detection and quantification limits of the analyte was studied for the first time.

3.8.Effect of HP-β-CD addition on the method precision

One of the most important factors to be borne in mind when an RP-HPLC method is developed is precision. In this work, the precision of the method was expressed by the relative standard deviations (RSD) of ten analysis of area and *Rt* of 2 μg/ml pterostilbene solutions using both UV-vis and fluorescence detection in the presence and absence of 12 mM HP-β-CD. Very interesting results for the RSD values can be observed in Fig. 4A. As regards the analysis of the pterostilbene *Rt*, no significant differences in RSD values were found between the detectors. However, reductions of 96% and 97% in the RSD values for UV-vis and fluorescence *Rt* determinations were observed when 12 mM HP-β-CD was added to the mobile phase. Indeed, this is the first study to show an increase in the precision parameter due to the presence of HP-β-CD in the RP-HPLC mobile phase.

As regards the area measurements, precision parameter differed substantially between UV-vis and fluorescence detection. In both the absence and presence of HP-β-CD, the RSD values obtained using the fluorescence detector was higher than those obtained using UV-vis. Furthermore, the precision of the area determinations was again higher when 12 mM HP-β-CD was added to the mobile phase than when this complexant agent was not present.

These results confirm that the use of 12 mM HP-β-CD in the mobile phase not only reduces the analysis time but increases the precision of the method when both UV-vis and fluorescence detection were used for the pterostilbene analysis.

3.9.Effect of HP-β-CD addition on the method linearity

The linearity of the response was examined by analyzing solutions in a range of concentration between 0.2-12 μg/ml. The linearity calibration curves were constructed for seven concentrations of each reference compound in triplicate. The regression equation was

calculated in the form of y = ax + b, where y and x were the values of peak area and concentration of each reference compound, respectively. The results of the regression analyses and the correlation coefficients (*r*) are shown in Table 2, which illustrates the substantial differences found in the absence and presence of HP-β-CD. Firstly, the results obtained show that the correlation coefficients of the linear regression of the standard curves are greater for the fluorescence detector than for the UV-vis detector in the absence (0.9985 vs 0.9977) and presence of HP-β-CD (0.9999 vs 0.9992). Moreover, in the presence of 12 mM HP-β-CD the new method presents higher correlation coefficient values ($r > 0.999$) than in the absence of the complexant agents for both detectors: UV-vis (0.9992 vs 0.9977) and fluorescence (0.9999 vs 0.9985). These results lead us to affirm that the combination of fluorescence detector and the presence of HP-β-CD significantly increases the linearity of pterostilbene determination by RP-HPLC.

3.10. Effect of HP-β-CD addition on the LOD and LOQ

To complete the study of the validation of the new method in the presence of CD, the limit of detection (LOD) was determined with a signal-to-noise ratio of 3, and the limit of quantitation (LOQ) with signal-to-noise ratio of 10. Our results show that LOD and LOQ showed different behaviours in the absence and presence of HP-β-CD for both detectors (UV-vis and fluorescence). Although they both showed high sensitivity in all chromatographic conditions assayed, two main conclusions can be extrapolated from the data obtained: i) in the absence and presence of HP-β-CD, LOD and LOQ presented lower values for the fluorescence detector than for UV-vis; ii) in the presence of 12 mM HP-β-CD both limits were strongly reduced, reflecting a significant increase in the sensitivity of the method described in this work. Thus, when 12 mM HP-β-CD was added to the reaction

medium, the LOD was reduced from 3.41 to 0.95 µM in the case of fluorescence detector and from 4.33 to 2.35 µM for UV-vis detector, representing increases in sensitivity of 72 % and 32 %, respectively, calculated at a signal-to-noise ratio of 3. A similar behaviour was observed for LOQ. Without any agent in the mobile phase, the value for this limit was 10.84 and 13.49 µM for the fluorescence and UV-vis detectors. However, the addition of HP-β-CD reduced these limits to 2.11 and 7.71 µM, respectively. These results confirm the increased sensitivity of 80% and 43% in the LOQ for the UV-vis and fluorescence detectors, respectively.

This is the first time that where a decrease in the LOD and LOQ has been demonstrated for a compound when CDs are added to the mobile phase, confirming, once again, the validity of the new method proposed in this work to measure pterostilbene by RP-HPLC.

3.11. Study of the complexation of pterostilbene by cyclodextrins. Stoichiometry of the complexes and determination of the apparent complexation constant

One advantage of the method proposed in this work is the possibility of calculate the apparent complexation constant (K_F) between the molecule analyzed (pterostilbene) and the optimum CD selected (HP-β-CD). In view of the results obtained in previous sections, a temperature of 25ºC, a pH of 7.0 and a modified type of CD such as HP-β-CD have been selected as the best physico-chemical conditions to determine the K_F between pterostilbene and HP-β-CD.

To determine the K_F value for the pterostilbene/HP-β-CD complex, equation 1, which relates the capacity factor, k, and the HP-β-CD mobile-phase concentration, [CD], is proposed [24]. In this equation we have assumed two conditions: 1) the complex presents a

1:1 stoichiometry and 2) interaction of the pterostilbene/HP-β-CD complex with the stationary phase is negligible.

$$\frac{1}{k} = \frac{1}{k_o} + \frac{K_F}{k_o}\left[\mathrm{CD}\right] \qquad (1)$$

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 HP-β-CD mobile-phase concentration.

Although several authors have claimed that stilbenes can not form CD complexes with a 1:2 stoichiometry, López-Nicolas et al. [21] 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 pterostilbene/HP-β-CD complex via a precursor 1:1 complex. Eq. 2 is an extension of eq. 1 and includes a second-order term that accounts for the possibility of 1:2 pterostilbene-HP-β-CD complex formation:

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

where k_0 is the capacity factor of pterostilbene in the absence of HP-β-CD modifier, K_{F1} is the apparent formation apparent for the 1:1 pterostilbene/HP-β-CD complex and K_{F2} is the apparent formation constant for the 1:2 pterostilbene/HP-β-CD complex.

Using equation 1 and the data obtained from the chromatogram presented in Fig. 3 in the presence of increasing concentrations of HP-β-CD, a plot of the reciprocal of k

versus [HP-β-CD] should give a straight line, indicating the formation of 1:1 pterostilbene/HP-β-CD complex. However, in the case of a 1:2 pterostilbene/HP-β-CD complex formation, a plot of reciprocal of *k versus* [HP-β-CD] should give a parabolic curve that fits eq. 2. In our study, a plot of 1/k vs. [HP-β-CD] gave a straight line with a linear correlation higher than 0.99, indicating that the presumed stoichiometry of the pterostilbene/HP-β-CD complexes formed was 1:1 (Fig. 4B, filled circles). On the other hand, when 1/k was plotted against $[HP\text{-}\beta\text{-}CD]^2$, a non-linear relationship was obtained (linear correlation of 0.82) (Fig. 4B, filled squares), which indicates that the stoichiometry of the inclusion complex is not 2:1. Fitting the data obtained to equation 1, the K_F value was calculated as $251 \pm 13\ M^{-1}$.

This K_F value determined at a methanol concentration of 50% is lower that the reported ($17520 \pm 981\ M^{-1}$) by our group for the interaction between pterostilbene and HP-β-CD in aqueous solution [21] determined by fluorimetric techniques. The strong dependence of apparent K_F values on the methanol concentration is due to the existence of a strong competition on the part of methanol and solute for the HP-β-CD cavity reported by different authors [24]. Moreover, the decrease in K_F values can be interpreted is 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 HP-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 HP-β-CD cavity and the eluent will become more intense. Consequently, complex formation will be even more strongly favoured.

3.12. Analysis of pterostilbene in blueberries and recovery study

In order to study the validity of the proposed method, it was applied to the determination of pterostilbene in blueberries. Although different varieties of blueberries present high concentrations of pterostilbene, other varieties not show significant values of this antioxidant [13]. For this reason it was of interest to determine whether the stilbene pterostilbene, which is also reported to have antioxidant activities, is present in blueberries variety *Duke*.

The use of photodiode array or fluorimetric detection meant that it was necessary to study the possible existence of a matrix effect. Fig. 5 shows the chromatograms obtained for blueberries samples using the photodiode array detector in the absence (II) and presence (I) of 12 mM HP-β-CDs in the mobile phase selected in the previous sections (50:50 (v/v) methanol:water mobile phase at 25ºC and pH 7.0). As can be seen, in the absence of 12 mM HP-β-CDs, the presence of pterostilbene in blueberries is practically imperceptible because of its low levels and the poor limits of both detection and quantification of traditional chromatographic methods (note the very low peak with poor definition at 44.6 minutes). However, due to the complexation of pterostilbene by CDs described previously, when 12 mM HP-β-CDs was added to the mobile phase, a well defined peak emerges at 10.6 minutes. The peak was identified by:

(a) comparing the retention data obtained for blueberries samples, the standard and the blueberries spiked with the standards under identical conditions; (b) using the photodiode array detector to continuously measure the pterostilbene UV–visible spectrum while the solute passed through the flow-cell; (c) comparing the information obtained using a fluorimetric detector.

Good agreement was found when the retention time of the pterostilbene peak obtained in the absence (44.6 minutes) and presence (10.6 minutes) of 12 mM HP-β-CDs in the mobile phase for the standard, blueberries sample and the spiked sample were compared. In addition, the absorption spectra obtained for the three samples also showed a good agreement. Fig. 5 *inset* A shows the absorbance spectrum of the pterostilbene found in blueberries samples. Absorption spectrum showed a single absorption band around 300 nm with a bandwidth of 30 nm and two small maxima centred at 304 and 335 nm. Finally, fluorimetric detection confirmed the identity of the compounds since the chromatographic profiles of blueberries and same sample spiked with the standard was identified. Good agreement was obtained for the fluorescence spectra of the chromatographic peak (Fig. 5 *inset* B). The excitation and emission spectra of pterostilbene showed a single excitation band at around 335 nm and a principal emission wavelength at around 420 nm, followed by another lower intensity emission wavelength at 400 nm and a shoulder at 378 nm.

The efficiency of both the extraction and analysis methods was confirmed by performing a recovery study. The samples were spiked at the beginning of the extraction procedure, and then spiked and unspiked samples were treated as described in the Experimental Procedures before being analyzed. Absolute recoveries were evaluated by comparing the concentrations found in blueberries samples spiked with a known amount of pterostilbene standard. These were submitted to the chromatographic procedure developed in this work and the concentrations were obtained using the calibration graphs. When the spike and recovery data were combined, an average recovery ±SD (n=6) of 97.89 ± 1.2% was obtained.

Once the pterostilbene was identified and the absence of a matrix effect had been confirmed, the stilbene in blueberries samples was quantified. Blueberries showed the presence a pterostilbene concentration of 110 ng/g dry sample. This concentration is similar to the obtained by Rimando et al. [13] in rabbiteye blueberry. Moreover, pterostilbene has been detected in fungos-infected grapes at levels of 0.2-4.7 µg/g fresh weight of skin [7] and also in healthy grape berries var. Gamay and Pinot Noir al levels of 14-74 ng/g and 120-530 ng/g fresh berries respectively [8].

These data confirm the validity of the novel RP-HPLC method described which uses cyclodextrins as mobile phase additives to determine pterostilbene in food samples.

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TABLES

Table 1. Comparison of the retention times, type and percentage of solvent organic used to determine pterostilbene in different works

Table 2. Correlation coefficients, limits of detection (LOD) and limits of quantitation (LOQ) for pterostilbene RP-HPLC analysis in the presence and absence of *HP-β-CD*

FIGURE LEGEND

Figure 1. Effect of the water content (%) on the pterostilbene retention time (flow-rate, 1.00 ± 0.01 mL/min, temperature, 25º C ± 1º C). (●) Without HP-β-CD in the mobile phase, (▲) with 8 mM HP-β-CD in the mobile phase. Each data point is the mean of 3 replicates *Inset*: Structure of pterostilbene.

Figure 2. Effect of pH (A) and temperature (B) on the pterostilbene retention time. Each data point is the mean of 3 replicates.

Figure 3. HPLC chromatograms of pterostilbene at different HP-β-CD concentrations: (A) no CD, (B) 1 mM, (C) 2 mM, (D) 4 mM, (E) 8 mM, (F) 12 mM. *Inset*: Effect of HP-β-CD concentration on the pterostilbene retention time.

Figure 4. *(A)* Relative standard deviations (RSD) for the pterostilbene measurements in the presence and absence of HP-β-CD using different detectors: (1) *Rt*, UV-vis, no CD (2) *Rt*, UV-vis, 12mM HP-β-CD, (3) *Rt*, fluorescence, no CD, (4) *Rt*, fluorescence, 12mM HP-β-CD, (5) Area, UV-vis, no CD, (6) Area, UV-vis, 12mM HP-β-CD, (7) Area, fluorescence, no CD, (8) Area, fluorescence, 12mM HP-β-CD.

(B) Effect of total HP-β-CD concentration on the reciprocal of retention factor (*k)* of pterostilbene for determining the stoichiometry of pterostilbene HP-β-CD complexes: $1/k$ versus [HP-β-CD] (assumption of 1:1 complex) (filled circles), (b) $1/(k)$ versus $[\text{HP-}\beta\text{-CD}]^2$ (hypothesis of 1:2 complex) (filled squares). Methanol-water (50:50 %) mobile phase.Each data point is the mean of 3 replicates.

Figure 5. HPLC analysis of blueberries in the absence (II) and presence (I) of 12 mM HP-β-CD concentrations (wavelength of monitoring: 306 nm). *Inset*: (A) Absorption spectra of pterostilbene, (B) Excitation and emission spectra of pterostilbene.

TABLE 1

Type of organic solvent	*% organic solvent*	*Retention time (minutes)*	*Reference*
Methanol	50	10.6	This work
Acetonitrile	5-80	50.0	17
Acetonitrile	10-85	21.2	15
Acetonitrile	60	20.7	12
Acetonitrile	50	15.0	3
Methanol:formic	50	26.3	16

TABLE 2

Detector	***Calibration***		***Detection limits***			
	No HP-β-CD	*12mM HP-β-CD*	*No HP-β-CD*		*12mM HP-β-CD*	
	Corr. Coeff.	*Corr. Coeff.*	*LOD (μM^{-1})*	*LOQ (μM^{-1})*	*LOD (μM^{-1})*	*LOQ (μM^{-1})*
Fluorescence	0.9985	0.9999	3.41	10.84	0.95	2.11
UV-vis	0.9977	0.9992	4.33	13.49	2.35	7.71

FIGURE 1

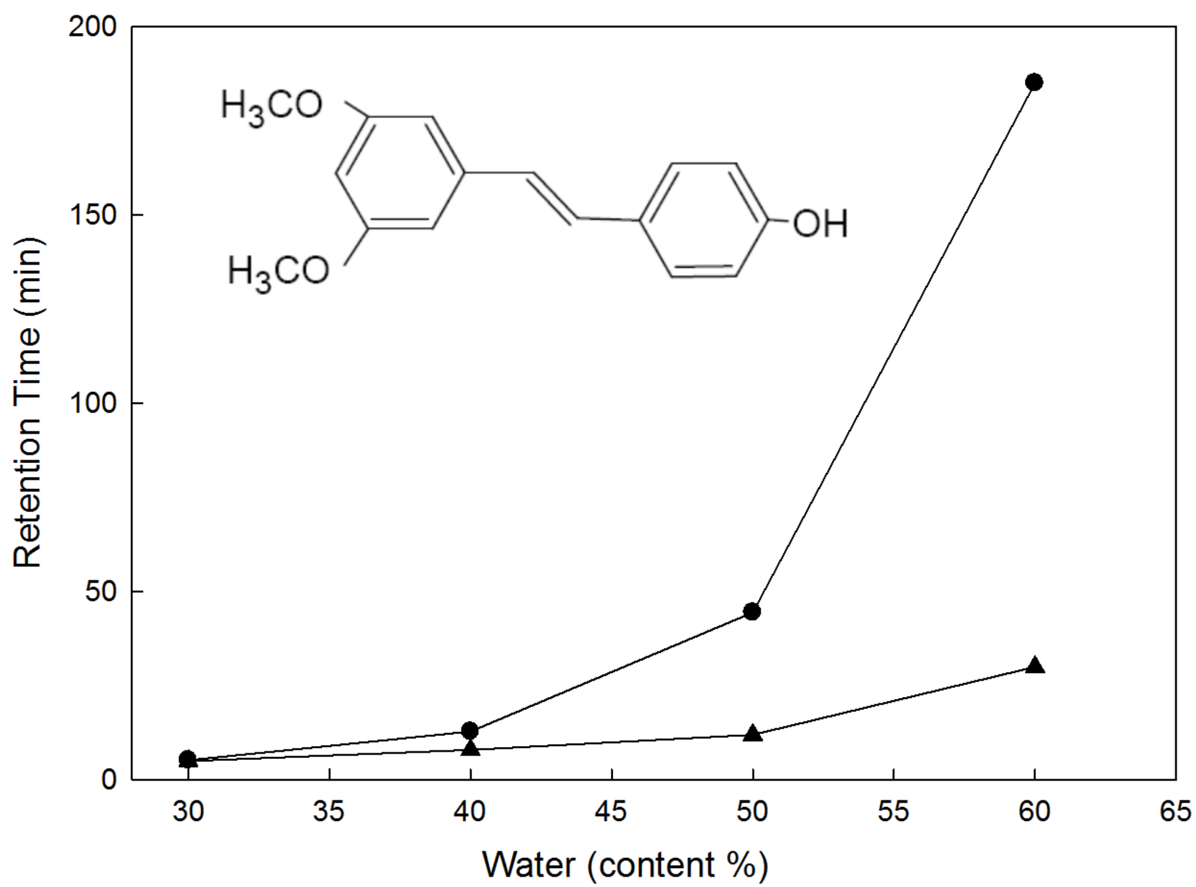


FIGURE 2

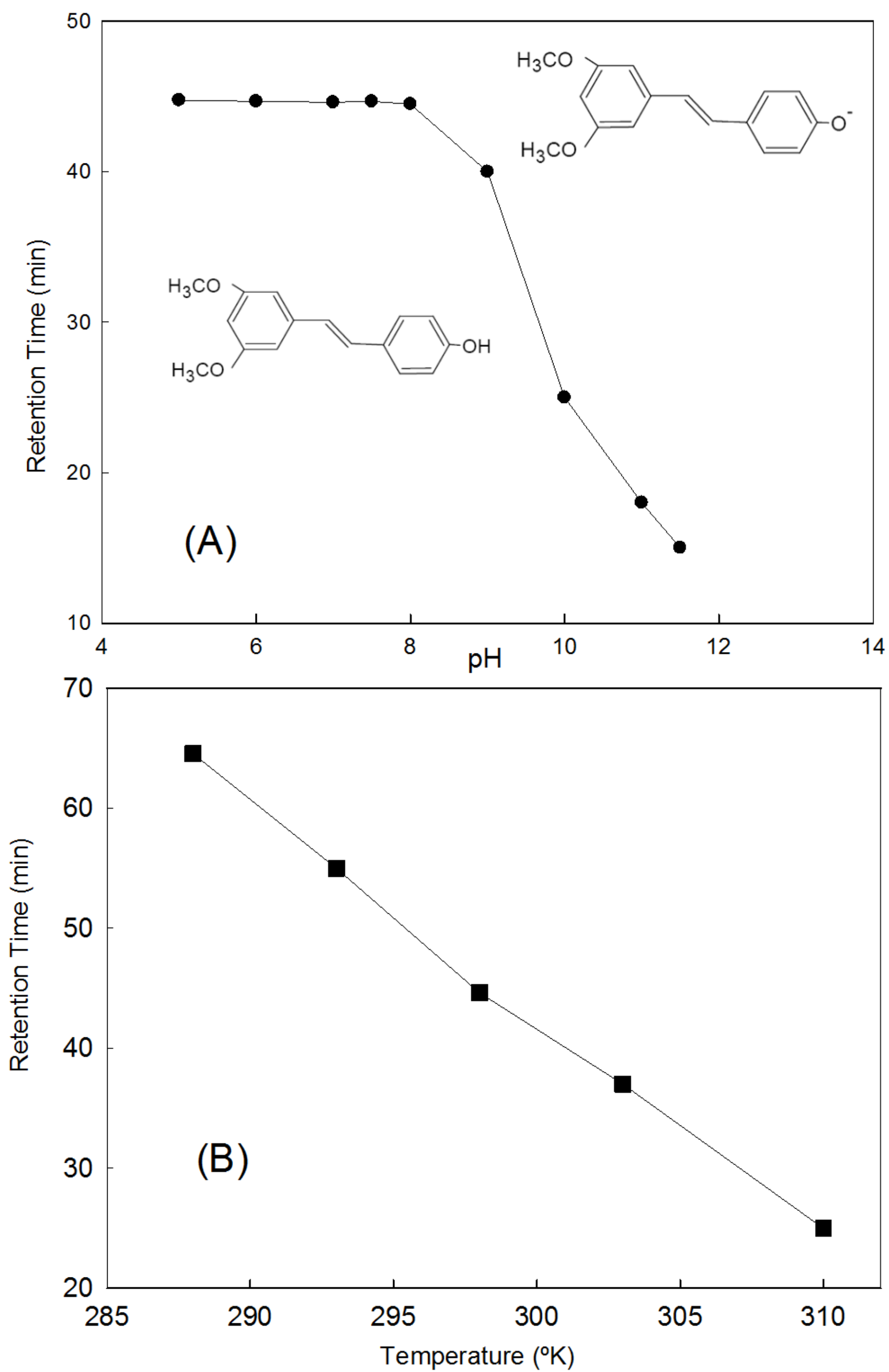


FIGURE 3

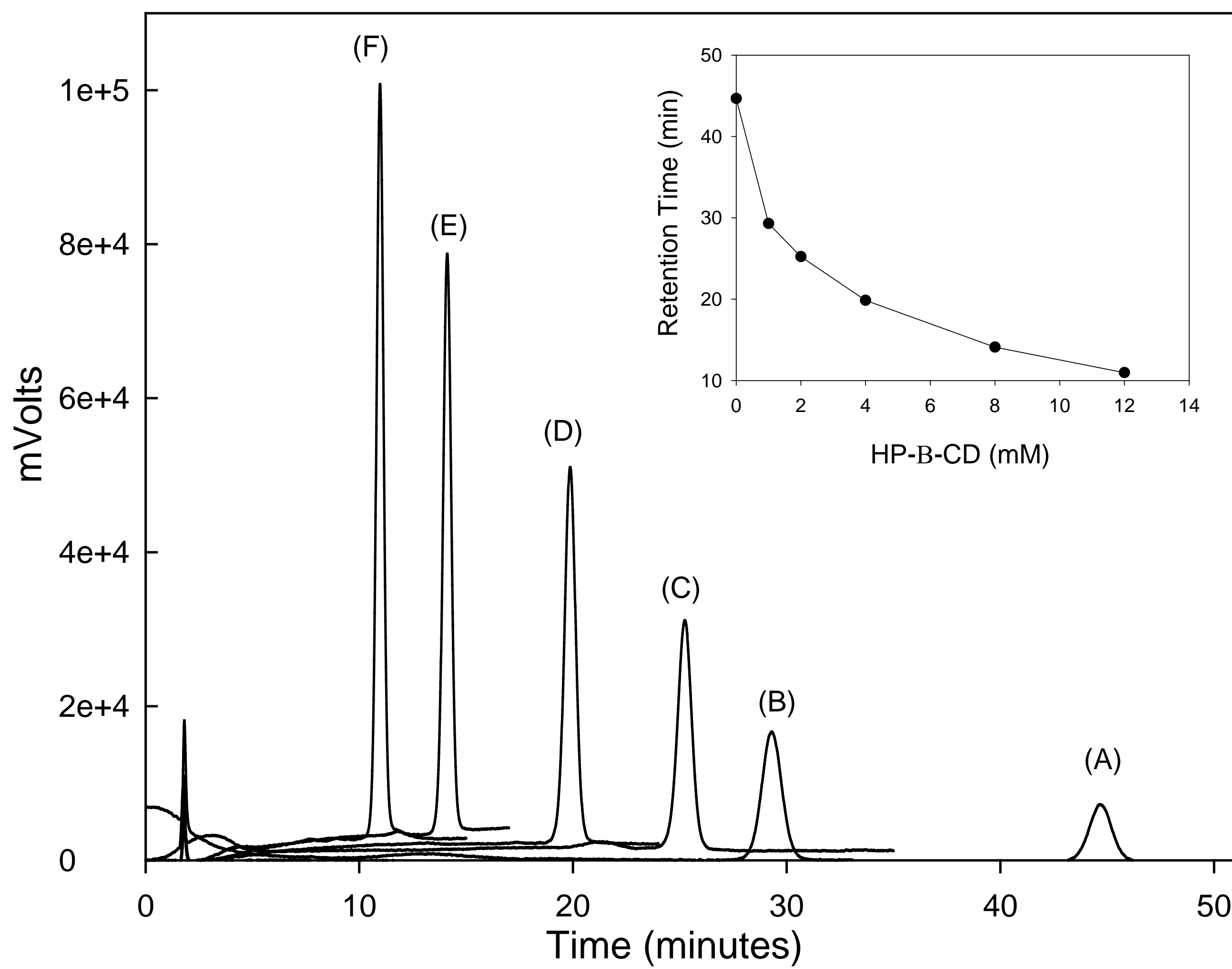


FIGURE 4

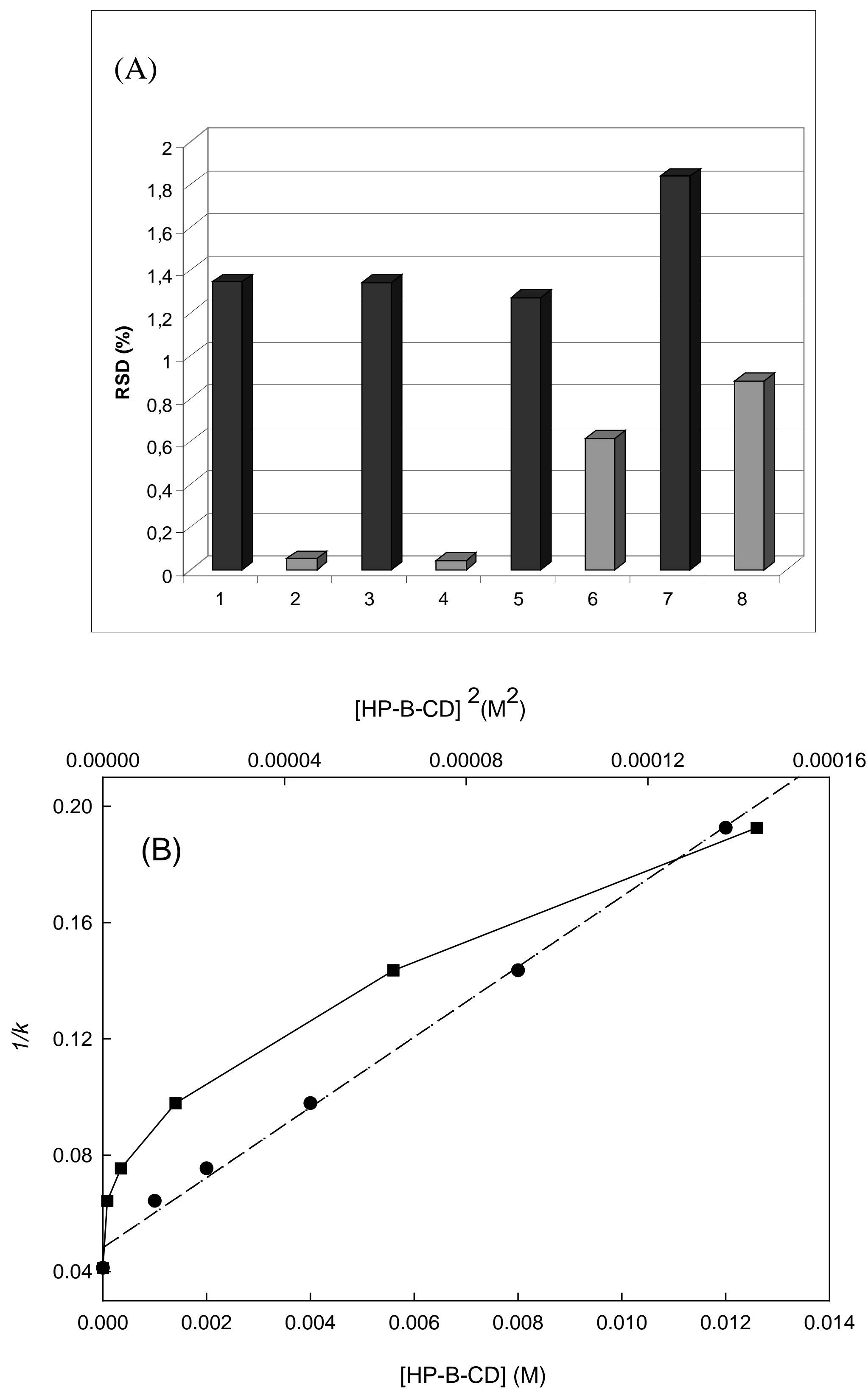
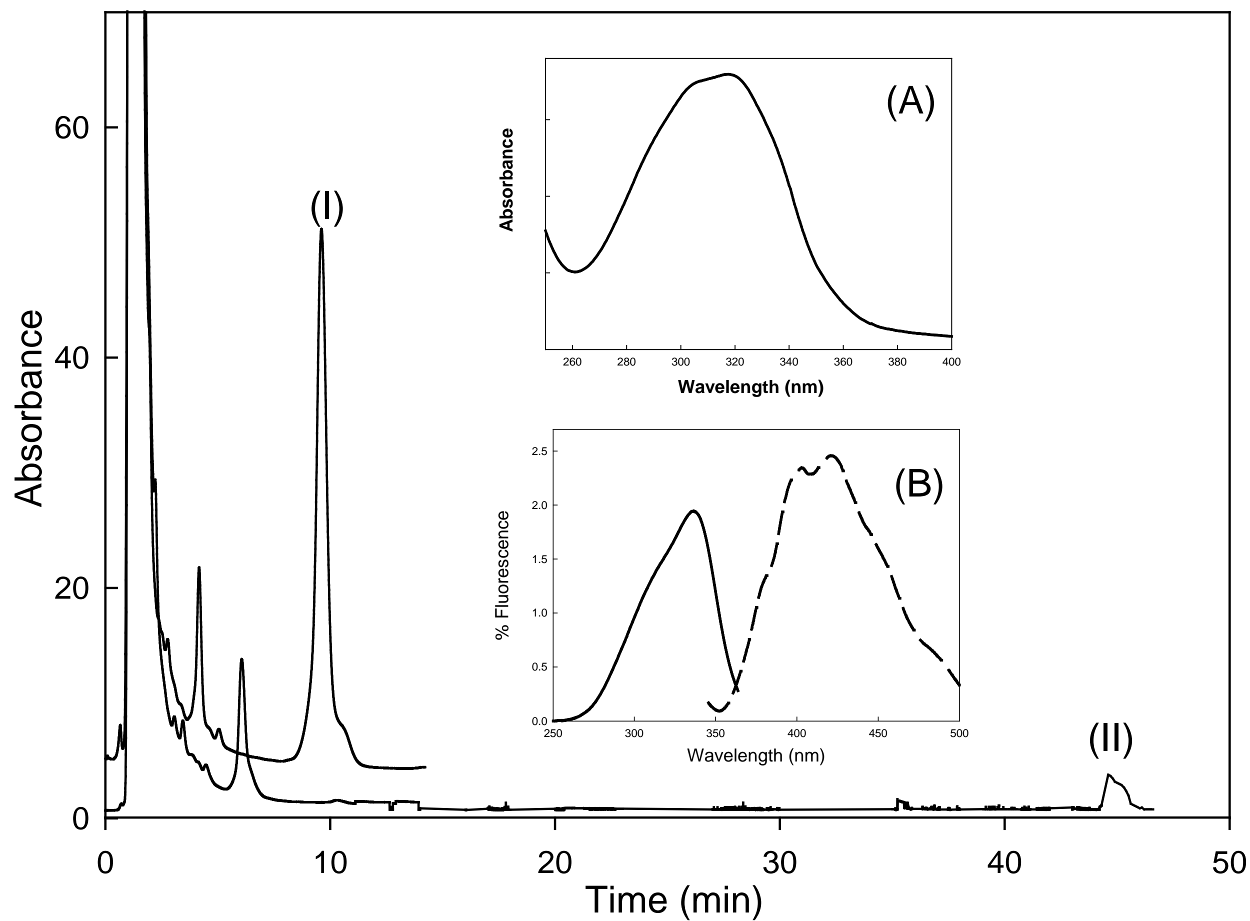


FIGURE 5



Capítulo VI

Estudio de la peroxidación enzimática de pterostilbeno como modelo de detoxificación de fitoalexinas

ABSTRACT

The enzymatic peroxidation of pterostilbene, a strong antifungal belonging to the stilbene family, by peroxidase (POX), is reported for the first time as a model of phytoalexin detoxification carried out by the enzymatic pool of pathogens. Kinetic characterization of the pterostilbene oxidation reaction pointed to an optimum pH of 7.0, at which value the thermal stability of POX was studied. Moreover, the data showed that pterostilbene inhibits POX activity at high concentrations of substrate. Several kinetic parameters, including V_{max}, K_m and K_I, were calculated and values of 0.16 ΔAbs min^{-1}, 14.61 μM, and 31.41 μM were reported. To understand the possible physiological role of this reaction in the phytoalexin detoxification process, the products of pterostilbene oxidation were identified using HPLC-MS and a radical-radical coupling reaction mechanism was proposed. Three main products with a high molecular weight and pronounced hydrophobicity were identified: pterostilbene *cis* dehydromer**,** pterostilbene *trans* dehydromer and pterostilbene open dimer. The dimeric structures of these molecules indicate that the pterostilbene oxidation reaction took place at the 4'-OH position of the hydroxystilbenic moieties and the three above mentioned dimeric products were found, due to the ability of electron-delocalized radicals to couple at various sites. Finally, the capacity of cyclodextrins (CDs) as starch model molecules in plants to complex both the substrate and the products of the oxidation reaction was evaluated. The inhibition process of POX activity was modified at high pterostilbene concentrations due to sequestering of the substrate reaction and to the different affinity of the reaction products for CDs.

1. INTRODUCTION

In response to infection by agents such as fungus, some plants can synthesize different antifungal compounds belonging to the phytoalexins family like resveratrol, piceid or some viniferins (Wilkens et al., 2010). Although a great number of authors have focused on the study of these three compounds belonging to the stilbenes family, the most important phytoalexins, in recent years the importance of pterostilbene (*trans*-3,5-dimethoxy-4′-hydroxystilbene), another stilbene-type phytoalexin, has been reported in different papers as a potent antifungal compound (e.g. Breuil et al., 1999). Pterostilbene, a naturally occurring phytoalexin belonging to a group of phenolic compounds known as stilbenes, has been identified in several plant species such as the heartwood of sandalwood (Sehadri et al., 1972*),* leaves of *Vitis vinifera* (Langcake et al., 1979*)*, peanuts (Medina-Bolivar et al., 2007), infected grape berries of *var*. Chardonnay and Gamay (Adrian et al., 2000), healthy and immature berries of *var*. Pinot Noir and Gamay (Pezet and Pont, 1988) and berries of some *Vacciunium* species (Pezet and Pont, 1988). It also appears to be a constituent of the bark of *Guibourtia tessmanii*, a tree found in central Africa and is commonly used in folk medicine (Fuendjiep et al., 2002). In addition to its antifungal role (Pezet and Pont, 1990; Mazullo et al., 2000), pterostilbene presents other biological activities, including antihyperglycemic (Manickam et al., 1997), antioxidative (Rimando et al., 2002; Katthryn et al., 2006; Remsberg et al., 2008), anticancer (Rimando et al., 2005; Kathryn et al., 2006; Remsberg et al., 2008; Pan et al., 2007; Nanjoo et al., 2007; Ferrer et al., 2005), antiinflammatory (Remsberg et al., 2008), anticholesterol (Mizuno et al., 2008; Mazullo et

al., 2000), hypolipidemic (Mazullo et al., 2000) and analgesic (Mazullo et al., 2000) activities.

It has already been reported that the pathogenicity of fungi like *Botrytis cinerea* or *Trabetes pubescens* is strongly associated with their ability to degrade stilbene phytoalexins such as pterostilbene or resveratrol (Ponzoni et al., 2007). These observations suggest that the enzymes of these pathogens attack the phytoalexins which represent the first line of the defense of the plant. Indeed, this type of fungus has an enzymatic pool able, for example, to lyse cell walls or to oxidize polyphenols (Gil-ad et al., 2000). Subsequently, direct evidence was obtained that resveratrol and pterostilbene can undergo degradation by enzymes such as the laccase produced by *B. cinerea* (Sbaghi et al., 1996).

Although most works published concerning the detoxification of phytoalexins by the enzymes present in *B. cinerea* only focus on the role of laccase-like stilbene oxidase produced by this fungus (Breuil et al., 1999; Ponzoni et al., 2007; Gil-ad et al., 2000), Gil-ad et al. (2000) showed that *B. cinerea* has a peroxidase (POX) (E.C. 1.11.1.7) enzymatic activity not reported previously. However, no work has reported, as is done for first time in this paper, on whether the enzymatic peroxidation of pterostilbene is involved in the detoxification of phytoalexins. Thus, although POX catalyzes the oxidation of a wide variety of substrates, the potential oxidation of pterostilbene by this oxidative enzyme as a model of phytoalexin degradation by *B. cinerea* has not been reported.

As occurs with the detoxification of other phytoalexins carried out by laccase (Ponzoni et al., 2007; Sbaghi et al., 1996; Pezet, 1998; Breuil et al., 1998, 1999), a knowledge of both the mechanism by which POX acts on pterostilbene and the nature of

the oxidation reaction products, is essential for understanding the possible physiological role of this reaction in the phytoalexin detoxification process. Indeed, metabolism of phytoalexins by *B. cinerea* or *T. pubescens* may manifest itself as an insolubilization of the products formed when different substrates are used. The fungus could, therefore, avoid the action of phytoalexins such as pterostilbene by transforming these compounds into other products of increasing molecular weight and hydrophobicity. Moreover, in the case of another phytoalexin-type stilbenes such as resveratrol, Dercks and Creasy (1989) stated that e-viniferin, a dimeric form of resveratrol analogous to the dimer produced by *B. cinerea* (Breuil et al., 1998), is less stable (soluble) in water than resveratrol itself, its biological activity against *B. cinerea* being lower. Similar results were found when pterostilbene was incubated in the presence of laccase from *B. cinerea*. These observations demonstrate the interest of indentifying the reaction products and underline the involvement of the enzyme-mediated oxidation of stilbenes in the pathogen-plant interaction.

Bearing the above in mind, and in order to study the possible role of POX in the detoxification of antigungal compounds and, consequently, in the pathogenesis of the organisms which contain POX activity, the four main objectives of this work were to: i) to determine the enzymatic kinetic parameters of the oxidation of pterostilbene by POX, ii) to identify the oxidation reaction products, iii) to propose a reaction mechanism to justify the results obtained, iv) to evaluate the use of cyclodextrins (CDs) as modulators of both the enzymatic activity and the oxidation products.

To perform the study, UV-VIS method which makes use of changes in the spectrophotometric parameters of pterostilbene in the presence of POX and an HPLC-MS tandem to identify the oxidation products in the absence or presence of CDs were used.

Moreover, horseradish POX, the most studied POX type, was used as an enzyme model for oxidising pterostilbene.

2. RESULTS AND DISCUSSION

2.1. Scanning spectrophotometric studies of the oxidation of pterostilbene by POX

Although many studies have focused on POX and its capacity to metabolize new substrates (O´Brien, 2000), no work has reported on the potential oxidation of the potent antifungal pterostilbene by this enzyme. For this reason, the first step in this investigation was to monitor the possible oxidation of pterostilbene by POX, observing the changes that occur in the UV spectrum of this phytoalexin with time.

The pterostilbene spectrum shows a single absorption band at around 300 nm with a bandwidth of 20 nm and two small maxima centred at 304 and 316 nm (Fig. 1). The changes in absorbance of a reaction medium when pterostilbene was incubated with POX and H_2O_2 pointed to maximal spectral changes in the 250 to 400 nm region, with a decrease in pterostilbene absorbance at 310 nm and an increase at 360 nm. Since the spectral changes observed were not detectable in the absence of H_2O_2, or in the absence of enzyme (data not shown), they were considered to be the result of POX activity. In addition, the dependence of these spectral changes on enzyme concentration confirms that the changes in the UV spectrum are a reliable measure of the enzymatic oxidation rate.

Finally, the nature of the spectral changes of pterostilbene with time at 250 to 400 nm (Fig. 1) indicated that the formation of pterostilbene oxidation products was proportional to time during the first 11 min of the reaction. Moreover, the presence of two isosbestic points at 268 nm and 342 nm in the consecutive spectra of the reaction medium suggests two things: i) the presence of one oxidation product formed during the course of pterostilbene oxidation by POX or ii) the presence of several oxidation products with a constant stoichiometry during the time of the reaction. As will be demonstrated in

following sections, the oxidation of pterostilbene by POX provides a mixture of products with a constant stoichiometry during the reaction.

2.2. Determination of the optimum physico-chemical conditions for studying the oxidation of pterostilbene by peroxidase

To ascertain the best physico-chemical conditions for kinetically characterizing the oxidation of pterostilbene by POX, the next step of this investigation was to study both the optimum pH and the thermal stability of the reaction. Reports in the literature describe how the optimum pH of any POX depends on different factors, such as the H-donor in the activity assay (Halpin et al., 1989), the substrate used (Chisari et al., 2007, 2008) or the POX source (Duarte-Vázquez et al., 2000). In the present study, the influence of the incubation medium pH on the rate of pterostilbene oxidation by POX is demonstrated (Fig. 2A). It was concluded that the rate of oxidation is strongly pH-dependent. As can be seen, the enzymatic activity showed an optimum at pH 7.0, above which it fell sharply. The pH profile depicted in Fig. 2A, with an optimum at pH 7.0 and a strong decrease in the region of basic pH, agrees with the observations of several authors (O´Brien, 2000). For these reasons, the optimum pH value selected for studying the oxidation of pterostilbene by POX was 7.0.

To continue the identification of the optimum physico-chemical parameters for studying the oxidation of pterostilbene by POX, the next step was to evaluate the thermal stability of POX at increasing temperatures from 15 to 90 ºC. For this, the enzyme solutions were incubated at ten temperatures for 40 min and, after heating, the samples were assayed

at 25 ºC. As can be seen in Fig. 2B, a strong dependence of the oxidation rate of pterostilbene by POX on the incubation temperature was observed, being stable between 15 and 50 ºC and strongly decreasing above 50 ºC. As a consequence of these results, 25 ºC was selected as the optimum temperature for the next sections.

Finally, as shown in Fig. 3A, pterostilbene oxidation was also seen to be dependent on the concentration of H_2O_2, being inhibited at high concentrations. These results are in good agreement with those described for asparagus and turnip peroxidases (Duarte-Vázquez et al., 2000; Rodrigo et al., 1996), and so a low H_2O_2 concentration of 114 µM was selected for the next steps of the investigation.

2.3. Study of the inhibition of POX activity at high pterostilbene concentrations

The results obtained in the previous sections show that the optimum reaction conditions for characterizing the oxidation of pterostilbene by POX are as follows: (i) pH: 7.0, (ii) temperature: 25 ºC, and (iii) H_2O_2 concentration of 114 µM. These conditions were selected to calculate the main kinetic parameters of the enzyme catalyzed-reaction, evaluating the changes in the oxidation rate with changes in pterostilbene concentration.

As can see in Fig. 3B, the dependence of the pterostilbene oxidation rate on a variation in the pterostilbene concentration was complex. Thus, although this parameter showed a Michaelis-Menten type kinetic at pterostilbene concentrations of less than 27 µM, it showed strong inhibition at higher concentrations. For this reason, equation 1, which calculates the main kinetic parameters of an enzymatic reaction when an inhibition of the oxidation rate at high concentrations of substrate is observed, was used.

$$v = \frac{v_{max}[S]}{\left(K_m + [S]*\left(1+ {[S]}/{K_I}\right)\right)} \qquad \text{(eq. 1)}$$

where V_{max} is the maximum enzyme velocity, K_m is the Michaelis-Menten constant and K_I is the constant for substrate inhibition.

Fitting the experimental data to equation 1, the kinetic constants V_{max}, K_m and K_I were calculated and values of 0.16 ΔAbs min^{-1}, 14.61 μM, and 31.41 μM were obtained.

2.4. Characterization of the reaction products of the oxidation of pterostilbene by peroxidase

Characterization of the pterostilbene metabolites allowed us to understand more about phytoalexin metabolism by different pathogens with POX activity and the relation with fungal pathogenicity. As mentioned above, the production of phytoalexins is considered to form part of a general defence mechanism of plants. In this species, such a response includes the formation, mediated by laccase enzyme, of a range of biosynthetically related di- and oligomers of different stilbenes. For this reason, the next step of our investigation was to identify the products of pterostilbene oxidation by POX.

The products formed upon incubation of pterostilbene with POX at pH 7.0 were analyzed by HPLC-MS. As shown in Fig. 4, the HPLC chromatogram resulted in the presence of three reaction products (peaks A,B,C).

The mass analysis of a sample of this less polar product (Fig. 4A inset) showed a quasi-molecular ion of 511 Da and a molecular formula $C_{32}H_{30}O_6$. Identical results were reported for the oxidation of pterostilbene by *B. cinerea* laccase (Breuil et al., 1999) and for the incubation of pterostilbene with the *T. pubescens* laccase (Ponzoni et al., 2007). For this reason, peak A shown in Fig. 4 is consistent with the structure of a pterostilbene *trans* dehydromer presented in the Fig. 5A. Moreover, mass analysis of peak B (Fig. 4) showed an identical quasi-molecular ion of 511 Da and a molecular formula $C_{32}H_{30}O_6$. According to the results reported by Breuil et al. (1999) for the oxidation of pterostilbene by laccase, this fact would be due to the existence of a photochemical isomerisation of the product presented in Fig. 5B, which is a typical reaction for *trans*-stilbenes. For this reason, although pterostilbene degradation to a major compound that was in the *trans* ethylenic form (Fig. 5A), the HPLC chromatogram shown in Fig. 4 also presented (as secondary product) a pterostilbene *cis* dehydromer (Fig. 5B) with an identical molecular ion and molecular formula.

To confirm that the structures of peaks A and B correspond to pterostilbene *trans* dehydromer and pterostilbene *cis* dehydromer respectively, the UV spectrum from 240 to 360 nm of these molecules are showed (Fig. 6). As can be seen in Figure 6A, the UV spectrum of pterostilbene *trans* dehydromer presents a single absorption band around 300 nm with a bandwidth of 20 nm and two small maxima at 309 and 325 nm. This type of spectrum is characteristic of the molecules belonging to the stilbene family which present a *trans* configuration as it is cited in different papers (Abert Vian et al., 2005; López-Nicolás et al., 2009a). However, the UV spectrum of molecules belonging to the stilbene family which present a *cis* configuration shows a single absorption band around 285 nm but with a

bandwidth of few nm (Abert Vian et al., 2005) identical to the spectrum presented in Figure 6B. These data confirm again the structures of pterostilbene *trans* dehydromer and pterostilbene *cis* dehydromer.

Finally, the HPLC study of the oxidation of pterostilbene by POX showed a third peak (C). The mass spectrum of the most polar product had a quasi-molecular ion of 529 Da (Fig. 4C inset), that is 18 amu more than the value obtained with the other products, and $C_{32}H_{31}O_7$ the formula molecular. This product was identified as an pterostilbene open dimer with two 3,5-dimethoxybenzoyl moieties (Fig. 5C) by Ponzoni et al. (2007) during the oxidation of pterostilbene by *T. pubescens* lacase.

2.5. Reaction mechanism of the oxidation of pterostilbene by peroxidase

On the basis of the results obtained, a mechanism for the synthesis of the different dimeric derivatives can be proposed. As stated above, phenols are among the most suitable POX substrates (O'Brien, 2000). Oxidations proceed via the formation of radical cations and the subsequent deprotonation of the phenolic hydroxy groups to give phenoxy radicals, which can undergo a broad variety of coupling reactions (Ponzoni et al., 2007). The main drawback of these biotransformations is the extensive polymerization that may occurs due to the radical mechanism of the oxidative process, which may frequently produce a complex mixture of polyphenolic oligomers.

As regards our investigation, the dimeric structures indicate that the pterostilbene oxidation reaction took place at the 4’-OH (4-OH) position of the hydroxystilbenic moieties. Due to the capability of electron-delocalized radicals to couple at various sites,

different dimeric products were found, depending on the structural features of the starting substrates. In turn, the phenoxy radicals formed could delocalize as reported in Fig. 7.

Successively, the coupling of one radical “B” and one radical “C”, followed by tautomeric rearrangement and intramolecular nucleophilic attack on the intermediate quinone, produced both *cis* and *trans* dihydrofuran pterostilbene dehydromers identified as the two least polar isomeric products in the HPLC chromatogram shown in Fig. 4 (peaks A and B).

In turn, the formation of the “open” hydroxylated (Fig. 4. peak C), the most polar of the reaction products, can be easily explained by the coupling of one radical “A” with one radical “C”, followed by addition of a water molecule to the intermediate quinones**.**

The results of our experiments clearly supports our hypothesis that the reaction proceeded through radical-radical coupling and not through radical addition to the double bond of a non-radical substrate. These results are in accordance with most of the literature reports (Ponzoni et al., 2007) and not with the hypothesis suggested by Szewczuk et al. (2005), who proposed a mechanism in which the another stilbene-type molecules dimerization occurs via radical attack on a second molecule of stilbene.

2.6. Effect of the presence of cyclodextrins on the rate of pterostilbene oxidation by peroxidase

Different studies have demonstrated that cyclodextrins (CDs) can modify the enzymatic activity of an oxidorreductase enzyme, such as LOX, when a stilbene is used as substrate (López-Nicolás et al., 2009a). As regards the physiological role in plants, several

works have reported that the oxidation of some antioxidant compounds forming inclusion complexes with CD by oxidative enzymes can be regarded as a model system of their oxidation in storage tissues complexed with starch (López-Nicolás et al., 1997). For these reasons, and in order to evaluate the effect of CDs on the structure and availability of this potent antifungal compound in the enzymatic reaction, the next step was to evaluate the response of POX to the presence of CDs in the reaction medium.

CDs are torus-shaped oligosaccharides made up of 6–8 glucopyranose units and originated by the enzymatic degradation of starch through the action of CD-glucano-transferase (Szente and Szejtli, 2004). Poorly water-soluble compounds and hydrophobic moieties of amphiphilic molecules interact non-covalently with the CD cavity to form so-called inclusion complexes, which are also highly water-soluble. Several publications have reported the aggregation behaviour of different enzymatic substrates such as fatty acids, phenols, stilbenes, etc, in the presence of CD, and evidence has been presented concerning the formation of guest/CD inclusion complexes with several stoichiometries (López-Nicolás et al., 1997, 2009a, b, c). Indeed, our group recently reported the formation of pterostilbene/CD complexes using fluorescence techniques (López-Nicolás et al., 2009b).

Fig. 8A shows the Hydroxy-propyl-β-CD dependence of POX at pH 7.0 when three pterostilbene concentrations were used. As can be seen, the addition of increasing concentrations of HP-β-CD produced a change in the POX enzymatic activity that depended on the pterostilbene concentration used. In the presence of the 24 μM pterostilbene in the reaction medium, the POX activity decreased when HP-β-CD concentration was increased (Fig. 8A filled circle). This typical behaviour is due to the ability of both natural and modified CDs to sequester part of the pterostilbene to form

soluble inclusion complexes (López-Nicolás et al., 2009b), thereby reducing the concentration of the free pterostilbene. Thus, CDs act as substrate reservoir in a dose-dependent manner. Indeed, free pterostilbene is the only effective substrate and the oxidation of the complexed substrate requires the prior dissociation of the complex.

Moreover, when a pterostilbene concentration of 47 µM was used, the POX activity practically remained constant when HP-β-CD concentrations below of 0.5 mM were tested. However, when the HP-β-CD concentration was above 0.5 mM, the POX activity decreased, as occurred at the pterostilbene concentration of 24 µM (Fig. 8A filled squares).

Finally, Fig. 8A shows that when a high substrate concentration of 140 µM was used, increasing HP-β-CD concentrations up to 1 mM produced a sharp increase in POX activity, which decreased again when the concentration of the complexation agent exceeded 1 mM (Fig. 8A filled triangles).

Since of this different pterostilbene concentration behaviour of POX activity in the presence of HP-β-CD has not been previously reported, we studied the possible effect of CDs on the inhibition of POX activity by the high pterostilbene concentrations evaluated in previous sections.

In Fig. 8B it can be seen that when increasing HP-β-CD concentrations (0, 0.5 and 1 mM) were added to the reaction medium, the previously observed inhibition by substrate excess occurred at a higher pterostilbene concentration than observed in the absence of any agent**.** Thus, the concentration of pterostilbene at which the POX activity was inhibited in the absence of HP-β-CD increased in its presence, reflecting the formation of inclusion complexes and extending the range in which the pterostilbene concentration does not

produce inhibition of the enzymatic activity. These results explain the different behaviour of POX activity dependent of pterostilbene concentration shown in Fig. 8A.

2.7. Effect of the presence of cyclodextrins on the stoichiometry of the pterostilbene oxidation products by POX

As shown in the previous sections, the oxidation of pterostilbene by POX produced three main products, which were identified as pterostilbene *cis* dehydromer**,** pterostilbene *trans* dehydromer and pterostilbene open dimer**.** Although CDs have usually been used for complexing the substrates of several enzymatic reactions, due to the importance of the pterostilbene oxidation products in the detoxification of phytoalexins, we studied the potential effect of the addition of CDs on the three main products of the oxidation of pterostilbene by POX.

Although in both the absence and presence of HP-β-CD, the sum of the three reaction products concentration remained constant (Fig. 9 filled diamonds), the presence of increasing concentrations of HP-β-CD had different effects on the concentration of the three reaction products determined by HPLC. For example, the area of the pterostilbene *trans* dehydromer decreased when increasing concentrations of HP-β-CD were added to the reaction medium (Fig. 9 filled circles), pointing to a significant interaction between this main reaction product and HP-β-CD. Moreover, the area of pterostilbene *cis* dehydromer increased (Fig. 9 filled squares) with increasing HP-β-CD concentrations due to the capacity of HP-β-CD to modify the isomerisation equilibrium shown in Fig. 10. Thus, pterostilbene *cis* dehydromer presents higher affinity for HP-β-CD than the observed for

pterostilbene *trans* dehydromer, and so a higher complexation constant ($K_{cis}>K_{trans}$). Finally, no significant variation in the area of pterostilbene *open* dimer was found when HP-β-CD was added to the reaction medium (Fig. 9 filled triangles), showing that this third pterostilbene oxidation product by POX can not been complexed by HP-β-CD.

Although further investigations are necessary to determine the biological activity of the three pterostilbene oxidation products identified in this work, the fact that HP-β-CDs, complexant agents defined as starch model molecules in plants (López-Nicolás et al., 1997), can form inclusion complex with both pterostilbene *cis* dehydromer and pterostilbene *trans* dehydromer pterostilbene may be used in the detoxification process of stilbene–type phytoalexins. As mentioned above, an increase in the solubility of the formed products is related with the detoxification process. Thus, metabolism of phytoalexins by the enzymes present in pathogens may manifest itself as an insolubization of the products formed. Bearing in mind that one of the most important properties of CDs is it ability for increasing the solubility of the guest molecule complexed (Szente and Szejtli, 2004), the inclusion complexes formed by the interaction between HP-β-CD and both the pterostilbene *trans* dehydromer pterostilbene and *cis* dehydromer products obtained by the oxidation of pterostilbene by POX may show higher solubility than these molecules in the absence of HP-β-CD and slow down the detoxification process thus increasing the defence mechanisms of the plant against fungal attack.

3. EXPERIMENTAL

3.1. Materials

Biochemicals were purchased from Fluka (Madrid, Spain). Pterostilbene was from Sequoia Research Products Limited (Pangbourne, United Kingdom) and was used without further purification. HP-β-CD was purchased from Sigma (Madrid, Spain). Hydrogen peroxide and POX type II from horsedish were obtained from Sigma (Madrid, Spain). Pterostilbene is sensitive to light, and intense irradiation of solutions of the analyte induces the formation of a highly fluorescent compound if the irradiation is intense. Because of this, the samples were stored in darkness. The hydrogen peroxide, POX and pterostilbene were freshly prepared every day.

3.2. Enzyme assay

POX activity was followed spectrophotometrically in a Jasco V-650 spectrometer (Applied Photophysics Ltd.) at 25 °C equipped with thermostated cells at the absorption maximum of the oxidation product of pterostilbene. The reaction was started by adding the indicated volume of enzyme to a standard reaction medium which contained the indicated concentration of pterostilbene, H_2O_2 and CDs.

3.3. pH studies

To study the effect of pH on the oxidation of pterostilbene by POX, several buffers were used: 0.1 M sodium acetate from pH 4.0 to pH 5.5, 0.1 M sodium phosphate from pH 5.5 to pH 8.5 and 0.1 M sodium borate from pH 8.5 to pH 10.0.

3.4. Thermal stability

The enzyme solutions (in Eppendorf tubes) were incubated in a circulating water bath Julabo Shake Temp SW 22 at ten temperatures (15 ºC, 20 ºC, 25 ºC, 30 ºC, 40 ºC, 50 ºC, 60 ºC, 70 ºC, 80 ºC and 90 ºC ± 1 ºC) for 40 min. After heating, the samples were cooled in ice water and assayed immediately at 25 ºC.

3.5. HPLC-MS analysis of pterostilbene oxidation products

For product analysis, 3 ml of a 100 µM solution of pterostilbene in 140 µM phosphate buffer (pH 7.0) was incubated with 100 µl enzyme extract at 4 °C and under constant aeration. After 30 min, the reaction was stopped by acidification to pH 4.0 with HCl, and the products were directly injected. These compounds were analyzed with an HPLC-MS AGILENT VL on a Kromasil C18 column (5 µm, 250 mm x 0.4) and detected at 306 nm. Products were eluted isocratically with methanol/water (80:20 v/v) at a flow rate of 0.7 ml/min. The ratio between the concentrations of the three reaction products was calculated from the peak areas. Parameters for mass spectrometric analysis: mass range mode: Std/normal; ion polarity: positive; ion source type: ESI; dry temperature: 350 ºC; nebulizer: 60.00 psi; dry gas: 9.00 l/min. HR-ESI-MS technique was used for calculating the molecular formula of pterostilbene oxidation products.

ACKNOWLEDGMENTS

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FIGURE 1

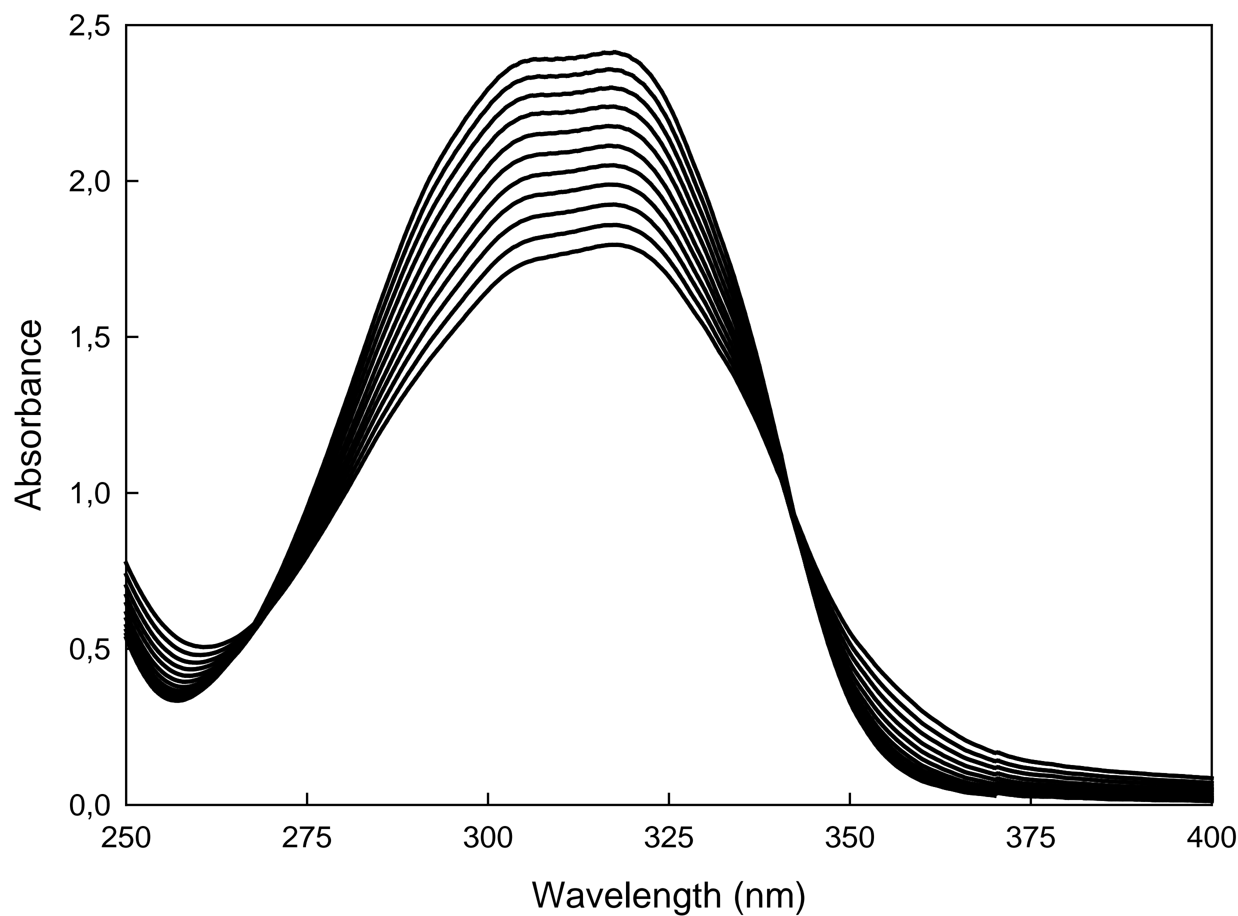


Figure 1. Evolution of the spectrum of the peroxidation of pterostilbene by POX in the presence of H_2O_2. The reaction medium at 25 °C contained 20 µM of pterostilbene in the presence of 114 µM H_2O_2 at pH 7.0. The scans were carried out every 0.5 min.

FIGURE 2

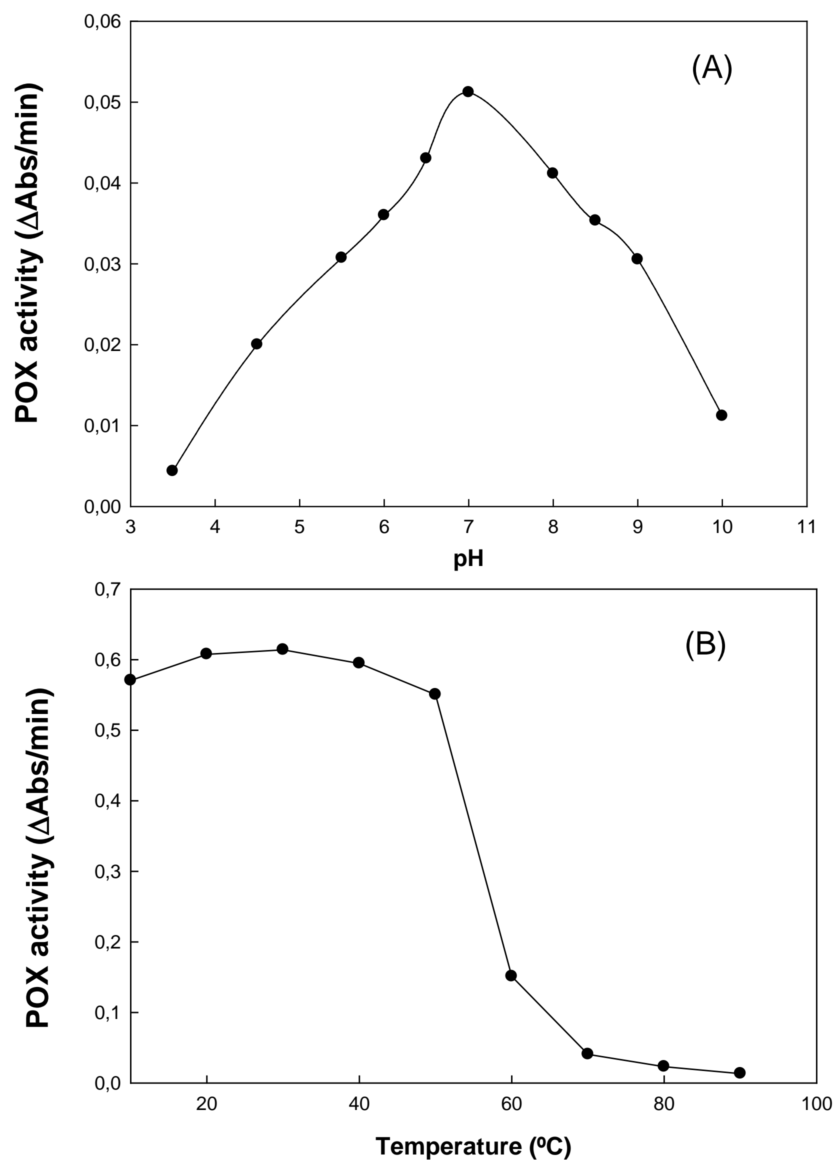


Figure 2. (A) Effect of pH on the peroxidation of pterostilbene by POX. The reaction medium consisted of 8 µM pterostilbene, 114 µM H_2O_2 at different pH values at 25 ºC. (B) Thermal stability of POX on pterostilbene at different temperatures for 40 min. The reaction medium consisted of 8 µM pterostilbene, 114 µM H_2O_2 at pH 7.0.

FIGURE 3

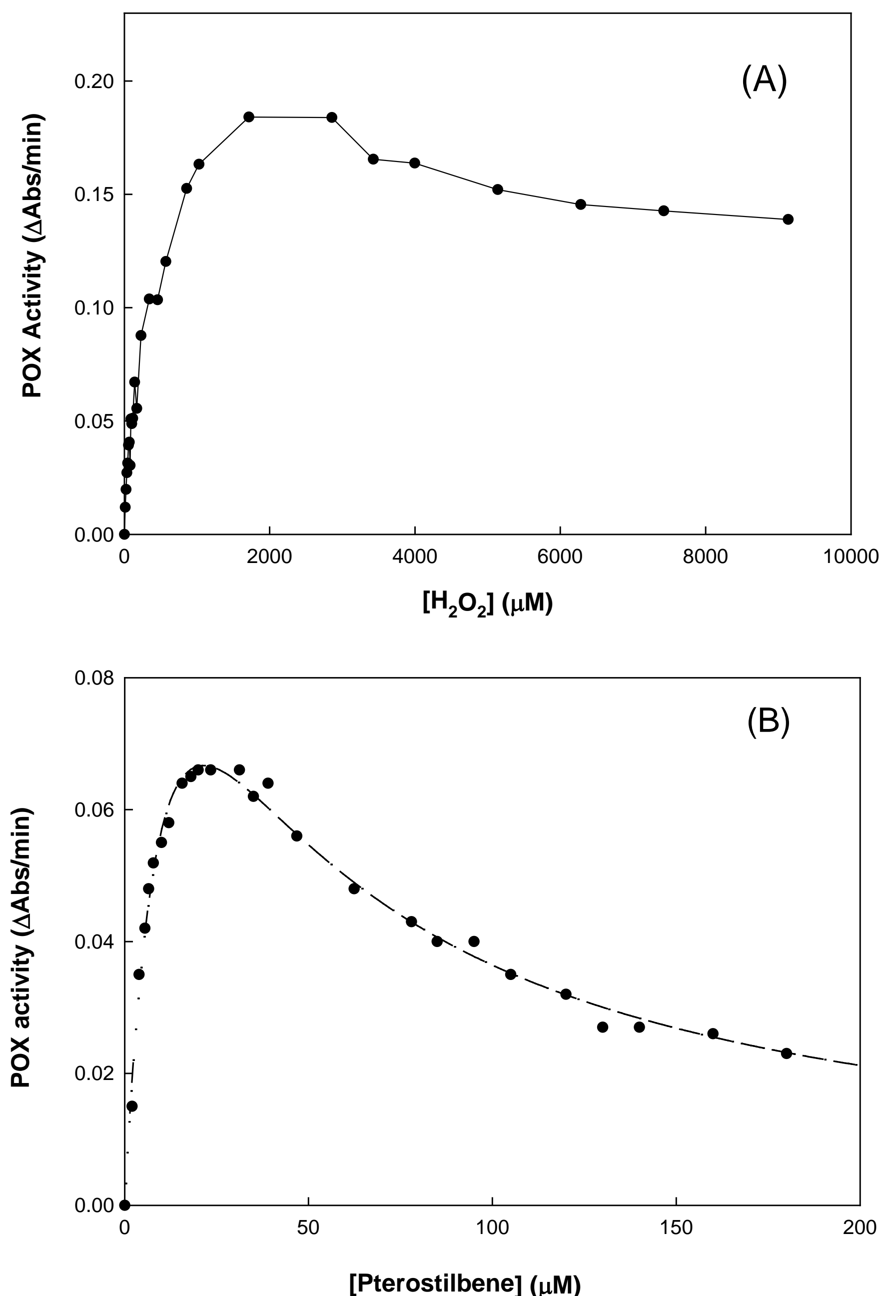


Figure 3. (A) Effect of H_2O_2 concentration on the peroxidation of perostilbene by POX. The reaction medium at 25 ºC contained 0.1 M phosphate buffer pH 7.0, 8 µM pterostilbene and increasing concentrations of H_2O_2 from 0 to 10 mM. (B) Effect of pterostilbene concentration on POX activity in the presence of 114 µM H_2O_2 at pH 7.0.

FIGURE 4.

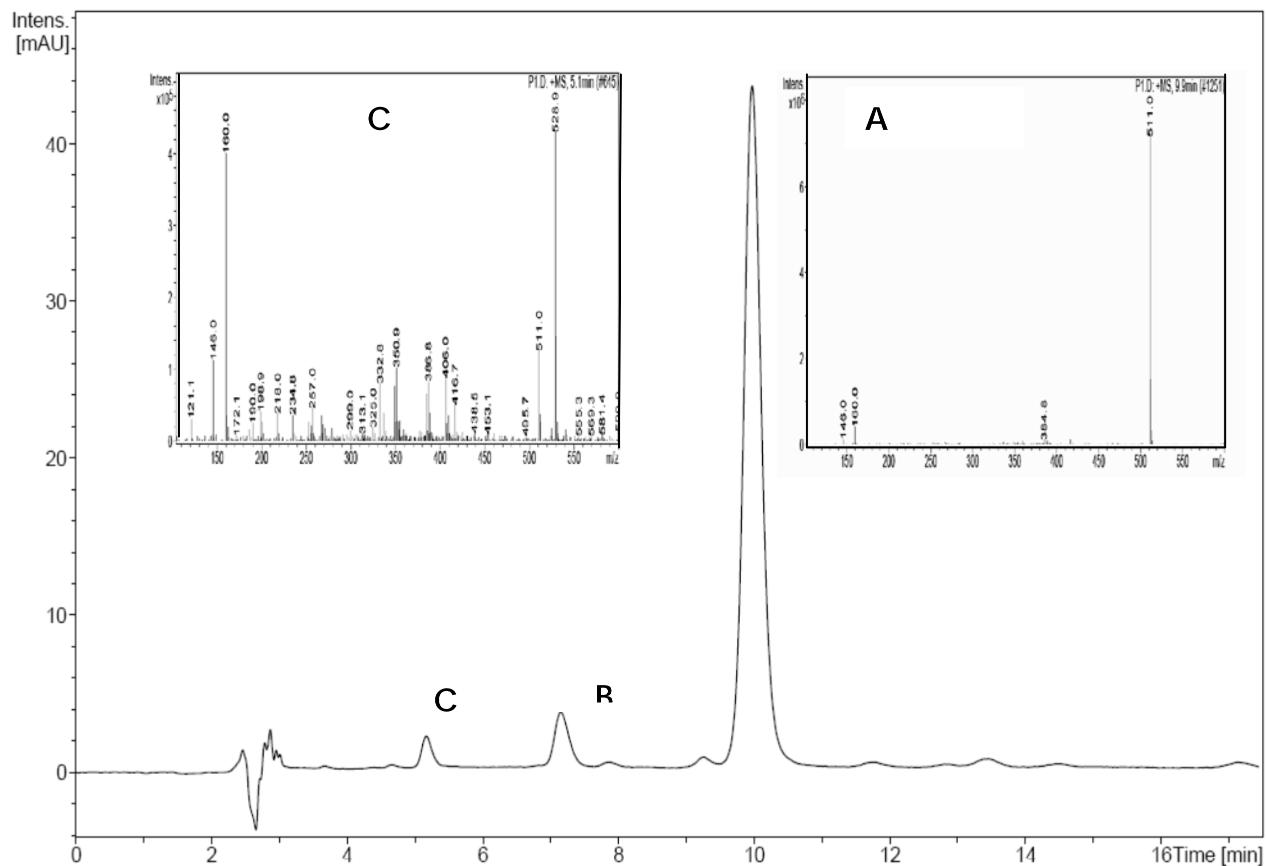
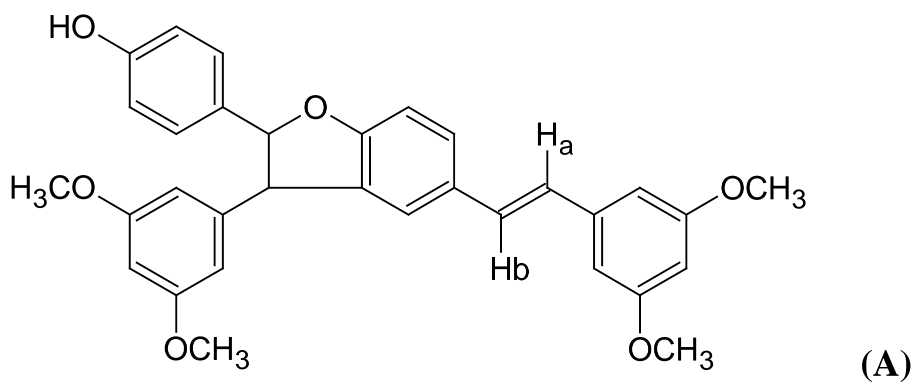
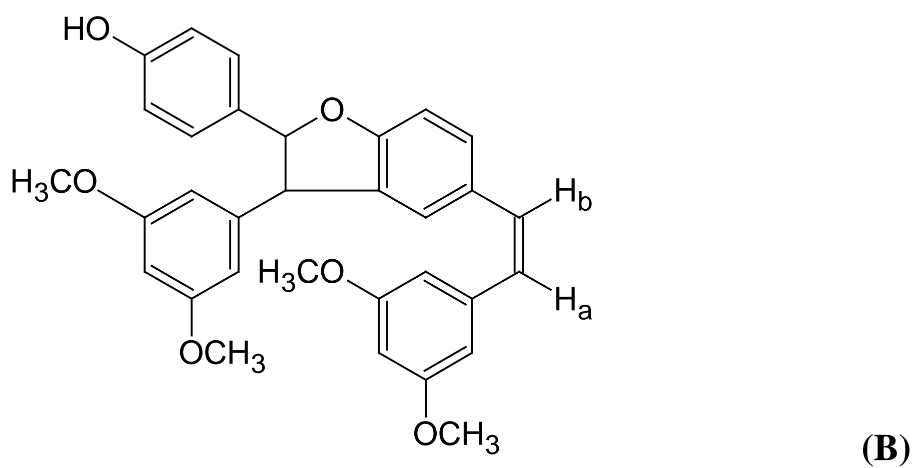


Figure 4. Reversed-phase HPLC analysis of pterostilbene oxidation products by POX. (A), *trans* dehydromer pterostilbene; (B) *cis* dehydromer pterostilbene and (C) pterostilbene open dimer. The eluent was methanol/water (80:20 v/v)**.** ***Inset*:** Mass spectrum of A and C pterostilbene oxidation products.

FIGURE 5





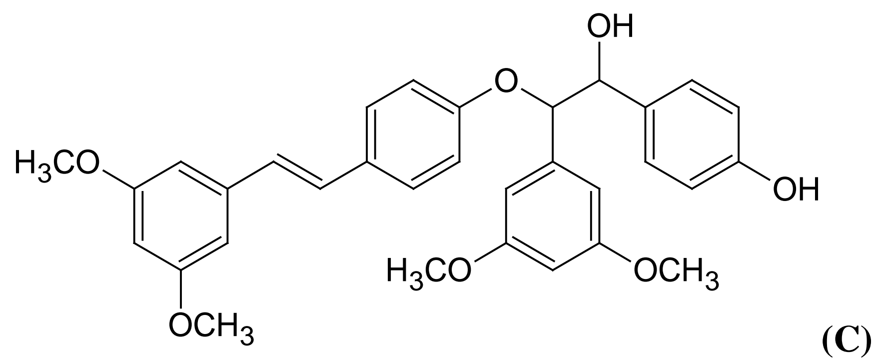


Figure 5. Reation´s products of the pterostilbene oxidation reaction products by POX. (A) pterostilbene *trans* dehydromer, (B) pterostilbene *cis* dehydromer and (C) pterostilbene open dimer.

FIGURE 6

Figure 6. (A) UV spectrum of pterostilbene *trans* dehydromer; (B) UV spectrum of pterostilbene *cis* dehydromer.

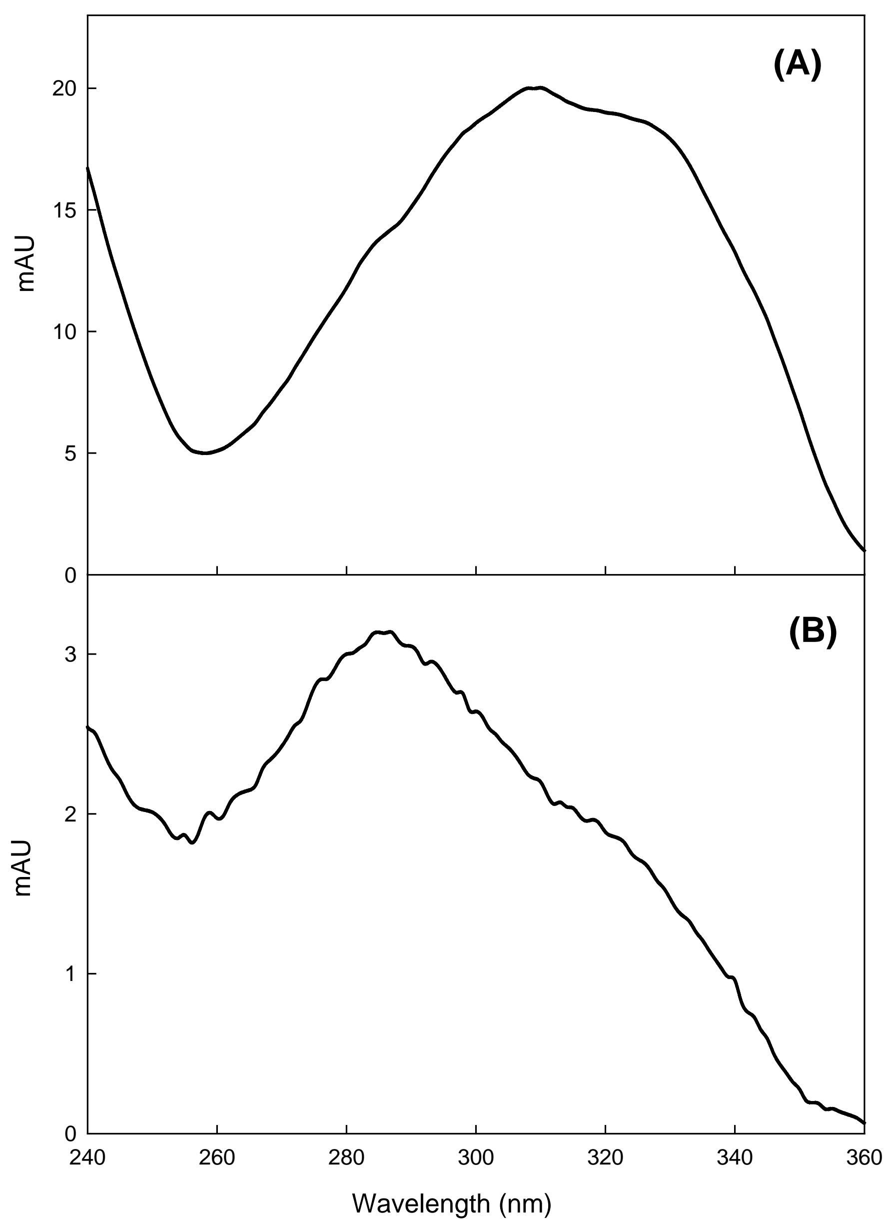


FIGURE 7

Figure 7. Radical-radical coupling reaction mechanism proposed for the synthesis of pterostilbene *trans* dehydromer, pterostilbene *cis* dehydromer and pterostilbene open dimmer.

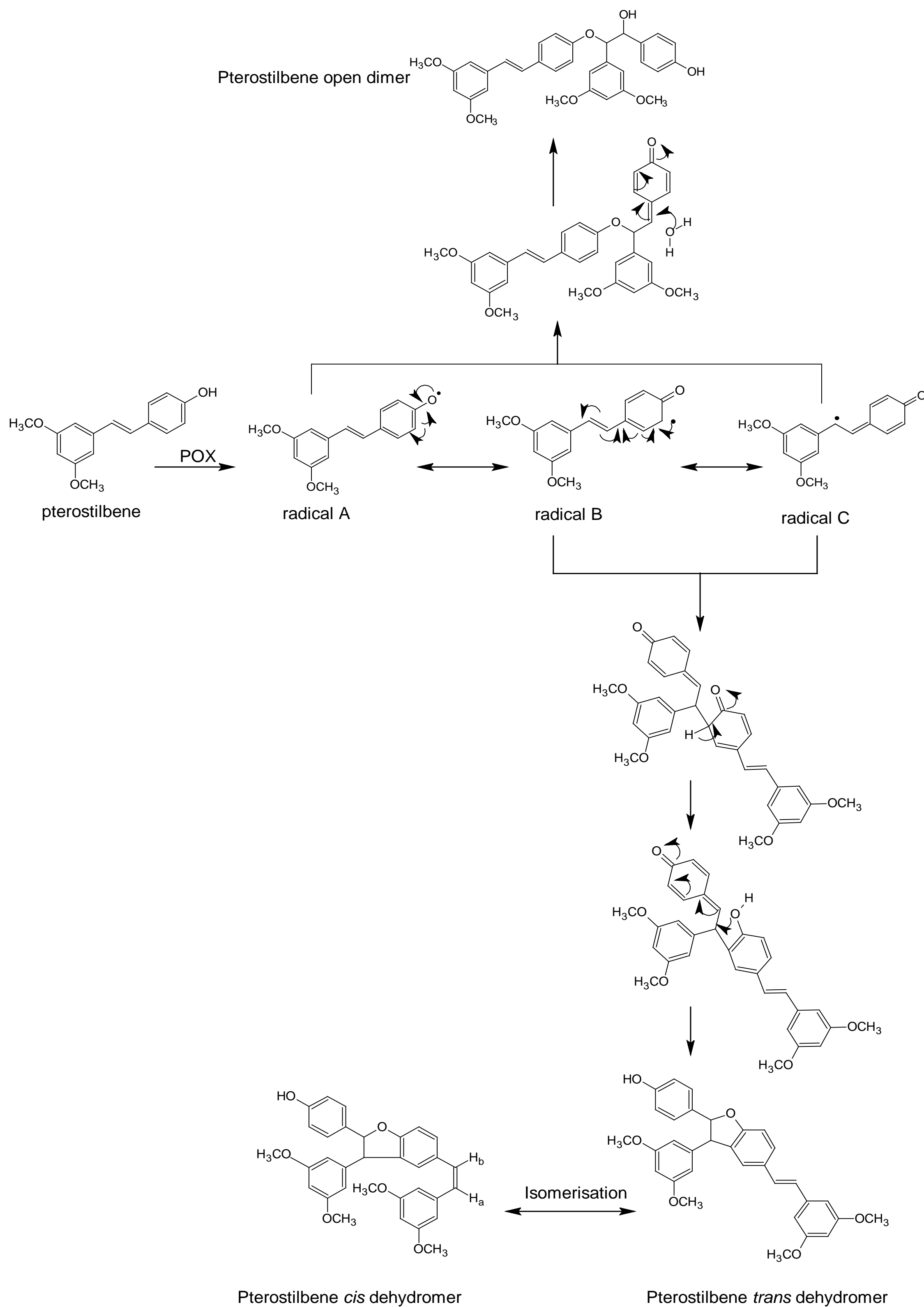


FIGURE 8

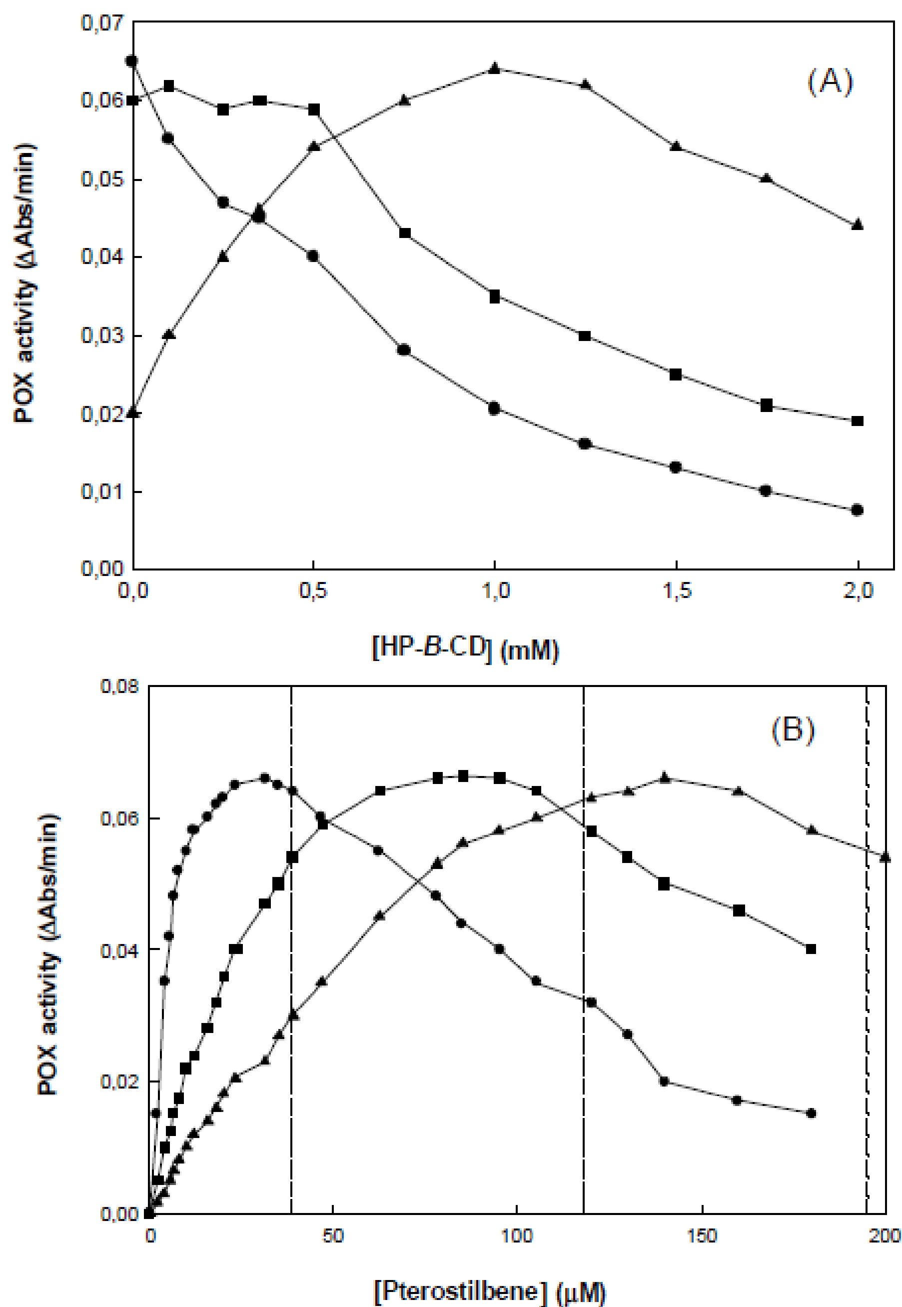


Figure 8. **(A)** Effect of HP-β-CD concentrations on the reaction rate of POX-catalyzed pterostilbene oxidation at different substrate concentrations: (●) 24 μM, (■) 47 μM, (▲) 140 μM. The reaction medium at 25 °C contained increasing HP-β-CD concentrations, 114 μM H_2O_2 in 0.1 M sodium phosphate buffer pH 7.0. **(B)** Effect of the addition of different HP-β-CD concentrations

on peroxidation of pterostilbene by POX: (●) no agent, (■) HP-β-CD 0.5 mM, (▲) HP-β-CD 1.0 mM. The reaction medium at 25 °C contained increasing pterostilbene concentrations, 114 μM H_2O_2 in 0.1 M sodium phosphate buffer pH 7.0.

FIGURE 9

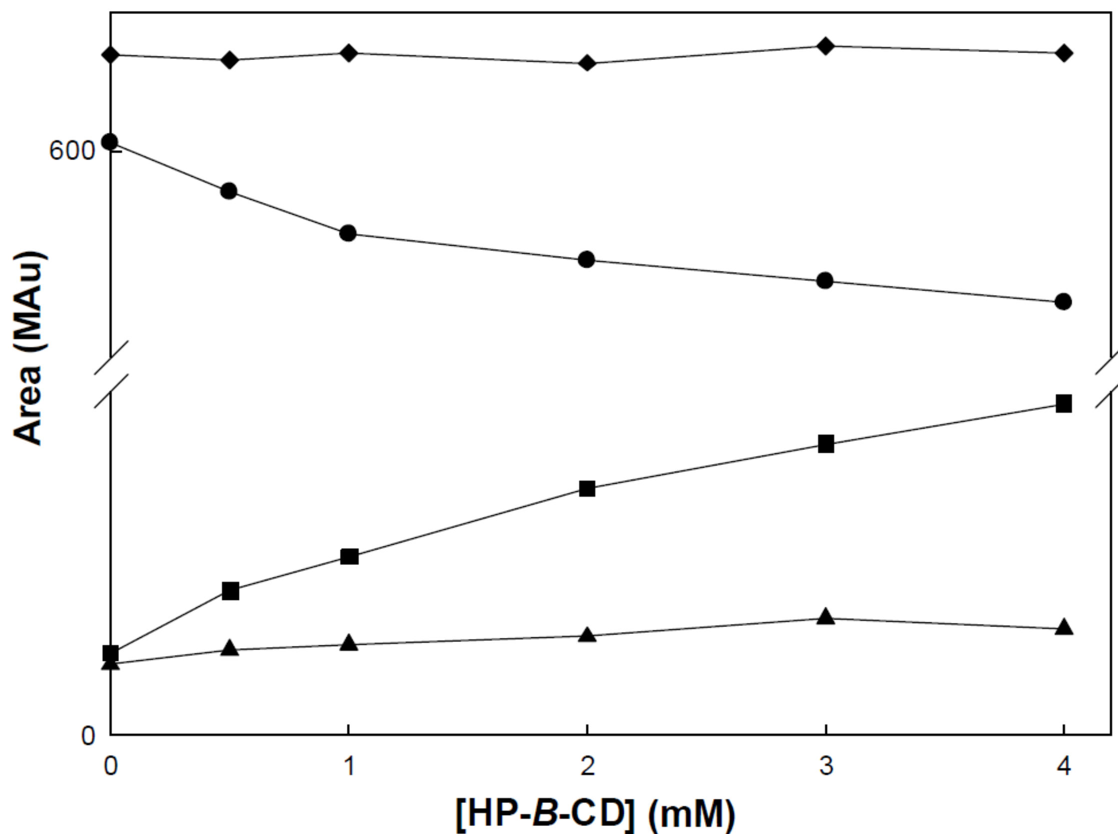


Figure 9. Effect of HP-β-CD concentration on the area of the three main pterostilbene oxidation products by POX determined by RP-HPLC. (●) pterostilbene *trans* dimer, (■) pterostilbene *cis* dimer and (▲) pterostilbene open dimer, (♦) sum of the concentrations of the three products. The eluent system is methanol/water (80:20 v/v). Mau: Miliabsorbance units.

Figure 10

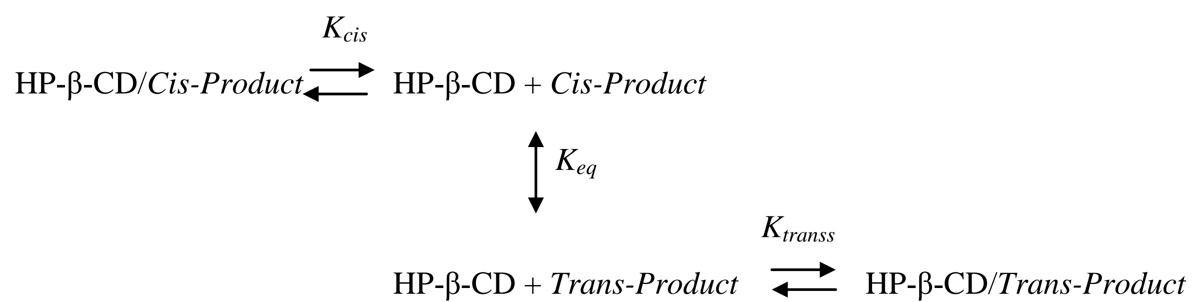


Figure 10. Isomerisation equilibrium between pterostilbene *trans* dehydromer and pterostilbene *cis* dehydromer in the presence of HP-β-CD.

Capítulo VII

Complejación de pinosilvina, un análogo del resveratrol con gran capacidad antifúngica y antimicrobiana, en diferentes tipos de ciclodextrinas

ABSTRACT

The complexation of pinosylvin, a potent antimicrobial and antifungal stilbenoid, by cyclodextrins (CDs) is described for first time in this work. Using steady state fluorescence, we demonstrated that natural (α-,β- and γ-CD) and modified (HP-β-CD, methyl-β-CD and ethyl-β-CD) CDs are able to complex pinosylvin following a 1:1 stoichiometry. However, substantial differences in the strength of the complexation exist between the CDs tested. Although among natural CDs, the interaction of pinosylvin with β-CD was more efficient than with α- and γ-CD, our results show that the complexation constants (K_F) were higher for all the modified CDs than for natural CDs, the highest K_F being that determined for HP-β-CD-pinosylvin complexes (12112 ± 761 M^{-1}). Moreover, deprotonation of the hydroxyl group of pinosylvin led to a sharp fell in the K_F values with respect to those observed for the complexes formed between the protonated structure of this stilbenoid and the CDs. Moreover, a pKa value is reported for first time for pinosylvin. Furthermore, when the temperature of the system was increased, a significant drop was observed in the complexation constant values. From these K_F values, and in order to throw light on the mechanism of pinosylvin affinity for HP-β-CD, we calculated three thermodynamic parameters, ΔH^o, ΔS^o and ΔG^o. The results show that the complexation of pinosylvin by HP-β-CD is a spontaneous and exothermic process with negative value for entropy changes. 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 such as *(E)*-resveratrol and pterostilbene was studied and compared with the results obtained for the HP-β-CD-pinosylvin complexes.

INTRODUCTION

CDs are non-reducing cyclic glucose oligosaccharides. There are three natural CDs: α-, β-, and γ -CD, with 6, 7, or 8 D-glucopyranonsyl residues, respectively, linked by α-1,4 glycosidic bonds *(1,2)*. The double characteristics of CDs, (a) the existence of a hydrophobic cavity and (b) the presence of two hydrophilic hydroxyl rims, give them the property to form inclusion complexes in water with a variety of organic molecules. Because CDs are able to increase the bioavailability of different compounds and to protect different molecules against the action of external agents, their use in both the pharmaceutical and food industries is increasing *(3,4)*.

Among these applications for CDs, recent years have seen an increased number of papers and patents concerning their use in packaging. Thus, the application of CDs in smart and active food packaging has been reviewed *(5)*. Over the past decade, active, controlled and intelligent packaging techniques have seen significant growth and change as new products have replaced the traditional forms of food and beverage packaging. By mixing CD-complexes of fragrances, dyes, insecticides, UV filters, etc. into molten thermoplastic polymers, improved packaging material (films, laminates, containers, trays, etc.) can be produced, in which the complexed substances are homogeneously dispersed and only slowly released from the polymer matrix *(6)*. The incorporation of CDs or CD complexes into a plastic packaging material makes it, at least partially, biodegradable *(7)*. The advantages of CD application in plastic packaging can be inclusion of by-products of polyethylene generated by heat seal *(8)*, the decreased release of impurities and undesired volatile by-products formed during manufacture of the packaging material into the food or beverages *(9)*, improvements of in barrier function of the packaging material entrapping

both penetrating volatiles (atmospheric pollutants migrating inwards) as well as the aroma substances, escaping outward, odour absorption when ‘‘empty’’ CD is used and controlled release of the active component (antimicrobial, antioxidant, etc.) when CD complex is applied *(9)*.

As regards this last application, several papers have reported, on the complexation of different antimicrobial compounds by CDs *(10)*. When a CD-antimicrobial compound molecular complex is exposed to water molecules, their interaction is weakened and the antimicrobial agent is passively released to the environment. These mechanisms could be used to generate an antimicrobial active packaging and therefore protect fresh-cut produce against bacterial and fungal growth.

In recent years, some papers have been published about the antimicrobial properties of a new type of stilbenoid, pinosylvin, for use in the packaging industry. Pinosylvin (*trans*-3,5-dihydroxystilbene) $C_{14}H_{12}O_2$, **(Scheme 1)** is a naturally occurring stilbene present in the wood pulp of pine and eucalyptus trees, and is present in tea oils and herbal remedies *(11-14)*. Several healthy benefits have been attributed to pinosylvin in recent years. Among its pharmacological properties are a wide range of biological activities, including antimicrobial *(15)*, antifungal *(16)*, anticancer *(17)*, antiinflammatory *(18),* antioxidative *(19)* and antibacterial *(20)* properties.

Despite these beneficial properties, several disadvantages of stilbenoids related with their poor solubility in water, their facility to be oxidized by different agents or their tendency to be photodegraded have meant that pinosylvin have no been used in active food packaging films *(21)*. For these reasons, the complexation of pinosylvin with different

types of molecule, for example cyclodextrins (CDs), has been examined in order to improve its suitability for use in the food packaging industry. However, the first step is the molecular characterization of the inclusion process of pinosylvin in CDs, as it has been realized in this work for first time.

To date, although CDs have been used to complex another type of stilbenoids, the effect of CDs on pinosylvin has not been described in any paper. Indeed, this is the first study in which the complexation between CD and this potent antimicrobial and antifungal is reported. Knowledge of the stoichiometric coefficients and of the complexation constants (K_F) of the CD-pinosylvin complexes is essential if this stilbene is to be used in the food industry.

Bearing the above in mind, the three main objectives of this work were:

i) to analyze the complexation mechanism of pinosylvin with different types of natural (α-, β- and γ-CD) and modified (*hydroxypropil*-β-CD, *hydroxyetil*-β-CD, methyl-β-CD or *hydroxypropil*-γ-CD) CDs under various experimental conditions of temperature and pH.

ii) to calculate the stoichiometry, K_F values and thermodynamic parameters for the CD-pinosylvin complexes.

iii) to compare the effect of the structure of some compounds of the stilbenoids family, such as *(E)*-resveratrol and pterostilbene, on both the stoichiometry and the K_F values.

To perform the study, a method which makes use of changes in fluorescence spectroscopic properties of pinosylvin in the presence of CDs, was used.

EXPERIMENTAL SECTION

Materials

Pinosylvin and pterostilbene were purchased from Sequoia Research Products Limited (Pangbourne, United Kingdom). *(E)*-resveratrol was from Sigma-Aldrich (Madrid, Spain). Stilbenes are sensitive to the light and irradiation of solutions containing the analyte induces the formation of other molecules, which leads to the formation of a highly fluorescent compound. Moreover, these stilbenes are also sensitive to light because of their (E) to (Z) diastereomerization. Because of this, the samples were stored in darkness.

All the CDs tested, natural (α-, β- and γ-CD) and modified (*hydroxypropil*-β-CD (HP-β-CD), *hydroxyetil*-β-CD (HE-β-CD), methyl-β-CD or *hydroxypropil*-γ-CD (HP-γ-CD)), were purchased from Sigma-Aldrich (Madrid, Spain) and used as received.

Equipment and Experimental Procedure

Fluorescence studies. The excitation wavelength for pinosylvin and pterostilbene was 330 and for *(E)*-resveratrol was 334, while the emission were 374 for pinosylvin and pterostilbene and 385 for *(E)*-resveratrol. The relative fluorescence intensity values were recorded at 25 ± 0.2 ºC. To avoid inner filter effects, 2-mm quartz cells were used. A Kontron SFM-25 spectrofluorimeter (Zurich, Switzerland) equipped with a xenon lamp source and thermostatically controlled cells was used to measure the fluorescence intensity in all the fluorescence experiments. Both the excitation and emission bandwidths were set at 2 nm.

Temperature studies. Four temperatures (15 ± 0.2 ºC, 20 ± 0.2 ºC, 25 ± 0.2 ºC, 30 ± 0.2 ºC and 37 ± 0.2 ºC) were used to determine the K_F values. To control the temperature, a Thermomixer Comfort (Eppendorf Ibérica, Madrid, Spain) was used. The thermodynamic parameters, ΔH^o, ΔS^o and ΔG^o, can be estimated using the following thermodynamic relationship equation:

$$\ln K_F = \frac{-\Delta H^o}{RT} + \frac{\Delta S^o}{R} \qquad \textit{(Equation 1)}$$

where K_F is the complexation constant of the inclusion complex, R is the gas constant, T is the temperature, and ΔH^o and ΔS^o are standard enthalpy and entropy changes of complex formation in the system. For a linear plot of ln K_F versus 1/T, the slope and intercept are $-\Delta H^o/R$ and $\Delta S^o/R$ respectively.

Using equation 2, the Gibbs free energy change for the interactions that take place during the inclusion process can be determined:

$$\Delta G^o = \Delta H^o - T\Delta S^o \qquad \textit{(Equation 2)}$$

RESULTS AND DISCUSSION

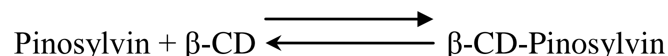
Determination of the stoichiometry and complexation constants of the inclusion process of pinosylvin by cyclodextrins.

Although the interaction between CDs and other stilbenes has been reported in several papers *(22-24)*, no work has reported the possible interaction between pinosylvin and CDs of any type.

In order to study the hypothetical interaction between this healthy stilbenoid and CDs, in this paper we selected the most widely used natural CD (β-CD). To quantify the interaction between pinosylvin and this type of natural CD, the K_F was determined using, as analysis technique, the steady state fluorescence which takes into account the changes in the physico-chemical state of this compound with the concentration of CD and following the Benesi-Hildebrand method *(25)*.

Two types of stoichiometry have been described for the CD-stilbenoids complexes, 1:1 and 2:1 *(22-24)*. For this reason, the next step of this investigation was to determine the stoichiometry of the β-CD-pinosylvin complex.

Assuming that the composition of the complex was 1:1, the following expression can be written:



The complexation constant, K_F is given by:

$$K_F = \frac{[B\text{ - }CD\text{ - }Pinosylvin]}{[Pinosylvin][B\text{ - }CD]} \qquad \textit{(Equation 3)}$$

where [β-CD], [Pinosylvin] and [β-CD- Pinosylvin] are equilibrium concentrations.

Using a Benesi–Hildebrand plot, we can calculate the stoichiometry and the K_F value for the 1:1 inclusion complex:

$$\frac{1}{F\text{ - }F_o} = \frac{1}{(F_\alpha - F_o)K_F[B\text{ - }CD]} + \frac{1}{F_\alpha - F_o} \qquad \textit{(Equation 4)}$$

where F is the observed fluorescence intensity at each β-CD concentration tested, F_0 the fluorescence intensity of pinosylvin in the absence of β-CD; F_∞ the fluorescence intensity when all of the pinosylvin molecules are essentially complexed with β-CD and [β-CD] denotes the β-CD concentration.

A similar expression can be used for the 2:1 inclusion complexes and a plot of 1/F-F_0 as a function of $1/[\beta\text{-CD}]^2$ was also analyzed because it was thought it might provide information about the presence of higher order complexes, especially at higher β-CD concentrations. Assuming the stoichiometry of the inclusion complex to be 2:1, the following expression is obtained *(30)*:

$$\frac{1}{F\text{ - }F_o} = \frac{1}{(F_\alpha - F_o)K_{F12}([\mathrm{B}\text{ - }CD])^2} + \frac{1}{F_\alpha - F_o} \qquad \textit{(Equation 5)}$$

To select which type of inclusion process is carried out between pinosylvin and β-CD, the first step was study the changes in the pinosylvin emission fluorescence when β-

CD was added to the reaction medium. **Figure 1** shows the emission fluorescence of pinosylvin values in the presence of increasing concentrations of β-CD. From these data, the difference in the intensity of the emission fluorescence of pinosylvin in the absence and presence of different amounts of β-CD was calculated. In order to determine the stoichiometry and the K_F of β-CD-pinosylvin complexes, a representation of the variation in fluorescence intensity at the wavelength band used as a function of β-CD concentration was analyzed by the Benesi-Hildebrand method (**Figure 1** *inset*).

In our study, a plot of $1/F - F_0$ vs. 1/[β-CD] gave a straight line with a linear correlation higher than 0.99, indicating that the presumed stoichiometry of the β-CD-pterostilbene complexes formed was 1:1 (**Figure 1** *inset*, filled circles). On the other hand, when $1/F - F_0$ was plotted against $1/([\beta\text{-CD}])^2$, a non-linear relationship was obtained (linear correlation of 0.82) (**Figure 1** *inset*, filled squares), which indicates that the stoichiometry of the inclusion complex is not 2:1.

This type of stoichiometry 1:1 have been reported for other CD-stilbenoid complexes such as *(E)*-resveratrol *(22,23)* or pterostilbene *(24)*, but differs from the stoichiometry 2:1 published for *trans*-stilbene *(24)*.

To quantify for the first time the interaction between pinosylvin and CD, the experimental data were fitted to equation 4, while the K_F value for pH 7.0 was calculated as 5181 ± 233 M^{-1}. These results are in good agreement with those previously obtained for the 1:1 complexes between β-CD and several compounds with structures similar to pinosylvin *(22-24)*.

Study of the complexation of pinosylvin by natural CDs. Effect of the cyclodextrin

structure on complexation constants.

In order to characterize the interaction between pinosylvin and the host CD at a molecular level, the next step of our investigation was to determine the K_F values between pinosylvin and several types of CD with different structure, size and glucose units number. Three types of natural CD with GRAS status and approved recently as additives in the European Union (α-, β- and γ-CD) were used to this end. Indeed, the three natural CDs have been included in the European lists of additives approved for food use and the corresponding E-numbers assigned are E-457, E-458, and E-459, respectively. As in the previous section when determining the K_F values between β-CD and pinosylvin, we fitted the values of relative intensity calculated experimentally to the previously equations mentioned. The K_F values for different natural species are shown in **Table 1**. It can be observed that the highest K_F value belonged to β-CD, followed by α-CD and, finally, γ-CD.

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

The fact that β-CD was the optimum natural CD for complexing pinosylvin is in good agreement with the most of the papers which compare the complexation of several stilbenoids compounds with CDs *(22,23)*.

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

Determination of the K_F values for the complexation of pinosylvin by modified cyclodextrins.

The effect of modifying β-CD by adding different functional groups to the macrocyclic ring on the K_F values was evaluated. Our results showed significant differences when HP-β-CD, HE-β-CD, methyl-β-CD or HP-γ-CD were used instead of β-CD. In all cases, the natural CDs showed lower K_F values than the modified CDs tested. As expected, the relative fluorescence values of pinosylvin increased when the concentration of all four modified CDs (**Figure 2**). However, the addition of increasing concentrations of modified CDs leads to greater increases in the relative intensity fluorescence values than when increasing concentrations of β-CD were used. Our data showed that HP-β-CD presented the highest K_F value of all the modified CD tested, followed by HE-β-CD, methyl-β-CD and, finally, HP-γ-CD (**Figure 2** *inset*). Several factors may influence the K_F values for the pinosylvin-modified CDs interactions. In this investigation, the length of the aliphatic chain of the β-CD substituent influenced the strength of the complexation. Thus, for a natural CD, the greater the number of carbon atoms in the substituent, the higher the K_F value for the resulting complex.

The hydrophobicity of the β-CD channel increases with the modification because occurs principally at position 2 of the sugar residues situated on one side of the torus at the edge and orientated inward. Indeed, the higher K_F observed for the modified CDs-pinosylvin complexes could be due to the hydrophobic interactions with one side of the CD molecule (that bearing the methyl, ethyl or hydroxypropyl groups). The behaviour of K_F may also be explained by the substantial changes resulting from the substitution of the internal –OH groups in the hydrophobicity of the CD torus.

The fact that HP-β-CD was the best type of CD to complex pinosylvin coincides with the results showed by other authors concerning the use of HP-β-CD to complex other stilbene compounds *(26)*.

For this reason, we selected HP-β-CD to study the complexation of pinosylvin by CDs.

Effect of temperature on the complexation of pinosylvin by HP-β-CD.

The effect of the temperature on the complexation process is one of the main factors to be taken into account when a host-guest complex is used in the food or pharmaceutical industry. Therefore, the next step in this work was to study the influence of the temperature on the K_F values of the HP-β-CD-pinosylvin complexes.

Different papers have reported that temperature can increase or decrease the strength of inclusion of a guest molecule. Generally, a increase in the medium temperature produces a dissociation of the inclusion complexes and the K_F values decrease *(27)*. However, the inclusion of another group of molecules such as polyunsaturated fatty acids in the cavity of CDs is favoured by high temperatures *(28)*. For this reason, the effect of temperature on the CD-pinosylvin complexes was clarified by studying the K_F values for the HP-β-CD-pinosylvin complexes at four different temperatures: 15 ± 0.2 ºC, 25 ± 0.2 ºC, 30 ± 0.2 ºC and 37 ± 0.2 ºC. The results can be observed in the **Figure 3** when we the K_F values obtained at 15 ± 0.2 ºC, 25 ± 0.2 ºC, 30 ± 0.2 ºC and 37 ± 0.2 ºC were 20460 ± 1036, 14800 ± 965, 12112 ± 761, 8364 ± 569 M^{-1} and 5839 ± 472 M^{-1}, respectively.

This strong decrease in the K_F values of the HP-β-CD-pinosylvin complexes when the temperature is increased might be interpreted as a lower degree of interaction at higher temperatures possibly due to the fact that hydrogen bonds are usually weakened by heating.

Thermodynamic study of the complexation of pinosylvin by HP-β-CD.

To obtain information on mechanistic aspects of pinosylvin´s affinity for HP-β-CD, a van´t Hoff plot (equation1) was used to calculate the main thermodynamic parameters of the complexation process (ΔH^o, ΔS^o and ΔG^o at 25 ± 0.2 ºC). To reach this objective, the Ln K_F was plotted versus 1/T and a lineal representation was obtained with correlation coefficient higher than 0.99 (**Figure 3** *inset*).

These results lead to three main conclusions concerning the nature of the complexation of pinosylvin by HP-β-CD:

i) The process is *exothermic*: the negative values obtained for enthalpy changes (–42 ± 1 kJ mol^{-1}) indicate the exothermic nature of the interaction processes of pinosylvin with HP-β-CD. This behaviour is typical of hydrophobic interactions, van der Waals interactions, the displacement of water molecules from the cavity of HP-β-CD or the formation of hydrogen bonds.

ii) The process presents a negative value for entropy changes (-64 ± 2 J $mol^{-1}K^{-1}$) due to a decrease in the translational and rotational degrees of freedom of the complexed pinosylvin compared with the free ones.

iii) The process is *spontaneous*: the negative value obtained for the Gibbs free energy change (–23 ± 1 kJ mol^{-1}) for the interactions that take place during the

inclusion process at 25 ± 0.2 ºC indicate that the inclusion process is spontaneous.

Effect of pH on the complexation of pterostilbene by HP-β-CD.

The behaviour of the HP-β-CD-pinosylvin complexes at different protonation states of the guest molecule must be taken into account when this CD complex is used in the food or pharmaceutical industry. For this reason, in this section we study the effect of the medium pH on the complexation of pinosylvin by HP-β-CD, calculating the K_F values for this type of complex in the pH range 5.5-10.0. When the medium pH was between 5.5 and 8.5, the K_F values remained stable, with a value around 12100 ± 760 M^{-1} (**Figure 4).** However, when the medium pH was increased from 8.5 to 11.5, the K_F values decreased to about 5000 ± 256 M^{-1}, as happens during the titration of a weak ionizable group. Since hydrogen bonding is one of the most important types of interaction in the stabilization of inclusion complexes, this strong decay in the complexation constant at high pHs may be attributed to the hypothetical formation of a hydrogen bond between one of the hydroxyl group of the pinosylvin and the hydrophilic groups of CD at pH values below the pK_a value. As can be observed in **Figure 4**, a significant decrease in the K_F value occurs in the pH region where the stilbenoids begin deprotonation of their hydroxyl groups.

Recently, López-Nicolás and García-Carmona (2008) *(29)* reported three pKa for *(E)*-resveratrol (pK_{a1}: 8.8; pK_{a2}: 9.8; pK_{a3}: 11.4). The first pKa is associated with the deprotonation of 4-OH because the abstraction of 4-H is easier than that of 3-H and 5-H. The second pKa indicates the deprotonation of 3-OH or 5-OH (the 3- and 5-positions have

the same structures because the molecule is symmetric). The third pKa indicates the deprotonation of 5-OH or 3-OH. Our results showed that the decay in the K_F value of pinosylvin in the pH 8.5-11.5 may indicate the existence of a pK_a of 9.8 for this stilbenoid, which coincides with the presented by López-Nicolás and García-Carmona (2008) *(29)* for the pK_{a2} of *(E)*-resveratrol.

The fact that the K_F values were higher at pH values lower than the pK_a value of pinosylvin knows that the complexes between HP-β-CD and the protonated form of pynosilvin were more stable than the interaction with the deprotonated forms of this antifungal and antimicrobial compound. These results are of great interest for both the food and pharmaceutical industries because several papers have reported that the protonated structures of stilbenoids have important beneficial biological effects for human health *(30)*.

Effect of the structure of different derivatives of stilbene on its complexation by HP-β-CD.

Recently, Perecko et al. *(31)* studied the structure-efficiency relationship in derivatives of stilbene, comparing *(E)*-resveratrol, pinosylvin, and pterostilbene. They concluded that the presence of different functional groups in the molecules of stilbenoids influence their antioxidative effect. Moreover, the modification of these functional groups may result in derivatives with the required antioxidative properties, targeting mainly extracellular reactive oxygen species which are responsible for tissue damage during chronic inflammation. For this reason, and due to the importance of the structure of these compounds in their potential health benefits, the influence of the structure of these three

stilbene derivatives on the K_F values needs to be studied. As cited preciously, in recent years, the complexation of different stilbenoids by several types of natural and modified CDs has been reported. However, the different methods used to study the inclusion process have resulted in strong differences in the observed K_F values. In our study, both the K_F values and the stoichiometry for the complexation of three derivatives of stilbene (pynosilvin, *(E)*-resveratrol and pterostilbene), which differ in the number of the hydroxyl groups and in the type of substituents of the aromatics ring, were determined (**Scheme 1**).

Figure 5 shows the relative K_F values for the complexes between HP-β-CD and the three derivatives of stilbene studied in this section. The complexes formed between all the stilbenoids tested with HP-β-CD presented a 1:1 stoichiometry. Moreover, a comparison of the K_F values (**Figure 5**) showed that the interaction was more effective for the HP-β-CD-*(E)*-resveratrol complexes, followed by the HP-β-CD-pterostilbene complexes and, finally, the lowest K_F value was found for the HP-β-CD-pinosylvin complexes. Among causes of this behaviour may be the hydrophobicity, resonance structure of the guest molecules or the type of substituent of the aromatic rings. Indeed, *(E)*-resveratrol, the stilbenoid that presents the highest K_F value, shows a resonance structure that produces a high stability in this type of stilbene, which is not shown by pterostilbene or pinosylvin, and may improve by its complexation by CDs. Concerning the K_F values determined for the complexes between HP-β-CD and pterostilbene or pinosylvin, the higher hydrophobicity of pterostilbene produced by the presence of two methyl substituents leads to a better fit of the guest molecule in the HP-β-CD cavity.

The use of antimicrobial, antifungal and antioxidant agents as components of active and intelligent packaging is one of the main objectives of the food industry. However, problems concerning both the physico-chemical properties of some of these components have meant that some potent antimicrobial, antifungal and antioxidant agents, such as pinosylvin, have not been used in active food packaging films. Indeed, pinosylvin shows very poor solubility in water and, more importantly, it is easily oxidized by several prooxidant agents. For these reasons, complexation of pinosylvin with types of molecule, such as cyclodextrins, which can reduce the release of impurities and undesired volatile products, improve the barrier function of the packaging material and protect the antimicrobial, antifungal and antioxidant agent from degradation, is desirable. Towards this aim, we have made a molecular characterization of the inclusion process of pinosylvin in CDs. Although the stoichiometry of the complex is 1:1 for all the conditions used, our results show that the K_F values for the stilbenoid-CD complexes are strongly dependent on several factors, such as temperature, pH, type of CD and structure of the guest molecule. Besides the above mentioned applications in the food packaging industry, another potential application for the resulting pinosylvin-CD complexes may be in the food and pharmaceutical ingredient industry as nutraceutical due their high solubility and stability and the because the pinosylvin-CD complexes may slow down the rapid metabolism and elimination of pinosylvin, improving its bioavailability, as has been demonstrated for other stilbenoid complexes.

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SCHEMES

Scheme 1. Structures of Pterostilbene, *(E)*-resveratrol and Pinosylvin.

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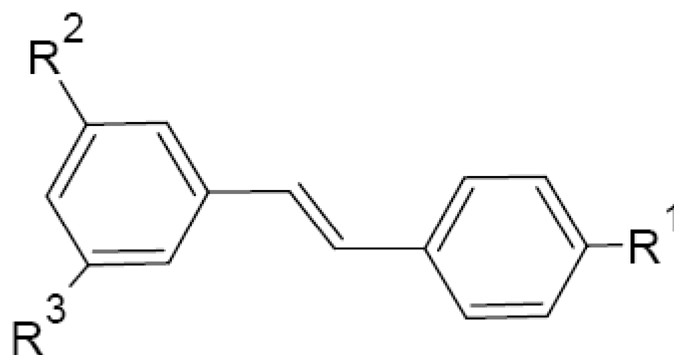

Pterostilbene: R1: OH; R2: OCH_3; R3: OCH_3

***(E)*-resveratrol:** R1: OH; R2: OH; R3: OH

Pinosylvin: R1: H; R2: OH; R3: OH

TABLES

Table 1. K_F values and correlation coefficients arising from equations (4) and (5) (for 1:1 and 1:2 pinosylvin-natural CD complexes, respectively) at 25 ± 0.2 ºC at pH 7.0.

Complex	K_F (M^{-1})	Inner diameter (Å)	Correlation coefficient	
			1:1 using Eq.(4)	*1:2 using Eq.(5)*
Pinosylvin/α-CD	4024 ± 135 M^{-1}	4.7-5.2	0.99	0.89
Pinosylvin/β-CD	5181 ± 233 M^{-1}	6.0-6.4	0.99	0.82
Pinosylvin/γ-CD	3084 ± 119 M^{-1}	7.5-8.3	0.99	0.84

FIGURE LEGENDS

FIGURE 1

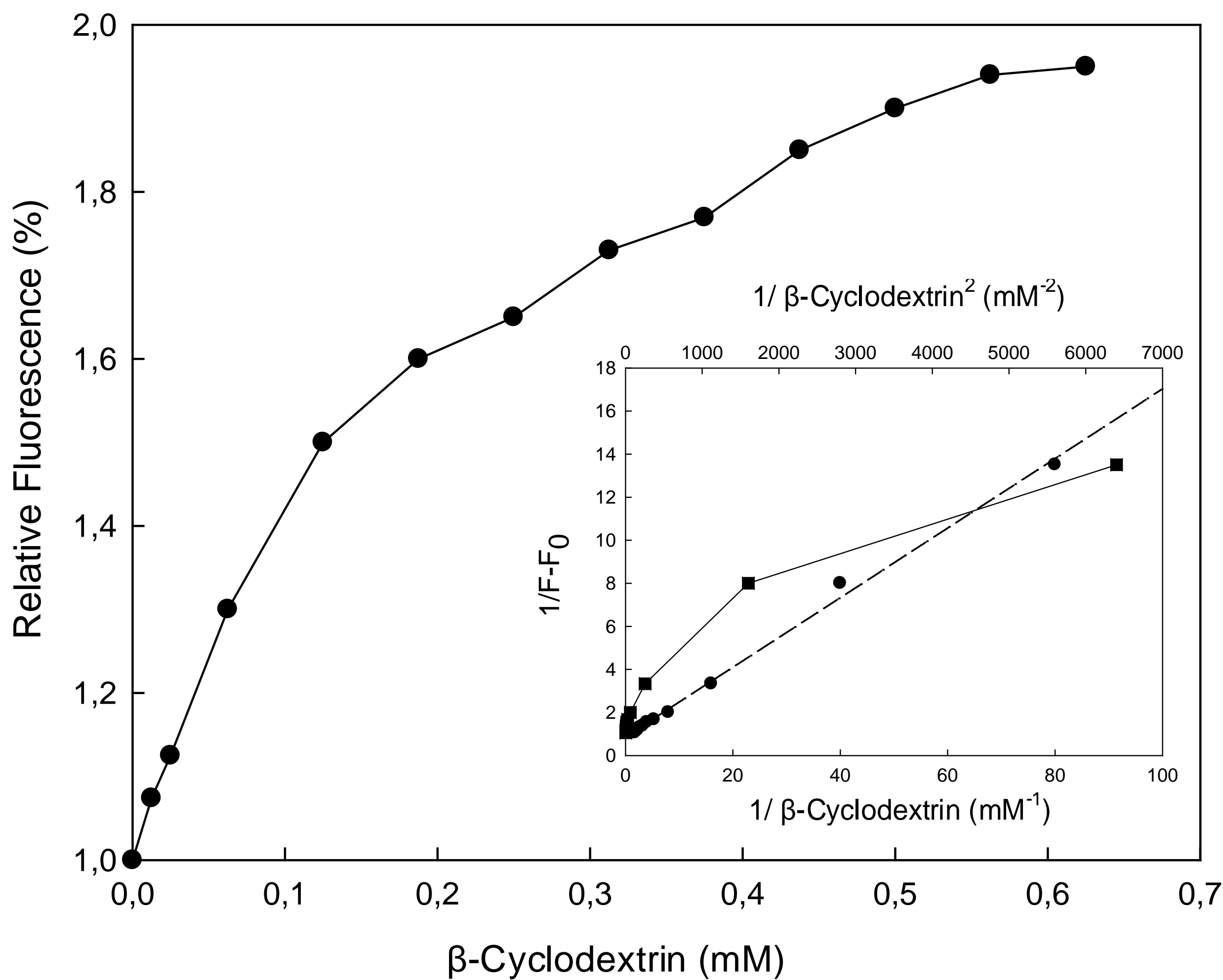

Figure 1. Dependence of emission fluorescence intensities of pinosylvin on β-CD concentrations. [Pinosylvin]: 30 μM. *Inset*: Double reciprocal plot of pinosylvin complexed

to β-CD for determining the stoichiometry of β-CD- pinosylvin complexes: $1/(F-F_0)$ versus $1/[\beta\text{-CD}]$ (assumption of 1:1 complex) (filled circles); (b) $1/(F-F_0)$ versus $1/[\beta\text{-CD}]^2$ (hypothesis of 1:2 complex) (filled squares).

FIGURE 2

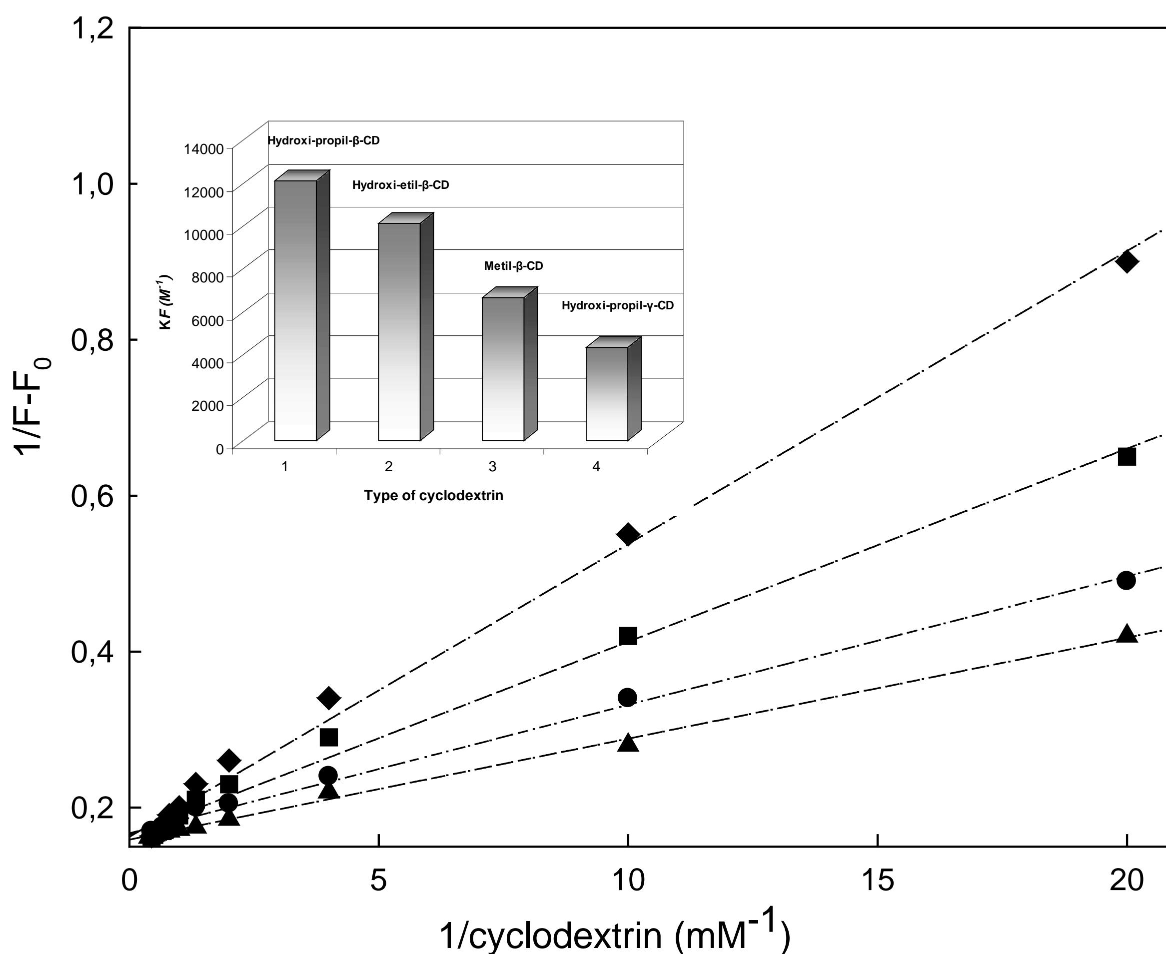

Figure 2. (A) Double reciprocal plot of pinosylvin complexed to modified CDs HP-β-CD (▲), HE-β-CD (●), methyl-β-CD (■) and HP-γ-CD (♦).*Inset*: Effect of the structure of modified and natural CDs on the complexation constant (K_F) values of pinosylvin-CD complexes at 25± 0.2 ºC in 0.1 M sodium phosphate buffer pH 7.0. [Pinosylvin]: 30 μM.

FIGURE 3

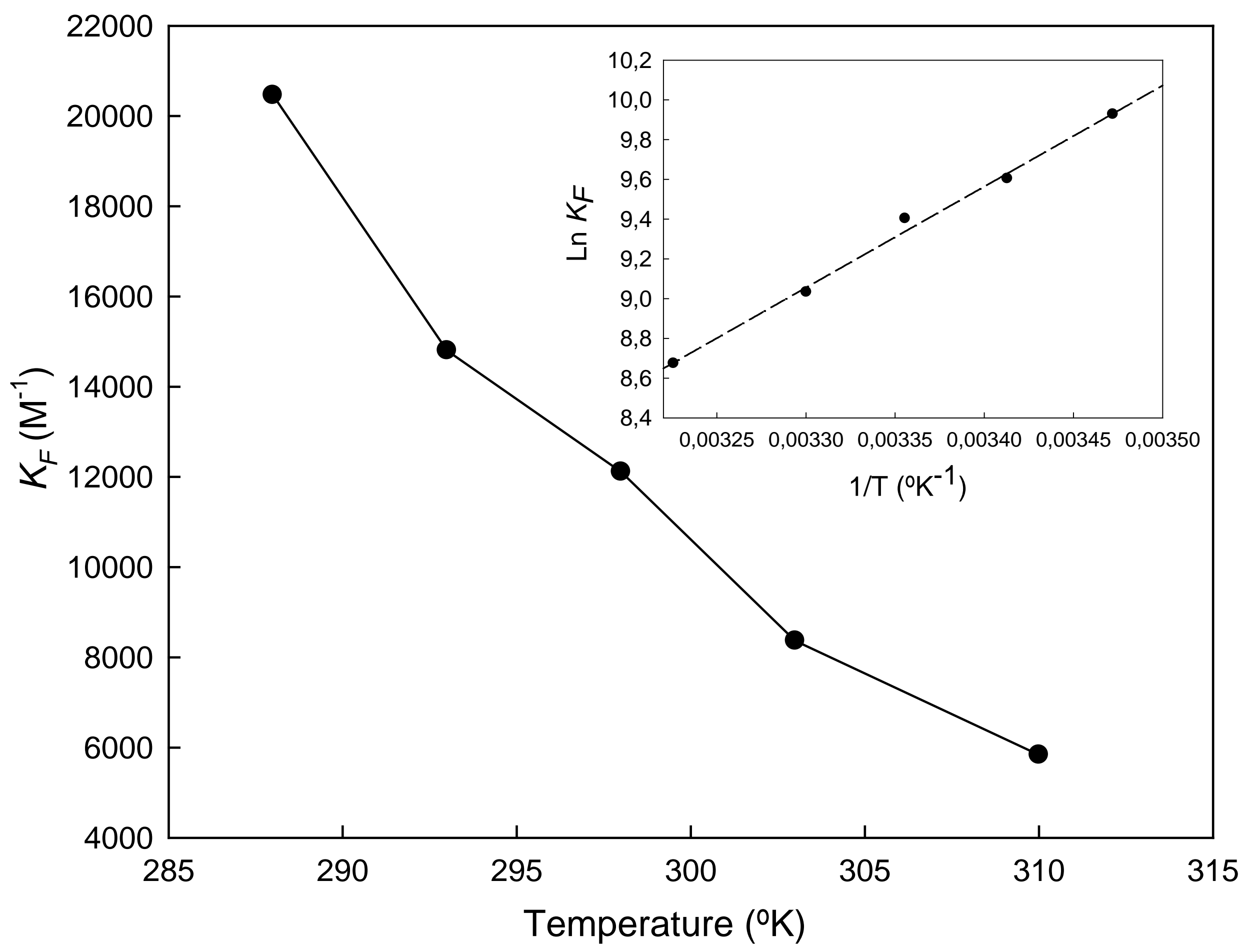

Figure 3. Effect of temperature on the complexation constant (K_F) values of pinosylvin-HP-β-CD complexes at pH 7.0. [Pinosylvin]: 30 μM. *Inset:* Van´t Hoff plot (ln K_F vs. 1/T) for pynosilvin-HP-β-CD complexes in 0.1 M sodium phosphate buffer pH 7.0.

FIGURE 4

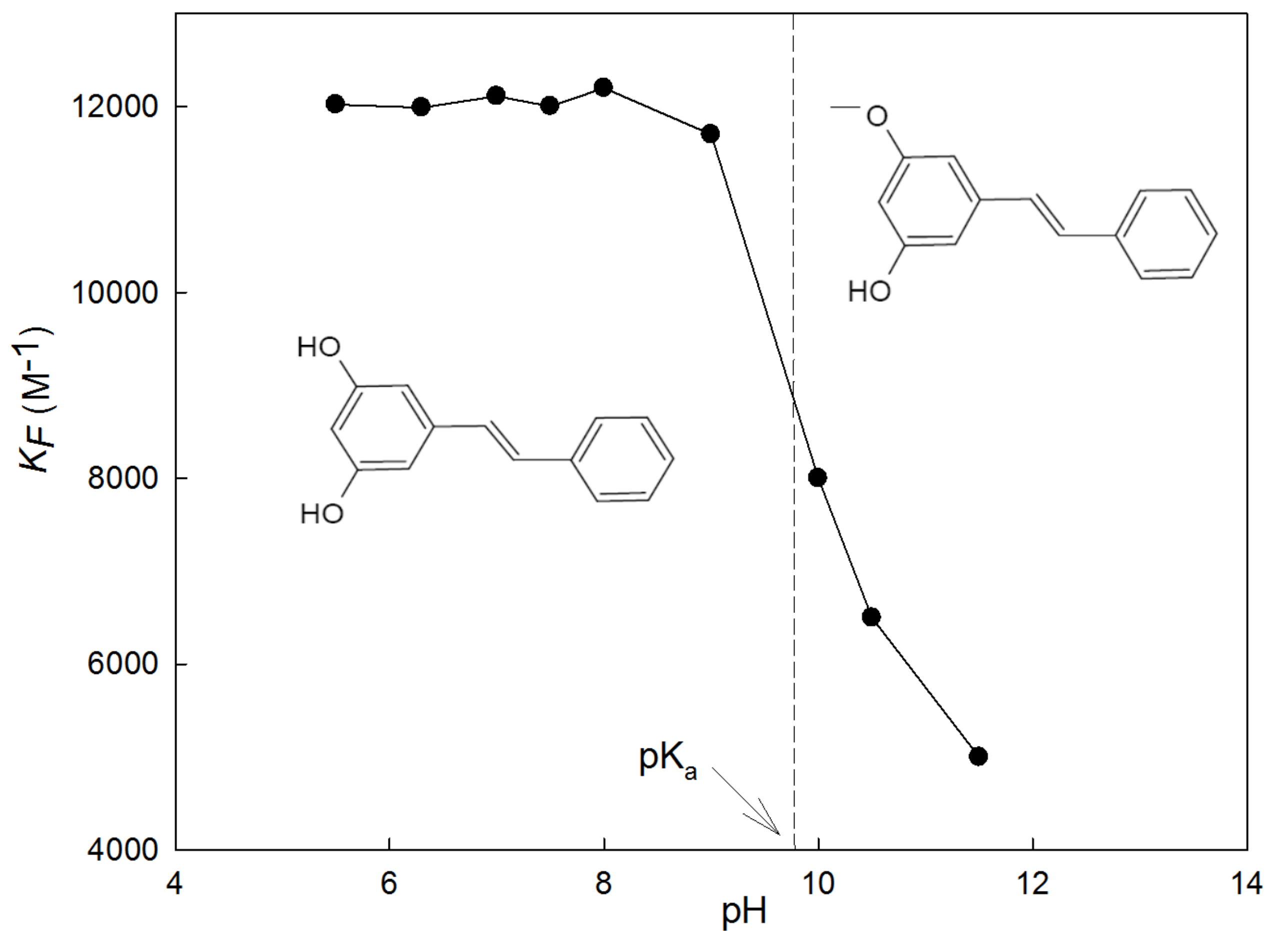

Figure 4. Effect of pH on the complexation constant (K_F) values of pynosilvin-HP-β-CD complexes at 25 ± 0.2 ºC . [Pinosylvin]: 30 μM.

FIGURE 5

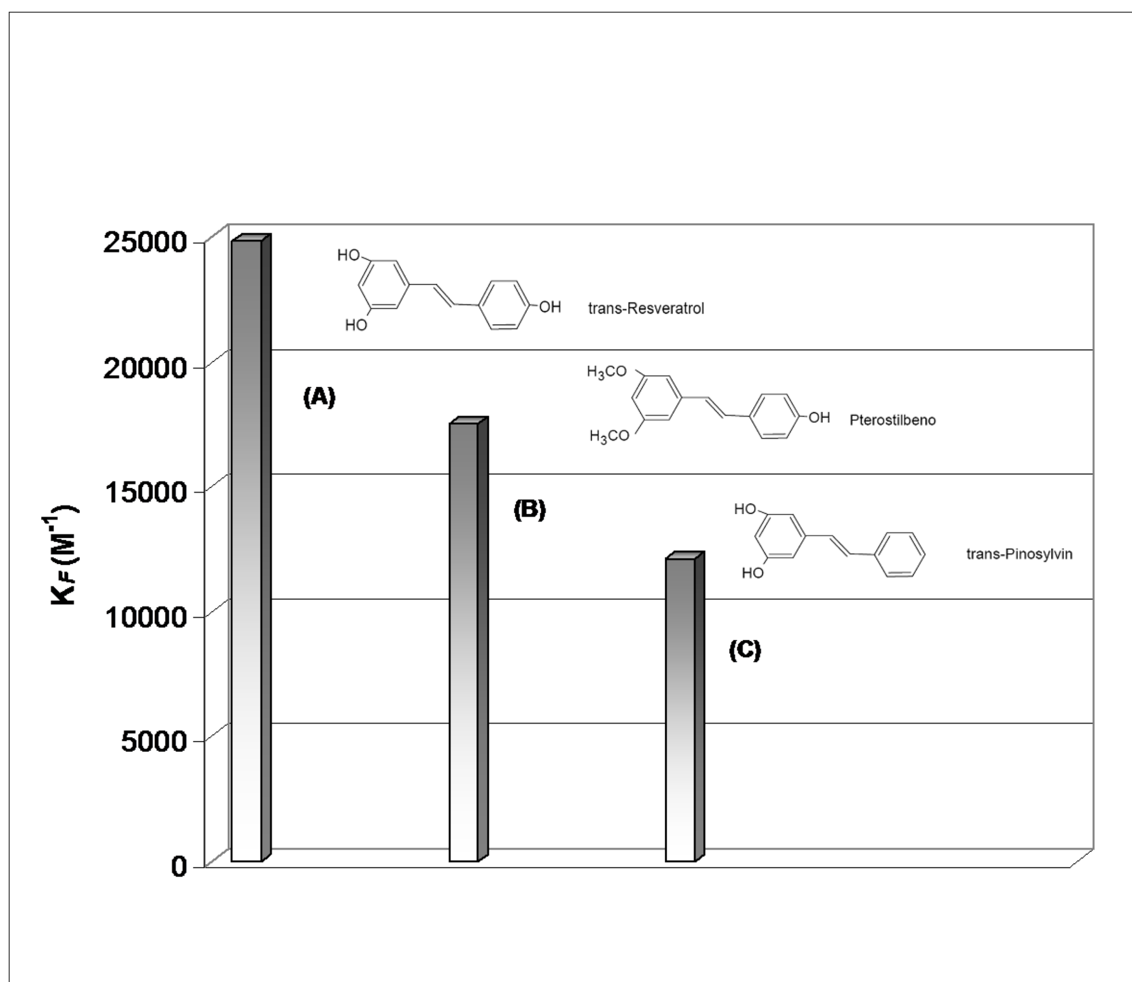

Figure 5. Effect of the structure of *(E)*-resveratrol (A), pterostilbene (B) and pinosylvin (C) on the complexation constant (K_F) values of stilbenoid-HP-β-CD complexes at 25± 0.2 ºC in 0.1 M sodium phosphate buffer pH 7.0. [Stilbenoids]: 30 μM

Capítulo VIII

Uso de cromatografía líquida de alta resolución para la caracterización físico-química y termodinámica del complejo oxyresveratrol/HP-β-ciclodextrina

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^o, ΔH^o and ΔS^o 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.

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 physico-chemical 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 [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^o, ΔH^o and ΔS^o) 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.

EXPERIMENTAL

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 to 50%, were used without further purification.

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 avalaible reversed-phase column LiChrospher RP18 (Agilent, Waldbronn, Germany) (150 x 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].

Temperature studies

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

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

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^o and ΔS^o 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 $-\Delta H^o/R$ and $\Delta S^o/R$. To determine the

Gibbs free energy change for the interactions that take place during the inclusion process may be found, we used equation 2:

$$\Delta G^{o} = \Delta H^{o} - T\Delta S^{o} \qquad (2)$$

RESULTS AND DISCUSSION

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 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.

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 *k* 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.

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 six units of glucose (β-CD: 6.0-6.4 Å) fitted oxyresveratrol better than the inner diameter of five units (α-CD: 4.7-5.2 Å) or seven 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.

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 2B 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, equation 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.

$$\frac{1}{k} = \frac{1}{k_o} + \frac{K_F}{k_o}\left[\mathrm{CD}\right] \qquad (3)$$

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 precursor 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:

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

where k_0 is the capacity factor of oxyresveratrol in the absence of β-CD modifier, K_{F1} is the apparent formation constant for the 1:1 oxyresveratrol-β-CD complex and K_{F2} is the apparent formation constant for the 1:2 oxyresveratrol-β-CD complex.

Using equation 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* [CD] should give a parabolic curve that fits eq. 4.

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 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 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 minutes), 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 to 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 4mM β-CD (Fig. 3 *inset*), confirming that the presence of β-CD does not affect the reversed-phase elution mechanism.

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 to 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 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.

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 equations 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 vs. [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 2:1.

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 $1/k$ *vs.* [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 is 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].

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 15º C and 30ºC (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.

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^o, ΔS^o and ΔG^o at 25 ± 0.2 ºC) 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 ºC.

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 hydrophobicity 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.

CONCLUSIONS

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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TABLES

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 ºC)

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

FIGURE LEGENDS

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

Figure 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 used in Fig 2A and Fig. 2B: 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_{F1}: formation constant for the oxyresveratrol-β-CD complex in a 1:2 model; K_{F2}: formation constant for the oxyresveratrol-(β-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-(β-CD)$_2$ complex between mobile and stationary phase; CD-M: β-CD-organic modifier interaction.

Figure 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, 25º C ± 1º C). Each data point is the mean of 3 replicates.

Figure 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, 25º C ± 1º 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 $[\beta\text{-CD}]^2$ (hypothesis of 1:2 complex) (filled squares).

Figure 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, 25º C ± 1º C). Each data point is the mean of 3 replicates.

Figure 6. Apparent formation constant (K_F) of oxyresveratrol-β-CD complexes as a function of temperature. *Inset:* Van´t Hoff plot (ln K_F vs. 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.

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

TABLE 1

Mobile phase (% H_2O)	*no addition*	*Retention time*			
		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

TABLE 2

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

FIGURE 1

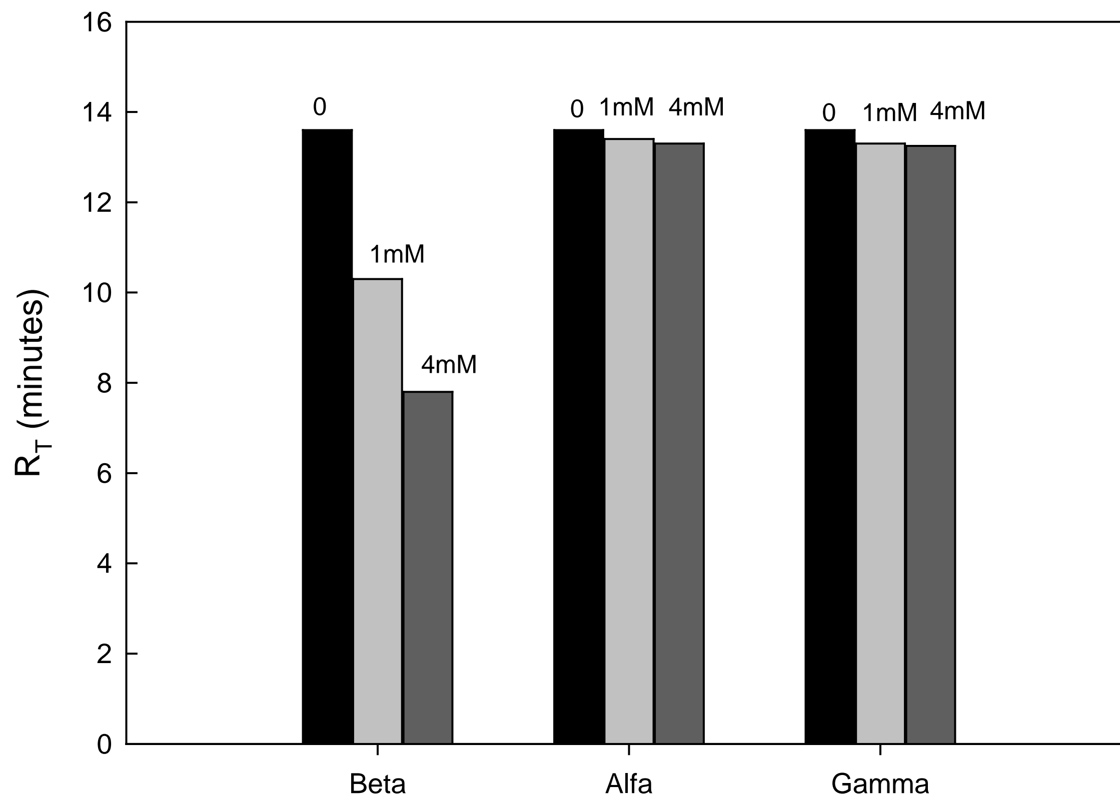
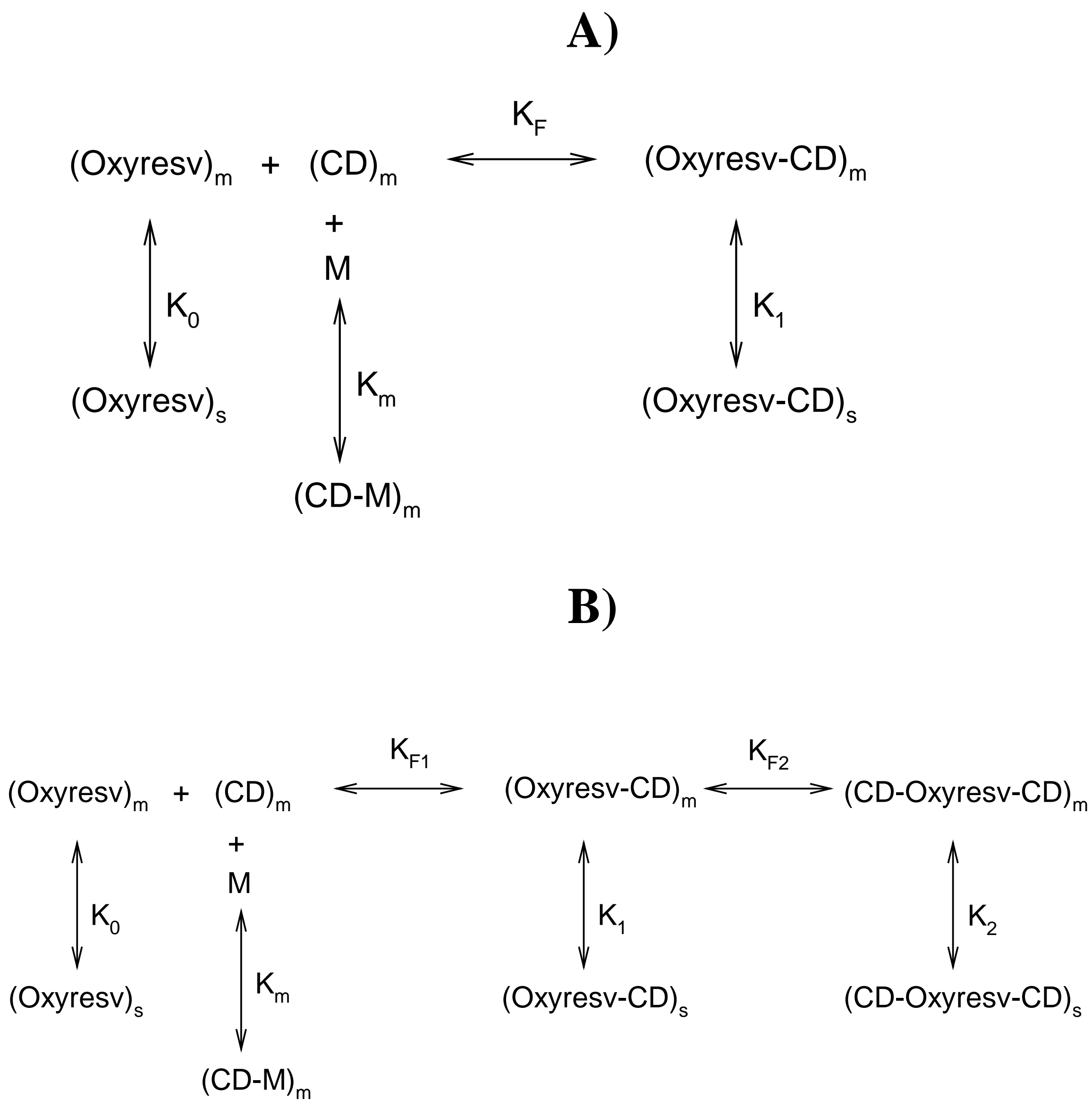


FIGURE 2



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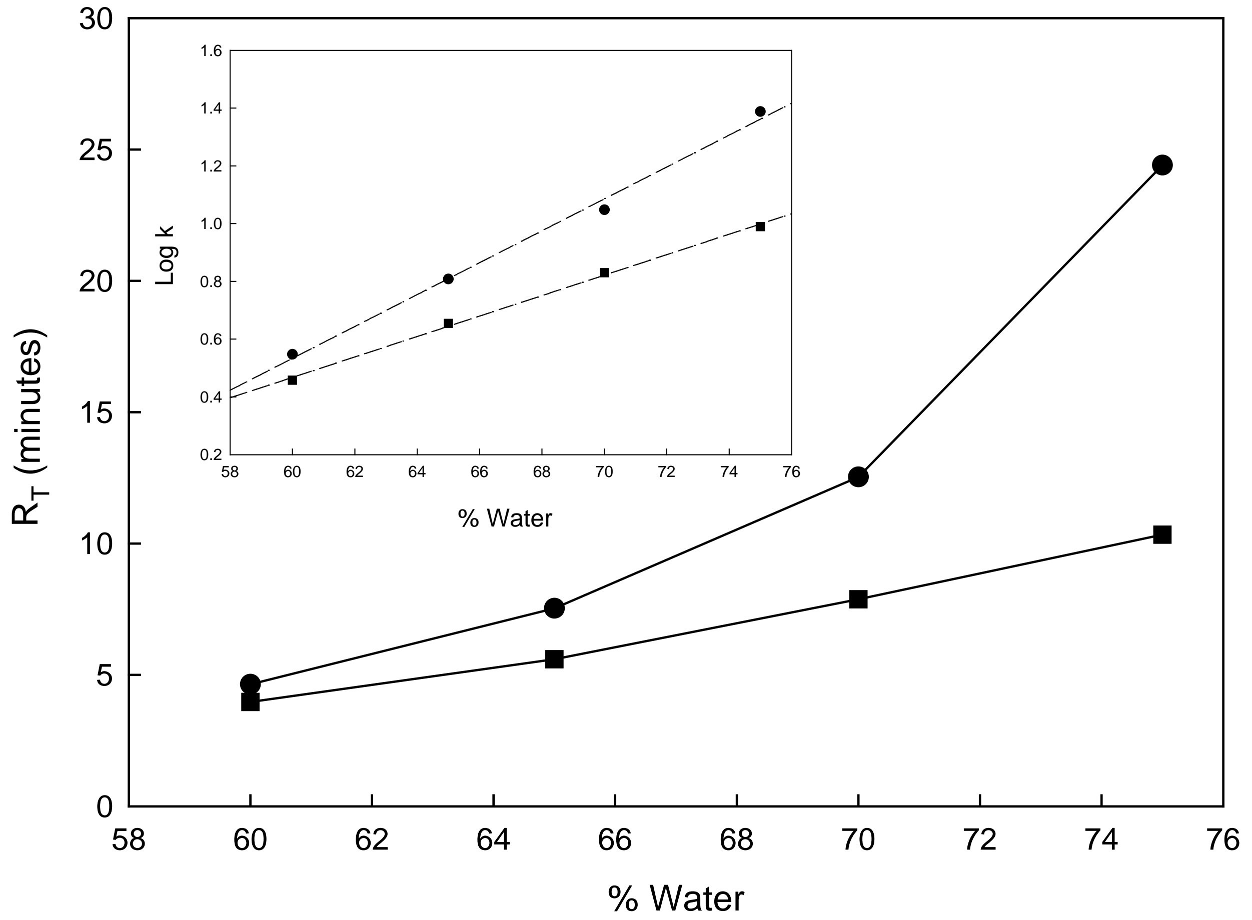

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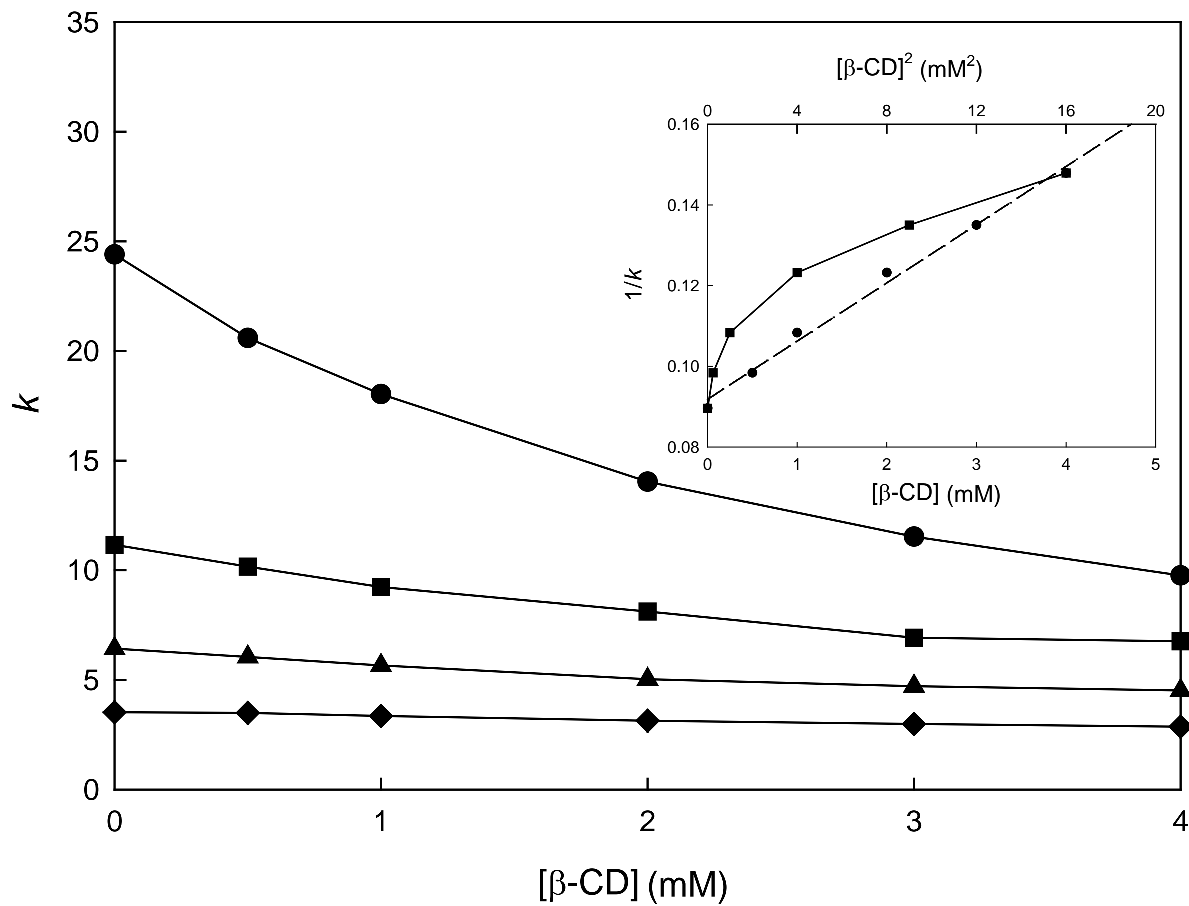

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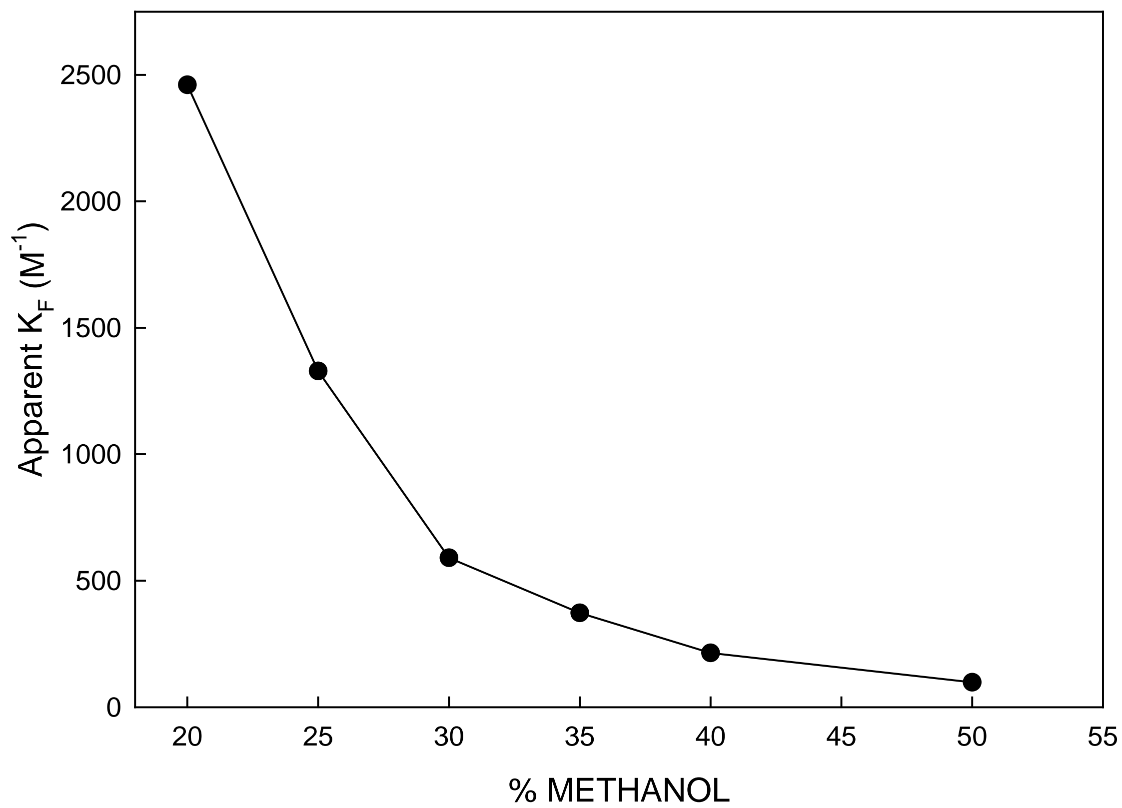

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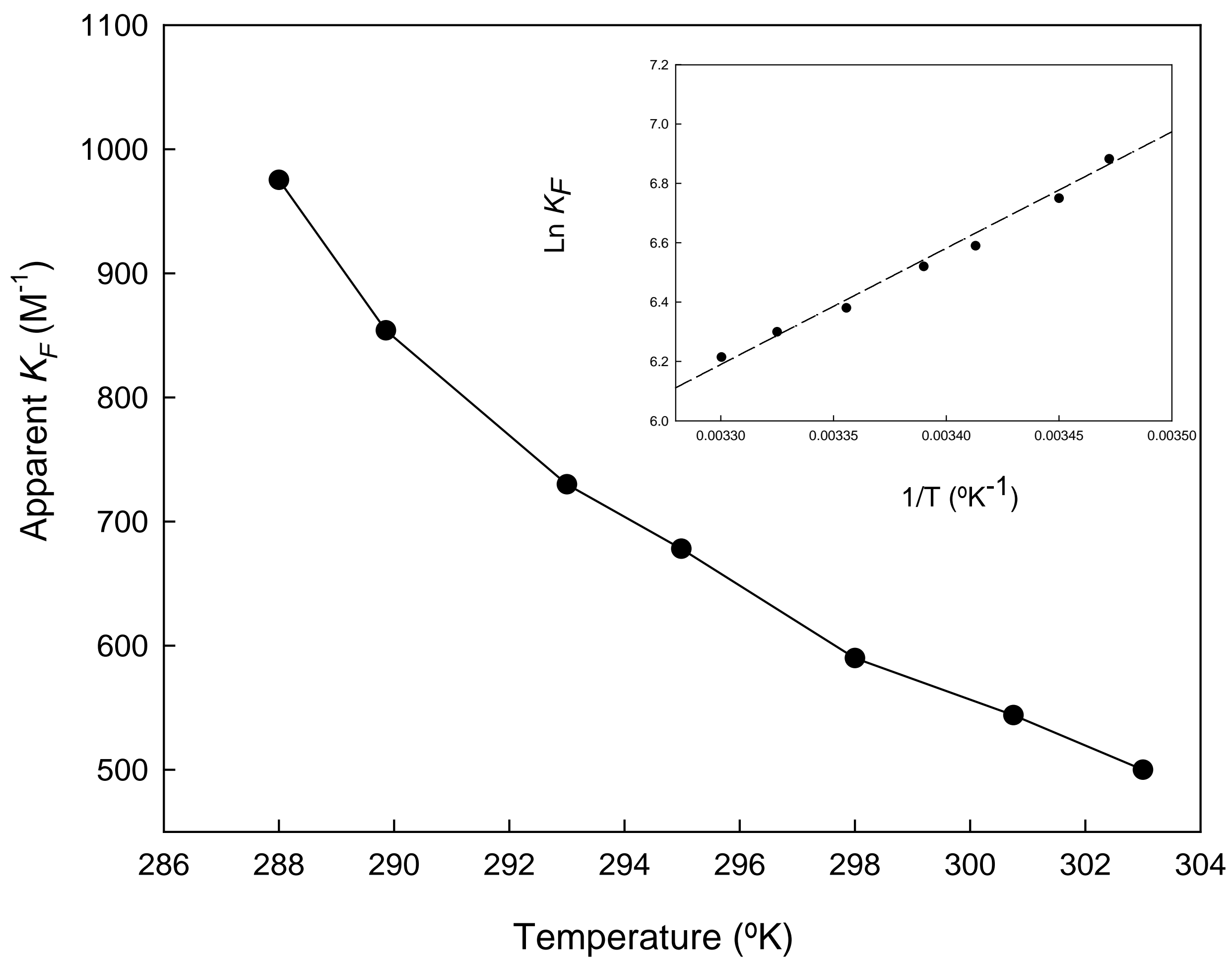
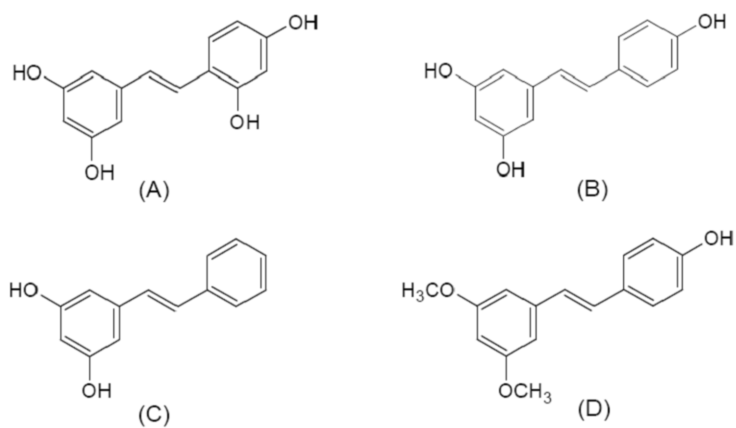


FIGURE 7



CAPÍTULO IX

Capacidad antioxidante y actividad antiradical de estilbenos

ABSTRACT

The family of stilbenes play important roles as health-protecting factors due their beneficial properties. Although the number of investigations about the antioxidant capacity of stilbenes family is high, no work has been focused on the comparison, in an unique study, of the antioxidant capacity of resveratrol, oxyresveratrol, pterostilbene and pinosylvin. Moreover, different researchers use different analytical techniques as ORAC, $ABTS^{\cdot+}$ or FRAP for measuring the antioxidant capacity. However, these authors do not have into account that those three techniques pursue different objective. In this study we used two types of tests to evaluate the antioxidant activity: the first one is based on a hydrogen atom transfer reaction, where the antioxidant and the substrate (probe) compete for free radicals (ORAC method). A second group is based on a single electron transfer reaction, monitored through a change in color as the oxidant is reduced ($ABTS^{\cdot+}$ and FRAP assays). For the ORAC assay, the greatest antioxidant activity is shown by resveratrol, followed by oxyresveratrol, pterostilbene and pinosylvin. However, for $ABTS^{\cdot+}$ assay, at a pH range 3.5-8.0, the highest antioxidant capacity presents the oxyresveratrol, followed by resveratrol, pinosylvin and finally pterostilbene. TEAC value in the $ABTS^{\cdot+}$ assay was determined. For oxyresveratrol it is 2.04±0.12, for resveratrol it is 1.57±0.1, for pinosylvin it is 1.23±0.1, and for pterostilbene it is 1.13±0.1. In FRAP assay all stilbenes present reducing activity below that obtained for trolox.This results are related to obtained with $ABTS^{\cdot+}$ at lower pH values.

INTRODUCTION

The nutritional protection that fruits, vegetables and various foodstuffs provide against several oxidative stress-based diseases has been attributed to various antioxidants including molecules belonging stilbene family, a group of compounds consisting of 2 aromatic rings joined by a methylene bridge which can be found in several foods and plants (Roupe et al., 2006). These molecules are a small family of plant secondary metabolites derived from the phenylpropanoid pathway, and produced in a number of unrelated plant species (Çelik et al., 2010). Moreover, they are involved in various ways in plant disease resistance (Chong et al., 2009).

The family of stilbenes includes four important molecules very studied due their beneficial properties: Oxyresveratrol (trans-2,3′,4,5′-tetrahydroxystilbene) is found in different sources such as mulberry (*Morus alba L.*) fruits and twig; trans-resveratrol (trans-3,4',5-trihidroxyestilbene) is a stilbene present in high concentrations in a wide variety of plants and fruits, including grapes, peanuts, blueberries, pine trees and plants used in oriental traditional medicine (Burns et al., 2002). Moreover, in plants trans-resveratrol is synthesized in response to stress conditions such as bumps, fungal infections, UV rays, heavy metals, etc; pinosylvin (trans-3',5'dihidroxistilbene) is a naturally occurring stilbene found in extracts of the wood of various species of pine (*Pinus sylvestris*) and eucalyptus. Pinosylvin formation occurs in pine needles, induced by infection or environmental stress (Antikainen et al., 2012). Finally, pterostilbene (trans-3,5-dimethoxy-4′-hydroxystilbene) a stilbenoid compound with many beneficial health effects. Pterostilbene is a naturally occurring phytoalexin which has been identified in some plant species and is found in different sources such as the leaves of *Vitis vinifera,* in

infected grape berries and in healthy and immature berries of var. Pinot Noir and Gamay (Pan et al., 2008). Moreover, pterostilbene is present in the heartwood of sandalwood (*Pterocarpus santalinus*) and *Pterocarpus marsupium* or in the berries of some Vacciunium species. Furthermore, this molecule also appears to be a constituent of the bark of Guibourtia tessmanii and, finally, high levels of pterostilbene were found in darakchasava (Rodríguez-Bonilla et al., 2011).

Several papers have showed that the consumption of these four stilbenes containing foods or nutraceuticals may be associated with improved health. These health benefits are related to a diverse range of biological activities such as anticancer (Huang et al., 2014; Laavola et al., 2015), antifungal (Caruso et al., 2011), antibacterial (Aslam et al., 2009), cardioprotection (Paul et al., 1999, Wu et al., 2001; Huang et al., 2014), antiviral (Sasivimolphan, et al., 2009), cytogenetic (Matsuoka et al., 2002), estrogenic (Gambini et al, 2013) and antiinflamatory (Robb E.L. & Stuart J.A., 2014; Nikhil et al., 2015; Perecko et al., 2015) activities and the inhibition of platelet aggregation (Yashiro et al., 2012; Kasiotis et al., 2013).

Among the health promoting properties of stilbene family, its antioxidant capacity has been studied in recent years due to the fact that this activity prevents the formation of reactive oxygen and nitrogen species, which play important roles in different pathologies. Indeed, resveratrol is one of the most studied antioxidants (de La Lastra & Villegas, 2007). Recently Gambini et al reported a review about the *in vitro* and *in vivo* effect of resveratrol on metabolism and its bioavailability and biological effects in animal models and humans. Moreover, the antioxidant activity of pterostilbene on brain and behaviour has been reported recently (Poulose et al., 2015). In 2015 Koskela et al. studied the pinosylvin-

mediated protection against oxidative stress. Furthermore, Choi et al, 2015 have recently published the mechanism of protection of oxyresveratrol on oxidative stress.

Although the number of investigations about the antioxidant capacity of stilbenes family is high, no work has been focused on the comparison, in an unique study, of the antioxidant capacity of resveratrol, oxyresveratrol, pterostilbene and pinosylvin.

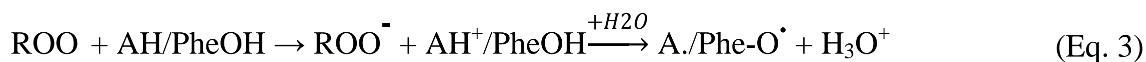
Moreover, different researchers use different analytical techniques as ORAC, $ABTS^{\cdot+}$ or FRAP for measuring the antioxidant capacity. However, these authors do not have into account that those three techniques pursue different objective. There is not an official standardised method, and therefore it is recommended that each evaluation should be made with various oxidation conditions and different methods of measurement (Frankel and Meyer, 2000). For this, to evaluate the antioxidant capacity of stilbenes in this study we used two types of tests (Huang et al., 2005): the first one is based on a hydrogen atom transfer reaction, where the antioxidant and the substrate (probe) compete for free radicals (ORAC method). A second group is based on a single electron transfer reaction, monitored through a change in color as the oxidant is reduced ($ABTS^{\cdot+}$ and FRAP assays).

This first transfer mechanism of a hydrogen atom to convert a radical into a stable species was recently named by Schaich (2013) as HAT-hydrogen atom transfer (H atom transferred to target radical), involving a possible secondary quenching by radical recombinations. (equations 1 and 2).

$$ROO + AH\ /PheOH \rightarrow ROOH + A^{\bullet}\ /Phe\text{-}O^{\bullet} \qquad \text{(Eq. 1)}$$

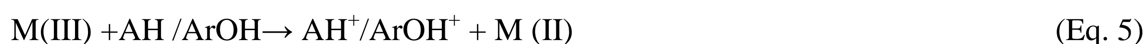
$$ROO^{\bullet} + A^{\bullet} \rightarrow ROOA \qquad \text{(Eq. 2)}$$

On the other hand, the second mechanism is called as SET (single electron transfer) and one or more electrons are transferred to reduce target compounds. It can be described with the following equations (Eq. 3-5):



$$ROO + AH/PheOH \rightarrow ROO^{-} + AH^{+}/PheOH \xrightarrow{+H2O} A./Phe\text{-}O^{\bullet} + H_3O^{+} \quad \text{(Eq. 3)}$$

$$ROO^{-} + H_3O \rightarrow ROOH + H_2O \quad \text{(Eq. 4)}$$



$$M(III) + AH/ArOH \rightarrow AH^{+}/ArOH^{+} + M(II) \quad \text{(Eq. 5)}$$

where AH = any antioxidant with donatable H, PheOH = phenol or polyphenol, M = redox-active metal.

Finally, although a systematic analysis of the structural features involved in the activity of stilbenes is necessary, no study has examined the relationship between the structure of resveratrol, oxyreveratrol, pterostilbene and pinosilvine and it antioxidant capacity. In the present study, the individual radical scavenging capacities and antioxidant properties of four relevant stilbenes are characterized in an attempt to comprare the different experimental approaches and to unveil the role of phenolic hydroxy groups in the antiradical activity of stilbenes.

EXPERIMENTAL SECTION

Materials

(E)-resveratrol was from Sigma-Aldrich (Madrid, Spain). Oxyresveratrol was kindly supplied by Dr. José Luis Cenis Anadón, IMIDA. Pinosylvin and pterostilbene were purchased from Sequoia Research Products Limited (Pangbourne, United Kingdom). Stilbenes are sensitive to the light and irradiation of solutions containing the analyte induces the formation of derived molecules, which leads to the formation of a highly fluorescent compounds. Moreover, these stilbenes are also sensitive to light because of their (E) to (Z) diastereomerization. Thus, all samples were stored in darkness. Other chemicals and reagents were obtained from Sigma (St. Louis, MO, USA). Solvents were from Merck Chemicals Ltd. (Dorset, England). Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

General Experimental Procedures

ORAC, $ABTS^{\cdot+}$ and FRAP methods were propped into two groups, depending on the type of measuring antioxidant activity:

1. Hydrogen atom transfer reaction

   1.1. ORAC-FL method

The aqueous oxygen radical absorbent capacity (ORAC-FL) assay was performed as described by Dávalos et al. 2004. Fluorescein (3´,6´-dihydroxy-spriro[isobenzofuran-

1[3H], 9´[9H]-xanthen]-3-one) was used as the fluorescent probe and AAPH (2,2'-Azobis(2-amidinopropane) dihydrochloride) was used as a peroxyl radicals generator.

The ORAC-FL analyses were carried out on a Synergy HT multi-detection microplate reader, from Bio-Tek Instruments, Inc. (Winooski, VT, USA), equipped with a thermostat. The temperature of incubation was 37ºC. Fluorescence was read with an excitation wavelength of 485/20 nm and an emission filter of 528/20 nm. The measurements were made using 96-well polystyrene microplates with black sides and clear bottom. In order to avoid a temperature effect, only the inner 60 wells were used for experimental purposes, while the outer wells were filled with 200 µL of distilled water. The reaction was carried out in 75 mM sodium phosphate buffer prepared (pH 7.4) and stock solutions of stilbenes (oxyresveratrol, resveratrol, pinosilvin and pterostilbene) were diluted in MeOH. The final reaction mixture was 200 µL. FL (100 µL; 60 nM, final concentration) and stilbenes (70 µL) solutions, were placed in the wells of the microplate. The mixture was preincubated for 30 min at 37 ºC, before rapidly adding the AAPH solution (30 µl; 200mM, final concentration) using a multichannel pipette. The microplate was immediately placed in the reader and the fluorescence recorded every 1.14 min for 120 min. The microplate was automatically shaken prior to each reading. A blank with FL and AAPH (using sodium phosphate buffer instead of the antioxidant solution, and eight calibration solutions using Trolox (6.25, 12.5, 18.75, 21.25, 27.5 y 31.25 µM) as antioxidant were also used in each assay.

All reaction mixtures were prepared in triplicate and at least three independent assays were performed for each sample.

The area under the fluorescence decay curve (AUC) was calculated by the following equation:

$$\text{AUC} = 1 + \sum_{i=1.14}^{i=120} \frac{fi}{f0}$$

Where:

f_o is the initial fluorescence read at 0 min and f_i is the fluorescence read at time i. The net AUC corresponding to the sample was calculated by subtracting the AUC corresponding to the blank. Data processing was performed using Sigmaplot software package 10.0 (Systat Software Inc., San Jose, CA, USA).

2. Single electron transfer reaction (ABTS and FRAP assays)

2.1. ABTS$^{\bullet+}$ assay

The antiradical capacity of oxyresveratrol, resveratrol, pinosylvin and pterostilbene was evaluated by following its effect on stable free radical ABTS$^{\cdot+}$ [2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)]. This assay is based on the reduction of the absorbance of ABTS$^{\bullet+}$ radical cation solutions, which has a broad absorption spectrum (Re et al., 1999; Katalinic et al., 2005). The ABTS$^{\bullet+}$ radical was prepared from 2mM ABTS through peroxidase activity (88 units/L commercial horseradish peroxidase type VI) in the presence of H_2O_2 (45 µM), in sodium acetate buffer, (pH 5.0; 0.2 M). The reactive was then diluted with the addition of samples, carrying out the reactions in sodium phosphate buffer,

pH 7.0. The final volume was 300 μL and the stilbenes concentration was 1 μM. Other conditions are specified in the text. The reaction was monitored spectrophotometrically at 414 nm (Escribano, et al., 1998). The measurements were carried out in 96 well plates at t = 0 and at different times of incubation until the final measurement after 24 hours of incubation at 20 °C in a plate reader Synergy HT plate reader (Bio-Tek Instruments, Winooski).

All experiments were performed in triplicate. The detector linearity under the assay conditions was confirmed (r = 0.999). Data analysis was carried out by using linear regression fitting using Sigma Plot Scientific Graphing for Windows version 10.0.

Antioxidant action is reported as: the antioxidant concentration that gives the same response as 1mM Trolox. Trolox equivalents are calculated by comparing (A_0-A_f) of the test antioxidant to (A_0-A_f) of Trolox standards.

2.2. FRAP assay

The antioxidant capacity of oxyresveratrol, resveratrol, pinosylvin and pterostilbene was determined by the reduction of Fe (III) a Fe (II), according to the method described by Benzie and Strain (1996). FRAP assay evaluates the antioxidant capacity of a compound based on its ability to directly reduce Fe (III) to Fe (II). At 3.6 pH, when ferric-pyridyltriazine (Fe^{III}-TPTZ) complex is reduced to the ferrous (Fe^{II}) form, an intense blue color with an absorption maximum at 593 nm develops. For reagent preparation $FeCl_3$ solutions at a final concentration of 1.48 mM, in 223 mM sodium acetate buffer, pH 3.6, were used. Fe(III) reduction to Fe(II) was observed through the addition of the reagent

2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) at a final concentration of 741 μM. The reaction was monitored at λ = 593 nm in a Synergy HT plate reader. Concentrations of stilbenes and Trolox were used as 1 μM. All measurements were performed in triplicate, and mean values and standard deviations were plotted.

RESULTS AND DISCUSSION

ORAC method

The Antioxidant activity of oxyresveratrol, resveratrol, pterostilbene and pinosylvin was evaluated by ORAC method. During the ORAC assay, fluorescence intensity (FI) was followed to monitor the decay of the fluorescence curve. A calibration curve was obtained by plotting the net curve against trolox solutions of concentrations in the 6.25–31.25 µM range.

The equation for the calibration curve was $y = 11.8338 + 1.6113\ x$ with a good correlation coefficient ($r2 = 0.998$). The instrumental precision and the precision of the method were confirmed by three consecutive repetitions of a single sample and by determination of the antioxidant capacity of three independent aliquots of the sample, respectively.

Figure 2 inset shows the fluorescence decay curve of resveratrol, oxyreveratrol, pinosylvin and pterostilbene at the same concentration (2 µM) for 90 minutes. Figure 2 shows that when increasing the concentration of antioxidant in the medium the net AUC increases linearly with a good correlation coefficient ($r2 > 0.998$).

The Trolox equivalent antioxidant capacity (TEAC) for each stilbene was determined. For oxyresveratrol it is 3.35±0.2, for resveratrol it is 3.67±0.2, for pinosylvin it is 1.71±0.1, and for pterostilbene it is 2.57±0.1.

According to the results, the greatest antioxidant capacity is shown by resveratrol (three hydroxy groups), followed by oxyresveratrol (four hydroxy groups), pterostilbene (one hydroxy groups) and pinosylvin (two hydroxy groups). These results show that the protection of the oxidative degradation of the fluorescent molecule is not only dependent of the presence of hydroxy groups in the stilbenes structure. Although, Xu et al., 2007 published a study on the reaction between trans-resveratrol and hydroperoxyl radical where they reported that resveratrol can scavenge free radicals mainly because phenoxy radicals are less reactive than many other radicals due to their resonance stabilization, other factors should also be considered.

$ABTS^{\cdot+}$ assay

The free radical scavenging activity of a molecule can be evaluated by its effect on stable colored solutions of radical $ABTS^{\cdot+}$. The assay is based on monitoring the decrease in the absorbance of the radical solution. Concentration of the antioxidant and the duration of reaction on the inhibition of the $ABTS^{\cdot+}$ radical cation should be taken into account to determine the antioxidant capacity of a molecule (Re et al., 1999). Figure 3A shows the recordings obtained for the activity of the four stilbenes. As can be seen, there is a decrease in the concentration of the radical with time after the addition of the corresponding compound.

The activities of the stilbenes were compared with that of trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a potent antiradical, water-soluble derivative of

vitamin E. Increasing concentrations of each stilbene or trolox was added to the $ABTS^{\cdot+}$ radical mixture, causing a linear response in the removal of the radical.

Figure 3B shows the effects of increasing the concentrations of the stilbenes and trolox in radical solutions at pH 7.0. Pterostilbene was used in a higher concentration so that the signal was registrable. As can be seen, all stilbenes exhibit a high free radical scavenging capacity at much lower concentrations than trolox.

Oxyresveratrol possesses the highest radical scavenging activity of the stilbenes assayed, followed by resveratrol, pinosylvin and pterostilbene. The order of the stilbenes activity when ABTS assay is used is different to the obtained in ORAC assay. This result shows that ORAC and ABTS techniques can not be used randomly to measure the antioxidant capacity of a molecule. This result is supported by the fact that the ABTS technique is based on a single electron transfer reaction and ORAC technique is based on a hydrogen atom transfer reaction.

These results are in agreement with Tian & Schaich (2013), due to the absorbance drop (A_0-A_f) upon wich TEAC calculatons are based provides reaction stoichometry (total mols $ABTS^{\cdot+}$ reduced per mol antioxidant after full reaction).

TEAC for each stilbene was determined. For oxyresveratrol it is 2.04±0.12, for resveratrol it is 1.57±0.1, for pinosylvin it is 1.23±0.1, and for pterostilbene it is 1.13±0.1.

Effect of pH on the free radical scavenging activity of stilbenes

As described Mikulski and Molski, 2010, the structure of stilbenes condition their ability to scavenge free radicals. As is shown in Figure 1 several hydroxyl groups, that can be

protonated or deprotonated depending of medium pH are present in the structure of stilbenes. Due to the importance of the pH on the structure of stilbenes, and thus on stilbenes activity, the next step was to evaluate the effect of pH of the medium on the free radical scavenging activity of oxyresveratrol, resveratrol, pinosylvin and pterostilbene. The pH was varied in the range 3.5 to 11 in the assay medium. Sodium acetate was used as a buffer for pH values ranging from 3.5 to 5.5 and sodium phosphate for 6.0 to 11.0.

Figure 4 shows the dependence of pH on antiradical activity of oxyresveratrol, resveratrol, pinosylvin and pterostilbene. At acidic pH values, there is a low free radical scavenging activity. However, at pH values above 8.0 there is a strong increase in free radical scavenging activity. This behavior may be due to the deprotonation of the first hydroxyl group existent in the structure of the stilbenes. In previous work of our investigation group it was reported the characterization of three pKa values for (E) resveratrol (pKa1, 8.8; pKa2, 9.8; and pKa3, 11.4). The first pKa is associated with the deprotonation of 4-OH because the abstraction of 4-H is easier than that of 3-H and 5-H. The second pKa indicates the deprotonation of 3-OH or 5-OH (the 3- and 5-positions have the same structures because the molecule is symmetric). The third pKa indicates the deprotonation of 5-OH or 3-OH. This indicates that the free radical scavenging activity is strongly dependent of the presence of hydroxyl groups in the structure of stilbenes.

As can also be checked in Figure 4, at pH 3.5-8.0 the highest antioxidant capacity presents the oxyresveratrol, followed by resveratrol, pinosylvin and finally pterostilbene. This suggests that the ability of radical scavenging activity may depend on the number and position of hydroxyl groups (4, 3, 2 and 1 hydroxyl groups, respectively).

Interestingly, all stilbenes present a maximum capacity at pH 9.0, but oxyresveratrol that presents the maximum at pH 8.0.

FRAP Method

The antioxidant activity of oxyresveratrol, resveratrol, pinosylvin and pterostilbene were characterized by their capacity to directly reduce Fe(III) to Fe(II). The FRAP assay was used (Benzie and Strain, 1996), monitoring the reduction reaction spectrophotometrically at λ=593 nm. Solutions of known concentrations of iron Fe (II) ($FeSO_4$) were used to set a calibration curve.

Figure 5 shows the values obtained for the ferric reduction by the different stilbenes assayed at varying concentration 0-50 µM assayed and Trolox, compared to the absorbance obtained for the calibration curve. All stilbenes present reducing activity below that obtained for Trolox. Taking into account that the FRAP assay is performed at pH 3.6, these observations are related to the results obtained with $ABTS^{\cdot+}$ at lower pH values (Figure 4).

Table 1 shows the Trolox molar equivalence in the reduction of Fe (III) for each stilbene.

CONCLUSIONS

Resveratrol, oxyresveratrol, pinosylvin and pterostilbene present high antioxidant and free radical scavenging activities not linked to the presence of phenolic hydroxy groups. This may be general to the four stilbenes studied which contain a similar electronic resonance system. In addition to their intrinsic activity, a clear enhancing effect on the scavenging of the free radical $ABTS^{\cdot +}$ and on the antioxidant power of stilbenes has been demonstrated to be linked to the presence of one or two phenolic hydroxy groups in their structure.

The structure of stilbenes is dependient of the pH of the medium. At pH values highest of 8.0, stilbenes suffer a deprotonation of their hydroxyl gropus. This fact increase the antioxidant activity of stilbenes. However, at lower pH values, all stilbenes present reducing antioxidant activity.

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TABLES

Table 1. TEAC of stilbenes calculated with FRAP assay.

FIGURE LEGEND

Figure 1. Structures for the stilbenes.

Figure 2. Effect of different concentrations of stilbenes in ORAC: (■) resveratrol, (●) oxyresveratrol, (♦) pterostilbene, (▲) pinosylvin. *Inset*: Fluorescence decay curve of different stilbenes [2 µM] during the ORAC assay.

Figure 3. (A) Absorbance decay curve during the ABTS assay. (x) trolox 1µM, (●) Oxyresveratrol 1µM, (■) Resveratrol, (♦) pterostilbene 5 µM, (▲) pinosylvin 1 µM. $ABTS^{\cdot+}$ was used at a concentration 47 µM. The reaction was followed spectrophotometrically at λ =414 nm, at pH 7.0. **(B)** ABTS radical depletion dependence on stilbene concentration as compared with trolox. $ABTS^{\cdot+}$ was used at a concentration 47 µM. (**x**) Trolox, (●) Oxyresveratrol, (■) resveratrol, (♦) pterostilbene, (▲) pinosylvin.

Figure 4. Percentage of the remaining stilbene as a function of different pH values. (●) Oxyresveratrol , (▲) Pterostilbene, (○) Pinosylvin, (■) Resveratrol, (--) Blank.

Figure 5. Antioxidant power of stilbenes through its ferric reducing ability and standard curve. The effect was evaluated spectrophotometrically at λ = 593 nm. (✕) Trolox, , (●) oxyresveratrol, (■) Resveratrol, (▲) Pinosylvin, (○) Pterostilbene. (r>0.99).

TABLE 1

STILBENE	TEAC
Oxyresveratrol	0.52±0.1
Resveratrol	0.47±0.1
Pinosylvin	0.31±0.1
Pterostilbene	0.05±0.1

FIGURE 1

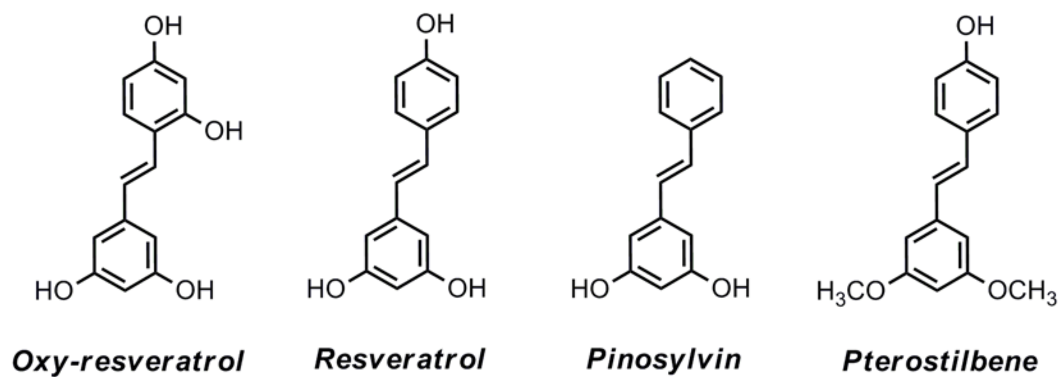


FIGURE 2

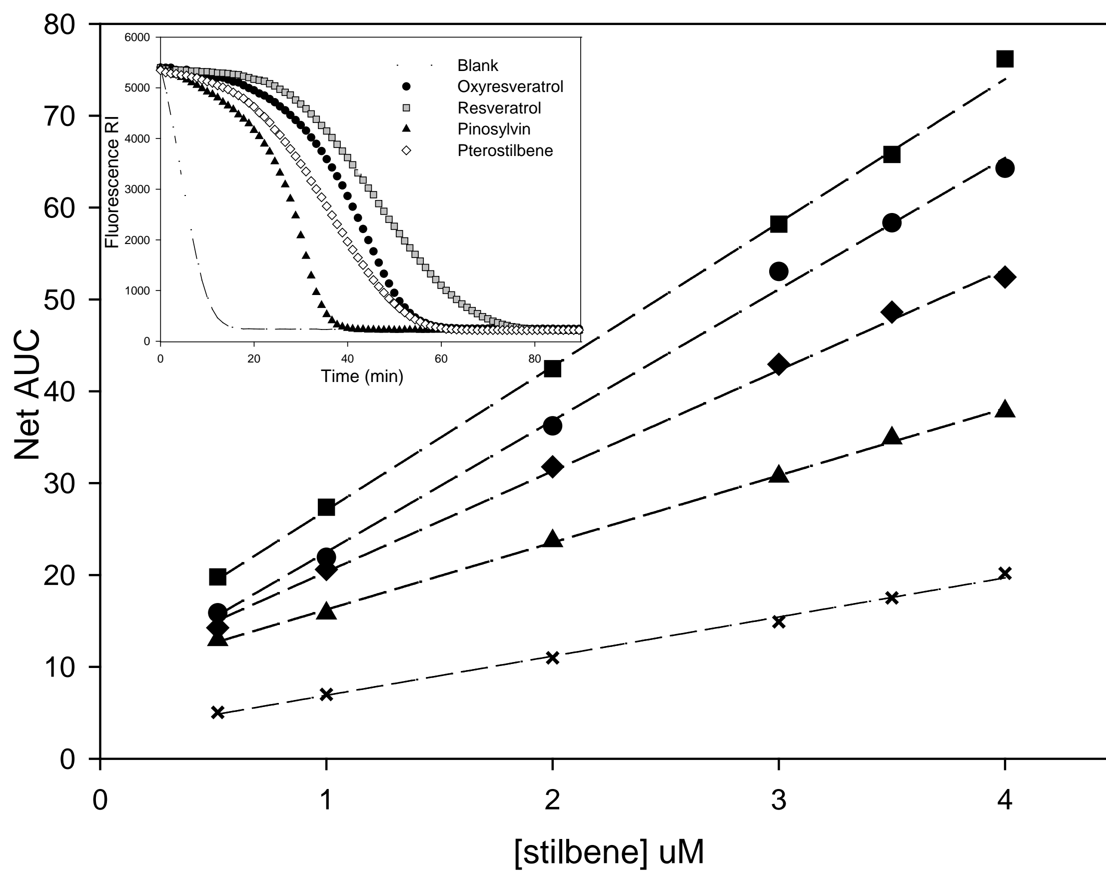


FIGURE 3

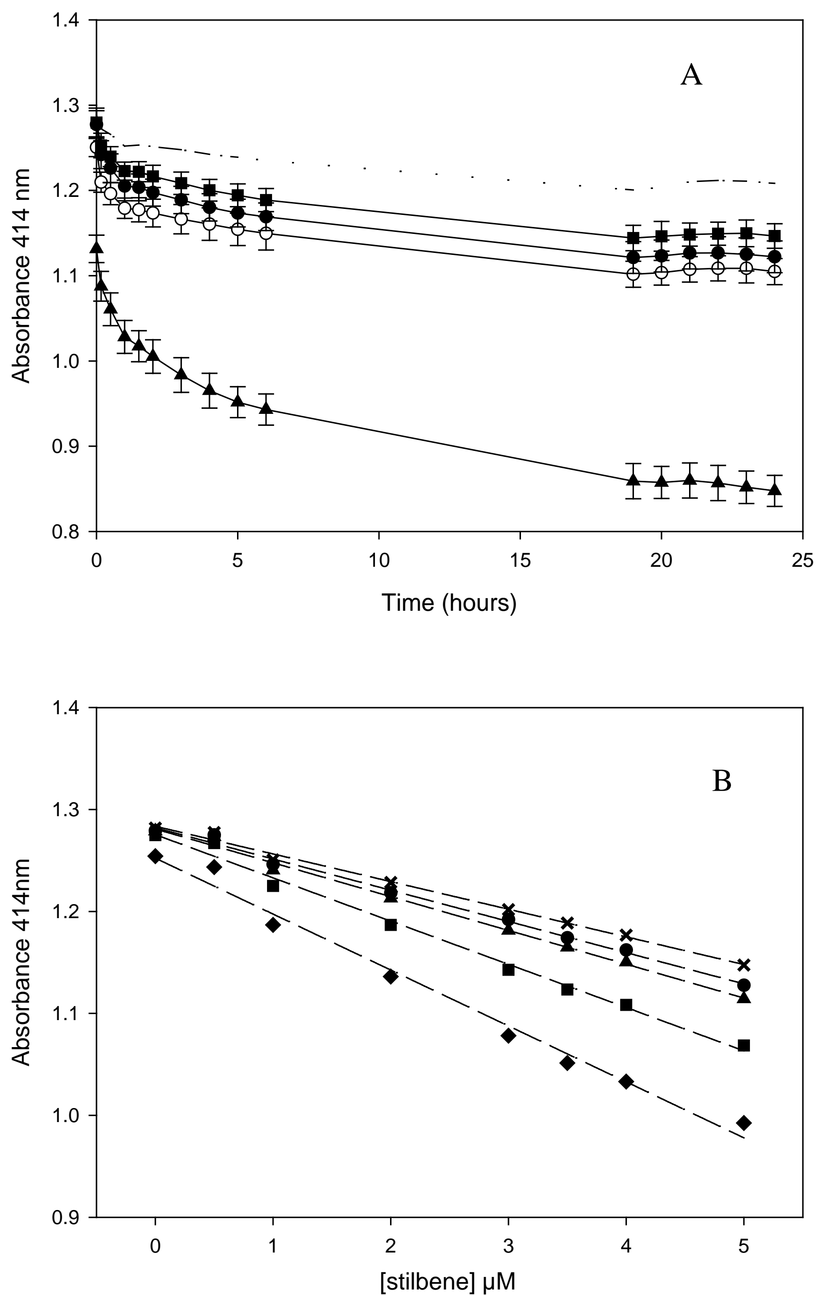


FIGURE 4

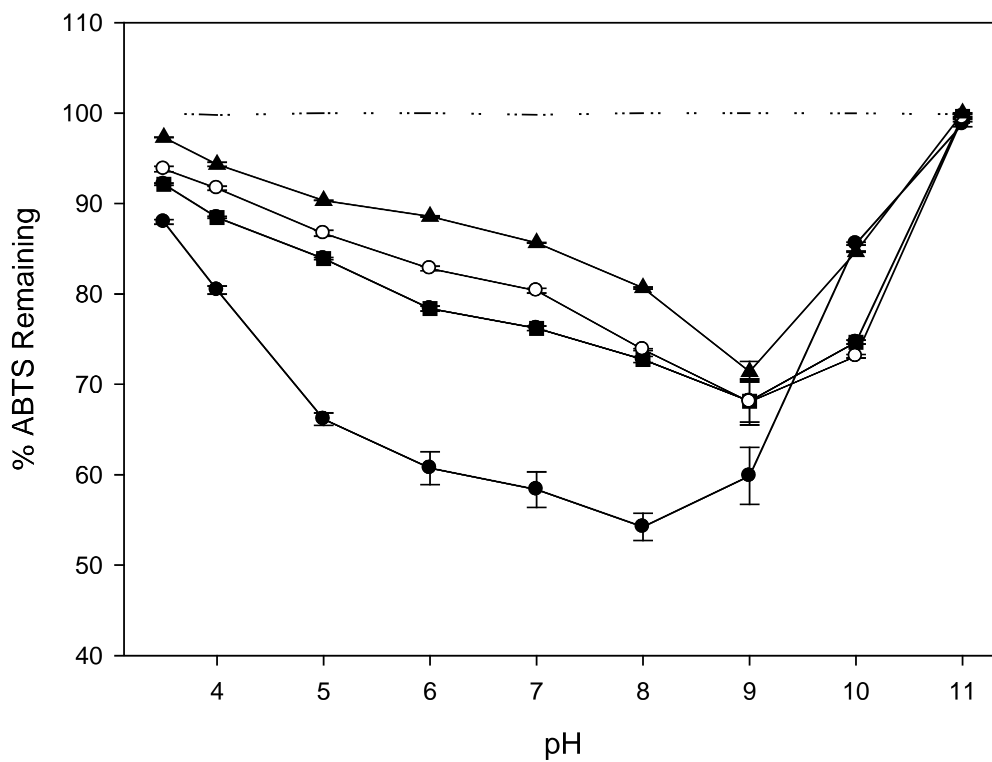
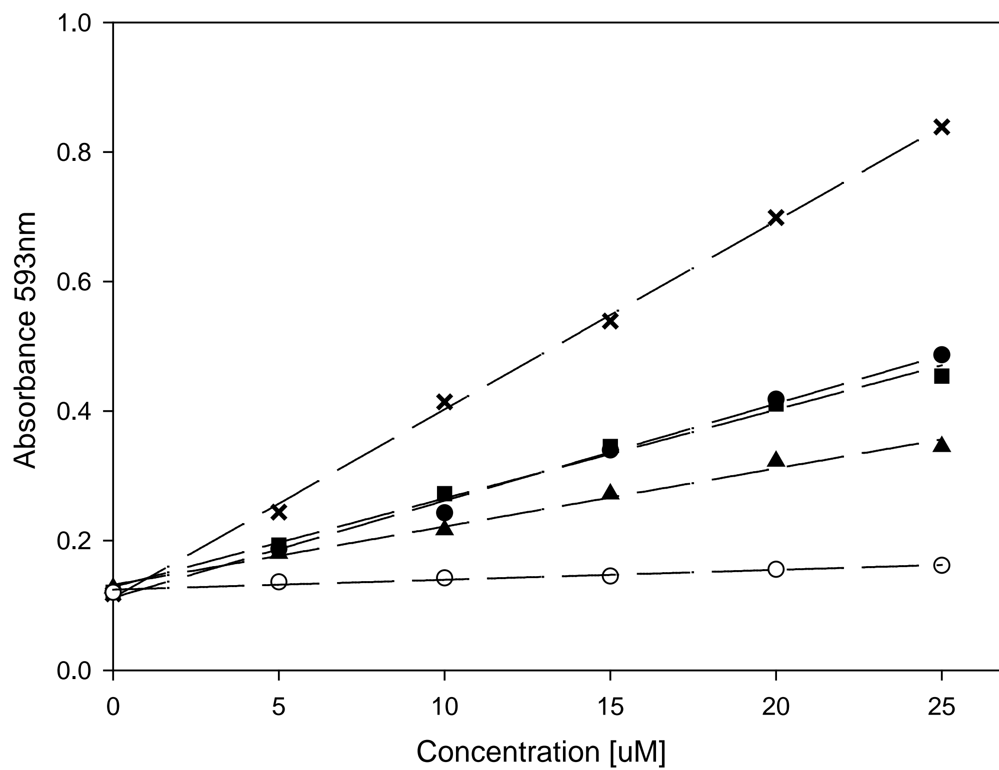


FIGURE 5



CAPÍTULO X

¿Pueden usarse los métodos ABTS, ORAC y FRAP para determinar la capacidad antioxidante de compuestos bioactivos encapsulados en ciclodextrinas?

ABSTRACT

This study evaluated the effect of the addition of different CDs on oxyresveratrol antioxidant capacity, an analogue of resveratrol, using three different methods: ORAC-FL, ABTS and FRAP.

In all methods, to the addition of different CDs in increasing concentrations to the medium seemed to increase oxyresveratrol antioxidant capacity. However, when assessing the effect of the CDs themselves on different methods, could be observed in ORAC-FL and ABTS methods produced different responses CDs alone, suggesting that they could produce an interference in such methods. However, that interference will not occur when using CDs in the FRAP method. With this method, it was observed that the response on the antioxidant capacity of oxyresveratrol when added CDs in the medium was dependent on the measurement time. Thus, it was observed that appeared to increase in the early stages of the reaction with the increasing concentration of CDs in the reaction medium, but as time passed it seemed not seen any noticeable effect.

INTRODUCTION

Antioxidants are molecules that have the ability to scavenge free radicals, delaying oxidative processes and increasing therefore the useful life of food products and pharmaceuticals (Halliwell, 1997). A family of compounds with high antioxidant activity described are stilbenes, a group of molecules consisting of 2 aromatic rings joined by a methylene bridge which can be found in several plants and foods (Roupe et al., 2006). These molecules are a small family of plant secondary metabolites derived from the phenylpropanoid pathway, and produced in a number of unrelated plant species (Çelik et al., 2010).

One of the stilbenes more studied in recent years is oxyresveratrol (trans-2,3´,4,5´-tetrahydroxystilbene), a molecule present in different sources such as mulberry (*Morus alba L.*) fruits and twig (Lorentz et al., 2003). Its pharmacological properties include a wide range of biological activities: antioxidant (Lorentz et al., 2003), antiviral (Sasivimolphan et al., 2009), hepatoprotective (Oh et al., 2002), and cyclooxygenase and tyrosinase-inhibitory (Kim et al., 2002; Li et al., 2007) activities.

These healthy properties recommend the use of this stilbene in both nutraceutical and food industry. However, problems concerning the physicochemical properties of oxyresveratrol have meant that no “novel food” has been fortified with this stilbene. Indeed, oxyresveratrol possesses low bioavailability and is easily oxidized by prooxidant agents. For these reasons, in a recent work (Rodríguez-Bonilla et al., 2010) our research group studied the complexation of oxyresveratrol with cyclodextrins, (CDs) a types of molecules which can increases the bioavailability and stability of oxyresveratrol in the face

of prooxidant agents is strongly desirable. CDs are a group of structurally related natural products formed during the bacterial digestion of starch (Szetjli, 1982). These cyclic oligosaccharides consist of α-(1–4) linked α -dglucopyranose units and contain a somewhat lipophilic central cavity and a hydrophilic outer surface. Natural α-, β-, and ɣ-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 (Del Valle, 2004; Challa et al., 2005). Because CDs are able to increase the bioavailability of different compounds with proven health properties, their use in food and pharmaceutical industries is increasing (Semeraro et al, 2015; Aktas et al., 2015).

Although in recent years we have demonstrated that CDs are able to increase the apparent solublitity of some stilbenes such as resveratrol (López Nicolás et al., 2008), pterostilbene (Rodríguez-Bonilla et al., 2011), oxyresveratrol (Rodríguez-Bonilla et al., 2010) and pinosylvin (López-Nicolás et al., 2009), no work has focused the effect of the addition of CDs on its antioxidant capacity and free radical scavenging of these stilbenes. Moreover, we found in the literature several papers which have contradictory conclusions about the effect of CDs on the antioxidant capacity of bioactive molecules. Firstly, several researchers have published that the addition of CDs increase the antioxidant activity of guest molecules (Álvarez-Parrilla et al, 2005; Lucas Abellán et al, 2011), others works conclude that CDs not are able to modify this health promoting property (Lucas Abellán et al, 2011) and, finally, different investigators affirm that the presence of CDs reduce the antioxidant activity of some bioactive compounds (Folch-Cano et al, 2010).

To throw light on this controversy, and because the majority of these investigations use different analytical techniques to measure the antioxidant activity, the main objective of this work is to analyze the antioxidant capacity and the free radical activity of oxyresveratrol encapsulated in CDs employing three techniques: ORAC, $ABTS^{\cdot +}$ and FRAP. Moreover, we determine which of these three methods is the optimum for analyzing the antioxidant activity of a molecule when CDs are present in the medium, showing that not all techniques can be used for measuring the antioxidant activity in the presence of CDs. Finally, molecular modelling studies were carried out to propose which molecular interactions are established in the complexation process of oxyresveratrol by CDs.

EXPERIMENTAL SECTION

Materials

Oxyresveratrol was kindly supplied by Dr. José Luis Cenis Anadón (IMIDA). Oxyresveratrol is sensitive to the light and irradiation of solutions containing the analyte induces the formation of derived molecules, which leads to the formation of a highly fluorescent compounds. Moreover, oxyresveratrol is also sensitive to light because of their (E) to (Z) diastereomerization. Thus, all the samples were stored in darkness. CDs (α-CD, β-CD, methyl-β-CD, ethyl-β-CD y HP-β-CD) was purchased to Sigma (Madrid, Spain). Other chemicals and reagents were also obtained from Sigma (Madrid, Spain). Solvents were from Merck Chemicals Ltd. (Dorset, England). Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

General Experimental Procedures

Molecular modeling and molecular docking:

Molecular structures used in this work was build using Avogadro Software (Hanwell et al., 2012) or by different databases. β-CD structure was extracted from a crystal of Protein Data Bank (PDB ID: 1Z0N). Oxyresveratrol was obtained from CACTUS database (NCI/CADD group, USA). HPβCD was built by adding hydroxypropyl group to our βCD. Molecular docking was done using Autodock Vina (Trott and Olson, 2010). All CDs was considered as rigid. Graphical representations of the docking results were prepared using PyMOL (Molecular GraphicsSystem, version 1.3, Schrödinger, LLC).

ORAC-FL method

The aqueous oxygen radical absorbent capacity (ORAC-FL) assay was performed as described by Dávalos et al. 2004. Fluorescein (3´,6´-dihydroxy-spriro[isobenzofuran-1[3H], 9´[9H]-xanthen]-3-one) was used as the fluorescent probe and AAPH (2,2'-Azobis(2-amidinopropane) dihydrochloride) was used as a peroxyl radicals generator.

The ORAC-FL analyses were carried out on a Synergy HT multi-detection microplate reader, from Bio-Tek Instruments, Inc. (Winooski, VT, USA), equipped with a thermostat. The temperature of incubation was 37ºC. Fluorescence was read with an excitation wavelength of 485/20 nm and an emission filter of 528/20 nm. The measurements were made using 96-well polystyrene microplates with black sides and clear bottom. In order to avoid a temperature effect, only the inner 60 wells were used for experimental purposes, while the outer wells were filled with 200 µL of distilled water. The reaction was carried out in 75 mM sodium phosphate buffer prepared (pH 7.4) and stock solutions of oxyresveratrol was diluted in MeOH. The final reaction mixture was 200 µL. FL (100 µL; 60 nM, final concentration) and oxyresveratrol (70 µL) solutions, were placed in the wells of the microplate. The mixture was preincubated for 30 min at 37 ºC, before rapidly adding the AAPH solution (30 µl; 200mM, final concentration) using a multichannel pipette. The microplate was immediately placed in the reader and the fluorescence recorded every 1.14 min for 120 min. The microplate was automatically shaken prior to each reading. A blank with FL and AAPH (using sodium phosphate buffer instead of the antioxidant solution, and eight calibration solutions using Trolox (6.25, 12.5, 18.75, 21.25, 27.5 y 31.25 µM) as antioxidant were also used in each assay.

All reaction mixtures were prepared in triplicate and at least three independent assays were performed for each sample.

The area under the fluorescence decay curve (AUC) was calculated by the following equation:

$$\mathrm{AUC} = 1 + \sum_{i=1.14}^{i=120} \frac{fi}{f0}$$

Where:

f_o is the initial fluorescence read at 0 min and f_i is the fluorescence read at time i. The net AUC corresponding to the sample was calculated by subtracting the AUC corresponding to the blank. Data processing was performed using Sigmaplot software package 10.0 (Systat Software Inc., San Jose, CA, USA).

Natural CDs (α-CD, β-CD) and modified CDs (HP-β-CD, Methyl-β-CD, Ethyl-β-CD) were diluted in 75 mM sodium phosphate buffer prepared (pH 7.4).

$ABTS^{\bullet +}$ assay

The antiradical capacity of oxyresveratrol was evaluated by following its effect on stable free radical $ABTS^{\cdot +}$ [2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)]. This assay is based on the reduction of the absorbance of $ABTS^{\bullet +}$ radical cation solutions, which has a broad absorption spectrum (Re et al., 1999; Katalinic et al., 2005). The $ABTS^{\bullet +}$

radical was prepared from 2mM ABTS through peroxidase activity (88 units/L commercial horseradish peroxidase type VI) in the presence of H_2O_2 (45 μM), in sodium acetate buffer, (pH 5.0; 0.2 M). The reactive was then diluted with the addition of samples, carrying out the reactions in sodium phosphate buffer, pH 7.0. The final volume was 300 μL and the oxyresveratrol concentration was 1 μM. Other conditions are specified in the text. The reaction was monitored spectrophotometrically at 414 nm (Escribano, et al., 1998). To evaluate the effect of addition of CDs on $ABTS^{\cdot+}$ were added increasing concentrations of Etil-β-CD [0-882 μM] diluted in sodium phosphate buffer (75 mM; pH 7.4) in a spectrophotometric cuvette with $ABTS^{\cdot+}$ and differents measurement were carried out.

The measurements were carried out in 96 well plates at t = 0 and at different times of incubation until the final measurement after 24 hours of incubation at 20 °C in a plate reader Synergy HT plate reader (Bio-Tek Instruments, Winooski, United States).

All experiments were performed in triplicate. The detector linearity under the assay conditions was confirmed (r = 0.999). Data analysis was carried out by using linear regression fitting using Sigma Plot Scientific Graphing for Windows version 10.0 (2001; SPSS, Chicago, IL).

Antioxidant action is reported as Trolox equivalents by comparing (A_0-A_f) of the test antioxidant to (A_0-A_f) of Trolox standards, or the antioxidant concentration that gives the same response as 1mM Trolox.

FRAP assay

The antioxidant capacity of oxyresveratrol, resveratrol, pinosylvin and pterostilbene was determined by the reduction of Fe (III) a Fe (II), according to the method described by Benzie and Strain (1996). FRAP assay evaluates the antioxidant capacity of a compound based on its ability to directly reduce Fe (III) to Fe (II). At 3.6 pH, when ferric-pyridyltriazine (Fe^{III}-TPTZ) complex is reduced to the ferrous (Fe^{II}) form, an intense blue color with an absorption maximum at 593 nm develops. For reagent preparation $FeCl_3$ solutions at a final concentration of 1.48 mM, in 223 mM sodium acetate buffer, pH 3.6, were used. Fe(III) reduction to Fe(II) was observed through the addition of the reagent 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) at a final concentration of 741 µM. The reaction was monitored at λ = 593 nm in a Synergy HT plate reader. Concentrations of stilbenes and Trolox were used as 1 µM. All measurements were performed in triplicate, and mean values and standard deviations were plotted.

To evaluate the effect of addition of CDs on FRAP assay were added increasing concentrations of HP-β-CD [0-4 mM] diluted in sodium phosphate buffer (75 mM; pH 7.4) in a spectrophotometric cuvette with $ABTS^{\cdot+}$ and differents measurement were carried out.

RESULTS AND DISCUSSIÓN

Molecular docking

In a recent work, we investigated the encapsulation of oxyresveratrol with natural CDs for first time using RP-HPLC and mobile phases to which naturals CDs were added (Rodríguez-Bonilla et al., 2010). However, up date no work about the encapsulation between modified CDs and oxyresveratrol has been published. As in the present work different modified CDs have been used, the first step was study the possibility of the encapsulation of oxyresveratrol in a modified CD (HPβCD).

In order to obtain more information about interactions between oxyresveratrol (ligand) and CD (receptor) in the complex, molecular docking technique was used to predict the orientation and interactions. Two CDs were selected, natural βCD and modified HPβCD.

Docking results (Figure 1) showed as Oxyresveratrol can enter in βCD almost totally (FIGURA A). On the other hand, Oxyresveratrol enters in HPβCD better than in βCD (FIGURA B) and, furthermore, Oxyresveratrol-HPβCD has more hydrogen bonds than Oxyresveratrol-βCD. The combination of these factors (a better insertion in CD and a more number of hydrogen bonds), as well as the correct orientation of the radicals of HPβCD, could justify differences in the strong of encapsulation. According to score results, HPβCD (-6.0) could be a better encapsulant agent than βCD (-5.7).

ORAC-FL Method

The next step in the study was to validate the ORAC-FL method as a system for measuring the oxyresveratrol antioxidant capacity. For this reason, we analyzed the response of the decrease of the fluorescence intensity presented by FL due to addition of AAPH (free radical generator) in the presence of increasing concentrations (0, 0.52, 1, 2, 3 and 4 mM) of oxyresveratrol. The values obtained by the oxyresveratrol were shown in **Figure 2.**

As it was expected an increase in oxyresveratrol concentration produced a decrease in intensity relative value of stilbenes fluorescence. The results were expressed as relative fluorescence respect to the initial measure. Figure 2 inset shows a regression of Net AUC obtained by oxyresveratrol different concentrations, resulting in a linear relationship (r2 = 0.998) between oxyresveratrol concentration and fluorescence decay curve.

Determination by ORAC-FL method of the antioxidant capacity of oxyresveratrol/CD nanocomplexes

After determining the existence of a linear relationship between the concentration of oxyresveratrol in the reaction medium and fluorescence decay curve of FL, the next step was to evaluate the effect produced by the addition of CDs on the antiradical capacity of oxyresveratrol when ORAC-FL method is used. To reach this objective various experiments were carried out:

a) Selection of type of CD: Because of the structure of the different CDs could have some effect on the antioxidant activity oxyresveratrol/CDs complexes, the first step was to evaluate the effect of the encapsulation of this stilbene by natural (α-CD and β-CD) and modified (HP-β-CD, Metil-β-CD y Etil-β-CD) CDs on the antioxidant activity.

As it was observed in **Figure 3**, the addition of α-CD at 0.7 mM had no significant effect on the FL decay curve. However, the presence of β-CD at the same concentration was apparently promoted and enhanced effect. As described as Rodriguez-Bonilla et al., 2010, 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 (ɣ-CD: 7.5–8.3 Å) of glucose.

Moreover, the use of modified β-CDs (HP-β-CD, methyl-β-CD and β-CD-Ethyl) produced fluorescence decay in all cases, being higher in for Ethyl-β-CD. These results suggest that modified CDs encapsulate and protect oxyresveratrol with higher efficiency than natural CDs and are in good agreement with numerous studies published by our group that compares the nature of the complexation of different stilbenes by natural and modified CDs (Rodríguez-Bonilla, López-Nicolás, García-Carmona, 2010; Rodríguez-Bonilla, 2011; (López-Nicolás et al., 2009 A; López-Nicolás et al., 2009 B).

b) Evaluation of the glucidic nature of CDs: To evaluate the effect of the use of CDs as additives on the ORAC-FL analysis, it is necessary to confirm that the effect of CDs on the FL decay curve is not due to the glucidic nature of the CDs but to their ability to complex hydrophobic compounds such as oxyresveratrol. To do this, we have studied the

effect on the fluorescence of oxyresveratrol due to the presence of D-glucose in the reaction medium because glucose is the monomer molecule included in the CD structure. Thus, various amounts of d-glucose (2, 3.5, 4.9 y 17.5 mM), corresponding to 0.28, 0.5, 0.7 y 2.5 mM β- CD as regards the number of glucose units (each molecule of β -CD contains seven units of D-glucose in a ring), were added to reaction medium and the fluorescence relativity intensity of ORAC-FL probe was checked. As shown in **Figure 4 and Figure 4 inset**, the addition of d-glucose dit not alter the fluoresecence relative intensity of ORAC probe, fluorescein, even though the concentration of d-glucose was the same as that of β-CD as regards the number of glucose units.

The effect of glucose was neglible even compared with the effect of the stilbene. For this reason, we discard that the effect of CDs on the fluorescence of oxyresveratrol is due.

c) Evaluation of effect of the addition of CDs on the ORAC-FL method in the absence of oxyresveratrol: In order to evaluate if the effect observed on fluorescence decay curve of FL could be due to the presence of CDs in the reaction medium and not because oxyresveratrol increases it antioxidant activity when is complexed by CDs, the next step was to evaluate the effect of CDs themselves on the ORAC-FL method. For this, an ORAC-FL assay was carried out with CDs without an antioxidant molecule. **Figure 5** shows the non expected fluorescence decay curves recorded when increasing concentrations of ethyl-β-CD are added to the medium.

According to the results obtained, when increasing concentrations of ethyl-β CD were added to the reaction medium, and in the absence of oxyresveratrol, a decrease in the initial fluorescence and a clear delay of the fluorescence decay curve was observed.

To the other hand, in **Figure 5** ***inset*** the relationship between net AUC presented against increasing concentrations of modified CDs, obtaining for all $r^2 > 0.99$ was plotted. These results led us to think that the CDs could interfere in the method due to the possible encapsulation of any of the reagents used in the ORAC-FL technique in the interior of CDs.

d) Evaluation of the cause of the interference: In order to explain the possible reactions occurring on the ORAC-FL method on addition of CDs to the reaction medium, we developed a equation model (SCHEME 1) about the possible actions of the CDs in the presence of the major reagents (APPH and FL) under the specific conditions of the method. Equation A) explains the possible reactions in the method if CDs formed inclusion complexes with APPH. On the other hand, Scheme B) shows the reactions that could occur if FL is complexed by CDs.

According to reaction scheme, and for evaluating whether CDs interfere with ORAC method, the effect of addition of CDs was evaluated. The initial fluorescence (F_0) at t = 0 against increasing concentrations of CDs (ethyl-β-CD, methyl-β-CD and HP-β-CD), without the presence of free radical generator (AAPH) was plotted. According to the results shown in **Figure 6**, a decrease in the initial fluorescence presented for each of the studied CDs was observed as the concentration of CDs was increased in the medium. These results suggested to us that the CDs may complex FL, interfering in the method.

The results showed in this section indicate that ORAC-FL is affected bhy the presence of CDs and it is not a good technique for measuring the antioxidant activity of a molecule when CDs are present in the reaction medium.

3.2. ABTS$^{\cdot+}$ METHOD

Several studies have described that stilbenoids compounds display antioxidant activity as a result of their capacity to scavenge free radicals (Gülçin., 2010; Piotrowska et al, 2012; Jung et al., 2009; Lee et al., 2004). Bearing the above in mind, our next aim was to evaluate the effect exerted by the addition of CDs to the medium on the oxyresveratrol antiradical activity.

The first step was to determine the optimal time of reaction measure. For this, the decrease in absorbance measured at λ = 414nm of the cationic radical in the presence of oxyresveratrol was monitored. As a reference antioxidant, trolox was used **(Figure 7).**

As can be seen at t = 20 h the absorbance presented for each of the molecules was stable. So, we select as the optimal measurement time for ABTS assay t = 24h.

3.2.1. Effect of pH on the free radical scavenging activity of oxyresveratrol:

As described Mikulski et al., 2010, the structure of stilbene conditioned its ability to scavenge free radicals. Due to the importance of the pH of the medium on the structure, and thus on oxyresveratrol activity, the next step was to evaluate the effect of pH of the medium on the antiradical capacity oxyresveratrol. To this, was used as measurement range pH 3.5-

11. Sodium acetate was used as a buffer for pH values ranging from 3.5 to 5.5 and sodium phosphate for 6.0 to 11.0.

Figure 8 shows the dependence of pH on the antiradical activity of oxyresveratrol. It was observed how the increased activity corresponded to pH 7.0-8.0. However, at pH values above 8.0 oxyresveratrol activity seemed strongly affected. This could be because at the pH we approach the area of deprotonation of the first of the four hydroxyl groups that compose the structure oxyresveratrol. This behavior coincides with that described for other stilbenes (López-Nicolás and García-Carmona 2008, López-Nicolás et al., 2009A, López-Nicolás, et al, 2009A).

Bearing the above in mind, we chose pH 7.0 as optimal for the following measurements.

3.2.2. Effect of the addition of CDs on the free radical scavenging activity of oxyresveratrol: To evaluate the effect produced of the addition of CDs on the oxyresveratrol antioxidant activity, increasing concentrations of HP-β-CD [0-1 mM] were added to a stable concentration of oxyresveratrol [1 uM]. **Figure 9** shows oxyresveratrol free radical scavenging versus concentration of HP-β-CD.

As can be seen, the free radical scavenging activity of oxyresveratrol represented as %ABTS remaining, was apparently increased when the concentration of CDs in the medium increased. These data were consistent with those reported by Lucas-Abellan et al., (2011).

However, as also is shown in **Figure 9**, when increasing concentrations of CDs without antioxidant was added to the medium, the response was similar to that of the stilbene. The reason may be that CDs interfere with the measurement method, complexing $ABTS^{\cdot+}$. As result, exist a positive interference of the assayed CD and there are additive activities when evaluating encapsulated stilbenes.

To verify the interference of the CDs on the ABTS method were carried out the following experiments:

a) Verification assays of the interference of CDs on $ABTS^{\cdot+}$: to evaluate the effect of CDs on the radical $ABTS^{\cdot+}$ a spectrophotometric assay in which increasing concentrations of ethyl-β-CD [0-2.5 mM] without antioxidant were added to a solution of $ABTS^{\cdot+}$ was carried out. As shown, the addition of increasing concentrations of CDs increased absorbance (**Figure 10**), demonstrating that the CDs may interfere with the measurement method.

Furthermore, a fluorimetric assay where increasing concentrations of ethyl-β-CD [0-2.5 mM] was used in the presence of ABTS was carried out and the relative fluorescence by each of the solutions was plotted. The data obtained were consistent with those obtained previously in the spectrophotometer (**Figure 10** ***inset***).

Bearing all in mind, these results demonstrate that there is an nteraction of CDs with the ABTS assay and therefore they would not be suitable to determine the antioxidant capacity of stilbenes using ABTS method.

3.3. FRAP METHOD

The ferric reducing antioxidant power (FRAP) assay measures the reduction of ferric iron (Fe3+) to ferrous iron (Fe2+) in the presence of antioxidants, which are reductants with half-reaction reduction potentials above Fe^{3+}/Fe^{2+}. This assay is also commonly used for the routine analysis of single antioxidants and total antioxidant activity of plant extracts (Gliszczynska, 2006).

3.3.1. Evaluation of the concentration on the antioxidant capacity of oxyresveratrol: Firstly, the relationship between the concentration in the reaction medium of oxyresveratrol and their absorbance at 593 nm was studied.

Figure 11 shows the dose-response lines for the antioxidants assayed. Solutions of known concentrations of iron Fe (II) (FeSO4) were used to set up a calibration curve. This figure shows the signals obtained for the ferric reduction by the oxyresveratrol [0-50 µM] assayed as well as for Trolox, as compared to the absorbance obtained for the calibration curve.

As can be seen, a similar effect of oxyresveratrol on the ferric reducing ability was found for Trolox. Because of the higher absorbance at 593 nm corresponded to a concentration of 50 µM, we decided to choose as optimal antioxidant concentration 50 µM for the following measurements.

3.3.2. Evaluation of time reaction on antioxidant capacity of oxyresveratrol.

Several authors have reported that the absorbance values for different compounds presented by FRAP method could vary depending on the time of measurement (Benzie and Strain,

1996; Anna Gliszczyn'ska-S'wigło, 2006). Therefore, the next step in our study was to evaluate whether the absorbance of the oxyresveratrol by FRAP method could be varied depending on the time of measurement.

In **Figure 12** can be seen the evolution in absorbance of oxyresveratrol over time. As shown, the absorbance increase gradually increasing reaching a plateau after 120 minutes. Therefore, the following measurements were carried out in the interval between t = 0 and 120 min.

3.3.2. Effect of addition of CDs on the antioxidant capacity of oxyresveratrol:

The next step in our study was to evaluate the effect that the addition of CDs produced due to the absorbance of the oxyresveratrol measured by FRAP. The effect of added increasing concentrations of HP-β-CD [0, 0.25, 0.5, 1, 2, 3 and 4 mM HP-β-CD] were monitored spectrophotometrically at λ=593 nm over time **Figure 13**.

As shown, at t = 0 the absorbance of oxyresveratrol increases as the concentration of CDs in the medium is increased. This could lead to the thought that the addition of CDs in the medium could increase the antioxidant capacity of the compound to be complexed. However, as it progresses the reaction time, the absorbance remains stable, although the HP β-CD concentration in the medium were increased.

3.3.3. Evaluation the effect of the addition of CDs on the FRAP method

To evaluate if the changes in the absorbance of oxyresveratrol were caused because CDs interfere on the measurement method, was carried out an assay in which increasing concentrations of HP-B-CD were used in presence and absence of antioxidant.

In **Figure 14** can be see how at t = 0 absorbance of oxyresveratrol increases as increasing concentrations of CDs in reaction medium. However, different concentrations of CD themselves does not produce changes in absorbance. These data lead us to conclude that the CDs do not interference on the FRAP method.

CONCLUSION

The effect of the addition of CDs on the antioxidant capacity of oxyresveratrol through ORAC, ABTS and FRAP methods was evaluated. The results show that CDs interfere with chemicals used in ORAC-FL and ABTS methods. For this reason these techniques should not be used to determine the antioxidant activity of a bioactive molecule in the presence of CDs. However, the presence of CDs does not interfere on the determination of oxyresveratrol antioxidant activity when FRAP method is used. For this, FRAP method appears to be a satisfactory method for determine the antioxidant activity of oxyresveratrol-β-CD inclusion complexes.

On the other hand, changes have occurred in the antioxidant activity of oxyresveratrol depending the reaction time of measures. Thus, it was observed that appeared to increase in the early stages of the reaction with the increasing concentration of CDs in the reaction medium, but as time passed it seemed not seen any noticeable effect. To end up, a molecular docking of the complex was done. The Score predict the encapsulation of oxyresveratrol by HPβCD. Molecular docking calculations provide insights into how the different interactions at the molecular level (hydrophobic, hydrogen bonds, and van der Waals) influence the encapsulation process.

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SCHEMES

Scheme 1. Possible actions of the CDs in presence of AAPH and FL under the specific conditions of the ORAC method.

FIGURE LEGEND

Figure 1. Docking results obtained in the complexation of oxyresveratrol with (A) β-CD and (B) HP-β-CD. Hydrogen bonds are in yellow.

Figure 2. Fluorescence decay curve during the ORAC-FL assay in the presence of increasing concentrations of oxyresveratrol: (●) blank, (□) 0.52 µM, (▲) 1 µM, (♦) 2 µM, (○) 3 µM, (▼) 4 µM. *Inset*: Regression of Oxyresveratrol on different concentrations

Figure 3. Effect on fluorescence decay curve of oxyresveratrol in the presence of different types of CDs. Final concentration of CDs was 0.7 mM.

Figure 4. Fluorescence decay curve during the ORAC-FL assay in the presence of different concentrations of glucose: (■) 2Mm, (○) 3.5 mM, (▲) 4.9 µM, (♦) 17.5 mM. *Inset*: Net area at different concentrations of glucose.

Figure 5. Fluorescence decay curve of increasing concentrations of ethyl-β CD: (▼) blank (no CD), (■) 1 mM, (○) 2 mM, (▲) 3 mM, (●) 4 mM, (□) 5 mM. *Inset*: Net AUC of increasing concentrations of different type of modified β-CDs: (▲)Etil-β CD, (●)Metil-β CD, (■) HP-β CD. The correlation coefficient was r^2 >0.99 for all the CDs. Net AUC= $AUC_{sample} - AUC_{blank}$

Figure 6. Effect of addition of CDs on the ORAC-FL method. **(A)** Consecutive spectra of increasing concentration of Etil-β CD on AAPH (12.5 mM, final concentration) in phosphate buffer (75 mM, pH 7.4). Concentrations of Etil-β CD used was: ( ) blank, ( ) 7.5 mM, ( )10.5 mM, ( ) 15 mM, ( ) 18.75 mM and) 22.5 mM. Assays were carried out without fluorescein. **(B)** Initial fluorescence activity showed by different CDs by the addition of increasing concentrations of: (●) Etil-β CD, (■) HP-β CD, (▲) Metil-β CD.

Figure 7. Absorbance decay curve during the ABTS assay. (■) Trolox 1μM, (●) Oxyresveratrol 1μM. The reaction was followed spectrophotometrically at λ =414 nm, at pH 7.0.

Figure 8. Oxyresveratrol free radical scavenging activity dependence on pH.

Figure 9: Effect of addition of HP-β-CD on the free radical scavenging (●) oxyresveratrol, (■)HP-β-CD alone.

Figure 10. Rate of increase in absorbance to increasing concentrations of Ethyl-β CD. The concentration used were: (-) no CD, (-) 0.25 mM, (-) 0.75 mM, (-) 1mM, (-) 2.5mM Ethyl-β CD. Inset: relative fluorescence of Ethyl-β CD.

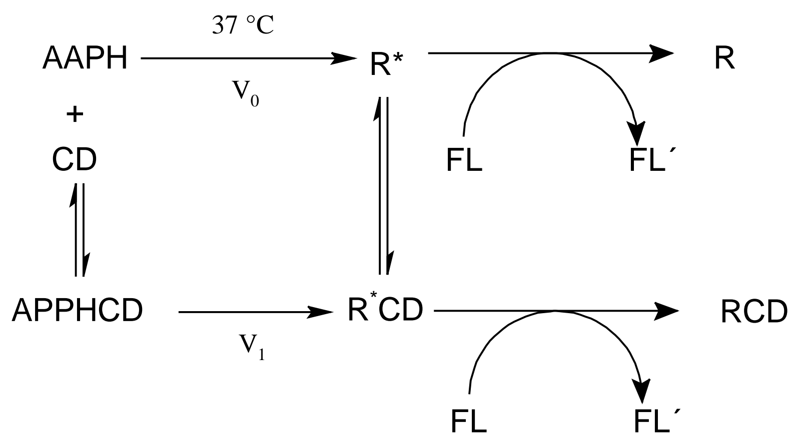
Figure 11. Antioxidant power of oxyresveratrol through its ferric reducing ability and standard curve. The effect was evaluated spectrophotometrically at $\lambda = 593$ nm. (***x***) trolox, (●) oxyresveratrol (r>0.99).

Figure 12. FRAP time course with oxyresveratrol (50 µM)

Figure 13. Evolution of oxyresveratrol [50µM] absorbance at λ=593 nm at different reaction times with increasing concentration of HP-β CD in the medium. Times of measurement were: (●) time 0 min, (■) 2 min, (Δ) 4 min, (♦) 6 min, (▼) 8 min, (○) 10 min, (■) 30 min, (▲) 60 min and (♦) 120 min.

Figure 14. Effect of addition of increasing concentrations of HP-β CD on the FRAP method. (●) Blank of CDs, (■) oxyresveratrol [50 µM], [▲] Fe (II) at a concentration

SCHEME 1



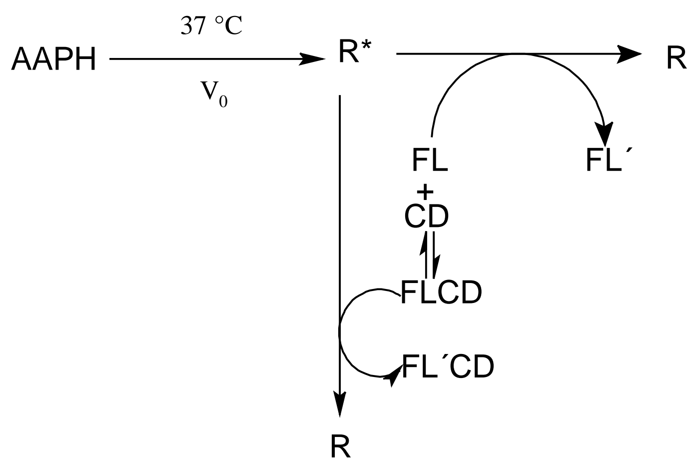


FIGURE 1

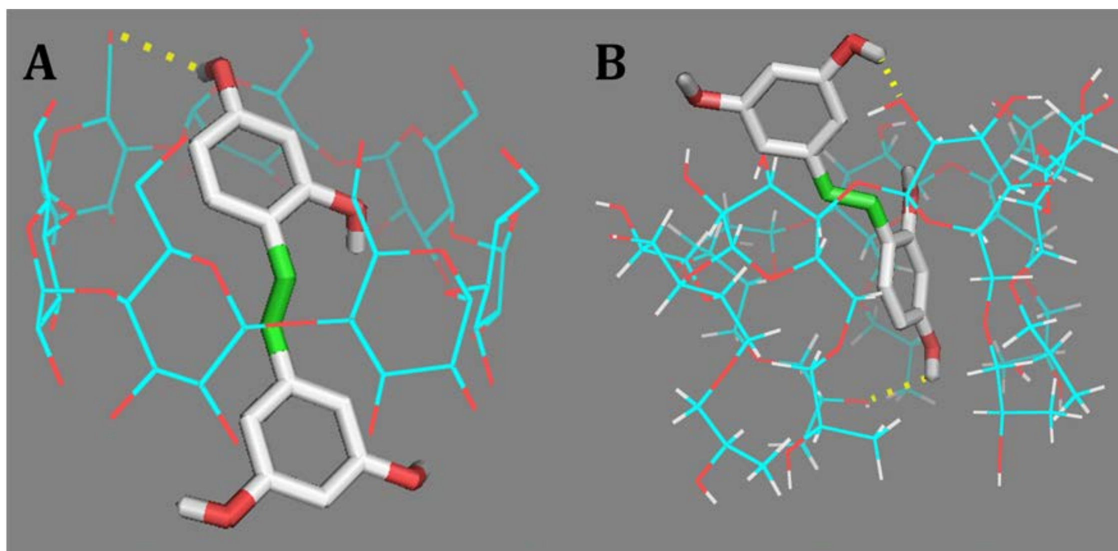
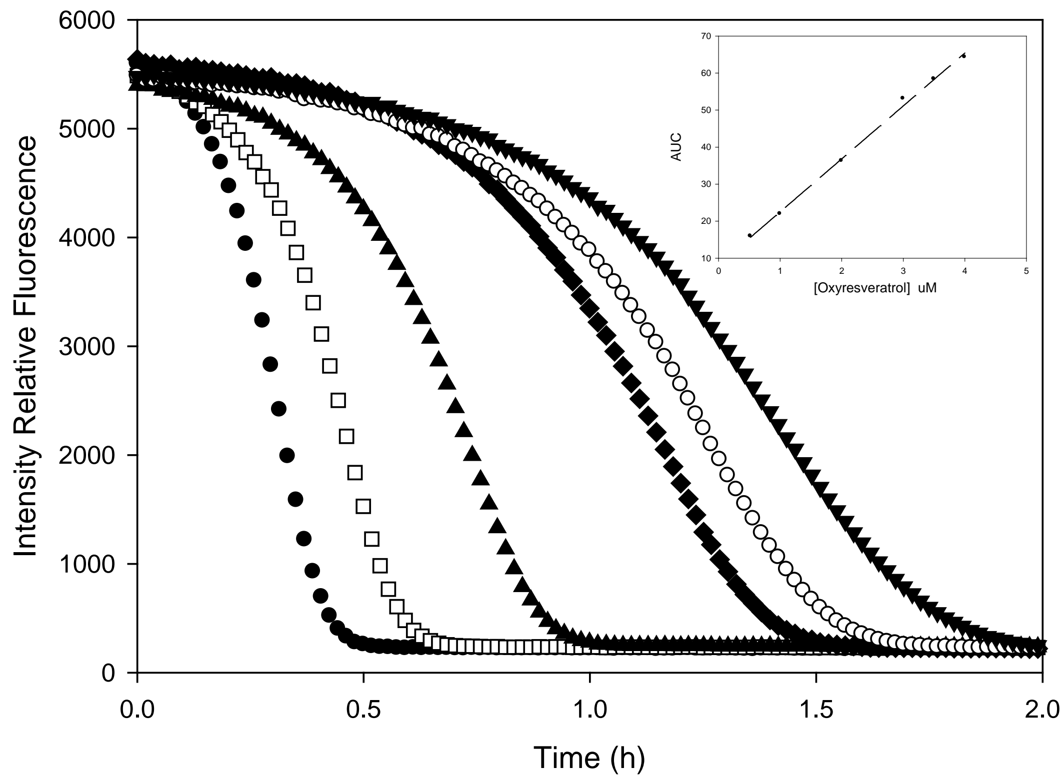


FIGURE 2



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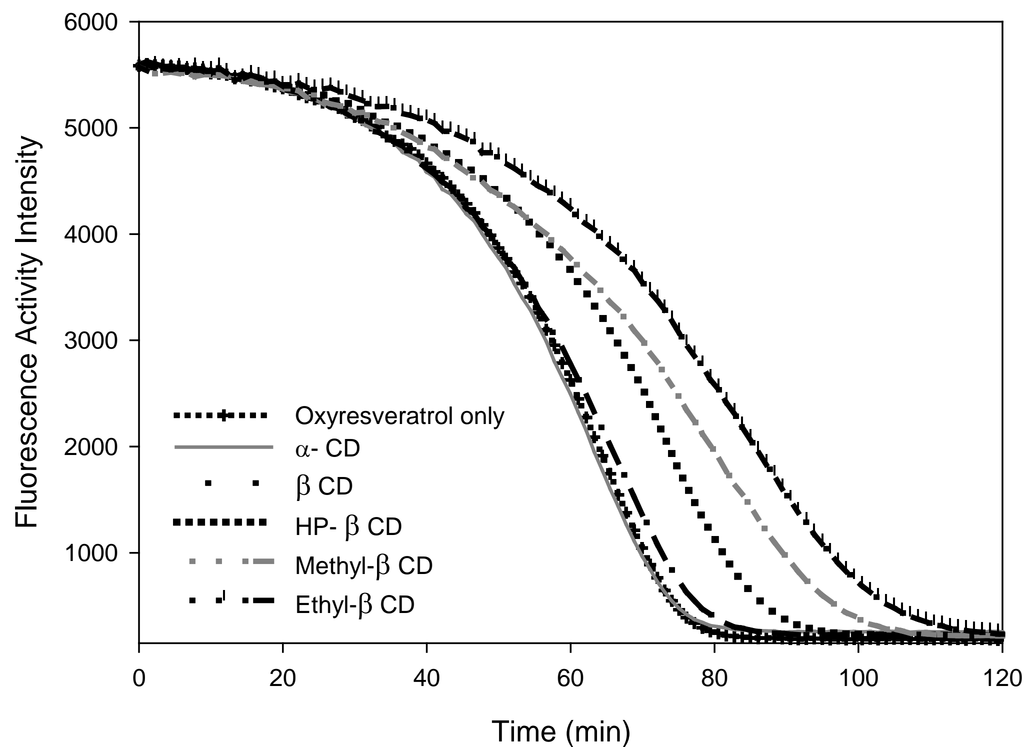

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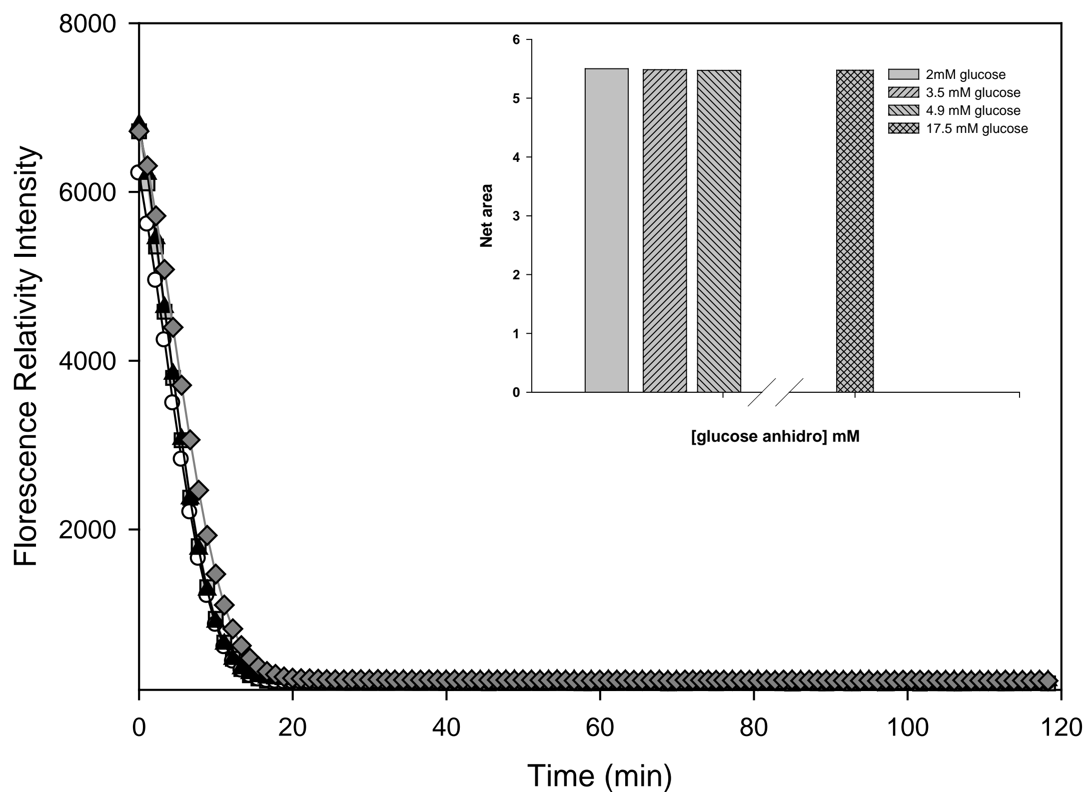


FIGURE 5

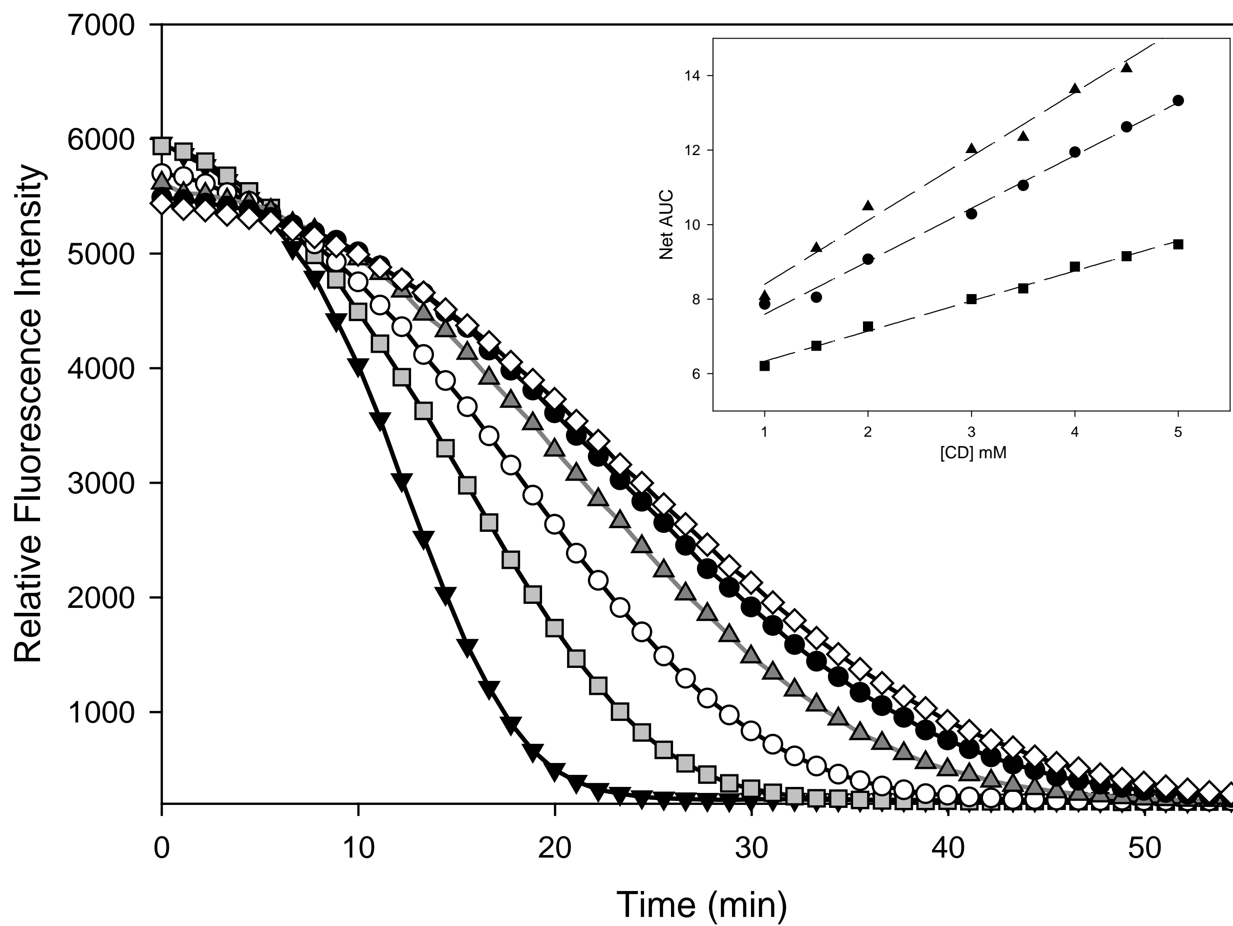


FIGURE 6

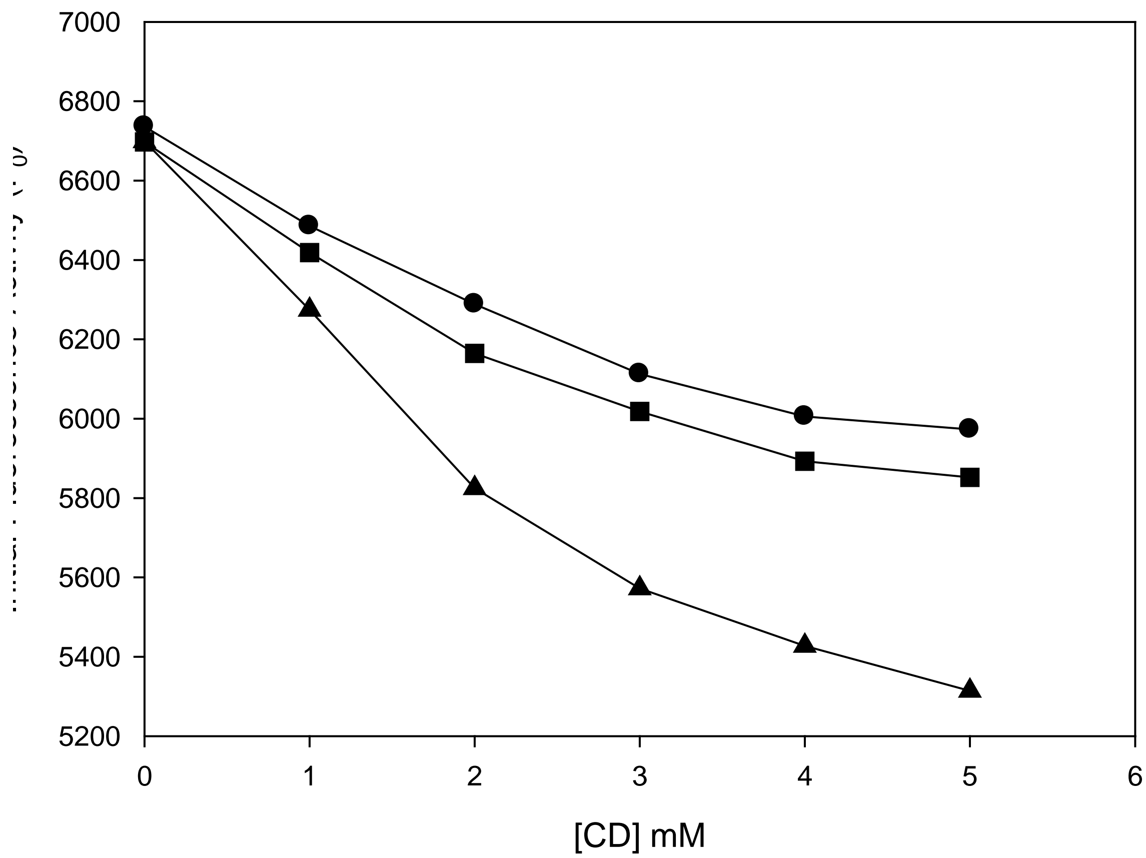


FIGURE 7

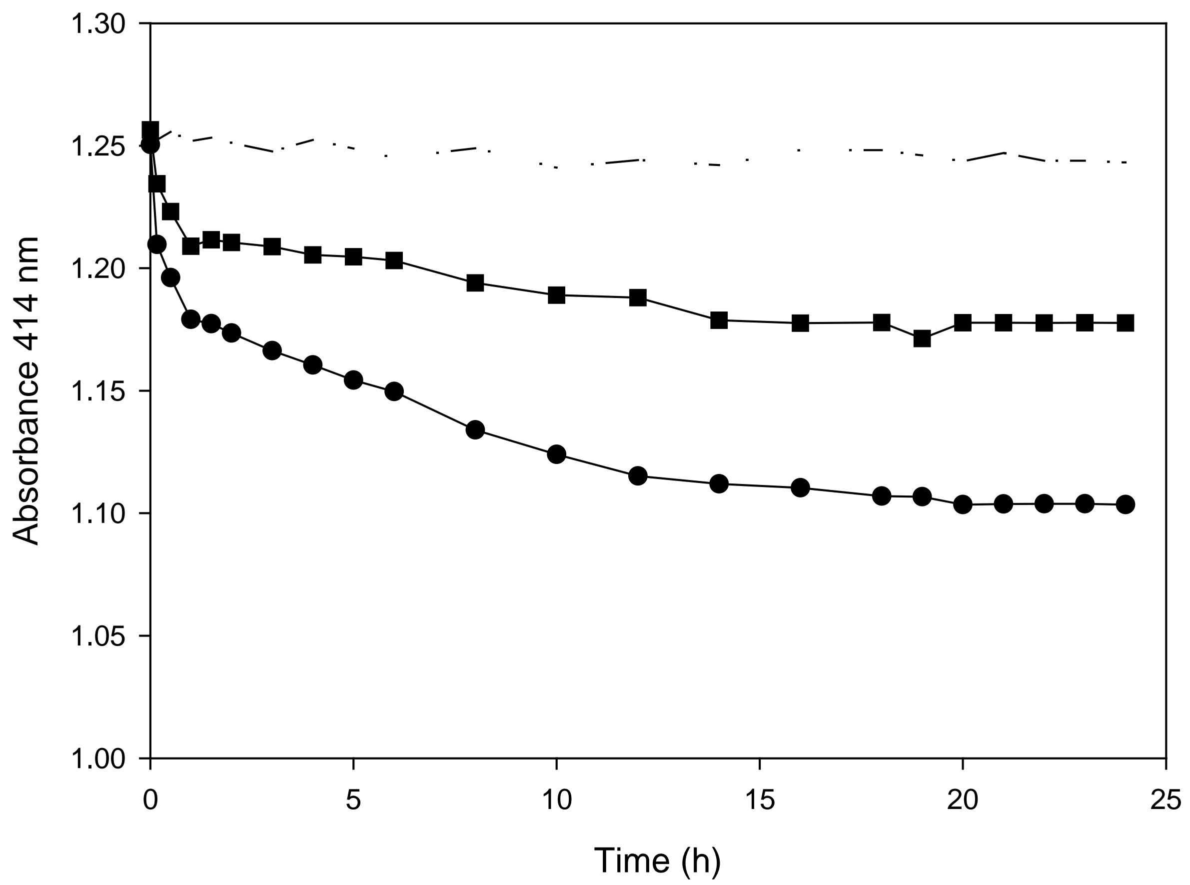


FIGURE 8

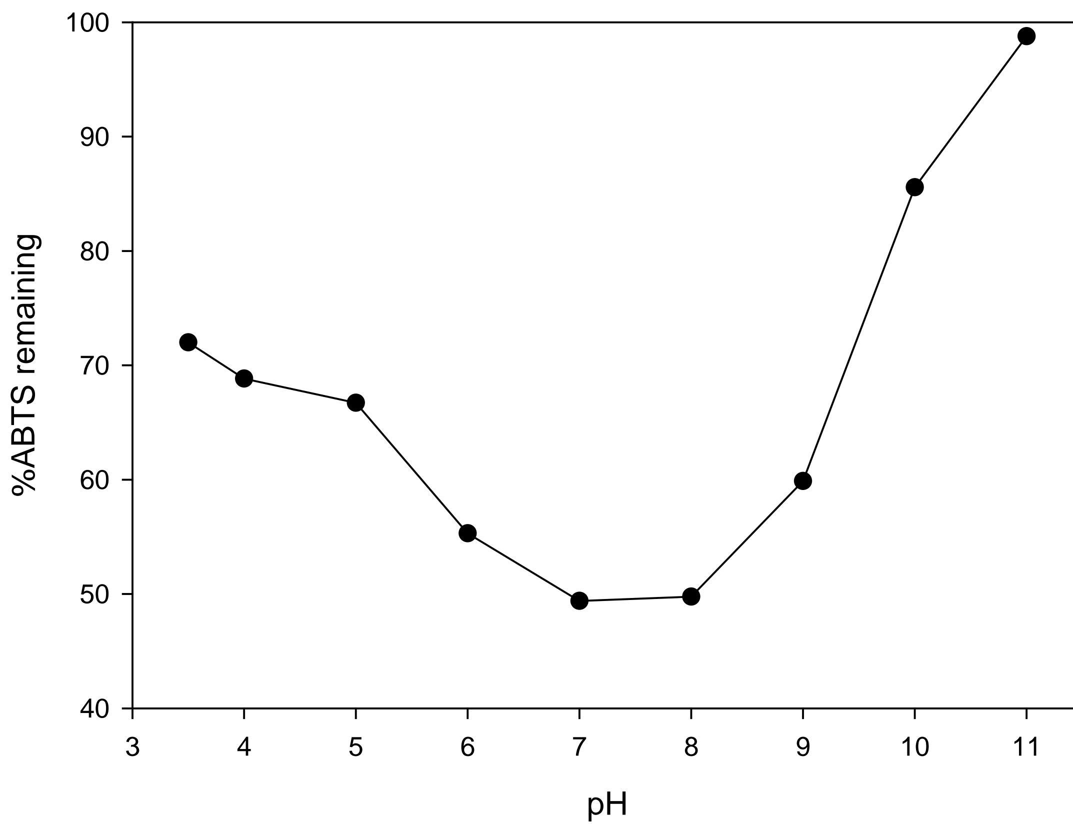


FIGURE 9

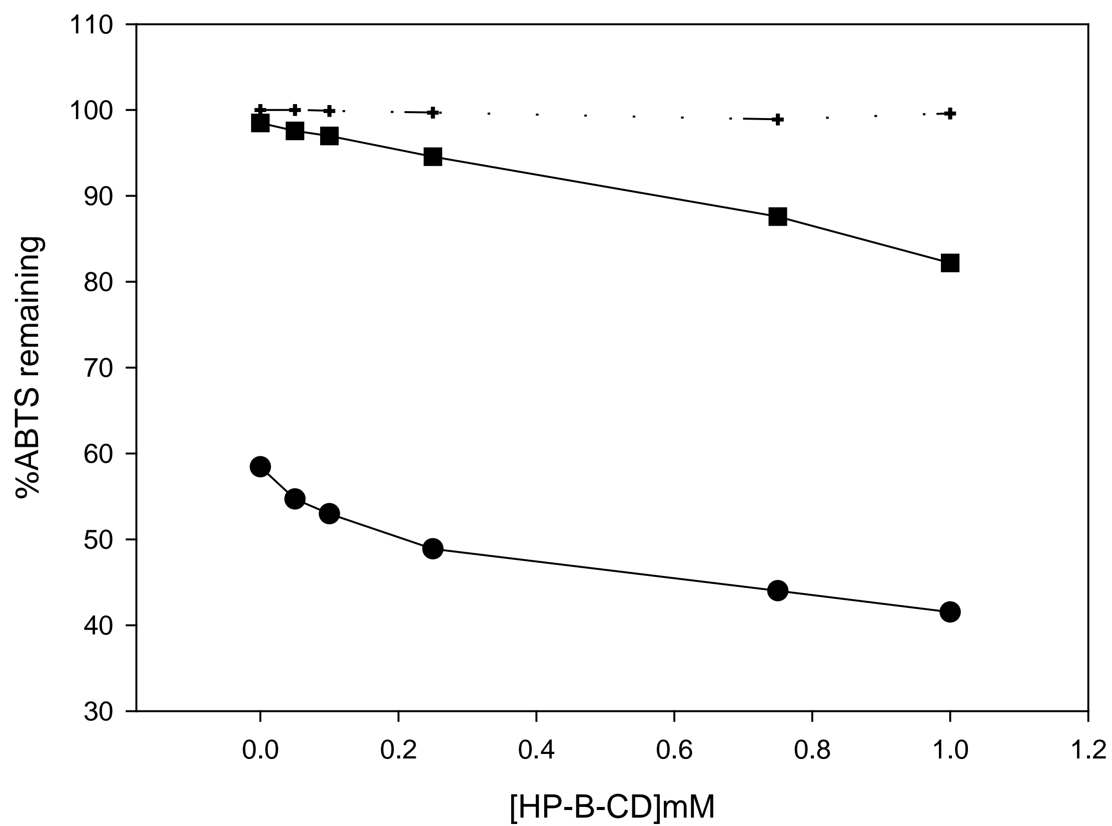


FIGURE 10

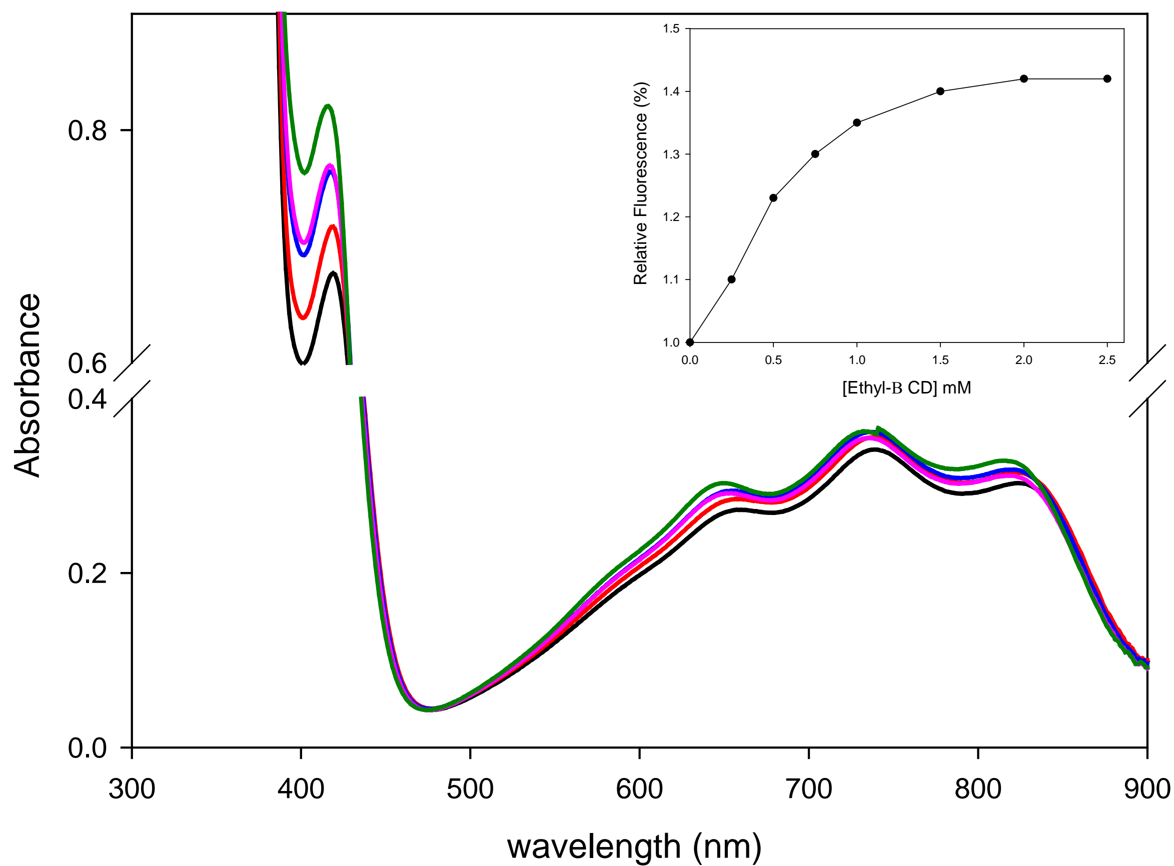


FIGURE 11

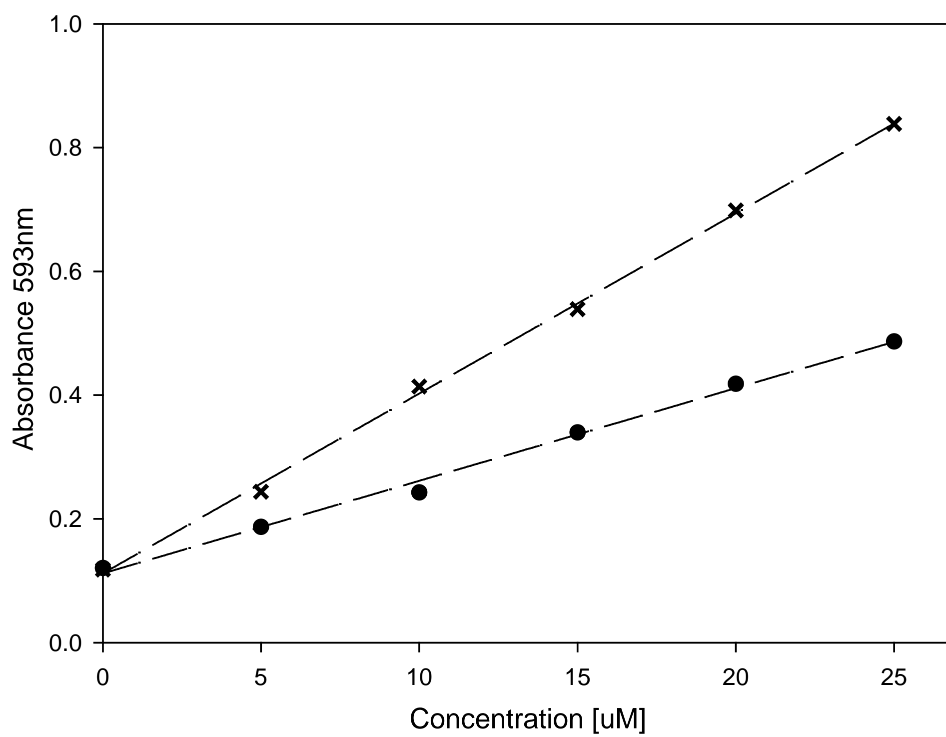


FIGURE 12

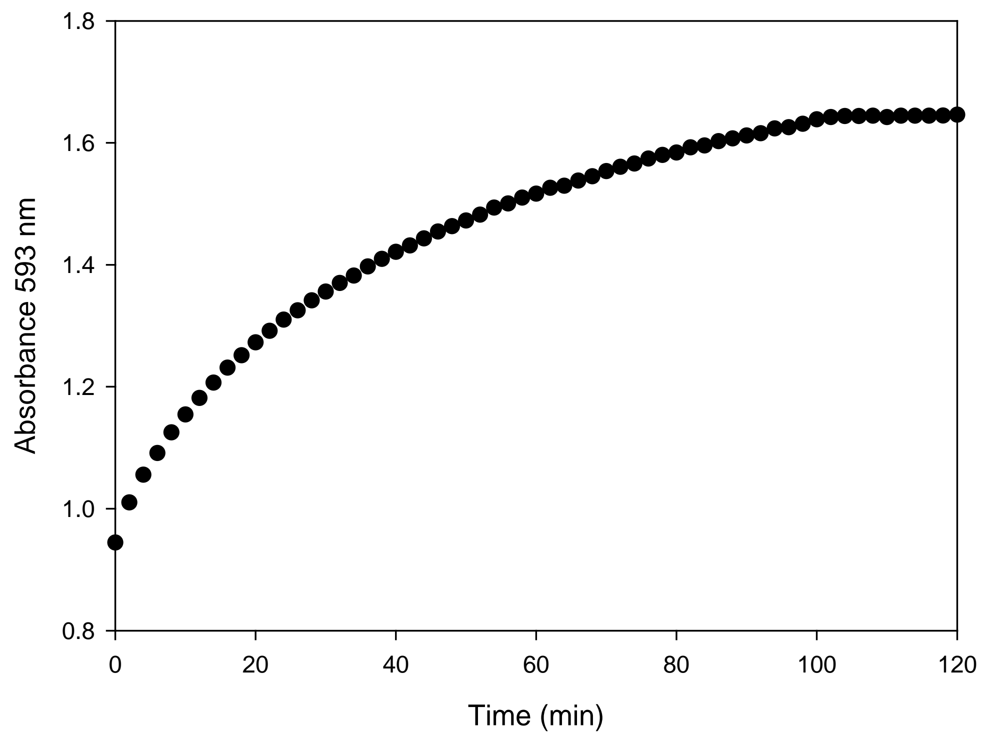


FIGURE 13

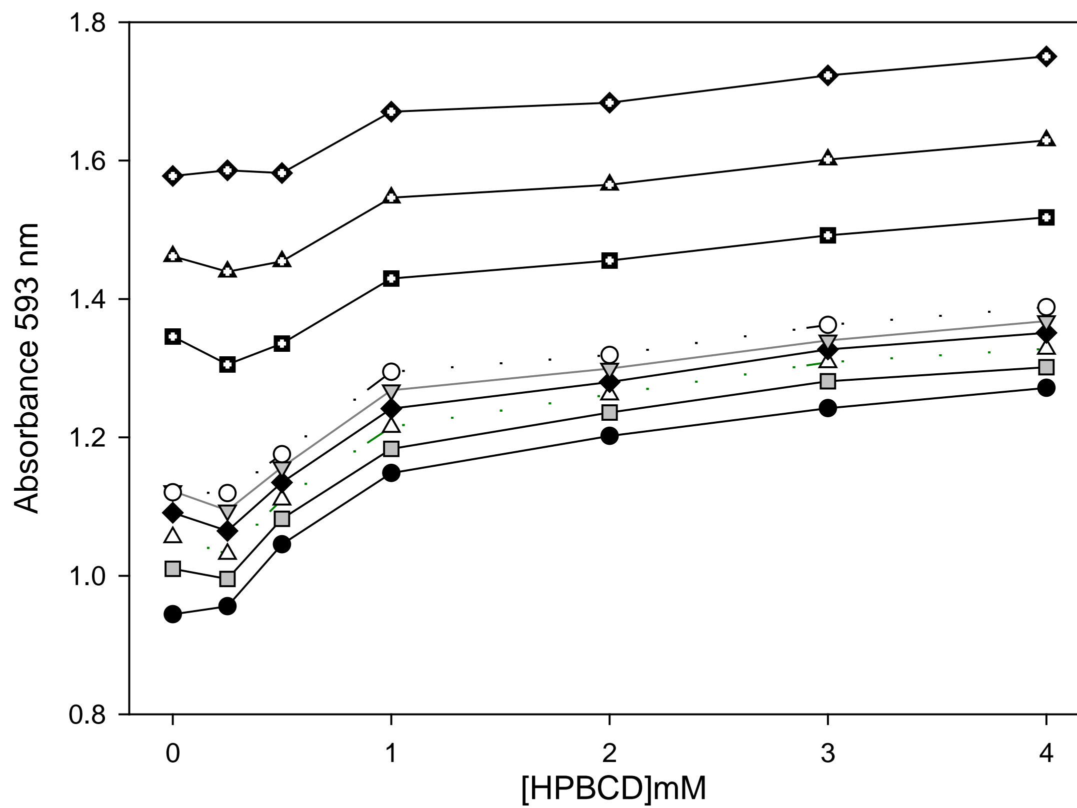
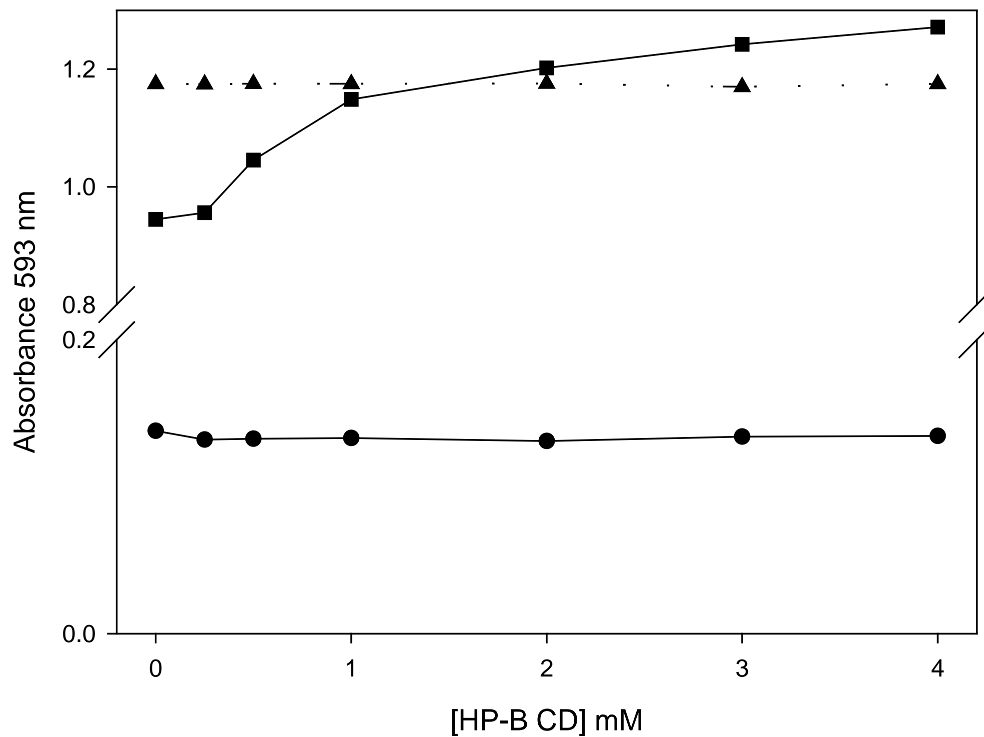


FIGURE 13



CAPÍTULO XI

Conclusiones

La conclusión principal de esta Tesis Doctoral es que se ha conseguido encapsular diferentes estilbenos (oxyresveratrol, resveratrol, pinosilvina y pterostilbeno) mediante distintos tipos de ciclodextrinas, lo que abre la oportunidad al uso de estos importantes compuestos bioactivos en la industria alimentaria, farmacéutica y cosmética.

Además, se han obtenido las siguientes conclusiones derivadas de los trabajos realizados:

1. Se ha estudiado la complejación de oxyresveratrol, resveratrol, pterostilbeno y pinosilvina mediante CDs naturales y modificadas, resultando la complejación con β-CD la más eficiente. Sin embargo, todas las CDs modificadas presentaban mayores constantes de complejación (K_F) que la β-CD. En particular, la mayor K_F presentada fue para el complejo de inclusión estilbeno–HP-β-CD.

2. La estequiometría de los complejos formados por oxyresveratrol- β-CD, pterostilbeno–HP-β-CD, pinosilvina–HP-β-CD y trans-resveratrol–HP-β-CD se corresponde con una estequiometría 1:1 mientras que el trans-estilbeno–HP-β-CD presentó una estequiometría 1:2.

3. Se ha evaluado la influencia de la estructura molecular de los estilbenos sobre la formación de complejos con CDs, resultando una mejor complejación para los estilbenos que presentan una estructura con más grupos hidroxilo, como son el resveratrol y el oxyresveratrol. Esto implica que no solamente la solubilidad del compuesto huésped influye en la encapsulación, sino que también es determinante la estructura resonante.

4. Se ha estudiado el efecto de algunos agentes físico-químicos sobre las K_F de estilbenos-CDs, resultando lo siguiente:

a. Se ha observado un efecto derivado por la temperatura sobre la complejación de los estilbenos mediante CDs, resultando una menor complejación a medida que se incrementaba la temperatura del medio, probablemente debido a la debilitación que producen las altas temperaturas sobre los enlaces de hidrógeno.

b. Se ha demostrado la fuerte dependencia de la concentración de disolventes orgánicos (metanol) en la fase móvil sobre K_F estilbenos-CDs. Esto es debido a la competencia entre el estilbeno y el metanol por la cavidad de la CDs.

c. Se ha evaluado el efecto del pH sobre las K_F de los estilbenos-CDs. Los resultados mostraron que la dependencia del pH sobre K_F podía ser debido a una hipotética formación de enlaces de hidrógeno entre los grupos hidroxilo de los estilbenos y los grupos hidrofílicos de CDs a valores de pH cercanos al pKa, ya que los puentes de hidrógeno son uno de los tipos más importantes de interacción en la estabilización de los complejos de inclusión. Por este motivo, es importante tanto para la industria farmacéutica como para la alimentaria, utilizar estos compuestos por debajo del pH al que se produce su deprotonación (pKa).

5. Se han identificado los tres principales productos de la peroxidación enzimática de pterostilbeno mediante peroxidasa (POX): pterostilbeno cis- dehydrómero, pterostilbeno trans dehydrómero y pterostilbeno como dímero abierto. La utilización

de CDs en el medio da lugar a la complejación del pterostilbeno como sustrato para la reacción enzimática, produciendo la inhibición de la actividad POX.

6. Se ha evaluado el uso de CDs sobre métodos cromatográficos que faciliten el análisis de compuestos alimentarios y farmacéuticos: A medida que se disminuía la concentración de disolvente orgánico (MeOH) de la fase móvil (MeOH:agua) para la determinación del tiempo de retención de los estilbenos mediante HPLC, se incrementaban los tiempos de retención de los mismos. Esto presentaba un problema para la determinación de ciertos compuestos hidrofóbicos. Sin embargo, la adición de CDs a la fase móvil daba lugar a un acortamiento de los tiempos de retención presentados, permitiendo, por tanto, un menor uso de disolventes orgánicos en la fase móvil y una mayor rapidez en los resultados.

7. Se ha evaluado el uso de ciclodextrinas en la determinación de la capacidad antioxidante de oxyresveratrol mediante diferentes técnicas: ORAC, ABTS y FRAP. El resultado ha sido una interferencia por parte de las ciclodextrinas en los métodos de medida ORAC y ABTS, por lo que no sería válido su uso con dichos métodos. Sin embargo, la adición de CDs al medio no produjo ninguna interferencia sobre el método FRAP, por lo que se considera apto para la determinación de la capacidad antioxidante de estilbenos.

8. Se ha observado que los resultados obtenidos mediante el método FRAP al adicionar CDs al medio depende del tiempo de medida. Al inicio de la reacción, la adición de CDs parece aumentar levemente la capacidad antioxidante del

oxyresveratrol, pero a medida que transcurre la reacción, ésta capacidad antioxidante permanece estable.

9. Se ha estudiado la capacidad antioxidante de diferentes estilbenos mediante diferentes técnicas de medida: ORAC, ABTS y FRAP. Los resultados dispares obtenidos demuestran que la capacidad antioxidante de una sustancia depende del método de medida con el que se analice. Se ha observado una baja actividad antioxioxidante para todos los estilbenos cuando el pH del medio de reacción se encontraba por debajo de 8.0.

10. A pH en el rango de 3.5-8.0 los resultados de capacidad antioxidante de los estilbenos obtenidos mediante los métodos de medida basados en la transferencia de electrones ($ABTS^{\cdot+}$ y FRAP) han seguido el mismo orden, siendo de mayor a menor: oxyresveratrol, resveratrol, pinosilvina y pterostilbeno (4, 3, 2 y 1 grupos hidroxilo), aunque los valores de TEAC obtenidos mediante $ABTS^{\cdot+}$ han sido mayores que los obtenidos mediante FRAP. Sin embargo, el orden registrado mediante el método ORAC, basado en la transferencia de H, ha sido: resveratrol, oxyresveratrol, pterostilbeno y pinosilvina.

CAPÍTULO XII

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