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POTENCIACIÓN DE LAS PROPIEDADES SALUDABLES DE LOS VINOS

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RESUMEN

En la relación alimentación-salud, la presencia de compuestos con actividad biológica en alimentos cobra especial relevancia. Cada vez más la evidencia científica avala los beneficios del consumo de alimentos con compuestos con actividad biológica. En este sentido existen numerosos alimentos que han sido enriquecidos en sustancia bioactivas, lo que les confiere un valor añadido. Los vinos enriquecidos en estilbenos, y más concretamente en resveratrol, son un nuevo producto de gran interés debido a las numerosas propiedades saludables de este compuesto.

En este trabajo se han estudiado tanto factores que afectan a la síntesis de estilbenos en uva, tales como la variedad o zona de cultivo, como tratamientos capaces de aumentar la concentración basal de estos compuestos. Así, se han estudiado tratamientos precosecha, postcosecha, y la combinación de ambos tipos buscando posibles sinergias.

Una vez optimizados factores y tratamientos para conseguir la máxima concentración de estilbenos en uva, se procedió a la elaboración del vino. El proceso de vinificación también estaba optimizado para minimizar las pérdidas de estilbenos, manteniendo la máxima la calidad.

Aunando lo anterior, uvas de la variedad Syrah cultivadas en Jerez de la Frontera fueron tratadas con metil jasmonato. Tras vendimiarlas fueron tratadas con ultravioleta C, y posteriormente vinificadas. El vino resultante contenía doble cantidad de estilbenos (piceatanol, *trans*-resveratrol y ε-viniferina), mayor concentración de antocianos y taninos, mejores parámetros de color (intensidad colorante, CieLAB) y mayor puntación en el análisis sensorial que el respectivo testigo.

ABSTRACT

Diet and health relationship depends on the content of bioactive compounds in food. Nowadays there are many researches on health-promoting properties of food which contain bioactive compounds. In this sense, there are many enriched-food in bioactives since considered as added-value food products. Stilbene-enriched wine is an interesting product due to the healthy properties of resveratrol.

In the present work, how stilbene concentration in grape is affected by variety and terroir is researched. Moreover, different pre and postharvest treatments have been tested to increase stilbene grape content. Evenmore treatment combinations have been tried.

Once factors and treatments had been optimized, wine was elaborated. Winemaking was performed to both avoid stilbene losses and maximaze quiality wine.

In concordance with above described, grapes cv. Syrah variety cultivated in Jerez de la Frontera were treated with methyl jasmonate preharvest treatment. After harvest, grapes were treated with UVC light, and finally winemaking was performed. The wine contained the double amount of stilbenes (piceatannol, *trans*-resveratrol and ε-viniferin) than control ones. Regarding to oenological parameters, anthocyanin and tannin concentration were achieved as well as CieLAB parameters. Additionally treated wines improved score in sensorial analysis.

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CAPÍTULO I

INTRODUCCIÓN

La vid es una de las plantas arbóreas más importantes cultivadas en la zona mediterránea. Su cultivo está sometido a una evolución constante, cada día más rápida por la disponibilidad de conocimientos para una mejor gestión de los viñedos. La uva (*Vitis vinífera)* es el nombre que recibe el fruto que crece formando racimos de la vid común o vid europea. Pertenece al género *Vitis* de la familia de las *Vitáceas*, que incluye unas 600 especies de arbustos, por lo general trepadores y que producen frutos en baya, propios de países cálidos y tropicales. Existen innumerables variedades de uvas con grandes diferencias entre sí en la forma, tamaño, tonalidad de los frutos, productividad, calidad, etc. La vid tiene especiales facultades para mutar genéticamente y adaptarse a condiciones climáticas y edafológicas diversas. De ahí el gran número de variedades que se conocen.

1. ESTRUCTURA Y COMPOSICIÓN DE LA UVA

El racimo de uva se compone de dos partes bien diferenciadas: el raspón, o parte leñosa, y las bayas o granos. Estos se unen al racimo mediante el pedicelo, a través del cual se nutren mediante un sistema vascular compuesto de elementos del xilema y el floema de la planta. Las bayas, a su vez, están formadas por una película exterior, denominada hollejo o piel, una masa que rellena interiormente la baya y de la que se extrae el mosto, conocida como pulpa y, en el centro de la misma, un número variable de semillas o pepitas (Figura I.1). Las proporciones entre ellos varían según la variedad y las condiciones de clima y cultivo. Por término medio las proporciones son: 89% de pulpa, 7% de hollejo y 4% de pepitas.

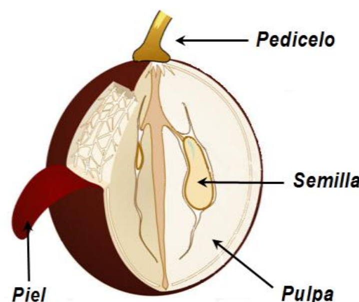


Figura I.1. Estructura de la baya

La composición de las uvas puede variar según sean tintas o blancas, pero ambas presentan un alto nivel de azúcar por lo cual se las considera una de las frutas más calóricas: 70-75 Kcal cada 100 g.

Según Flanzy (2003), podemos decir que por cada 100 g de ingesta tenemos: agua (83 g), carbohidratos (16 g), grasa (0.16 g), proteínas (0.72 g), fibra (0.8 g), potasio (300 mg), sodio (2 mg), fósforo (185 mg), calcio (16 mg), magnesio (10 mg), hierro (0.6 mg), zinc (0.6 mg), vitamina C (4 mg), vitamina B1 (0.05 mg), vitamina B6 (0.16 mg), vitamina B2 (0.03 mg), vitamina A (10 µg), y vitamina E (0.7 mg).

La mayor parte de las propiedades bioactivas de la uva se puede atribuir a los compuestos fenólicos encontrados principalmente en la semilla y la piel. Bertelli y Das (2009) han descrito por lo menos 500 diferentes antioxidantes en varias partes de este fruto.

1.1. COMPUESTOS FENÓLICOS

Los compuestos fenólicos o polifenoles constituyen un amplio grupo de sustancias químicas, considerados metabolitos secundarios de las plantas, con diferentes estructuras químicas y actividades.

La presencia de polifenoles en las plantas es muy variada, dependiendo de la especie vegetal, variedad, parte de la planta considerada (frutos, semillas, hojas, tallos, etc.), condiciones agroclimáticas del cultivo, así como aspectos tecnológicos relacionados con el procesado y conservación de los alimentos que los contienen.

Sus principales funciones en las células vegetales son las de actuar como metabolitos esenciales para el crecimiento y reproducción de las plantas, y como agentes protectores frente a la acción de patógenos, siendo secretados como mecanismo de defensa de la planta (Harborne, 2001). También son a menudo cruciales en la determinación de la calidad en los atributos de los alimentos (color, sabor y aroma), así como en los colores y pigmentos de las plantas ornamentales (Kashif y col., 2010).

Los compuestos polifenólicos han sido estudiados en profundidad en los últimos años debido a que ejercen numerosos efectos beneficios sobre la salud, ya que por su gran capacidad antioxidante, contribuyen a reducir el riesgo de sufrir diversas enfermedades.

1.2. Clasificación de los compuestos fenólicos

Se han llegado a identificar más de 8.000 compuestos fenólicos con estructura muy variada, por lo que su clasificación es una tarea compleja. Comprenden desde moléculas simples, como los ácidos fenólicos, hasta polímeros complejos de elevada masa molecular como son los taninos hidrolizables y condensados.

Son estructuras químicas formadas por un anillo aromático unido a uno o más grupos hidroxilo, incluyéndose también derivados funcionales como ésteres, metil ésteres, glicósidos, etc. (Harborne y col., 1989). Habitualmente se encuentran conjugados con uno o más residuos de azúcar unidos a los grupos hidroxilo, aunque en algunos casos se pueden producir uniones directas entre una molécula de azúcar y un carbono aromático. Los azúcares pueden ser tanto monosacáridos como disacáridos o incluso oligosacáridos. Los más comunes son la glucosa, galactosa, arabinosa, ramnosa y xilosa, así como los ácidos glucurónido y galacturónico (Manach y col., 2004).

La clasificación consta de dos grandes grupos, que se subdividen de la siguiente manera (Waterhouse, 2002; Figura I.2):

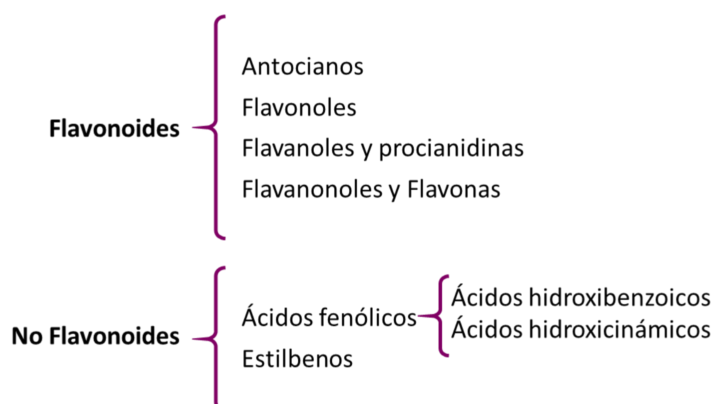
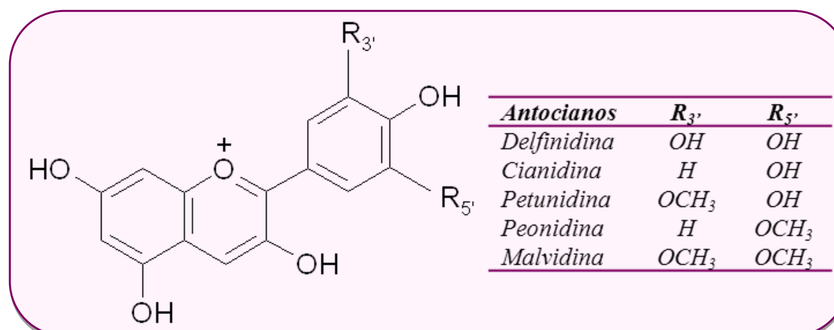


Figura I.2. Clasificación de los compuestos fenólicos.

1.2.1. Antocianos

Las antocianinas son los pigmentos más abundantes en la piel de las uvas rojas (Figura I.3). Estos pigmentos solubles en agua son responsables de color azul, rojo y púrpura en la piel de uvas rojas y vino tinto. Se encuentran fundamentalmente localizados en el hollejo, y en la pulpa solamente en las variedades tintoreras.

Los antocianos, pueden encontrarse en forma glicosilada, recibiendo el nombre de antocianinas, o en forma aglicona, las antocianidinas, siendo más estables las primeras. La glucosa puede a su vez ligarse a un ácido orgánico (cumárico, cafeico, acético) para formar las denominadas antocianinas aciladas. En el género *Vitis vinifera* se han identificado cinco moléculas de antocianidinas: cianidina, peonidina, delfinidina, petunidina y malvidina.



Antocianos	$R_{3'}$	$R_{5'}$
Delfinidina	*OH*	*OH*
Cianidina	*H*	*OH*
Petunidina	OCH_3	*OH*
Peonidina	*H*	OCH_3
Malvidina	OCH_3	OCH_3

Figura I.3. Estructura química de las formas aglicona de los antocianos más comunes en el vino y uva.

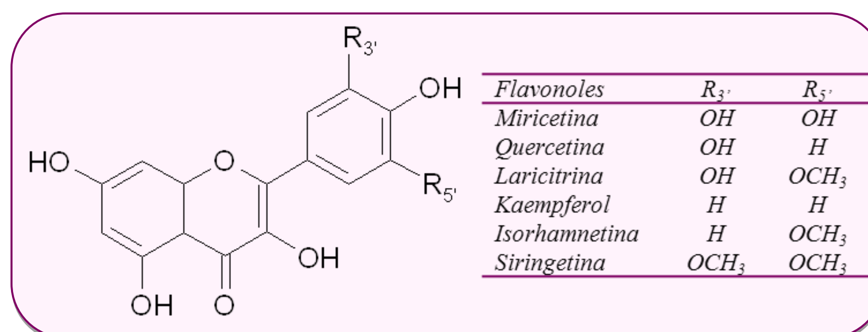
Malvidina 3-glucósido es la antocianina principal en todas las variedades de *Vitis vinifera*. La cantidad de antocianinas en las uvas, así como en todos los compuestos fenólicos, depende de la variedad vid y está muy influenciada por factores vitícolas y ambientales tales como luz, temperatura, altitud, tipo de suelo, el agua, el estado nutricional, la patogénesis y diversos procesos del desarrollo (Downey y col., 2006). La temperatura tiene una gran influencia en la biosíntesis de antocianinas. Los niveles de antocianinas en uvas de Cabernet Sauvignon son mayores cuando las temperaturas

diurnas se mantienen constantes en torno a 20 ºC en lugar de 30 ºC. Por lo tanto, un mayor contenido de antocianinas se asocia con uvas cultivadas en las zonas altas. Sin embargo, esta relación se complica por el efecto de las diferencias en la temperatura diurnas: bajas temperaturas de la noche dan como resultado una mayor acumulación de antocianinas (Mori y col., 2005). La acumulación de antocianinas se inicia en la fase de envero con descensos ocasionales hacia el final de la fase de madurez, especialmente en climas cálidos (Fournand y col., 2006). De hecho, en estaciones más cálidas se observa una disminución en el color de la baya. No se conoce si esta disminución se produce debido a la degradación de las antocianinas o por la reducción de su biosíntesis. De forma general, el rango de concentración varía de 500 a 5000 mg/Kg peso fresco (Tabla I.1).

1.2.2. Flavonoles

Estos compuestos están presentes en formas glicosiladas, unidos a un azúcar que suele ser una glucosa o una ramnosa, pero también pueden estar implicados otros como la galactosa, arabinosa, xilosa o el ácido glucurónido (Manach y col., 2004).

Las agliconas correspondientes (Figura I.4) se pueden encontrar en el vino, junto con los 3-glucósidos.



Flavonoles	*R3'*	*R5'*
Miricetina	*OH*	*OH*
Quercetina	*OH*	*H*
Laricitrina	*OH*	*OCH_3*
Kaempferol	*H*	*H*
Isorhamnetina	*H*	*OCH_3*
Siringetina	*OCH_3*	*OCH_3*

Figura I.4. Estructura química de las formas aglicona de los flavonoles más comunes en vino y uva.

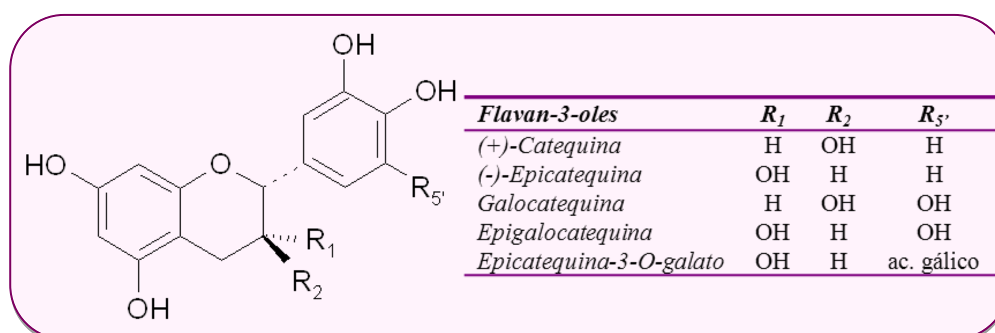
Existe una relación positiva entre la exposición al sol y el aumento de la acumulación de flavonoles (Downey y col., 2004; Cortell y col., 2006).

Los flavonoles se encuentran exclusivamente en pieles de las uvas tintas y blancas, y sus rangos de concentración van desde trazas hasta 300 mg/kg peso fresco (Tabla I.1).

1.2.3. Flavan-3-oles y procianidinas

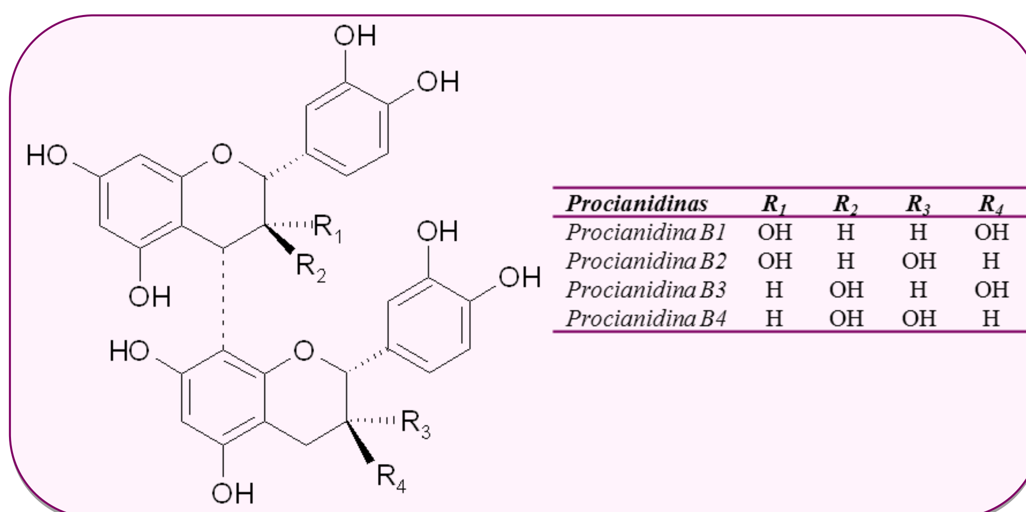
Los flavan-3-oles, también llamados comúnmente catequinas, se encuentran en forma monomérica (principalmente catequina y epicatequina) (Figura I.5) y en su forma polimérica (proantocianidinas también llamados taninos condensados o no-hidrolizables) (Figura I.6).

Se han identificado una veintena de procianidinas dímeros y trímeros en las semillas y en el hollejo de la uva, siendo las principales la B1, B2, B3 y B4 (Fulcrand y col., 1999).



Flavan-3-oles	R_1	R_2	$R_{5'}$
(+)-Catequina	H	OH	H
(-)-Epicatequina	OH	H	H
Galocatequina	H	OH	OH
Epigalocatequina	OH	H	OH
Epicatequina-3-O-galato	OH	H	ac. gálico

Figura I.5. Estructura química de los flavan-3-oles.



Procianidinas	R_1	R_2	R_3	R_4
Procianidina B1	OH	H	H	OH
Procianidina B2	OH	H	OH	H
Procianidina B3	H	OH	H	OH
Procianidina B4	H	OH	OH	H

Figura I.6. Estructura química de las procianidinas.

Las procianidinas presenten en la semilla de la uva se caracterizan por tener menor grado de polimerización que las que se encuentran en el hollejo. Sin embargo, las procianidinas del hollejo se extraen más fácilmente durante la elaboración del vino, lo que confiere las características organolépticas sobre el vino como la astringencia y el amargor (Souquet y col., 1996).

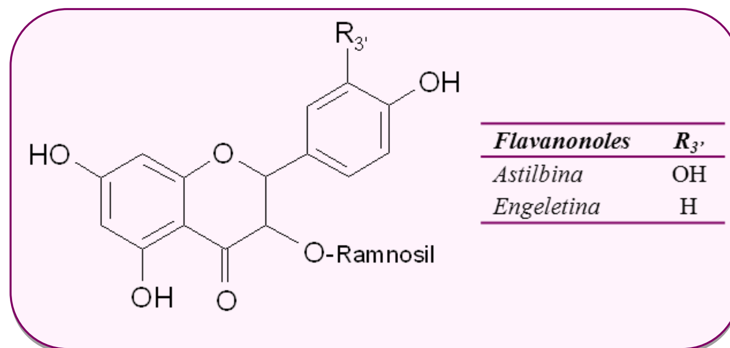
A diferencia de los grupos anteriores no se encuentran en forma glicosilada, aunque sí forman ésteres con ácido gálico, siendo la epicatequina-3-*O*-galato uno de los flavan-3-oles más importantes en las semillas de uva. Los flavan-3-oles más representativos de la uva se encuentran en piel y mayoritariamente en semillas, y son los siguientes: (+)-catequina, (-)-epicatequina, galocatequina, epigalocatequina y epicatequina 3-*O*-galato (Figura I.5) (Piñeiro y col., 2004).

A diferencia de antocianinas y flavonoles, las condiciones climáticas tienen poco efecto sobre flavan-3-oles, ya que estos compuestos se encuentran mayoritariamente en la semilla. De hecho, el clima parece tener mayor efecto sobre la composición que en la cantidad (Downey y col., 2006).

La cantidad total de procianidinas varía de 1.7 hasta 4.4 g/kg de bayas en el hollejo, de 1.1 a 6.4 g/kg en las semillas, y de 0.2 a 1 g/kg en la pulpa (Mane y col., 2007).

1.2.4. Flavanonoles y flavonas

Los compuestos pertenecientes a esta familia han sido identificados en el hollejo de uva blanca. Las agliconas más ampliamente distribuidas son la astilbina y la engelatina (Figura I.7). Sus concentraciones en la piel son del orden de 10.7 y 2.4 mg/Kg de peso fresco para astilbina y engelatina, respectivamente; lo que representa el 5% de los compuestos fenólicos totales del hollejo de la uva (Trousdale y Singleton, 1983).

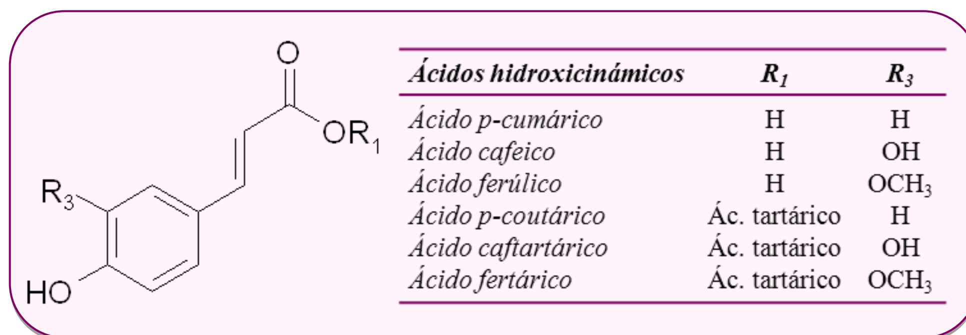


Flavanonoles	$R_{3'}$
Astilbina	OH
Engeletina	H

Figura I.7. Estructura química de dos flavanonoles.

1.2.5. Ácidos hidroxicinámicos

Los ácidos hidroxicinámicos son el tercer grupo más abundante de compuestos fenólicos en uvas y comprenden principalmente los ácidos cafeico, cumárico, ferúlico y sus correspondientes ésteres tartáricos (Figura I.8).



Ácidos hidroxicinámicos	R_1	R_3
Ácido p-cumárico	H	H
Ácido cafeico	H	OH
Ácido ferúlico	H	OCH_3
Ácido p-coutárico	Ác. tartárico	H
Ácido caftartárico	Ác. tartárico	OH
Ácido fertárico	Ác. tartárico	OCH_3

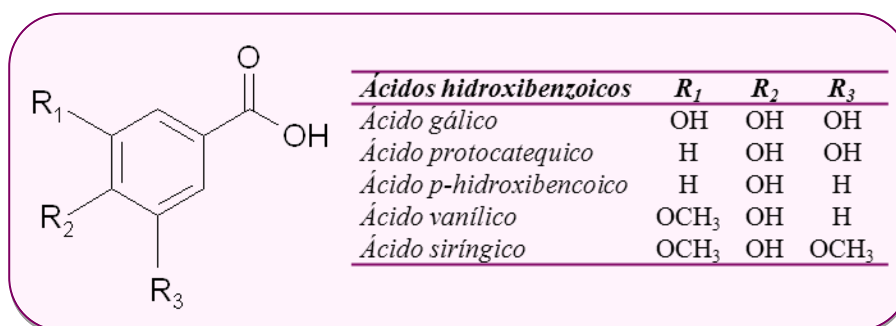
Figura I.8. Estructura química de los ácidos hidroxicinámicos.

Los ácidos hidroxicinámicos son el tercer grupo de polifenoles más abundantes en uva y los mayoritarios en zumo y vino blanco. Son fácilmente oxidables y, por tanto, responsables en parte del pardeamiento de los vinos (Kallithraka y col., 2009).

A diferencia de los flavonoides, que se encuentran fundamentalmente en la piel de la uva, los ácidos hidroxicinámicos se encuentran mayoritariamente en la pulpa (Ong y Nagel, 1978; Singleton y col., 1986; Rodríguez y col., 2006).

1.2.6. Ácidos hidroxibenzoicos

Los ácidos hidroxibenzoicos son un grupo minoritario de los compuestos fenólicos en la uva (Figura I.9). Los más comunes son el ácido gálico, ácido protocatéquico, ácido *p*-hidroxibenzoico, ácido vanílico, y el ácido siríngico, que se encuentran principalmente en forma libre (Pozo-Bayon y col., 2003; Vanhoenacker y col., 2001). El ácido hidroxibenzoico mayoritario es el ácido gálico, que se encuentra libre y como sustituyente acilo en flavan-3-oles. Se encuentran en la piel de uva, y sus rangos importe total de 2 a 5 mg/Kg de peso fresco (Tabla I.1).



Ácidos hidroxibenzoicos	R_1	R_2	R_3
Ácido gálico	OH	OH	OH
Ácido protocatequico	H	OH	OH
Ácido p-hidroxibencoico	H	OH	H
Ácido vanílico	OCH_3	OH	H
Ácido siríngico	OCH_3	OH	OCH_3

Figura I.9. Estructura química de los ácidos hidroxibenzoicos.

1.2.7. Estilbenos

Se verán detalladamente en el apartado 2.

Tabla I.1. Compuestos fenólicos en uva tinta y blanca.

Familia	Compuestos fenólicos	Uva tinta (mg/kg peso fresco)	Uva blanca (mg/kg peso fresco)	Referencias
Antocianos	Delfinidina-3- glc Cianidina-3- glc Petunidina-3- glc Peonidina-3- glc Malvidina-3- glc Derivados acetilados Derivados *p*-cumaroil	500-5000	_	Mattivi y col., 2006 Mazza y col., 1995 Cantos y col., 2002 Guerrero y col., 2009
Flavonoles	Quercetina-3-glc/glu/gal/rut Kaempferol -3-glc/glu/gal Miricetina -3-glc/glu Isorhamnetina -3-glc/glu/gal Laricitrina -3-glc/gal Siringetina -3-glc	3-300	1-200	Mattivi y col., 2006 Guerrero y col., 2009 Castillo-Muñoz y col., 2007, 2010 Rodríguez Montealegre y col., 2006
Flavan 3-oles	Catequina Epicatequina Galocatequina Epigalocatequina Epicatequina-3-*O*-galato Procianidinas B1, B2, B4, C1	2500-11000	2500-11000	Guendez y col., 2005 Mane y col., 2007 Rodríguez Montealegre y col., 2006
Derivados ácido hidroxicinámico	Cafeoil tartárico Cumaroil tartárico Feruloil tartárico	1.5-50	4-45	Mazza, 1995 Cantos y col., 2002 Rodríguez Montealegre y col., 2006 Gómez-Alonso y col., 2007
Ácidos Hidroxibenzoicos	Ácido gálico Ácido protocatequico Ácido *p*-Hidroxibenzoico Ácido vanílico Ácido siríngico	2-5	2-5	Gómez-Alonso y col., 2007
Flavanonoles y flavonas	Astilbina Engelatina	---	2.4-10.7	Trousdale y Singleton, 1983

Abreviaturas: glc, glucosido; glu, glucuronido; rut, rutinosido; gal, galactosido.

2. ESTILBENOS

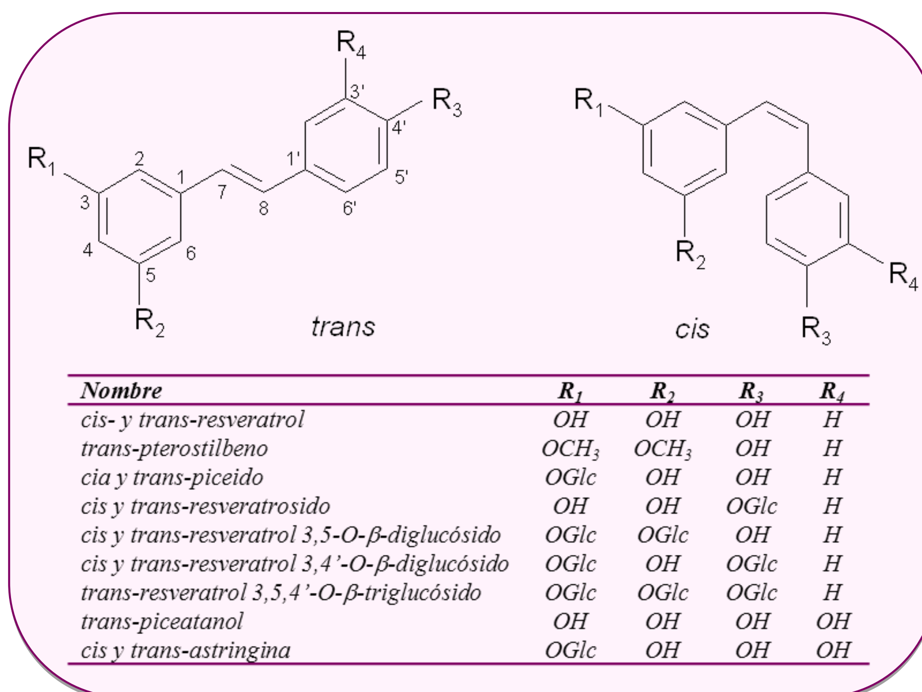
Los estilbenos son polifenoles no flavonoides que son sintetizados por una amplia variedad de plantas de las familias *Pinaceae, Moraceae, Liliaceae, Myrtaceae, Fagaceae, Gnetaceae, Cyperaceae, Dipterocarpaceae Leguminoseae* y *Vitaceae* (Harborne, 1999). Dentro de la familia *Vitaceae* existen varios géneros en los que se han encontrado estilbenos, concretamente en *Ampelopsis, Cissus, Cyphostemma, Phartenocissus y Vitis* (Pawlus y col., 2012). Sin embrago la mayoría de estas plantas no son normalmente consumidas en la alimentación Las principales fuentes de estilbenos en la dieta son las uvas y sus derivados, zumo y vino tinto, aunque existen otras fuentes minoritarias (Tabla I.2).

Tabla I.2. Fuentes de resveratrol en la dieta.

Alimento	*trans*-Resveratrol	Bibliografía
Arándanos	0.02 µg/g	Lyons y col., 2003
Fresas pulpa	0.09 µg/g	Wang y col., 2007
Cacahuetes	0.84 µg/g	Tokusoglu y col., 2005
Pistachos	1.15 µg/g	Tokusoglu y col., 2005
Cacao en polvo	1.85 µg/g	Hurst y col., 2008
Chocolate negro	0.35 µg/g	Hurst y col., 2008
Tomate	3.55 µg/g*	Ragab y col., 2006
Uva de mesa	0.65 µg/g	Cantos y col., 2002
Cerveza	0.01 mg/L	Chiva-Blanch y col., 2011
Zumo de uva tinta	0.5 mg/L	Romero-Perez y col., 1999
Vino blanco	0.13 mg/L	Romero-Perez y col., 1996
Vino rosado	0.41 mg/L	Romero-Perez y col., 1996
Vino tinto	1.9 mg/L	Landrault y col., 2002 Stervbo y col., 2006

* Piel liofilizada

La estructura química está formada por dos anillos aromáticos unidos por un puente etileno (C6-C2-C6). A partir de esta estructura relativamente simple, hay una gran variedad de compuestos: i) monómeros, para lo cual el número y posición de los grupos hidroxilo, la sustitución con azúcares, metilo, metoxi y otros residuos y la configuración estérica de las moléculas varían (Figura I.10); ii) oligómeros, que son el resultado de diferentes condensaciones del monómero resveratrol (por ejemplo, dímeros, trímeros, tetrámeros) (Figura I.11).



Nombre	R_1	R_2	R_3	R_4
cis- y trans-resveratrol	*OH*	*OH*	*OH*	*H*
trans-pterostilbeno	OCH_3	OCH_3	*OH*	*H*
cia y trans-piceido	*OGlc*	*OH*	*OH*	*H*
cis y trans-resveratrosido	*OH*	*OH*	*OGlc*	*H*
cis y trans-resveratrol 3,5-O-β-diglucósido	*OGlc*	*OGlc*	*OH*	*H*
cis y trans-resveratrol 3,4'-O-β-diglucósido	*OGlc*	*OH*	*OGlc*	*H*
trans-resveratrol 3,5,4'-O-β-triglucósido	*OGlc*	*OGlc*	*OGlc*	*H*
trans-piceatanol	*OH*	*OH*	*OH*	*OH*
cis y trans-astringina	*OGlc*	*OH*	*OH*	*OH*

Figura I.10. Estructura química de monómeros de estilbenos en *Vitis vinífera* (Waffo-Teguo y col., 2008)

El resveratrol ha sido identificado como el compuesto con mayor actividad biológica, por lo que la mayoría de los estudios se han centrado en él. Las dos formas isómeras del resveratrol (*cis* y *trans*) tienen características químicas y actividades biológicas diferentes. El isómero *trans* es generalmente el más estable y la conversión a *cis* puede producirse en presencia de luz o por radiación ultravioleta (Sieman y Creasy, 1992; Leiro y col., 2004; Chen y col., 2007; Blache y col., 1997), resultando una mezcla de los dos isómeros (Goldberg y col., 1995a, 1995b; Jeandet y col., 1995; Trela y Waterhouse, 1996).

Además del resveratrol y de los estilbenos simples mencionados anteriormente se han aislado dímeros como el palidol, la ε-viniferina, la δ-viniferina, el ampelosin A, D y F; trímeros como la α-viniferina y tetrámeros como el isohopeafenol, el hopeafenol o la vitisina A, entre otros (Pawlus y col., 2012).

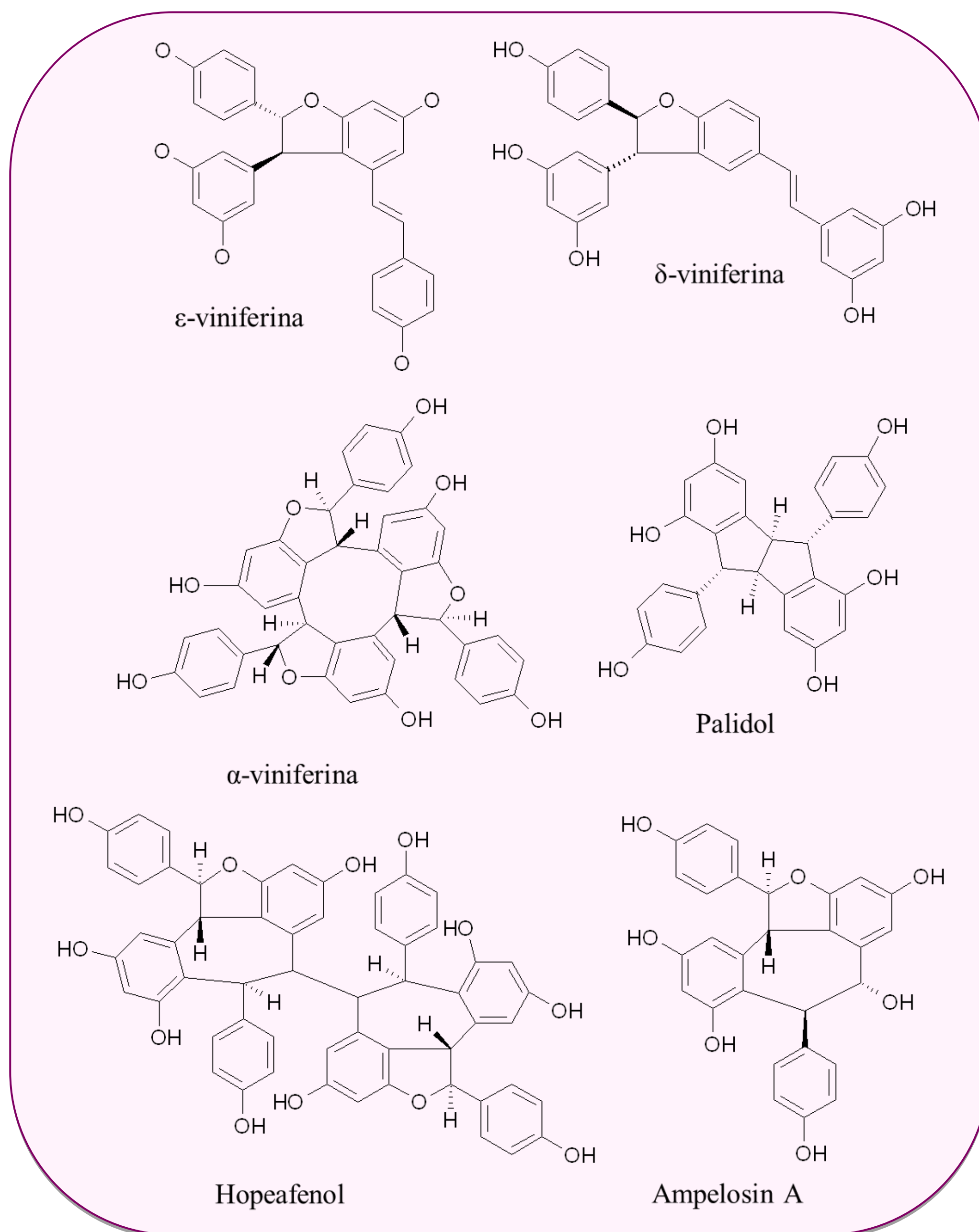


Figura I.11. Estructura química de algunos oligómeros presentes en *Vitis vinífera* (Pawlus y col., 2012)

2.1. BIOSÍNTESIS DE ESTILBENOS

La síntesis de estilbenos es inicialmente común a la de los flavonoides, a través de la ruta del shikímico. En primer lugar se produce la pérdida del grupo amino de la fenilalanina mediante desaminación oxidativa, catalizada por la enzima específica *fenilalanina-amonio-liasa*, dando lugar al ácido cinámico, el cual es hidroxilado a ácido *p*-cumárico por acción de la enzima *cinamato-4-hidroxilasa*, y finalmente transformado en *p*-cumaril-CoA, por acción de una *CoA-ligasa*. El cumaroil CoA reacciona con tres moléculas de malonil CoA en una reacción catalizada por la enzima *estilbeno sintetasa* para formar el resveratrol (3,5,4´-trihidroxiestilbeno), estilbeno de estructura más sencilla que es el punto de partida de la síntesis del resto de los estilbenos (Figura I.12).

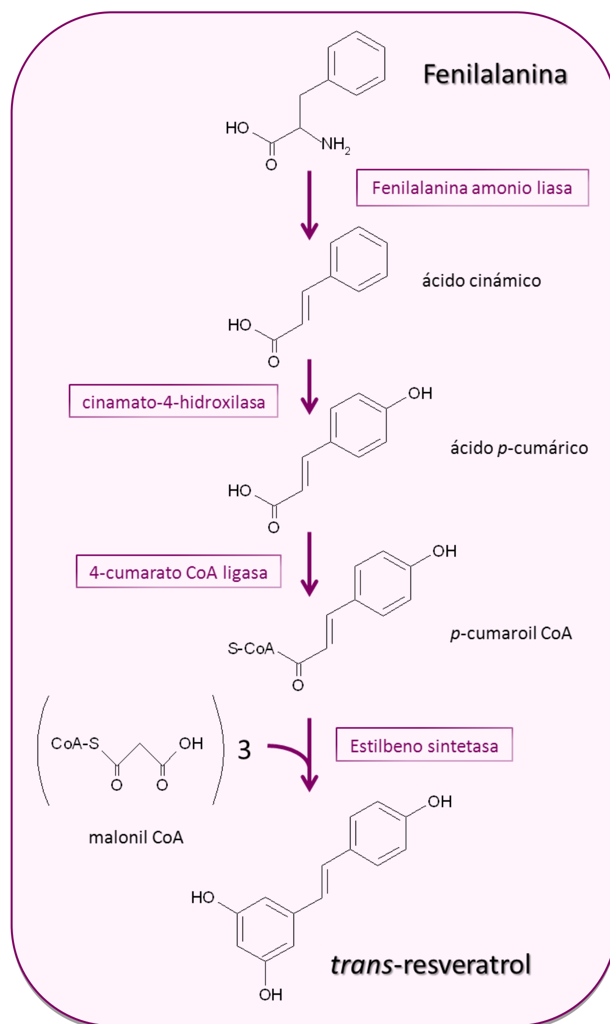


Figura I.12. Ruta biosintética de los estilbenos.

La *estilbeno sintetasa* es una enzima codificada por varios genes, ocho en el caso de la vid, cuyo cADN ha sido descrito (Sparvoli y col., 1994), y se sabe que codifican para una proteína homodímera de 90 Kda (Liswidowati y col., 1991). Esta enzima es inducible por diversos factores.

La síntesis de estilbenos en uva tiene lugar en la piel, y depende de la variedad, de las condiciones climáticas y de los procedimientos empleados en el cultivo así como del proceso de vinificación (Creasy y Creasy, 1998; Tomás-Barberán y Espín, 2001).

2.2. AUMENTO DE ESTILBENOS EN UVA

Las fitoalexinas fueron definidas como metabolitos secundarios de plantas con actividad antimicrobiana que eran sintetizados *de novo* y entraban dentro del funcionamiento básico de mecanismos de defensa (Müller y Börger, 1940). En 1980, se dio una nueva definición donde las fitoalexinas se definían como moléculas antimicrobianas de bajo peso molecular que eran sintetizadas y acumuladas en plantas después de estar expuestas a microorganismos (Paxton, 1980). Presentan una función defensiva y se inducen cuando la planta se ve amenazada ante un ataque o estrés de tipo biótico (patógenos y no patógenos) o abiótico (luz UVC, corte, daño, ozono, etc.), ya sean pre- o postcosecha (Langcake y Pryce, 1976; Calderón y col., 1993; Sarig y col., 1996).

Los estilbenos, como fitoalexinas, tienen modulada su síntesis por distintos factores, y por tanto, la concentración de éstos en el caso específico de la uva, el zumo y el vino varían ampliamente (Gürbüz y col., 2007; Bavaresco y Vezzulli, 2006). Uno de estos factores está asociado a la genética de la planta. La capacidad de sintetizar resveratrol es diferente según la especie de *Vitis* de la cual se trate, siendo más elevada en *Vitis rupestris* y en *Vitis cinerea* que en *Vitis vinifera* (Doulliet- Breuil y col., 1999). Dentro de una misma especie también se pueden observar diferencias entre distintas variedades (Cantos y col., 2002; Guerrero y col., 2010). Otros factores que pueden afectar a la capacidad de inducción de estilbenos en uva son los factores edafoclimáticos (Siemann y Creasy, 1992; Bais y col., 2000; Andrés-De Prado y col., 2007) y el grado de madurez existiendo una correlación negativa entre el estado de madurez y la capacidad de

inducción de síntesis de resveratrol (Jeandet y col., 1991) Por el contrario, recientemente otros autores han encontrado un aumento de la expresión genética de las enzimas de la biosíntesis de estilbenos durante el periodo de maduración (Gatto y col., 2008).

Por tanto existen numerosos factores externos que no se pueden modular y que también afectan notablemente a la síntesis de estilbenos (Dercks y Creasy, 1989; Cantos y col., 2000), y por ello estandarizar la concentración de resveratrol en uva y vino es extremadamente complejo.

A continuación se exponen los tratamientos más utilizados para aumentar la concentración de resveratrol en uva, divididos en tratamientos precosecha y postcosecha.

2.2.1. Tratamientos precosecha

2.2.1.1. Infecciones fúngicas

El resveratrol, así como sus dímeros y trímeros (ε-, δ- y α-viniferina), son sintetizados en la piel de las bayas, y en menor medida en las semillas, en respuesta al estrés o presión de infecciones fúngicas como *Botrytis cinerea*, *Plasmopara vitícola* o *Uncinula necator* (Jeandet y col., 2002; Breuil y col., 1998; Bavaresco y col., 1997; Pérez y col., 1991). El resveratrol producido inhibe la progresión de la infección fúngica. Tanto es así, que la cantidad de resveratrol sintetizada por la planta está relacionada con la resistencia que presentan diferentes variedades a la infección por *Botrytis cinerea* (Pool y col., 1981; Pezet y col., 2004).

A pesar de que la síntesis de resveratrol se estimula por la infección de *Botrytis cinerea*, un aumento descontrolado de la infección degrada el resveratrol producido por la planta debido a la acción exocelular de enzimas oxidasas del resveratrol parecidas a las enzimas lacasas segregadas por *Botrytis* (Perez y col., 1991; Breuil y col., 1998).

Jeandet y sus colaboradores analizaron en 1995 el nivel de resveratrol en uvas infectadas por *Botrytis cinerea* y observaron que los granos visiblemente afectados

tenían un nivel de resveratrol bajo en comparación con los que estaban alrededor de la zona afectada. Esta aparente contradicción en cuanto a la inducción por parte del hongo y la disminución en el contenido de resveratrol, podría explicarse por la dimerización y/o trimerización oxidativa del *trans*-resveratrol mediada por peroxidasas vegetales y lacasas de *Botrytis cinerea* (Langcake y Pryce, 1977; Calderón y col., 1994). Por tanto, es de esperar una baja concentración de resveratrol en vendimias donde se produzcan infecciones fuertes de *Botrytis cinerea* (Jeandet y col., 1995; Roldan y col., 2003).

En uvas infectadas con Mildiu y Oídio, la concentración de resveratrol y de piceido está directamente correlacionada con la presión fúngica (Romero-Perez y col., 2001).

El nivel de resveratrol también aumenta notablemente con la exposición a diferentes agentes bióticos no patógenos como *Thichoderma viride* o bacterías del género *Bacillus* aisladas del suelo, por lo que algunos autores han postulado la utilidad de estos agentes como tratamiento de la vid frente la infección por *Botrytis cinerea* (Calderón y col., 1993).

2.2.1.2. Tratamientos químicos

El **benzotiadiazol** (BTH) es un fungicida sistémico análogo al ácido salicílico (hormona endógena de las plantas), que puede ser utilizado en tratamientos precosecha como activador de mecanismos de defensa de la planta mediante la producción de resveratrol. Es un compuesto con una baja toxicidad, y de rápida degradación en los tejidos de las plantas por lo que su uso no tendría el impacto medioambiental de otros tratamientos de cultivo (Iriti y col., 2004; 2005). La aplicación de un spray sintético activador de la resistencia de las plantas, como el benzotiadiazol, en cepas antes de la vendimia puede incrementar la concentración de resveratrol en bayas hasta aproximadamente un 40% (Iriti y col., 2004).

El **quitosano** (CHIT) es un polisacárido de N-acetilquitina con carácter fungicida que se ha usado en uva de mesa, tanto en precosecha como en postcosecha, para mejorar la calidad (Meng y col., 2007). Pero además de su carácter fúngico, este compuesto resulta ser un inductor de la síntesis de estilbenos, efecto que se ve incrementado si se combina con un tratamiento UVC (Romanazzi y col., 2006).

El ácido jasmónico **(AJ)** y **el metil jasmonato (MEJA)** son un grupo de hormonas vegetales que ayudan a regular el crecimiento de las plantas y su desarrollo. Han sido propuestos como compuestos clave de la vía de transducción de señales involucradas en la activación de la biosíntesis de metabolitos secundarios que participa en las reacciones de defensa de las plantas (Gundlach y col., 1992).

Algunos experimentos (Larronde y col., 2003) han demostrado que niveles muy bajos en la atmósfera de MEJA puede aumentar la concentración de estilbenos en hojas y bayas. En bayas tratadas 15 días después del envero hubo un aumento en el contenido de *trans*-resveratrol mientras que los tratamientos realizados 30 días después no tuvo ningún efecto. Parece que conforme avanza la maduración, la uva pierde su capacidad para responder al MEJA. En las hojas, *trans*-resveratrol mejora independiente de la fase, aunque el principal compuesto inducido fue el piceido. Por otra parte, los tratamientos UV de las hojas pre-tratadas con MEJA dieron lugar a un aumento del contenido de *trans*-resveratrol y de piceido. Esto sugiere que *trans*-resveratrol se produce por estimulación directa de la *estilbeno sintasa* y no por desglicosilación del piceido.

En otros estudios (Belhadj y col., 2006) sólo se encontró *trans*-piceido en las hojas control mientras que en las tratadas con MEJA se encontraron además del *trans*-piceido, *trans*-resveratrol, ε-viniferina, pterostilbeno y δ-viniferina. Por otro ladoobservó que sólo en cepas de la variedad Merlot tratadas previamente con MEJA se produjo una reducción de la infección de oidio del 75% respecto a su control además de un aumento en la producción de estilbenos.

De acuerdo con Tassoni y col. (2005) los jasmonatos y, en particular, MEJA representan elicitores adecuados para la producción de *cis* y *trans*-resveratrol en cultivos celulares de la variedad Barbera.

Unos años más tarde, Vezzulli y col. (2007) rociaron con MEJA la variedad Barbera en diferentes etapas: cuajado, envero y maduración. Demostraron que tratamientos acumulativos aumentaron significativamente las concentraciones de resveratrol y viniferinas en las bayas.

El ácido salicílico (SA) también ha sido utilizado como elicitor en la síntesis de resveratrol. Los resultados del estudio mostraron que pulverizando las bayas con SA

aumentaron el contenido de resveratrol a pesar de que sus efectos variaron un poco dependiendo de la variedad (Li y col., 2008).

Otros estudios han demostrado que la dimetil-β-ciclodextrinas induce la síntesis de estilbenos cuando se añade a cultivos celulares (Morales y col., 1998; Bru y col., 2006; Zamboni y col., 2006). Además se ha demostrado que cuando se combina la ciclodextrina con otro elicitor como el MEJA la acumulación de resveratrol se incrementa tres veces en comparación con la adición de la ciclodextrina sola, habiendo por tanto un efecto sinérgico (Lijavetzky y col., 2008).

Un estudio realizado sobre cultivos celulares de la variedad Barbera demostró que tras ser tratadas con sacarosa se produjo una inducción y liberación al medio de cultivo del resveratrol y sus glucósidos piceido y resveratrosido (Ferri y col., 2011).

El ácido abscísico (ABA) también actuó como elicitor de fitoalexinas en las uvas de la variedad Kyoho, mejorando la concentración del resveratrol (Ban y col., 2000).

Finalmente, los iones metálicos, tales como lantano, europio, calcio, plata y cadmio también pueden inducir la biosíntesis de fitoalexinas en cultivos celulares vegetales (Radma y col., 2003).

2.2.2. Tratamientos postcosecha

2.2.2.1. Tratamiento con UV

En 1977, Langcake y Pryce observaron que la síntesis de resveratrol era máxima en hoja cuando la longitud de onda de luz UV de la fuente de irradiación era de 260-270 nm. No observaron este efecto a longitudes de onda por encima de 300-310 nm (Langcake y Pryce, 1977). Otros autores también corroboraron que la síntesis de resveratrol respondía a la irradiación ultravioleta (Jeandet y col., 1991). Posteriormente Doulliet-Breuil y col. (1999) señalaron igualmente que la inducción de resveratrol se daba en las hojas de la vid como respuesta a la irradiación ultravioleta C (254 nm), manteniéndose el elevado nivel de resveratrol durante dos días.

Fue en 2001 cuando se describió por primera vez la inducción de resveratrol por tratamiento postcosecha UVC para obtener uvas enriquecidas en *trans*-resveratrol, entre

otros estilbenos. Para ello, emplearon lámparas germicidas con un máximo de emisión de 254 nm con una potencial total de 510 W, una distancia de lámparas-muestra de 40 cm y una duración de tratamiento de 60 segundos (Patente WO/2002/085137; ES2177465). Tras la irradiación, las uvas debían permanecer a temperatura ambiente durante un periodo de tiempo que podía fluctuar (según variedad de uva y grado de madurez) siendo el valor medio 5 días, con un rango de 3 a 7 días. De esta forma, el *trans*-resveratrol en uva se inducía entre 100 y 2000 veces según la variedad tratada (Cantos y col., 2001).

Otros estudios más recientes han demostrado que en vino con este mismo tratamiento de luz UVC, se aumentó la concentración de resveratrol un 320% y de piceatanol un 2600% (Guerrero y col., 2010a, 2010b).

2.2.2.2. Tratamiento con ozono

El ozono es un gas azulado con un olor acre característico, siendo el segundo agente oxidante más potente detrás del flúor. La propiedad que hace al ozono particularmente interesante es que puede autodescomponerse, debido a que es muy inestable, convirtiéndose rápidamente en oxígeno. Además fue declarado por la FDA (Food and Drug Administration) sustancias GRAS (generalmente conocida como segura) para su empleo como desinfectante o higienizante en el procesado de alimentos (Kim y col., 1999). En su forma gaseosa o disuelta en agua se ha empleado en diversas aplicaciones y en diferentes productos vegetales.

A pesar de todas las aplicaciones que tiene el ozono, un uso más novedoso podría ser el tratamiento de uvas con la finalidad de provocar un estrés y de esta forma inducir la síntesis de fitoalexinas. Así, se ha puesto de manifiesto que la biosíntesis de resveratrol es inducida mediante tratamientos con ozono (Sarig y col., 1996). Más recientemente, otros ensayos con tratamientos postcosecha de ozono han revelado un aumento de resveratrol, piceatanol y viniferinas (Artes-Hernández y col., 2003; González-Barrio y col., 2006).

2.2.2.3. *Tratamientos anóxicos*

Cortos tratamientos anóxicos (bayas coloca en una cámara de vacío lleno de gas nitrógeno a temperatura ambiente durante 6 a 24 h) dieron lugar a diferencias en la síntesis de estilbeno. Cuando el tratamiento anóxico dura entre 6 y 15 h mejoró considerablemente la cantidad de resveratrol en los frutos y no se detectaron daños organolépticos. Cuando el tratamiento dura entre 24 y 48 h causó daños considerables al fruto a la vez que se produce un descenso rápido del contenido en estilbenos. Estos tratamientos anóxicos controlados también mejoraron la calidad post-cosecha y la salud de las bayas (Jiménez y col., 2007).

2.3. INFLUENCIA DE LOS FACTORES VITÍCOLAS Y DE LA VINIFICACIÓN EN EL CONTENIDO RESVERATROL EN UVA Y VINO

La síntesis de estilbenos, tanto en la hoja como en la baya puede ser desencadenada por varios factores bióticos y abióticos, los cuales están directamente relacionados con la acumulación de estilbenos, como se discutió en la sección anterior. También hay un número de prácticas vitícolas, que interactúan con los elicitores, por lo que las concentraciones de estilbenos varían ampliamente. Algunos de éstos están bien documentados en la literatura. Factores vitícolas incluyen: variedad, portainjerto, localización geográfica, condiciones meteorológicas y las prácticas en campo. La comprensión de las diferentes funciones de estos factores es crucial, ya que, sólo con esta información, será posible el desarrollo de las prácticas culturales encaminadas a mejorar el contenido de estilbenos en uva y vinos (Figura I.13).

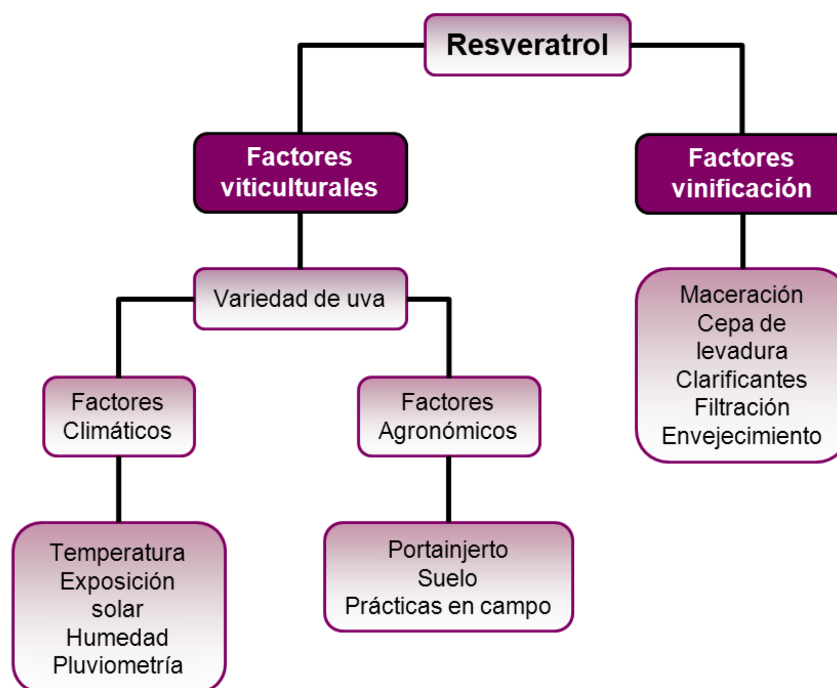


Figura I.13. Factores que afectan la concentración de resveratrol en uva y vino.

2.3.1. Factores Vitícolas

2.3.1.1. Variedad

La capacidad de sintetizar estilbenos es distinta según la especie de *Vitis* de que se trate; en *Vitis rupestris* y en *Vitis cinerea* la capacidad de síntesis es mayor que en *Vitis vinifera* (Douillet-Breuilt y col., 1999). Lamikanra y col. (1996) realizaron un estudio donde se observó que las concentraciones de resveratrol eran mayores en vinos de *V. rotundifolia*, especie con mayor resistencia a las enfermedades, que en vino de *V. vinifera* (que generalmente tienen baja resistencia a las enfermedades).

Dentro de una especie también se observan diferencias como se demostró en el estudio realizado por Cantos y col. (2002), en el cual se estudiaron siete variedades tintas y cada una de ellas obtuvo diferentes concentraciones de estilbenos.

Según algunos autores las variedades más productoras de resveratrol son la Pinot noir y la Cabernet sauvignon (Soleas y col., 1995). Se han encontrado valores medios para la concentración de resveratrol en vinos tintos de la variedad Pinot noir de 7.15 mg/L (Mattivi, 1993). También se han encontrado altas concentraciones en variedades como la Nero d´Avola de Sicilia (Bavaresco, 2003).

Sun y col. (2006) compararon tres variedades tintas, Castelao, Syrah y Tinta Roriz y demostró que en la piel no se encontraron diferencias significativas en el contenido de *trans*-resveratrol, pero que tienen niveles muy diferentes de *trans*-piceido y *cis*-piceido.

En el 2010a, Guerrero y col. realizaron un estudio sobre el contenido de estilbenos en 12 variedades tintas. Demostraron que las *Vitis viniferas silvestris* tienen mayores concentraciones que *las Vitis vinifera sativa* y que dentro de la subespecie sativa las que presentaron mayor contenido de estilbenos fueron la Syrah y la Jaén tinto.

Como norma general, las variedades tintas tienen niveles mayores de estilbenos que las variedades blancas. De acuerdo con Okuda y Yokotsuka (1996), quienes estudiaron 33 variedades cultivadas en Japón en 1994, el genotipo tiene un efecto significativo en la concentración de resveratrol en bayas, con un rango entre 0.06 mg/kg (Pizutello bianco) a 1.76 mg/kg peso fresco (Müller thurgau)

Un estudio realizado sobre 120 variedades demostró que el contenido en resveratrol variaba dependiendo del portainjerto. Se encontró que los niveles de resveratrol eran mayores para las variedades de uva con semillas que para las variedades sin semilla y además, que las variedades de uvas de vinificación contienen niveles más altos de resveratrol en pulpa y piel que en las variedades de uva de mesa (Li y col. 2006).

Romero-Pérez y col. (1999) encontraron diferencias en la concentración de piceido en zumos comerciales de uva. La concentración en los zumos de variedades tintas fue de 3.38 mg/L para el *trans*-piceido y 0.79 mg/L para el *cis*-piceido, mientras que para los zumos de variedades blancas fue de 0.18 mg/L para el *trans*-piceido y 0.26 mg/L para *cis*-piceido. Además, Bavaresco y col. (2007b) encontraron mayores concentraciones de

resveratrol y piceido en las bayas de Barbera y Croatina (tintas) que en Malvasia di Candia aromatica (blanca).

Por otro lado, de acuerdo con Soleas y col. (1995b) y Eder y col. (2001), el contenido de resveratrol es mayor en los vinos de variedades de *V. vinifera* que en los de híbridos interespecíficos. En otros estudios similares, no se encontraron diferencias significativas en cuanto a la concentración de resveratrol (Korbuly y col., 1998). Es difícil de explicar estos resultados contradictorios, pero además de la variedad existen otros factores importantes que deben ser tenidos en cuenta.

2.3.1.2. Factores climáticos

El efecto del clima parece ser crucial. El clima debe ser considerado como viene determinado por la posición geográfica de la viña (altitud, elevación, etc.), y las variaciones metereológicas para un área determinada.

De acuerdo con Goldberg y col. en 1995 y 1996, los vinos, especialmente de Cabernet sauvignon, producidos en zonas consideradas de clima frío tienen mayores niveles de resveratrol que vinos de áreas más cálidas. Igualmente, las temperaturas altas, comparadas con las bajas temperaturas, y la insolación redujeron la concentración de resveratrol en vinos de Cabernet sauvignon (Adrian y col., 2000). Sin embargo, el mismo autor (Goldberg y col., 1995, 1996, 1999) sugiere que no hay una relación clara entre los niveles de estilbenos en vinos y el clima.

La elevación del viñedo, que afecta a la temperatura y a la irradiación UV, tiene un efecto positivo en la síntesis de resveratrol, probablemente debido a una mayor radiación UVB (Fregoni y col., 1994). Por otro lado, Bavaresco y col. (2007) realizaron un ensayo en Pienza (norte de Italia) donde investigaron el papel de las condiciones meteorológicas y la elevación de la viña en el contenido de estilbenos de uvas maduras. La concentración de *cis*-piceido se correlacionó positivamente con la humedad relativa durante la maduración y negativamente con la temperatura (grados-día de crecimiento). La elevación del terreno parece afectar también a los niveles de resveratrol y piceido en las uvas, produciéndose un aumento entre 150-320 m a.s.l y una disminución para 420 m a.s.l.

Las condiciones metereológicas para un área determinada, especialmente las condiciones de humedad y los valores de precipitación, afectan al contenido en estilbenos debido su relación con la presión de enfermedades fúngicas en la viña (Jeandet y col., 1995; Martínez-Ortega y col., 2000). Condiciones de extrema sequedad durante el periodo de maduración son desfavorables a la síntesis de estilbenos (Vezzulli y col. 2007a). Para conseguir estimular la síntesis de estos compuestos es necesario una presión fúngica baja (indetectable a simple vista), como ocurre en climas húmedos durante el periodo de maduración.

Se han medido concentraciones altas de resveratrol en vinos elaborados en climas fríos, como Ontario (Canadá) o Burdeos, y menores concentraciones en vinos con clima relativamente cálidos y secos, aunque se encontraron diferencias entre sub-regiones (Stervbo y col., 2007). Faustino y col. en 2003 realizaron un estudio de vinos tintos chilenos y canadienses en el que se encontró una concentración media de resveratrol de 2.5 mg/L que fue comparada con los 1.25 mg/L detectada en vinos tintos producidos en los Estados Unidos. Esta relación entre clima y concentración de resveratrol presumiblemente refleja, como se ha comentado, una mayor presión fúngica asociada a las regiones de clima más frío.

2.3.1.3. Suelo

Existen pocos estudios sobre el efecto del suelo en la concentración de resveratrol. Las viñas con suelos arenosos tienen mayor concentración en estilbenos que aquellas con suelos arcillosos. Incluso el contenido en caliza del suelo parece estar relacionado con el nivel de estilbenos: suelos calcáreos inducen menos la síntesis (Gebbia y col., 2003). Por el contrario, Bavaresco y col. (2005) observó que el suelo calcáreo mejoró significativamente el contenido en estilbenos en uvas maduras de la variedad Merlot injertadas sobre patrón C3309.

En otro estudio se evaluó el efecto del tipo de suelo en el contenido de estilbenos, en dos viñedos de la variedad Garnacha (Andrés-de Prado y col., 2007). Se observó que los viñedos que tenían suelos más fértiles y con una mayor capacidad de retención de agua, producía vinos con un menor contenido fenólico y con una menor intensidad del color, aunque la concentración de estilbenos fue mayor.

2.3.1.4. Prácticas en campo

La fertilización es un factor importante que interacciona con la fisiología de la planta, incluyendo la resistencia a enfermedades. El nitrógeno es el elemento mineral más reactivo ya que puede ser absorbido fácilmente por la planta, que reacciona rápidamente y de manera eficaz ante cambios en el contenido en nitrógeno del suelo. Altos contenidos de nitrógeno en suelo reducen las concentraciones de resveratrol en uva y vino (Bavaresco y col., 2001, 2007a).

Los tratamientos con cobre utilizados contra enfermedades fúngicas (mildiu o podredumbre gris, por ejemplo) aumentan el contenido en estilbenos en uvas y vinos (Coulomb y col., 1999). La comparación entre los efectos producidos por tratamientos con cobre y otros productos sintéticos contra el mildiu son contradictorios. Los productos químicos distintos del cobre aumentaron la concentración en vinos de la variedad Monastrel con respecto a los tratados con cobre (Albert y Gauchi., 2002). Sin embargo, vinos elaborados de manera ecológica mostraron concentraciones de resveratrol mayores que otros vinos (Zafrilla y col., 2003; Tinttunen y Lehtonen., 2001).

La intensidad de la poda en invierno no afectó las concentraciones de resveratrol en uvas de la región de Valpolicella (noreste de Italia) (Celotti y col., 1998), mientras que en el caso de uvas cultivadas en Sicilia, las vides con mayor número de yemas alcanzaron mayores concentraciones de estilbenos en sus vinos (Gebbia y col., 2003).

El sistema de conducción también parece tener efecto en los contenidos finales de resveratrol en vinos, ya que afectan al microclima producido en los racimos (Threlfall, 1996; Bertamini y col., 2002). El mantenimiento de los racimos en la sombra no afecta a la concentración de resveratrol en uvas (Ban y col., 2000), mientras que una baja exposición de los racimos a la radiación solar favorece la síntesis de resveratrol en años cálidos. Justo lo contrario ocurre en años fríos (Bertamini y col., 2002).

Bavaresco y col. 2008a llevaron a cabo un estudio durante 4 años en la zona vitivinícola de Pienza. El estudio consistió en ver como afectaba el deshojado en el contenido de estilbenos. Se observó que cuando las condiciones meteorológicas eran frías se producía cambios en la concentración de estilbenos, mientras que, cuando las condiciones eran cálidas no se apreciaba ninguna diferencia. Por último, Prajitna y col. (2007)

demostraron que un aclareo de racimos aumentaba el contenido de resveratrol en la baya.

Los vinos con alta concentración de estilbenos se producen en viñas con bajo rendimiento por hectárea (ha) y sin riego (Gebbia y col., 2003). Esto sugiere que la viticultura orientada a la calidad no sólo produce vinos de mayor calidad sino con mayores concentraciones de resveratrol.

2.3.2. Factores de Vinificación

2.3.2.1. Maceración

El contacto del mosto fermentando con los hollejos es un punto clave en la vinificación de vinos con alta concentración de resveratrol. El resveratrol se encuentra en la piel de las uvas y pasa al mosto/vino durante la fermentación alcohólica conforme se aumenta el tiempo de contacto y el mosto adquiere una mayor concentración alcohólica, al igual que ocurre para la mayoría de compuestos fenólicos (Soleas y col., 1995).

2.3.2.2. Cepa de levadura

Se pueden emplear estrategias para aumentar la concentración de resveratrol mediante la elección de la cepa de levadura.

Se conocen cepas de levadura del género *Saccharomyces cerivesae* que segregan enzimas que liberan resveratrol al mosto (Vrhovsek y col., 1997). El uso de levaduras con actividad β-glucosidasa produce vinos con concentraciones comparativamente más altas de resveratrol que las convencionales por la rotura de los enlaces glucosídicos del piceido. La ruta biosíntetica de los polifenoles puede ser reconstruida en levaduras convencionales de vino. Se puede insertar el gen que codifica el sustrato 3 malonil Co-A de la coenzima-A ligasa que codifica el gen (4CL216) y el gen de la enzima sintetasa de resveratrol (vst1) que introduce la ruta biosintética de los fenilpropanoides en la levadura, de manera que la levadura puede sintetizar más resveratrol (Becker y col., 2003). Si bien, la utilización de productos genéticamente modificados en la elaboración de vinos no está permitida por la Unión Europea.

2.3.2.3. Clarificante

Los clarificantes como bentonita, caseina, carbón activo, albúmina, gelatina y polivinilpolipirrolidona (PVPP) se unen con los polifenoles y disminuyen sus concentraciones en el vino. La caseina, el carbón activo y la PVPP en particular, se unen al resveratrol y pueden llegar a retener más de un 90% (Bambalov, 1997; Baron y col., 1998; Revilla y col., 1997; Vrhovsek y col., 1997; Castellari y col., 1998). Limitar el uso de estos clarificantes en la elaboración de los vinos para no disminuir el contenido en polifenoles es un punto crítico dentro del proceso que compromete el concepto de calidad del usuario quien asocia la calidad de un vino con su clarificación y estabilización. Aun así la tendencia actual en las elaboraciones de vinos de alta gama es la de reducir la utilización de clarificantes.

2.3.2.4. Filtración

Los cambios más dramáticos en la concentración de resveratrol ocurren durante la filtración, ya que el resveratrol se une a las sustancias que componen los filtros. Se produce una retención mayor del isómero *trans*-resveratrol que su forma *cis*-, llegándose a un 58% de retención en el primer caso. La reducción de la concentración depende del tipo de filtros (Soleas y col., 1995).

2.3.2.5. Envejecimiento

Durante el envejecimiento en madera se producen pérdidas debidas a la precipitación, oxidación y/o absorción de resveratrol. Incluso manteniéndose condiciones de oscuridad y baja temperatura. Algunos autores han descrito aumentos en la concentración de resveratrol durante esta etapa, pero son debidos a las mermas sufridas durante este proceso (Soleas y col., 1995). En 1997, Roggero demostró que los vinos de la variedad Mourvédre conservaron tanto el resveratrol como el piceido independientemente de su añada. Finalmente, Moreno-Labanda y col. (2004) encontraron que en la variedad Monastrel, la concentración total de resveratrol en los vinos envejecidos era muy similar a la observada en los vinos jóvenes.

2.4. BIODISPONIBILIDAD DEL RESVERATROL

La biodisponibilidad del resveratrol es muy baja. Una vez absorbido, al menos un 70% es metabolizado en forma de glucurónido y sulfato. Los metabolitos del resveratrol alcanzan su máxima concentración en plasma a los 30 minutos tras su consumo, siendo su vida media 9,2 horas (Walle y col., 2004). Tras la ingesta de vino tinto, se han identificado en orina cinco metabolitos distintos: resveratrol monosulfato, dos isómeros del resveratrol monoglucurónido, dehidroresveratrol monoglucurónido, dehidroresveratrol monosulfato y dehidroresveratrol (Vitaglione y col., 2005). En otro estudio, se identificaron hasta seis metabolitos distintos adheridos a la estructura de las LDL de voluntarios, tras la ingestión de 250 mL de vino tinto: *trans*-resveratrol-3-*O*-glucurónido, *cis*-resveratrol-3-*O*-glucurónido, *cis*resveratrol-3-*O*-glucósido, *trans*-resveratrol libre, resveratrol-4'-*O*-glucurónido y *trans*-resveratrol-4-O-glucósido (Urpí-Sarda y col., 2005). Dado que la concentración de los metabolitos del resveratrol puede ser más alta que el resveratrol en sí, se hace necesario determinar la actividad de dichos metabolitos.

Bertelli y col., 1996 realizaron un estudio en el cual pretendían evaluar la absorción, la concentración de resveratrol en los diferentes órganos tras la administración de vino tinto, por vía oral a las ratas. Un primer grupo de animales recibió una dosis de 4 ml de vino tinto (6.5 mg/L de resveratrol total). Un segundo grupo de ratas recibió una dosis diaria de 2 ml de vino tinto (6.5 mg/L de resveratrol) durante quince días. Las concentraciones totales de resveratrol se midieron en plasma, orina, corazón, hígado y riñón. Comparando ambos resultados determinaron que, aunque la cantidad de resveratrol encontrado en los diferentes tejidos fue menor que la requerida para la actividad farmacológica, la administración prolongada de vino tinto en la dieta puede conducir a un aumento de concentración de resveratrol en diferentes tejidos y esto podría explicar sus propiedades beneficiosas frente a enfermedades cardíacas (Bertelli y col., 1998). Estudios posteriores demuestran que el resveratrol se une a la albúmina, pudiendo ser ésta un reservorio *in vivo* de resveratrol, recirculando desde el torrente sanguíneo a los distintos órganos diana como corazón, riñones, pulmón y estómago (Jannin y col., 2004).

Otra posible explicación a su gran bioactividad, a pesar de su relativamente baja concentración en la dieta, es su efecto sinérgico cuando se encuentra con otros polifenoles bioactivos. Por ejemplo, se ha estudiado la eficiencia del resveratrol junto a catequina y quercetina en distintas matrices, observándose efectos sinérgicos entre dichos compuestos (Goldberg y col., 2003). Además, se ha demostrado su sinergia con la quercetina y el ácido elágico en la inducción de la apoptosis en células de leucemia humana (Mertens-Talcott y Percival, 2005), con el etanol en la inhibición de la expresión de la iNOS (Chan y col., 2000), con la vitamina E en la prevención de la peroxidación lipídica (Fang y col., 2002), con catequina en la protección de las células PC12 frente al efecto toxico de los péptidos β-amiloides sobre las neuronas (Conte y col., 2003), con análogos de nucleósidos en la inhibición de la replicación del VIH-1 en cultivos de linfocitos T (Heredia y col., 2000), y con tirosol y β-sitoesterol en la modulación de los efectos de LDLox sobre el estrés oxidativo y la síntesis de prostaglandinas (Vivancos y Moreno, 2008).

Con respecto al consumo de estilbenos, un estudio realizado sobre la población adulta española (Zamora-Ros y col., 2008), demostró que el 32% de la población no consumen resveratrol ni piceido en su dieta. Los mayores consumidores españoles son individuos de las regiones norte, con necesidades altas de aporte energético, un nivel educacional alto y fumadores. El consumo de resveratrol representa, en general, un 21% de los estilbenos consumidos mientras que el piceido llega al 54%. En poblaciones con patrones dietéticos distintos a los españoles estos porcentajes pueden variar.

Conviene destacar que la toxicidad del resveratrol resulta prácticamente nula, no observándose ningún efecto adverso en casos de ingesta alta y prolongada de este compuesto (Juan y col., 2002).

2.5. ACTIVIDAD BIOLÓGICA DE LOS ESTILBENOS

2.5.1. Actividad biológica del resveratrol

El resveratrol es uno de los polifenoles más estudiados en los últimos años debido al creciente interés por sus propiedades beneficiosas sobre la salud (Figura. I.14). El resveratrol puede prevenir o retrasar la progresión de diversas enfermedades, incluyendo enfermedades cardiovasculares (Bradamante y col., 2004), neurodegenerativas (Jang y Surh, 2003; Zamin y col., 2006) y cancer (Jang y col., 1997; Athar y col., 2007).

El resveratrol, además de presentar propiedades anti-inflamatorias (Leiro y col., 2010), antioxidantes (Filip y col., 2003) y antimicrobianas (Docherty y col., 2003) evita procesos de envejecimiento y aumenta la longevidad (Baur y Sinclair, 2006).

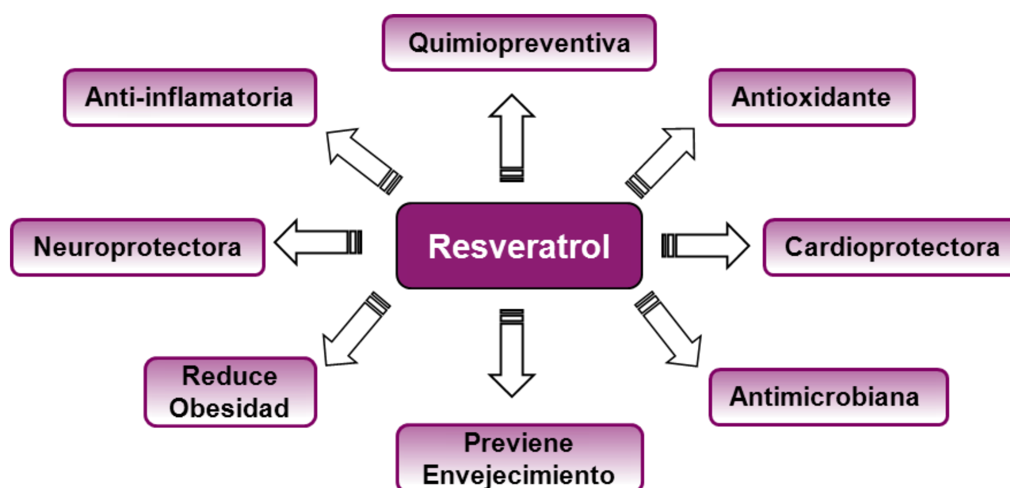


Figura I.14. Propiedades beneficiosas para la salud del resveratrol.

Su actividad **anticancerígena** es una de las bioactividades más prometedoras del resveratrol. En 1997, Jang y col. descubrieron la capacidad del resveratrol para inhibir el proceso de carcinogénesis en sus tres etapas: iniciación, promoción y progresión del tumor. Su hallazgo de que la aplicación tópica de resveratrol reduce hasta en un 98% el cáncer de piel en ratones extendió la investigación del resveratrol en todo el mundo.

Sus efectos anti-proliferativos y pro-apoptóticos en líneas celulares cancerígenas se han mostrado ampliamente in vitro (Aggarwal y col., 2004) y son apoyados por una regulación de las proteínas del ciclo celular (Schneider y col., 2001) y el aumento de la apoptosis (Garvin y col., 2006) en modelos de tumores in vivo.

Sin embargo, en algunos experimentos in vivo el resveratrol no produjo ningún efecto sobre el cáncer, lo que sugiere que factores como la dosis, su posología, el origen del tumor, y otros componentes de la dieta podrían contribuir a la eficacia del tratamiento con resveratrol. En general, los estudios in vivo muestran claramente un uso prometedor de esta molécula en el tratamiento del cáncer.

También, se ha demostrado que el resveratrol mejora la resistencia del organismo frente al estrés, tiene efecto **antienvejecimiento** y prolonga el tiempo de vida en numerosos organismos, desde levaduras (Howitz y col., 2003) a vertebrados (Valenzano y col., 2006). Otras investigaciones han demostrado que ratones obesos cuya dieta era suplementada con resveratrol no solo son más longevos, sino que además, son más activos, presentando en menor medida los efectos adversos de una dieta hipercalórica (Baur y Sinclair, 2006).

El interés por el carácter **cardioprotector** del resveratrol surgió a partir de un estudio llevado a cabo en 1992, en el que se comparó la tasa de mortalidad por enfermedad cardiovascular (ECV) y el nivel de colesterol sérico en distintas poblaciones del mundo (Renaud y De Lorgeril, 1992). La correlación fue altamente positiva para las poblaciones estudiadas, excepto para Toulouse, la cual mostraba los mismos niveles de colesterol en sangre que Glasgow, pero una tasa de mortalidad por ECV mucho menor. Esta aparente discrepancia entre el riesgo de sufrir ECV (alto colesterol en sangre) y muerte por ECV, se denominó "Paradoja Francesa". Tras el estudio minucioso de las variables que podrían contribuir a esta discrepancia, se encontró el elevado consumo de vino tinto en Toulouse como presunto responsable de dicha evidencia. Posteriormente, numerosos estudios han corroborado que el elevado contenido polifenólico del vino tinto, le confiere carácter cardioprotector (Wollin y Jones, 2001; De Gaetano y Cerletti, 2001; De Gaetano y col., 2002), y otros muchos señalan al resveratrol como principal agente responsable de esta actividad (Renaud y Gueguen, 1998; Vidavalur y col., 2006;

Das y Das, 2007; Wang y col., 2005). El resveratrol actúa a distintos niveles en el organismo, de ahí su demostrada efectividad (De Gaetano y col., 2002). Uno de los puntos más importantes es que inhibe la apoptosis celular a dosis muy bajas, previniendo varias enfermedades entre las que se incluye: daño miocárdico por isquemia-reperfusión, aterosclerosis y arritmias ventriculares. A dosis altas, es capaz de inducir apoptosis, actuando como agente quimiopreventivo (Das y Das, 2007).

La agregación plaquetaria es uno de los factores que más afecta al proceso de arterosclerosis. Las plaquetas pueden activar la formación de trombos y su agregación produciendo oclusión vascular. En 1995, Bertelli y col. demostraron que el resveratrol es capaz de inhibir, *in vitro*, la agregación plaquetaria. Años después se demostró esta actividad del resveratrol también *in vivo*, concretamente en conejos sometidos a una dieta hipercolesterolémica (Wang y col., 2005).

La capacidad vasodilatadora del resveratrol ha sido atribuida a su capacidad de estimular los canales Ca^{2+} y K^{+}, y a la mejora de la señalización del óxido nítrico (NO) en el endotelio. Esta última actividad fue atribuida a la inhibición de la actividad NADH/NADPH oxidasa, permitiendo una reducción de la producción basal de superóxido, y consecuentemente un descenso en la inactivación del óxido nítrico (Orallo y col., 2002; Li y col., 2000). *In vivo*, el resveratrol aumenta la expresión de óxido nítrico sintetasa, tanto la endotelial (eNOS) como la inducible (iNOS) (Das y col., 2005). En arterias de enfermos cardiovasculares, la vasodilatación a través del mecanismo del NO activado por resveratrol no se observa, aunque sí existen otros mecanismos independientes del NO, lo que sugiere que la acción vasodilatadora del resveratrol ocurre a través de distintas rutas bioquímicas (Cruz y col., 2006).

La oxidación de proteínas de baja densidad (LDL) está directamente asociada a la probabilidad de sufrir enfermedades coronarias e infarto de miocardio (Holvoet, 2004). Se ha demostrado que el resveratrol inhibe la peroxidación de las LDL más que un extracto de vino tinto administrado a voluntarios sanos (Frankel y col., 1993). Así, se ha descrito que tras la ingestión de vino tinto es posible detectar resveratrol en las LDL (Urpí-Sarda y col., 2005). Experimentos *in vivo* realizados en ratas sometidas a hipertensión, propensas a infartos de miocardio, demuestran que el resveratrol

disminuye los marcadores de estrés oxidativo como albúmina glicosilada en suero y 8-hidroxiguanosina en orina (Mizutani y col., 2001). Éstas y otras investigaciones sugieren que el resveratrol puede inhibir la oxidación de LDL entre otras macromoléculas *in vivo*; sin embargo, si el mecanismo de acción es directo o indirecto está aún por determinar.

Por otro lado, el resveratrol interfiere en el metabolismo de lípidos, inhibiendo la lipogénesis a nivel hepático (Arichi y col., 1982).

El resveratrol podría actuar contra el daño isquémico en infartos de miocardio. En ratas, la perfusión del corazón con resveratrol (10 µM) antes de un daño isquémico provoca una mejora en la recuperación de la presión y el flujo aórtico, reduciendo tanto la concentración de malonaldehído como el tamaño del infarto (Ray y col., 1999). Este efecto podría deberse, al menos en parte, a la capacidad antioxidante del resveratrol. Esta hipótesis cobra fuerza al observar que el piceatanol, estilbeno similar al resveratrol pero con un grupo hidroxilo más en posición 3', y por tanto con mayor capacidad captadora de radicales libres, mejora aún más la protección frente al daño isquémico (Hung y col., 2000). De nuevo, el mecanismo propuesto se basa en el aumento de eNOS e iNOS, y por tanto, de la concentración de NO en suero (Hung y col., 2000).

En un estudio realizado en ratas, a las que se había adicionado resveratrol en el agua de bebida durante 15 días (1 mg/kg de peso), se observó una mejor recuperación de la funcionalidad cardíaca tras la inducción experimental de un daño por isquemia-reperfusión (Bradamante y col., 2003). En otro estudio realizado en cobayas, se observó un aumento significativo de la NADPH-quinona reductasa y de catalasa, o lo que es lo mismo, de la capacidad de eliminar oxidantes del músculo cardíaco cuando se les dio para beber agua enriquecida con resveratrol (14 mg/kg de peso) durante 16 días (Floreani y col., 2003).

El resveratrol podría, por tanto, aumentar la concentración de NO mediante un aumento de expresión de la NO sintetasa y disminuir su inactivación por radicales libres. Esto sugiere que podría ser un potente protector *in vivo* contra el daño isquémico durante infartos de miocardio.

También se ha estudiado la actividad **neuroprotectora** del resveratrol, demostrando que disminuye el estrés oxidativo en células neuronales, reduciendo por tanto la toxicidad de los radicales libres (Tredici y col., 1999).

Además, varios estudios en modelos animales sugieren que el resveratrol puede reducir la incidencia de enfermedades neurodegenerativas como la enfermedad de Alzheimer y la enfermedad de Parkinson (Parker y col., 2005). Igualmente se ha observado *in vitro* que el resveratrol puede activar algunas de las enzimas neuronales (AMP-kinasa) que se estimulan bajo la restricción calórica, proporcionando un efecto neuroprotector (Rasouri y col., 2007; Dasgupta y Milbrandt, 2007).

El resveratrol también tiene efecto **antiinflamatorio**, ya que inhibe enzimas que son claves en las rutas biosintéticas de prostaglandinas y eicosanoides, compuestos responsables de la respuesta inflamatoria (Jang y col., 1997; Martínez y Moreno, 2000).

También se ha descrito en la literatura que el resveratrol presenta actividad **antibacteriana y antivírica**. Inhibe el crecimiento de bacterias de forma selectiva no siendo efectivo frente a *Escherichia coli, Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa, Candida albicansy, Helicobacter pylori* (responsable de numerosos trastornos gástricos), *Neisseria gonorrhoeae y Neisseria meningitidis* (causantes de la gonorrea y de la meningitis, respectivamente) (Mahady y Pendland, 2000; Docherty y col., 2001). En cuanto a su actividad antivírica, inhibe de forma reversible y dosis-dependiente la replicación de los virus herpes simple (HSV-1 y HSV-2) (Docherty y col., 1999) y potencia la acción de algunos fármacos usados contra el virus del SIDA (VIH-1) sin mostrar efectos tóxicos para las células (Heredia y col., 2000).

2.5.2. Actividad biológica de otros estilbenos

El **piceido** (5,4´-dihidroxiestilbeno-3-O-*β*-glucósido) es el glucósido del resveratrol, forma menos susceptible a la oxidación. Es el estilbeno mayoritario del zumo de uva. La forma *cis*-piceido, como *cis*-resveratrol, se encuentran normalmente a menores concentraciones y son menos activas biológicamente que sus formas *trans* (Orallo y col., 2006).

trans y *cis*-Piceido son moléculas activas frente la oxidación de LDL *in vitro* (Merillon y col., 1996). En 1982, el piceido se describió como inhibidor de la lipogénesis a nivel hepático (Arichi y col., 1982) y un poco más tarde se demostró que también actúa como inhibidor de la síntesis de eicosanoides (Kimura y col., 1985) y como inhibidor de la agregación plaquetaria (Chung y col., 1992; Orsini y col., 1997; Shan y col., 1990). Otra de las propiedades bioactivas más destacable de este estilbeno, es que al igual que el resveratrol, el piceido inhibe la polimerización de péptidos β-amiloides de forma dosis dependiente, por lo que podría tener valor terapéutico en la enfermedad de Alzheimer (Riviere y col., 2007).

El **piceatanol** (3,3´,4,5´-tetrahidroxiestilbeno) está presente tanto en uva como en vino. Es el resultado en humanos de la hidroxilación del *trans*-resveratrol en el hígado. Igualmente, puede ser producido por la hidrólisis intestinal de su forma glucosilada, la *trans*-astringina (Vitrac y col., 2003). Los análogos hidroxilados del resveratrol, especialmente aquellos con el grupo *O*-hidroxilado, exhiben una alta capacidad antioxidante (Murias y col., 2005; Cai y col., 2004), lo que puede explicar la alta bioactividad del piceatanol (Larrosa y col., 2004; Wieder y col., 2001),

Además, se ha demostrado que el piceatanol posee propiedades anticancerígenas y antileucémicas, contribuyendo a la inducción de apoptosis en varias líneas celulares y en animales e inhibiendo la tirosina quinasa implicada en la proliferación celular (Larrosa y col., 2002; Ovesna y col., 2006). Un estudio ha demostrado en parte que las propiedades anticancerígenas del resveratrol están relacionadas con su transformación en piceatanol por la enzima CYP1B1, que está presente en una amplia variedad de tumores humanos (Potter y col., 2002).

La **astringina** (5,4´,3´-trihidroxiestilbeno-3-*O*-β-glucósido) ha sido descrita tanto en uva como en vino en su forma *trans* y *cis* (Buiarelli y col., 2007). La astringina es un gran captador de radicales libres, presentando carácter protector frente a lesiones isquémicas (Hung y col., 2001). Está considerado como un compuesto potencialmente quimiopreventivo y activo frente a la oxidación de LDL *in vitro* (Waffo-Teguo y col., 1998). También se le atribuyen propiedades terapéuticas *in vitro* en la enfermedad de Alzheimer (Riviere y col., 2007).

Químicamente el **pterostilbeno (**3,5-dimetoxi-4'-hidroxiestilbeno) es muy similar al resveratrol. Fue aislado por primera vez de la madera del sándalo y posteriormente se encontró en frutas, como las uvas y los arándanos, conocidas por sus efectos beneficiosos sobre la cognición y la función neuronal durante el envejecimiento (Casadesus y col., 2004).

El pterostilbeno es un potente antioxidante y agente anti-inflamatorio que ha demostrado tener efectos beneficiosos en el envejecimiento del cerebro (Joseph y col., 2008; Remsberg y col., 2008; Rimando y col., 2002). Es interesante saber que, *in vitro*, tiene una mayor potencia en la inducción de la apoptosis en células cancerígenas que el resveratrol (Mikstacka y col., 2007; Tolomeo y col., 2005), y muestra poderosas propiedades agonistas (Rimando y col., 2005) sobre un complejo receptor (PPAR) que está íntimamente relacionado con el metabolismo de ácidos grasos, la inflamación y la regulación del estrés oxidativo (Pyper y col., 2010).

Las **viniferinas** son producidas por la oxidación de monómeros de resveratrol por la acción de la *4-hidroxiestilbeno peroxidasa* (Ros-Barcelo y col., 2003), particularmente la ε-viniferina. Algunos de estos derivados del resveratrol tienen una alta bioactividad. Aunque menos contrastadas que el resveratrol, la ε-viniferina (dímero de resveratrol) ha mostrado propiedades hepatoprotectoras (Oshima y col., 1995) y antioxidantes (Privat y col., 2002), así como capacidad de inducir apoptosis en células tipo B leucémicas (Billard y col., 2002), de inhibir enzimas citocrómicas P450 involucradas en la activación de numerosos procesos cancerígenos (Piver y col., 2003) y de inhibir la adsorción de noradrenalina y 5-hidroxitriptamina (serotonina), además de inhibir la

actividad enzimática de la monoamina oxidasa (relacionado con la degradacion de neutrotramisores) (Yañez y col., 2006).

Además de poseer propiedades bioactivas, los estilbenos ejercen una función protectora frente a enfermedades. La ε-viniferina poseen actividades contra los patógenos como son el mildiu (*Plasmophara viticola*), la pudrición gris (*Botrytis cinerea*), *Poma medicaginis*, *Rhizopus stolonifer*, y un amplio espectro de microorganismos presentes durante el periodo de almacenamiento de frutas y vegetales cosechados (Rayne y col., 2008).

Las publicaciones correspondientes a este Capítulo se encuentran en el Anexo 1 y 2.

CAPÍTULO II

JUSTIFICACIÓN Y OBJETIVOS

JUSTIFICACIÓN Y OBJETIVOS

Cada vez resulta más evidente la importancia de la alimentación en la calidad de vida. Esta idea ha ocasionado una serie de modificaciones en los hábitos alimentarios a favor de la llamada “dieta mediterránea”. El vino, consumido con moderación, es uno de los alimentos de esta conocida dieta que más atención ha recibido.

Al vino se le atribuyen efectos beneficiosos para la salud desde la antigüedad. Los trabajos de investigación emprendidos en los últimos años han permitido establecer bases científicas sobre la actividad de los diferentes compuestos del vino, y demostrar mediante pruebas y ensayos biológicos sus efectos beneficiosos. Actualmente, un gran número de resultados científicos avalan que los polifenoles del vino ejercen propiedades biológicas múltiples tales como antioxidantes, antisépticas, vasoprotectoras, antivíricas, anti-inflamatorias y anticancerígenas, entre otras.

Las propiedades saludables del vino están fuertemente relacionada con su concentracions en compuestos fenólicos. El perfil de los compuestos fenólicos de las uvas, y por tanto de sus vinos, es muy variable. No sólo varía en función de la variedad de uva y su estado de madurez, sino también por las condiciones de cultivo, el tipo de suelo, el clima, presión fúngica, etc. Asimismo, las condiciones de vinificación influyen notablemente en el contenido polifenólico del vino.

Esta Tesis Doctoral es el resultado de la realización del Proyecto INIA, titulado “El reto de la calidad en vinos enriquecidos en estilbenos bioactivos: tratamientos pre y postcosecha para la obtención de uva con alta concentración en estilbenos” (RTA2008-00014).

Este trabajo de investigación tiene como **principal objetivo** potenciar las propiedades saludables de los vinos. Para ello nos hemos centrado en conseguir un aumento de la concentración de una familia de compuestos polifenólicos: los estilbenos. Se persigue así obtener un vino de calidad enriquecido en estilbenos bioactivos, y por tanto con valor añadido.

Para la consecución de dicho objetivo principal se plantean los siguientes objetivos específicos:

1) Determinar la contribución de los factores variedad y zona de cultivo andaluza sobre la concentración de estilbenos, así como sobre su capacidad de inducción por tratamiento postcosecha UVC, para establecer la combinación variedad/zona óptima para obtener uva enriquecida en estilbenos dentro del territorio Andaluz.
2) Determinar el efecto de distintos tratamientos precosecha (benzotiadiazol, quitosano, botrydial, metil jasmonato) sobre la inducción de estilbenos en uva de vinificación.
3) Combinar los anteriores tratamientos precosecha con el tratamiento postcosecha con UVC, buscando sinergias sobre la capacidad de inducción de estilbenos, para obtener una uva de calidad enriquecida en estilbenos.
4) Seleccionar todas las condiciones optimizadas en los objetivos anteriores para que combinando éstas (zona/variedad óptima, tratamiento precosecha solo ó combinado con UVC) se obtenga una uva de calidad enriquecida en estilbenos, y a partir de esta obtener un vino tinto de calidad con alta concentración de estilbenos bioactivos.

CAPÍTULO III

CONTENIDO BASAL E INDUCCIÓN DE ESTILBENOS POR TRATAMIENTO POSTCOSECHA UVC EN VARIEDADES DE UVA CULTIVADAS EN DISTINTAS ZONAS GEOGRÁFICAS ANDALUZAS

1. INTRODUCCIÓN

Los compuestos fenólicos de la uva juegan un papel fundamental en enología debido a su influencia sobre la calidad de los vinos. En los últimos años, estos compuestos han sido objeto de un creciente interés debido a sus propiedades antioxidantes y sus potenciales efectos beneficiosos sobre la salud.

Sin embargo, el contenido de estos compuestos fenólicos en la uva depende de varios factores como la variedad de la vid (Katalinić y col., 2010; Dimitrovska y col., 2011; Navarro y col., 2008; Yang y col., 2009; Guerrero y col., 2009b), las condiciones edafoclimáticas (Gambelli y Santaroni, 2004; Goldberg y col., 1998; González-San y col., 1990; Mc-Donald y col., 1998; Rastija y col., 2009) y las técnicas culturales aplicadas al viñedo. Las condiciones climáticas y la composición del suelo tienen una gran importancia sobre la concentración de estos compuestos en la uva y por tanto en el vino (Downey y col., 2006). Este es el concepto de terruño (*Terroir*) que condiciona, que en determinados lugares una misma variedad de uva dé lugar a vinos con distintas propiedades organolépticas.

Ahora bien, centrándonos en los estilbenos, en especial en el *trans*-resveratrol podemos decir que su contenido en la uva depende en gran medida de los factores comentados con anterioridad, como la variedad (Katalinić y col., 2010; Gatto y col., 2008; Guerrero y col., 2010a; Lee y Rennaker, 2007), el clima, la zona geográfica y las condiciones de cultivo (Bavaresco, 2003; Bavaresco y col., 2008). Por un lado, Goldberg y col. (1995) y Abril y col. (2005) determinaron que los climas fríos parecían estimular la biosíntesis de estilbenos, mientras que otros autores como Li y col. (2011) describen lo contrario. Respecto como afecta el suelo a la concentración de estilbenos existen pocos estudios. Por último, todas las prácticas culturales, que de alguna manera hacen que la viña se estrese, parecen aumentar el contenido de resveratrol (Bavaresco, 2003; Bavaresco y col., 2001; Fregoni y col., 2001; Dani y col., 2007; Deluc y col., 2011).

Además, la síntesis de estilbenos puede ser inducida por elicitores bióticos y abióticos. Entre todos los elicitores abióticos descritos, el tratamiento postcosecha con luz UVC ha

sido considerado uno de los estreses más eficaces para aumentar el contenido de estilbenos en la uva (Guerrero y col., 2010a).

Respecto al terruño, Andalucía posee una tradición milenaria en el cultivo de la vid y en la elaboración de vinos de gran prestigio y fama en todo el mundo. La topografía, geología y clima de los suelos andaluces resultan excelentes para el cultivo de la vid. El clima Mediterráneo y los diferentes microclimas, las suaves temperaturas medias (16ºC), las numerosas horas de sol al año junto con un fuerte contraste altitudinal, crean vinos de gran calidad, a la par que muy variados y de gran tipicidad.

Para este trabajo se eligieron cuatro zonas vitivinícolas importantes andaluzas: Jerez de la Frontera, Cabra, Ronda y Cádiar. Jerez de la Frontera está situada en la provincia de Cádiz y se encuentra a diez kilómetros de la costa atlántica; Cabra está situada al sur de la provincia de Córdoba, en las estribaciones de la Sierra Subbética; Ronda se sitúa en la zona más noroccidental de la provincia de Málaga, en una cuenca rodeada de montañas de gran continuidad y mediana altitud. Por último, Cádiar situada en la Sierra de la Contraviesa, localizada en La Alpujarra (Granada) entre el Mar Mediterráneo y Sierra Nevada a unos 1368 metros sobre el nivel del mar.

El objetivo de este estudio fue determinar la contribución de la variedad y el terruño en la concentración basal de estilbenos, así como en su capacidad de inducción después del tratamiento postcosecha con luz UVC con el fin de encontrar la combinación terruño/ variedad más adecuada para obtener un vino enriquecido en estilbenos.

2. MATERIALES Y MÉTODOS

2.1. Material vegetal.

Para este estudio se seleccionaron cuatro variedades tintas internacionales: Syrah, Merlot, Cabernet Sauvignon y Pinot noir.

Las cuatro zonas andaluzas elegidas presentan condiciones edafoclimáticas claramente diferenciadas. Participaron dos centros IFAPA, uno situado en Jerez de la Frontera (Cádiz) y otro en Cabra (Córdoba), ambos con viñedos experimentales y dos fincas privadas, Cortijo Barranco Oscuro situado en Cádiar (Alpujarra Granada) y Cortijo Los Aguilares situado en Ronda (Málaga).

Todos los viñedos se plantaron entre los años 1995 y 2000. La densidad de plantación varió entre 3000 y 4000 cepas/ha. El sistema de conducción fue en espaldera de 2-3 alambres y el sistema de poda fue en cordón doble.

El ensayo se llevó a cabo durante la campaña 2009. La maduración de la uva fue monitorizada semanalmente desde el envero hasta la cosecha en cada terruño para determinar la fecha óptima de la vendimia.

2.2. Parámetros edafoclimáticos

Los datos meteorológicos (temperatura, humedad relativa, radiación solar y precipitación) del período de la cosecha fueron proporcionados por el Servicio de información agroclimática de Andalucía.

(http://www.juntadeandalucia.es/agriculturaypesca/ifapa/ria/servlet/FrontController).

Los datos edafológicos fueron proporcionados directamente por el IFAPA o las empresas colaboradoras en su caso. A continuación se muestra una tabla resumen donde se muestra tanto los datos edafológicos como los meteorológicos de Junio a Septiembre (Tabla III.1).

2.3. Parámetros enológicos de la uva y del mosto

En el día de la vendimia, siguiendo los métodos oficiales de la OIV (1990) se determinaron los siguientes parámetros enológicos: residuo seco, peso medio de la baya, grado Brix, acidez total, pH, ácido tartárico, ácido málico, potasio, índice de Folin-Ciocalteau (IFC), índice de maduración; antocianos totales, antocianos extraíbles, extractabilidad, índice de polifenoles totales (IPT), taninos, taninos de hollejos, taninos de semilla y madurez de la semilla.

Tabla III.1. Tipo de suelo, coordenadas geográficas y parámetros climáticos de los cuatros terruños estudiados (Periodo Junio-Septiembre).

	JEREZ	CABRA	RONDA	CADIAR
Tipo de suelo	Caliza	Caliza	Caliza	Pizarra
Textura suelo	Arena (19%) Arcilla (38.5%) Limo (42.5%) Franco limo arcilloso	Arena (45.5%) Arcilla (27.8%) Limo (26.8%) Franco limo arcilloso	Arena (63%) Arcilla (23%) Limo (14%) Franco limo arcilloso	Arena (67%) Arcilla (11%) Limo (22%) Franco arenoso
Caliza activa (%)	20.5	11.7	1.4	0
Capacidad retención agua (mm)	131.6	107.0	90.5	77.4
Latitud (N)	36:45:29	37:29:58	36:46:47	36:55:27
Longitud (W)	06:00:58	04:25:46	04:53:24	03:10:57
Altitud a.s.l. (m)	35	560	540	1300
Tª máxima (ºC)	33.12	38.77	29.94	35.63
Tª mínima (ºC)	16.55	13.02	15.41	13.25
Tª media (ºC)	24.96	25.96	22.57	23.14
h.r. máxima (%)	80.79	86.47	60.94	90.90
h.r. mínima (%)	32.08	10.39	24.36	9.27
h.r. media (%)	56.72	38.61	40.80	45.20
Radiación Solar (MJ/m^2)	28.98	27.41	23.58	22.43
Pluviometría Jul-Sept (mm)	0.02	23.8*	0.62	13.40

*Sólo una tormenta en Agosto; Tª, temperatura; h.r., humedad relativa

2.4. Tratamiento con luz UVC y periodo de almacenamiento

Una vez determinada la fecha óptima de vendimia, las uvas vendimiadas se dividieron en dos grupos, el primer grupo (CT) no fueron tratadas y el segundo grupo (UVC) fueron irradiadas con luz UVC.

El sistema de luz UVC utilizado se basó en el dispositivo descrito por Cantos y col. en 2001 (patente WO/2002/085137; ES 2177465) con algunas modificaciones. El sistema comprende una bandeja, donde se depositaron los racimos, iluminada con 34 lámparas (Potencia teórica = 1020 W) colocadas en dos paneles de 17 lámparas (uno encima y otro debajo de la bandeja, situándose ambas a 42 cm de ésta) recubiertas con láminas protectoras reflectantes. Los racimos se iluminaron con luz UVC de λ = 254 nm durante 60 s, dando lugar a una velocidad de flujo de radiación promedio de 15.41 mW/cm^2.

Tanto las muestras CT como las UVC se almacenaron en tanques de acero inoxidable manteniéndose a 20ºC de temperatura y 80% de humedad relativa, durante 7 días (periodo postratamiento). Durante este periodo, se realizó una toma de muestra diaria al azar de unos 100 g de uvas. Las uvas fueron peladas y la piel se congeló a -80ºC para su posterior análisis.

El parámetro "día máximo" (Dm) se definió como el número de días transcurridos después del tratamiento con luz UVC para alcanzar la máxima concentración de *trans*-resveratrol en la uva. La velocidad de inducción del *trans*-resveratrol (IV_{RESV}) se definió como la diferencia entre las concentraciones de resveratrol en el Dm ($C_{Dm\ RESV}$) y el día de la cosecha ($C_{0\ RESV}$), dividido por el número de días necesarios para alcanzar el contenido máximo de resveratrol (ver detalles en Guerrero y col., 2010a).

2.5. Método de extracción de estilbenos

El método de extracción empleado fue el descrito por Bavaresco y col. (2001) con algunas modificaciones.

La piel previamente congelada a -80ºC se liofilizó con ayuda de un liofilizador (Cryodos -80ºC, Telstar). 0.25 g de piel liofilizada se extrajeron dos veces con 5 ml de hidrogeno carbonato de sodio (5%) y 5 ml de acetato de etilo. Se homogenizó la mezcla

utilizando para ello un equipo Ultraturrax T-25 (Jankel and Kunkel, Ika-Labortechnick, Alemania). Posteriormente, la muestra se mantuvo en agitación magnética durante 20 min. Una vez pasados los 20 min, la muestra se centrifugó a 5000 rpm durante 5 min en una centrífuga Digicen 20-R (Orto Alresa, España).

La fase orgánica se llevó a sequedad mediante una concentrador centrífugo a vacío, se redisolvió en 2 ml de metanol y finalmente se filtró por 0.22 µm. Todas las extracciones se realizaron por triplicado y se conservaron a oscuridad y a baja temperatura para evitar posibles oxidaciones e isomerizaciones. Los datos se expresan en mg/Kg de peso fresco de bayas.

2.6. Identificación y cuantificación de estilbenos

Los estilbenos se identificaron y cuantificaron siguiendo el protocolo descrito por (Guerrero y col., 2010a).

Para la cuantificación los extractos (20 µl) fueron analizados mediante un equipo Waters HPLC compuesto por bomba binaria modelo 1525, controlador de temperatura TCM y un detector de fotodiodo Waters 996. Para la separación realizada a 30ºC se utilizó como fase estacionaria una columna Mediterránea Sea_{18} (Teknokroma, España) (RP-18, 25x0.46 cm; 5 µm de tamaño de partícula) con precolumna del mismo material.

Las fases móviles consistieron en una mezcla agua:metanol:ácido acético de 88:10:2 para la fase A y 8:90:2 para la fase B. El programa de elución comenzó con un flujo de 1ml/min, con 35% de B durante 3 min para llegar al 50% B a 10 min, al 70% B a 20 min y al 100% de B a 23 min para posteriormente mantenerse constante hasta 28 min (Jiménez y col., 2005). El software usado fue Empower suministrado por Waters. Los estilbenos fueron cuantificados como *trans*-resveratrol a 306 nm.

2.7. Análisis estadístico

Todas las muestras se analizaron por triplicado. El test Z-Score se empleó para descartar aquellos triplicados de muestra cuyas desviaciones estándar fueran anómalas en el conjunto de los datos, eliminándose aquellos datos con $Z \geq |2|$ para una misma

muestra. Se utilizó el software Statistica 6.0 para el análisis de los datos resultantes. Por medio del Análisis de Componentes Principales (PCA), se excluyeron las variables redundantes que ofrecen una información similar. Para el análisis estadístico posterior se seleccionaron sólo aquellas variables que contribuyen a la variación de la matriz de los datos. El método Ward empleado en el análisis de cluster nos permitió explorar la tendencia de los datos. Finalmente, el análisis discriminante se llevó a cabo para crear las funciones de clasificación para discriminar muestras de acuerdo con los terruños.

3. Resultados y Discusión

3.1. Vendimia

Para determinar la fecha de vendimia se controlaron semanalmente los parámetros enológicos (datos no mostrados). Los parámetros enológicos en el día de la vendimia se muestran en la Tabla III.2. Como era de esperar, la fecha de la vendimia para una misma variedad varió en función de la zona. La zona donde se realizó la primera vendimia fue Cabra seguida de Jerez, Ronda y Cádiar.

El residuo seco varió entre 18.30 y 24.55, tanto la variedad como el terruño parecen no tener efecto alguno sobre este parámetro. El peso de la uva fue similar, excepto para la zona de Cádiar donde el peso de las uvas fue ligeramente superior.

La fecha de vendimia se determinó en función del índice de maduración (IM) (azúcar/acidez), siendo muy similar en todos los casos. La Pinot noir de Jerez se vendimió antes de tener el contenido óptimo en azúcares debido al proceso de deshidratación que se observó. Esta variedad es muy sensible al clima cálido por lo que en lugar de seguir una maduración normal, las uvas comenzaron a deshidratarse.

Tabla III.2. Parámetros enológicos de las cuatro variedades de uva en los cuatro terruños estudiados en el día de la vendimia.

	SYRAH				MERLOT				CABERNET SAUVIGNON				PINOT NOIR			
	Jerez	Cabra	Ronda	Cádiar	Jerez	Cabra	Ronda	Cádiar	Jerez	Cabra	Ronda	Cádiar	Jerez	Cabra	Ronda	Cádiar
Fecha vendimia	20-ago	13-ago	10-sep	17-sep	20-ago	13-ago	31-ago	17-sep	27-ago	27-ago	31-ago	25-sep	04-ago	27-jul	21-ago	17-sep
Residuo seco (%)	21.42	19.15	21.23	20.84	21.61	20.87	24.55	22.29	22.00	23.80	22.27	22.47	18.30	22.12	22.40	21.73
Peso uva (g)	1.65	1.64	1.44	1.91	1.39	1.21	1.07	1.64	1.04	1.03	1.08	1.30	1.10	1.03	0.98	1.58
Grado Brix	23.2	21.5	23.0	22.3	22.7	22.5	26.0	24.8	22.8	24.2	24.0	24.7	18.5	23.4	21.8	22.2
AT (g/L TH_2)	5.01	8.49	8.05	9.00	4.98	7.71	9.60	9.56	7.56	8.17	9.37	9.57	8.78	11.44	9.85	9.39
pH	3.64	3.26	3.24	3.11	3.55	3.25	3.04	3.02	3.35	3.23	3.11	2.94	3.22	3.09	3.14	3.03
Ác. Tartárico (g/L)	5.44	7.81	8.57	7.53	6.31	9.67	9.19	8.98	7.37	6.47	9.57	9.04	8.82	9.83	10.40	8.11
Ác. Málico (g/L)	2.60	3.33	1.65	2.37	1.48	1.70	1.77	1.24	2.17	2.05	2.19	2.82	3.27	4.49	3.05	2.06
Potasio (mg/L)	2151	2502	1841	1606	2122	2536	1517	1489	2060	2096	1562	1649	2073	2056	1964	1427
IFC	20.06	17.64	19.07	19.00	10.96	11.42	10.60	10.23	15.02	16.58	12.76	17.90	9.63	12.20	13.53	11.73
IPT	21.75	18.73	19.11	16.81	19.64	11.51	21.19	18.67	15.36	15.93	13.65	15.28	12.46	21.99	15.65	12.36
IM	47.15	25.34	29.07	25.13	45.83	29.46	28.23	26.99	30.56	30.23	26.03	26.63	20.28	20.90	22.14	23.75
Antoc.Tot. (mg/L)	767	684	1052	1556	742	630	978	1419	619	500	841	1078	295	1004	645	881
Antoc.ext. (mg/L)	417	274	483	532	416	251	474	468	315	284	413	344	124	460	315	248
Extractabilidad	45.4	61.9	54.1	65.7	43.8	60.4	51.5	67.0	49.1	43.2	50.8	68.1	58.6	54.1	51.1	72.1
Antoc.ext. (mg/L)	417	274	483	532	416	251	474	468	315	284	413	344	124	460	315	248
Taninos (g/L)	3.04	2.62	2.68	2.35	2.75	1.61	2.97	2.61	2.15	2.23	1.91	2.14	1.74	3.08	2.19	1.87
Tan. hollejos (g/L)	1.17	0.34	1.35	1.49	1.16	0.7	1.33	1.31	0.88	0.79	1.16	0.96	0.35	1.29	0.88	0.69
Tan. semillas (g/L)	1.88	1.06	1.32	0.86	1.59	0.91	1.64	1.30	1.27	1.44	0.75	1.18	1.40	1.79	1.31	1.18
Madur.semillas	61.59	75.39	49.48	36.6	57.66	56.71	55.27	49.9	59.03	64.29	39.44	55.07	80.44	57.94	59.71	62.89

AT, acidez total; IFC, índice de Folin-Ciocalteu; IPT, índice de polifenoles totales; IM, índice de maduración; Antoc.Tot., antocianos totales; Antoc.ext., antocianos extraíbles; Tan. Hollejos, taninos hollejos; Tan. Semillas, taninos semillas; Madur.semillas, madurez de semillas.

La variedad Merlot alcanzó un mayor contenido en azúcares en Ronda y Cádiar. En estos casos la vendimia se retrasó para poder disminuir los valores tan altos de acidez total.

Algunos parámetros parecían estar afectados tanto por la variedad como por el terruño. La acidez total varía ampliamente entre los terruños y las variedades. La variedad Pinot noir alcanzó los valores más altos en los 4 terruños estudiados, que podría deberse a su alto contenido tanto en ácido tartárico como málico, siendo el contenido en ácido málico especialmente alto. Por el contrario, Jerez fue la zona donde las variedades tuvieron valores más bajos de acidez total. Esto podría explicarse debido al alto nivel de radiación solar sufrido en esta zona.

La concentración de potasio en la uva también estuvo influenciada por la zona debido a las diferencias que existen en la composición del suelo (Tabla III.1), como han descrito otros autores (Kodur, 2011; Gómez-Míguez y col., 2007). Los valores de potasio en Cabra y Jerez fueron mayores que los obtenidos en Ronda y Cádiar. Los datos de pH estuvieron en concordancia con los datos de la acidez y del potasio.

Los valores de IFC fueron bastantes similares en los 4 terruños independientemente de la variedad.

En cuanto al contenido de antocianos totales, se alcanzaron valores más altos en Cádiar excepto para la variedad Pinot noir, donde su concentración máxima se obtuvo en Cabra. La razón puede estar en que Cádiar al ser la zona con mayor altura (Tabla III.1), la uva sufre durante el periodo de maduración grandes diferencias de temperatura entre el día y la noche, lo que induce la síntesis de antocianos (Mateus y col., 2001; Yamane y col., 2006). Sin embargo, esta zona muestra el menor porcentaje de antocianos extraíbles, y por tanto una extractabilidad más alta, si lo comparamos con el resto de zonas.

Para el caso de los taninos y el IPT, no se encontraron diferencias significativas para ninguna de las variedades y terruños. De hecho, los taninos se ven más afectados por el clima en la composición que en su propia concentración (Mateus y col., 2001).

3.2. Contenido basal de estilbenos en uvas: variedad frente a terruño.

En el día de vendimia, previo al tratamiento postcosecha con luz UVC, se determinó la concentración de estilbenos en las 4 variedades cultivadas en las cuatro zonas nombradas con anterioridad (Concentración basal, C_0; Tabla III.3).

En las muestras analizadas se encontraron los siguientes estilbenos: piceatanol, *trans*-resveratrol, ε-viniferina y δ-viniferina (Tabla III.3). Estos resultados coinciden con estudios realizados previamente donde los estilbenos se identificaron por UPLC-DAD-TQD (Guerrero y col., 2010a).

Tabla III.3. Concentraciones de estilbenos en el día de la vendimia y el día de máxima inducción.

		C_0 resv*	C_0 pictnol*	C_0 vinif*	C_0 estilb*	C_{Dm} resv*	C_{Dm} pictnol*	C_{Dm} vinif*	C_{Dm} estilb*	CIresv (C_{Dm}/C_0)	ΔCresv ($C_{Dm}-C_0$)*	VI resv ($C_{Dm}-C_0$)/Dm	Dm (día)
SYRAH	**Jerez**	1.88	0.32	0.23	2.43	7.04	2.27	0.61	9.92	3.75	5.16	1.03	5
	Cabra	11.45	1.35	0.27	13.07	23.55	6.13	3.46	33.14	2.06	12.10	2.02	6
	Ronda	0.15	n.d.	n.d.	0.15	4.67	1.25	0.80	6.72	31.12	4.52	0.65	7
	Cádiar	1.61	0.41	0.34	2.36	7.80	2.61	1.90	12.31	4.85	6.19	0.88	7
MERLOT	**Jerez**	0.43	0.12	n.d.	0.55	2.30	1.17	0.26	3.73	5.32	1.87	0.37	5
	Cabra	0.64	0.10	n.d.	0.74	3.11	0.91	0.61	4.63	4.87	2.47	0.62	4
	Ronda	0.16	n.d.	n.d.	0.16	5.89	1.94	0.65	8.48	36.33	5.72	0.82	7
	Cádiar	0.28	n.d.	n.d.	0.28	2.01	0.76	0.27	3.04	7.20	1.73	0.35	5
CABERNET SAUVIGNON	**Jerez**	0.64	0.11	n.d.	0.75	6.15	2.24	1.63	10.02	7.56	4.20	0.60	7
	Cabra	2.17	0.30	0.20	2.67	9.34	2.86	1.65	13.85	4.30	7.16	1.02	7
	Ronda	0.04	n.d.	n.d.	0.04	1.62	0.60	0.95	3.17	37.77	1.58	0.23	7
	Cádiar	0.37	0.11	n.d.	0.48	2.36	0.95	1.19	4.50	6.47	1.99	0.29	7
PINOT NOIR	**Jerez**	1.35	0.34	0.18	1.87	7.25	1.73	1.02	10.00	5.38	5.90	0.98	6
	Cabra	1.95	0.38	0.14	2.47	6.37	1.67	0.63	8.67	3.27	4.42	0.74	6
	Ronda	0.14	n.d.	n.d.	0.14	0.89	0.22	0.35	1.46	6.32	0.75	0.12	6
	Cádiar	1.08	0.27	0.32	1.67	2.05	0.58	0.83	3.46	1.90	0.97	0.14	7

* Datos expresados en mg/Kg bayas; C_0resv, concentración basal resveratrol; C_0pictnol, concentración basal piceatanol; C_0vinif, concentración basal viniferinas; C_0estilb, concentración basal estilbenos; C_{Dm}resv, concentración resveratrol a Dm; C_{Dm}pictnol, concentración piceatanol a Dm; C_{Dm}vinif, concentración viniferinas a Dm; C_{Dm}estilb, concentración estilbenos a Dm; CIresv(C_{Dm}/C_0), capacidad de inducción resveratrol; ΔCresv($C_{Dm}-C_0$), incremento concentración resveratrol; VIresv, velocidad inducción resveratrol; Dm, día de máxima inducción de resveratrol; n.d., no detectado.

El contenido basal de estilbenos de mayor a menor en cada variedad fue el siguiente: Cabra>Jerez>Cádiar>Ronda en todas las variedades estudiadas (C_0 resv, Tabla III.3).

En el día de la vendimia, se detectó piceatanol en todas las variedades de Cabra y de Jerez. Sin embargo, en Cádiar sólo tres (Syrah, Cabernet Sauvignon y Pinor noir) de las cuatro variedades presentaban piceatanol, y en Ronda no se observó en ninguna de las variedades. Las viniferinas sólo se encontraron en algunas de las muestras: Syrah y Pinot noir de Jerez, Cabra y Cádiar y Cabernet Sauvignon de Cabra. La variedad Merlot no presentó ningún contenido basal de viniferinas en ninguna de las zonas estudiadas. Esto coincide con estudios previos hechos en nuestro laboratorio donde la variedad Merlot ha sido descrita como una variedad con un bajo contenido en estilbenos (Guerrero y col., 2010a). Por otro lado, el *trans*-resveratrol fue el único compuesto presente en todas las muestras de vendimia (aunque se repita).

Como comentamos anteriormente Cabra fue la zona donde todas las variedades mostraron un mayor contenido basal de estilbenos. Estos resultados podrían deberse a que Cabra fue la zona que mostró la temperatura media más alta, una mayor diferencia de temperatura entre el día y la noche y una baja humedad relativa durante el periodo Junio-Septiembre (Tabla III.1). Por otra parte, otro factor que pudo ocasionar estrés a la planta e inducir la síntesis de estilbenos durante el desarrollo de las bayas fueron las precipitaciones (Andrés de Prado y col., 2007; Li y col., 2006). También, es importante decir que no se observó ningún síntoma de enfermedad en el viñedo.

Por el contrario, Ronda fue la zona que mostró mayor escasez de precipitaciones, las diferencias de temperaturas entre el día y la noche fueron menores y la humedad relativa media fue baja (Tabla III.1), presentado así un bajo contenido basal de estilbenos. Además, el único estilbeno que se encuentra de forma basal en este terruño en bajas concentraciones es el *trans*-resveratrol.

En lo que respecta a la radiación solar y a los valores medios de temperatura, Cabra y Jerez mostraron los valores más altos en comparación con Ronda y Cádiar, factores que también pueden inducir la síntesis de estilbenos al proporcionar estrés al viñedo (Tabla III.1).

Teniendo en cuenta todas las afirmaciones anteriores, se pudo establecer que una mayor variación en las condiciones climáticas podría estimular la biosíntesis natural de estilbenos. Sin embargo, el efecto del clima no puede ser aislado del resto de factores. De hecho, el efecto del suelo en la cantidad estilbenos ha resultado ser tan importante como el efecto climático (Andrés de Prado y col., 2007). El terruño de Cabra presenta un suelo pobre en nutrientes. El suelo que presenta Jerez (llamado albariza) es similar al suelo de Cabra pero con diferencias en su textura (Tabla III.1). Los terruños de Cabra y Jerez mostraron una alta capacidad de retención de agua (Tabla III.1). Se ha descrito que los suelos con esta capacidad pueden estimular la biosíntesis de estilbenos en la uva (Andrés de Prado y col., 2007; Koundouras y col., 2006; Bavaresco y col., 2009). Ronda y Cádiar mostraron suelos con niveles muy bajos de caliza activa, debido a su textura arenosa. Sin embargo, existen pocos estudios en este sentido.

El término terruño implica tantos factores que resulta muy difícil comparar con otros estudios. Li y col. (2011) estudiaron el efecto del terruño en la concentración de estilbenos en la variedad Cabernet Sauvignon. En ese estudio se determinó que ese terruño, con suelo arenoso, baja altitud, clima semi-húmedo y ligeras diferencias de temperaturas, era idóneo para obtener altas concentraciones basales de estilbenos. Las condiciones son tan diferentes para cada terruño que es difícil hacer una comparación directa.

3.3. Capacidad de inducción de estilbenos en uvas: variedad frente a terruño.

Tras el tratamiento postcosecha con luz UVC se determinaron los estilbenos presentes en cada variedad y terruño durante los siete días siguientes al tratamiento. Todas las variedades incrementaron su contenido en *trans*-resveratrol, piceatanol y en viniferinas (Figura III.1).

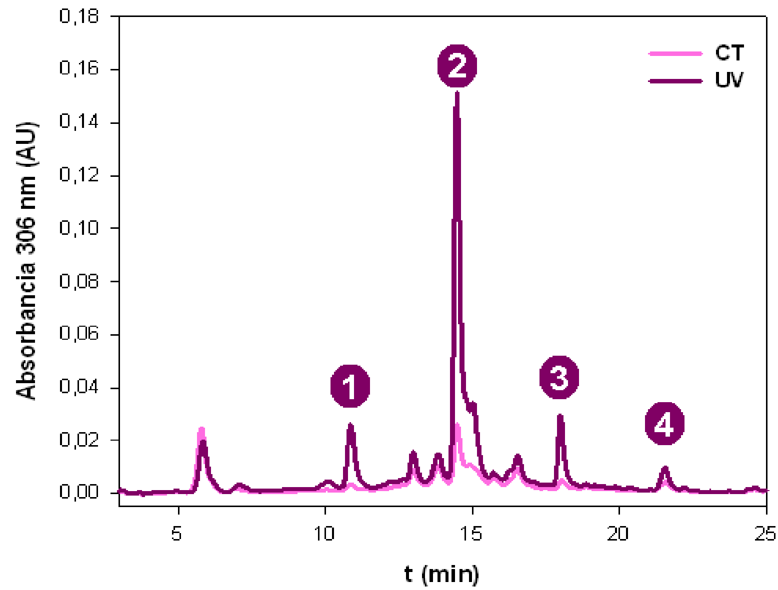


Figura III.1. Comparación cromatogramas HPLC-DAD entre el CT y el UVC a 306nm de la variedad Syrah de Cabra. (1) Piceatanol, (2) *trans*-resveratrol, (3) ε-viniferina y (4) δ-viniferina.

La concentración alcanzada por cada compuesto fue diferente dependiendo de la variedad y de la zona de cultivo (Figura III.2, Tabla III.3).

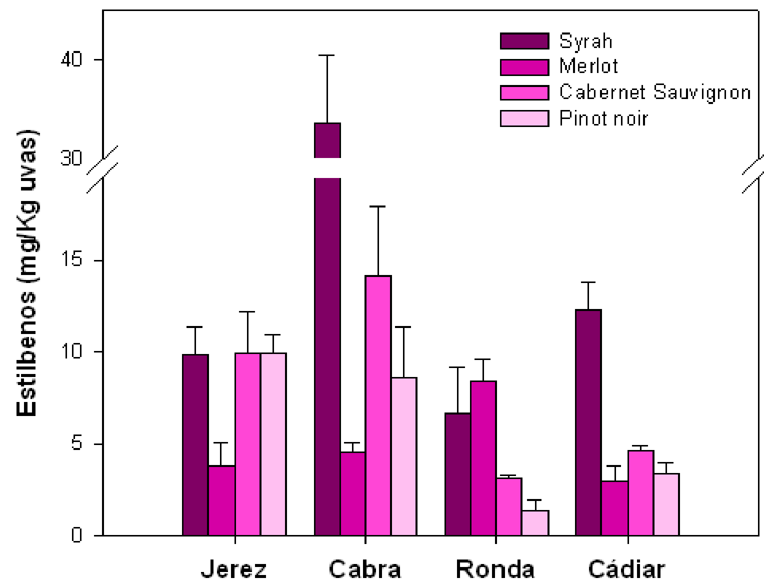


Figura III.2. Concentración de estilbenos totales (mg/Kg bayas) en día de máxima inducción.

En la Tabla III.3 se muestra la máxima concentración alcanzada (C_{Dm}, concentración en el día de máxima inducción), así como el día de máxima inducción (Dm), el incremento en la concentración (ΔC), capacidad de inducción (CI) y la velocidad de inducción (VI).

Cabra fue el terruño donde todas las variedades mostraron una mayor capacidad de inducción (Figura III.2), especialmente la Syrah que alcanzó la mayor concentración de *trans*-resveratrol (23.55 mg/kg bayas), piceatanol (6.13 mg/kg bayas) y de viniferinas (3.46 mg/kg bayas). La velocidad de inducción fue de 2.02, lo que significa que la variedad Syrah de Cabra sintetizó de media 2.02 mg/Kg por día después del tratamiento postcosecha con luz UVC.

Esto coincide con resultados obtenidos en estudios anteriores, donde se estudiaron treces variedades cultivadas en un mismo terruño (Jerez de la Frontera), resultando la variedad Syrah la más destacada por su gran aumento en las concentraciones de estos compuestos tras el tratamiento postcosecha con luz UVC (Guerrero y col., 2010a).

Tras la Syrah de Cabra, dos variedades destacaron también por su alto contenido en estilbenos totales. Una de ellas fue la Cabernet Sauvignon de Cabra que alcanzó 13.85 mg/Kg bayas, y la otra fue la Syrah de Cádiar que obtuvo una concentración ligeramente inferior (12.32 mg/Kg bayas).

Las variedades cultivadas en Jerez, excepto la Merlot, obtuvieron aproximadamente 7 mg/Kg bayas y 10 mg/Kg bayas de *trans*-resveratrol y de estilbenos totales respectivamente en el día de máxima inducción (C_{Dm}). Estas concentraciones pueden considerarse altas si se comparan con las obtenidas en las uvas no tratadas (concentraciones basales).

Al igual que ocurrió con el contenido basal de estilbenos, las variedades cultivadas en Ronda y Cádiar obtuvieron un menor incremento en la concentración de *trans*-

resveratrol (ΔCresv) tras el tratamiento postcosecha con luz UVC, excepto en el caso de la Syrah de ambas zonas y la Merlot de Ronda (Tabla III.3).

Con respecto al piceatanol y a las viniferinas, se obtuvieron mayores concentraciones en aquellas variedades que alcanzaron niveles más altos de *trans*-resveratrol, lo que es lógico ya que el resveratrol es el precursor de otros estilbenos (Coutos-Thévenot y col., 2001). Así, el resveratrol condiciona la presencia o no de otros estilbenos y así como la concentración de estilbenos totales.

De forma general podemos decir que la velocidad de inducción es mayor en todas las variedades cultivadas en Cabra y Jerez (Tabla III.3). Aunque se observaron excepciones, la Syrah de Ronda y de Cádiar con una velocidad de inducción de 0.65 mg/Kg y 0.88 mg/Kg respectivamente y la Merlot de Ronda con 0.82 mg/Kg.

En cuanto al día de máxima inducción (Dm), observamos que para las variedades Syrah y Merlot varió entre 4 y 7 días dependiendo de la zona de cultivo (Tabla III.3). Esto complica la estandarización del tratamiento postcosecha con luz UVC, ya que el día de máxima inducción debería establecerse por cada zona y variedad. Sin embargo, para las variedades Cabernet Sauvignon (Dm=7) y Pinot noir (Dm=6) se mantuvo constante en cada zona, excepto para la Pinot noir de Cádiar que se retrasó un día más.

A modo de resumen, y centrándonos solamente en las variedades, podemos decir que la variedad Syrah mostró mayor capacidad de inducción de estilbenos. La Merlot excepto en el terruño de Ronda fue la variedad que mostró una menor capacidad de inducción. Por último, la Cabernet Sauvignon y la Pinot noir de los terruños de Cabra y Jerez mostraron una mayor capacidad de inducción que las cultivadas en Ronda y Cádiar.

3.4. Análisis de componentes principales y análisis de Cluster.

La matriz de datos incluyó un conjunto de dieciséis muestras correspondientes a las cuatro variedades en las cuatro zonas geográficas distintas. Se realizaron 24 determinaciones analíticas de cada muestra por triplicado, por lo que en el análisis estadístico tenemos en cuenta 24 variables, que son: Concentración basal resveratrol (C_0resv), Concentración resveratrol en el día de máxima inducción (C_{Dm}resv), día de

máxima inducción (Dm), velocidad de inducción de resveratrol (VIresv), incremento en la concentración de resveratrol ΔCresv ($C_{Dm} - C_0$), capacidad de inducción de resveratrol CIresv(C_{Dm}/C_0), residuo seco, peso de la uva, grado Brix, acidez total, pH, ácido tartárico, ácido málico, potasio, índice de Folin-Ciocalteu (IFC), índice de polifenoles totales (IPT), índice de maduración (IM), taninos totales, antocianos totales, antocianos extraíbles, extractabilidad, taninos del hollejo, taninos de semillas y madurez de las semillas.

En primer lugar realizamos un análisis de componentes principales (PCA). Cuando las variables se sometieron a dicho análisis se redujo la matriz de datos a dos factores principales. Con estos dos factores se explicaba el 52.38% de la varianza total. El primer factor explicó el 31.75% de la varianza total, estando compuesto por los siguientes descriptores: C_0resv, C_{Dm}resv, VIresv, potasio, antocianos totales, antocianos extraíbles, taninos del hollejo y madurez de la semilla. Estas variables se relacionan principalmente con la capacidad de inducir *trans*-resveratrol y con el contenido polifenólico en la uva. El segundo factor explicó el 20.63% de la varianza total, aportaba factores relacionados principalmente con la madurez de la uva como el índice de madurez, el pH, la acidez total y el ácido tartárico.

En la Figura III.3, podemos ver la representación gráfica de los dos factores principales obtenidos del análisis de componentes principales. Dicha representación nos es de interés para visualizar la tendencia de los datos.

Se observó una separación lineal de las muestras en base a su origen geográfico. Las muestras se dividen en dos grupos, un primer grupo (izquierda de la imagen) formado por las variedades pertenecientes a Ronda y Cádiar y un segundo grupo (derecha de la imagen) formado por las variedades de Jerez y Cabra. Las muestras del segundo grupo están más dispersas en comparación con las del primero.

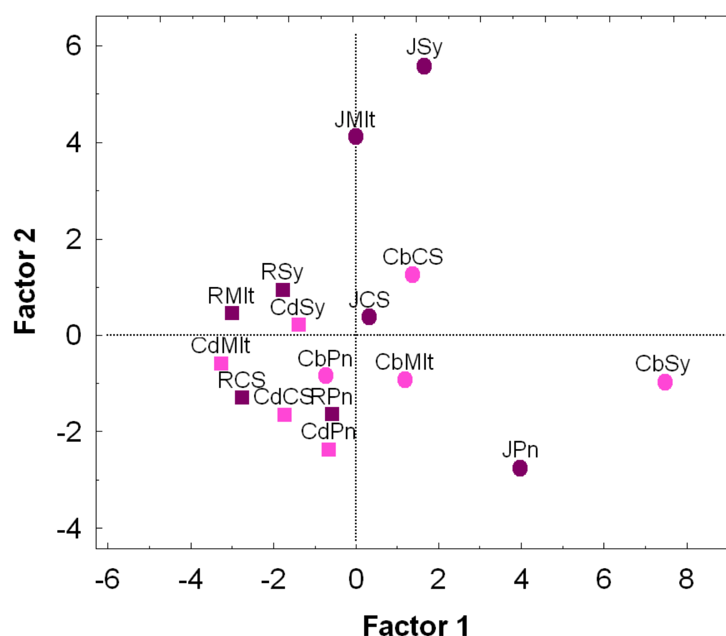


Figure III.3. Análisis de Componentes Principales (JSy, Jerez Syrah; JMlt, Jerez Merlot; JCS, Jerez Cabernet Sauvignon; JPn, Jerez Pinot noir; RSy, Ronda Syrah; RMlt, Ronda Merlot; RCS, Ronda Cabernet Sauvignon; RPn, Ronda Pinot noir; CbSy, Cabra Syrah; CbMlt, Cabra. Merlot; CbCS, Cabra Cabernet Sauvignon; CbPn, Cabra Pinot noir; CdSy, Cádiar Syrah; CdMlt, Cádiar Merlot; CdCS, Cádiar Cabernet Sauvignon; CdPn, Cádiar Pinot noir).

Estos resultados demuestran las tendencias generales que se expusieron en los apartados anteriores (Tablas III.2 y III.3), en la que ya observábamos como las variables se agrupaban en función de su zona de cultivo.

Los valores de antocianos totales y de acidez total fueron mayores para el primer grupo que para el segundo grupo. Al contrario pasó con el potasio donde sus concentraciones fueron menores. Sólo la Pinot noir de Cabra no se ubicaba donde correspondería, lo que podría deberse a su alto contenido en antocianos así como su alta acidez total que son características propias del grupo contrario.

Por otro lado, las características del terruño entre Jerez y Cabra son más similares, como ocurre entre Cádiar y Ronda (Tabla III.1). En cuanto al tipo de suelo, Jerez y Cabra tienen suelos menos arenosos, mayor proporción de caliza activa y mayor capacidad de

retención de agua en comparación con las otras zonas. Igual ocurre con las condiciones climáticas, las cuales son más similares entre Cabra y Jerez por un lado y entre Ronda y Cádiar por otro, coincidiendo con lo observado en apartados anteriores.

Además del análisis de componentes principales se realizó el análisis de cluster con la idea básica de agrupar el conjunto de muestras en un número dado de clusters o grupos. Las muestras se agruparon en dos grupos principalmente cuando se tuvieron en cuenta sólo las variables que formaron el primer factor del PCA (C_0resv, C_{Dm}resv, VIresv, potasio, antocianos totales, antocianos extraíbles, taninos del hollejo y madurez de las semillas) (Figura III.4).

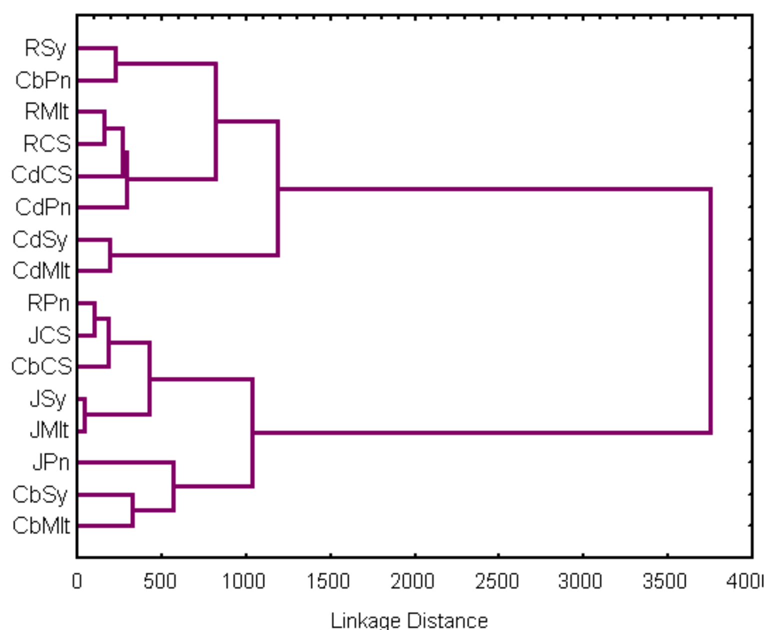


Figura III.4. Análisis de Cluster teniendo en cuenta las variables del primer factor del PCA. (JSy, Jerez Syrah; JMlt, Jerez Merlot; JCS, Jerez Cabernet Sauvignon; JPn, Jerez Pinot noir; RSy, Ronda Syrah; RMlt, Ronda Merlot; RCS, Ronda Cabernet Sauvignon; RPn, Ronda Pinot noir; CbSy, Cabra Syrah; CbMlt, Cabra. Merlot; CbCS, Cabra Cabernet Sauvignon; CbPn, Cabra Pinot noir; CdSy, Cádiar Syrah; CdMlt, Cádiar Merlot; CdCS, Cádiar Cabernet Sauvignon; CdPn, Cádiar Pinot noir).

Cada grupo originado se compuso del 50% de las muestras. Uno de ellos incluyó el 75% de las variedades de Ronda y el 100% de las variedades de Cádiar y el otro grupo estuvo formado por el 100% de las variedades de Jerez y el 75% de las de Cabra. Es evidente, igual que en el PCA, que las muestras se agruparon conforme a la zona

geográfica en lugar de por la variedad, de manera similar a lo descrito con el *terroir* de los vinos de Croacia (Rastija y col., 2009). En este análisis hubo dos excepciones o dos variedades que no se clasificaron en sus correspondientes grupos, estas fueron la Pinot noir de Ronda y la de Cabra. Sus comportamientos difieren respecto a las demás y esto pudo deberse, en comparación con las otras dos zonas, a sus altos contenidos en antocianos, los cuales son un factor con mucho peso a la hora de obtener una clasificación.

Al realizar el análisis de cluster con las variables del segundo factor, que estaban relacionadas con la madurez de la uva, no se observó ninguna clasificación en función de la zona geográfica. Esto pudo deberse al bajo peso que tienen las variables del factor 2, donde según el PCA sólo explicaba el 20.63% de la varianza.

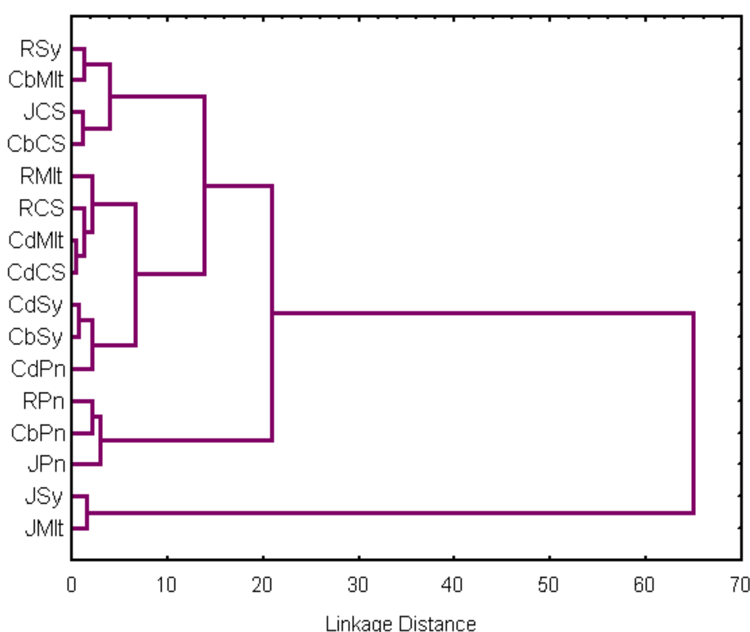


Figura III.5. Análisis de Cluster teniendo en cuenta las variables del segundo factor del PCA. (JSy, Jerez Syrah; JMlt, Jerez Merlot; JCS, Jerez Cabernet Sauvignon; JPn, Jerez Pinot noir; RSy, Ronda Syrah; RMlt, Ronda Merlot; RCS, Ronda Cabernet Sauvignon; RPn, Ronda Pinot noir; CbSy, Cabra Syrah; CbMlt, Cabra. Merlot; CbCS, Cabra Cabernet Sauvignon; CbPn, Cabra Pinot noir; CdSy, Cádiar Syrah; CdMlt, Cádiar Merlot; CdCS, Cádiar Cabernet Sauvignon; CdPn, Cádiar Pinot noir).

El análisis de la varianza (ANOVA) demostró que existían diferencias significativas ($p<0.05$) entre las zonas si considerábamos el conjunto total de variables. Por otro lado, se aplicó el análisis discriminante para clasificar las muestras según las zonas. Aplicando el criterio de selección “forward” se seleccionaron cinco variables: $C_{0\ RESV}$, $C_{Dm\ RESV}$, IV_{RESV}, potasio y antocianos totales. La matriz de clasificación mostró que el 93.7% de las variedades se clasificaban correctamente. De las 16 muestras sólo una de ellas no se clasificó correctamente. La variedad Cabernet Sauvignon de Cabra se clasificó como si perteneciese a Jerez (Tabla III.4).

Tabla III.4. Matriz clasificación de los casos tras el análisis discriminativos por zonas.

	Observación	1	2	3	4
R Sy	R	R	J	Cd	Cb
R Mlt	R	R	Cd	J	Cb
R CS	R	R	Cd	J	Cb
R Pn	R	R	J	Cb	Cb
C Sy	Cd	Cd	R	J	Cb
C Mlt	Cd	Cd	R	J	Cb
C CS	Cd	Cd	R	J	Cb
C Pn	Cd	Cd	R	J	Cb
J Sy	J	J	Cb	R	Cd
J Mlt	J	J	Cb	R	Cd
J CS	J	J	Cb	R	Cd
J Pn	J	J	Cb	R	Cd
C Sy	Cb	Cb	J	R	Cd
C Mlt	Cb	Cb	J	R	Cd
*C CS	Cb	J	Cb	R	Cd
C Pn	Cb	Cb	R	J	Cd

(J, Jerez; R, Ronda; Cb, Cabra; Cd, Cádiar; Sy, Syrah; Mlt, Merlot; CS, Cabernet Sauvignon; Pn, pinot noir)

Así, el análisis discriminante de las zonas con estas variables mostró que un 94% de las muestras estaban correctamente clasificadas (Tabla III.5). Sin embargo, cuando se aplica a las variedades, la clasificación correcta era sólo de un 50% (Tabla III.6).

Tabla III.5. Matriz clasificación tras el análisis discriminativos por zonas.

	Porcentaje	Ronda	Cádiar	Jerez	Cabra
Ronda	100	4	0	0	0
Cádiar	100	0	4	0	0
Jerez	100	0	0	4	0
Cabra	75	0	0	1	3
Total	**93.75**	4	4	5	3

Tabla III.6. Matriz clasificación tras el análisis discriminativos por variedades.

	Porcentaje	Cabernet Sauvignon	Pinot noir	Syrah	Merlot
Cabernet Sauvignon	25	1	2	0	1
Pinot noir	75	0	3	0	1
Syrah	75	0	0	3	1
Merlot	25	0	2	1	1
Total	**50**	1	7	4	4

Podemos por tanto concluir, que tanto la variedad como el terruño determinan tanto la concentración basal de estilbenos como la capacidad de inducción de estos por tratamiento UVC postcosecha en uva. Sin embargo, el terruño parece tener un mayor peso que la variedad. Los terruños de Cabra y Jerez (para dar paso al siguiente capítulo), fueron los más idóneos para cultivar uvas con alto contenido en estilbenos, especialmente la variedad Syrah.

La publicación correspondiente a este Capítulo se encuentra en el Anexo 3.

CAPÍTULO IV

ESTUDIO DE TRATAMIENTOS PRECOSECHA PARA OBTENER UVAS ENRIQUECIDAS EN ESTILBENOS. COMBINACIÓN DE ESTOS CON UVC POSTCOSECHA

1. INTRODUCCIÓN

Los estilbenos son polifenoles no flavonoides que son sintetizados por una amplia variedad de plantas (distintas especies de las familias *Pinaceae, Moraceae, Liliaceae, Myrtaceae, Fagaceae, Vitaceae, Gnetaceae, Cyperaceae, Dipterocarpaceae* y *Leguminoseae*), aunque la mayoría de ellas no son normalmente consumidas en la alimentación (Harborne, 1999). A pesar de encontrarse ampliamente distribuidos en el Reino Vegetal, su presencia en la dieta es escasa. Las principales fuentes de estilbenos en la dieta son las uvas y sus derivados, zumo y vino tinto; y en menor medida los cacahuetes y el chocolate (Guerrero y col., 2009).

Sin embargo, la concentración de estos compuesto puede aumentar debido a que son fitoalexinas y, por tanto, inducibles por distintos tipos de estrés de tipo biótico y/o abiótico.De entre los estreses bióticos destaca la infección por *Botrytis cinérea* (Roldan y col., 2003). La infección controlada de este hongo patógeno puede llegar a cuadruplicar la cantidad de estilbenos en uva, sin embargo su aplicación en campo resulta muy arriesgada ya que el control de la infección dependeria de las condiciones climáticas. Existen ensayos previos *in vitro* que indican que la aplicación de Botrydial (BOT) (Figura IV.1), toxina más activa de *Botrytis* es activa directamente sobre la uva cuando se aplica a concentraciones entre 1-1000 ppm (Colmenares y col., 2002), evitándose de este modo una infección descontrolada del patógeno.

Por otra parte también se han descrito numerosos elicitores de tipo abiótico tales como ozono (González-Barrio y col., 2006), radiación ultravioleta C (Cantos y col., 2001) y tratamientos químicos entre los que destacan el fungicida benzotiadiazol (Iriti y col**.,** 2004), el metil jasmonato (Vezulli y col., 2007) y el quitosano (Romanazzi y col., 2006).

El benzotiadiazol (BTH) (Figura IV.1) es un fungicida sistémico análogo al ácido salicílico (hormona endógena de las plantas), que puede ser utilizado en tratamientos precosecha como activador de mecanismos de defensa de la planta mediante la producción de resveratrol (Iriti y col., 2004).

Es un compuesto con una baja toxicidad, y de rápida degradación en los tejidos de las plantas por lo que su uso no tendría el impacto medioambiental de otros tratamientos de cultivo (Iriti y col., 2004 y 2005). La aplicación de BTH en cepas antes de la vendimia puede incrementar la concentración de resveratrol en bayas hasta aproximadamente un 40% (Iriti y col., 2004).

El quitosano (QUIT) (Figura IV.1) es un polisacárido de N-acetilquitina con carácter fungicida que se ha usado en uva de mesa, tanto en precosecha como en postcosecha, para mejorar la calidad (Olivas y Barbosa-Cánovas, 2005; Meng y col., 2007). Pero además de su carácter antifúngico, este compuesto resultó ser un inductor de la síntesis de estilbenos, efecto que se ve incrementado si se combina con un tratamiento UVC (Romanazzi y col., 2006).

El ácido jasmónico (AJ) y el metil jasmonato (MEJA) (Figura IV.1) son un grupo de hormonas vegetales que ayudan a regular el crecimiento de las plantas y su desarrollo. Han sido propuestos como compuestos clave de la vía de transducción de señales involucradas en la activación de la biosíntesis de metabolitos secundarios, que participan en las reacciones de defensa de las plantas (Gundlach y col., 1992).

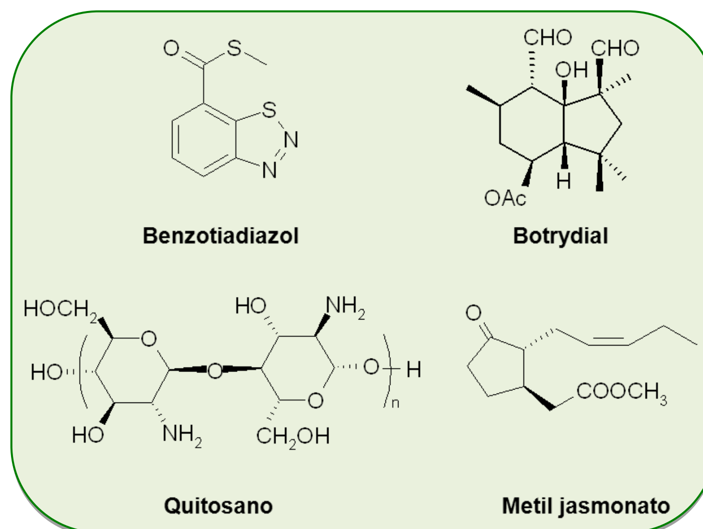


Figura IV.1. Estructura química del Benzotiadiazol, Botrydial, Quitosano y Metil Jasmonato.

El objetivo de este trabajo fue investigar la eficacia de diferentes tratamientos precosecha (benzotiadiazol, botrydial, metil jasmonato y quitosano) y su combinación con el tratamiento postcosecha UVC (buscando sinergias) para inducir la síntesis de estilbenos en uvas.

2. MATERIALES Y MÉTODOS

2.1. Material vegetal.

Este trabajo se ha desarrollado en una viña experimental del Centro IFAPA "Rancho de la Merced", ubicada en Jerez de la Frontera bajo condiciones de secano y clima cálido. El terreno es de albariza, calizo y arcilloso, con gran capacidad de retención de la humedad. El sistema de conducción fue en espaldera de 2-3 alambres y el sistema de poda fue en cordón doble.

Durante la campaña 2009, el ensayo se realizó sobre la variedad Jaén Tinto. Se eligió esta variedad ya que en estudios anteriores obtuvo buena respuesta al estrés (Guerrero y col., 2010b).

Durante la campaña 2010, el ensayo se llevó a cabo en la variedad Syrah como continuación al estudio realizado en el Capítulo III de esta Tesis.

2.2. Parámetros enológicos

Semanalmente se realizaron los controles de maduración (ºBrix, acidez total, pH, ác. Tartárico, ác. Málico, potasio, índice de maduración) para determinar la fecha óptima de la vendimia (Datos no mostrados).

En el día de vendimia se determinaron los siguientes parámetros enológicos según el método oficial de la OIV (1990): peso medio de la baya, grado Brix, acidez total, pH, ácido tartárico, potasio, índice de polifenoles totales (IPT), antocianos totales, antocianos extraíbles y taninos.

2.3. Tratamientos precosecha y postcosecha

Todos los tratamientos se aplicaron por triplicado y bajo un diseño de bloques completos al azar (10 cepas por réplica). Tras los diferentes tratamientos precosecha se tomó muestra cada 48 h hasta el día de vendimia y en el caso del tratamiento postcosecha se tomó muestra cada 24 horas durante siete días tras terminar éste, con el fin de determinar la cinética de la síntesis de estilbenos.

2.3.1 Vendimia 2009

2.3.1.1. Tratamiento precosecha con Benzotiadiazol

Se aplicaron dos concentraciones (0.3 mM y 1 mM) de BTH (BION W.G.; Syngenta, Madrid) siguiendo el protocolo descrito por Iriti y col. (2004). Se realizaron tres aplicaciones: la primera aplicación se realizó 24 días antes de la fecha de vendimia, la segunda y la tercera se dieron al cuarto y séptimo día tras la primera aplicación. Las cepas que se utilizaron como control se trataron con agua.

2.3.1.2. Tratamiento precosecha con Botrydial

El Botrydial (toxina *Botrytis cinérea*) fue suministrado por el Departamento de Química Orgánica de la Universidad de Cádiz. El metabolito puro fue disuelto en 40% de acetona (v/v) en agua con 10 µl de Tween 80. El tratamiento se realizó siete días antes de la fecha de vendimia. Se ensayaron dos concentraciones (100 ppm y 200 ppm) en dos partes de la planta (hojas y bayas). Las plantas control se trataron con 40% de acetona.

2.3.1.3. Tratamiento precosecha con Quitosano

El Quitosano (Sigma-Aldrich, St Louis) se aplicó según lo descrito por Romanazzi y col., 2006. Se aplicó una concentración de 10g/L diez días antes de la fecha de vendimia. Las plantas control se pulverizaron con agua deionizada a pH 5.6

2.3.1.4. Tratamiento postcosecha con UVC

El tratamiento postcosecha UVC se realizó siguiendo el protocolo descrito en el Capítulo III.2.4.

2.3.2. Vendimia 2010

2.3.2.1. Tratamiento precosecha con Benzotiadiazol

En la campaña 2010 se repitió el ensayo realizado en la campaña anterior pero utilizando sólo una de las concentraciones (0.3 mM) (Capítulo IV.2.3.1.1). Se aplicó 3 semanas antes de la fecha de vendimia.

2.3.2.2. Tratamiento precosecha con Metil Jasmonato

El tratamiento con MEJA (Sigma-Aldrich, Madrid) se realizó de acuerdo con el protocolo establecido por Vezzulli y col., 2007. Se aplicó una concentración de 10mM. Se realizaron tres aplicaciones, la primera se realizó 20 días antes de la fecha óptima de vendimia, dándose la segunda y tercera aplicación, al cuarto y séptimo día después de la primera aplicación. Las cepas control fueron pulverizadas con etanol.

2.3.2.3. Tratamiento precosecha con Quitosano

Se aplicó una concentración de 10 g/L de una solución basada en un 1.25% (w/v) de Quitosano (Biorend®, Idebio S.L., Salamanca) diez días antes de la vendimia. Las plantas control se pulverizaron con agua deionizada a pH 5.6 (Romanazzi y col., 2006).

2.3.2.4. Tratamiento postcosecha con UVC

El tratamiento postcosecha UVC se realizó siguiendo el protocolo descrito en el Capítulo III.2.4.

2.5. Extracción de estilbenos.

La extracción de estilbenos se llevó a cabo siguiendo el protocolo descrito en el Capítulo III.2.5

2.6. Identificación y cuantificación de estilbenos.

La identificación y cuantificación se realizó según la metodología descrita en el Capítulo III.2.6.

2.7. Análisis estadístico

El análisis de los datos se realizó utilizando el software estadístico Statistix versión 8.0. Los datos se sometieron al análisis de la varianza (ANOVA) y se analizaron las diferencias significativas según el criterio de Tukey con un nivel de significación de $p<0.05$.

3. Resultados y Discusión

3.1. Vendimia 2009

3.1.1. Efecto de los tratamientos en los parámetros enológicos de las uvas

En el día de vendimia se estudió el efecto de los tratamientos sobre la calidad de la uva. Los parámetros enológicos se midieron en cada tratamiento y se compararon con su respectivo control (Tabla IV.1).

El tratamiento con BOT no afectó a los parámetros de la uva (Tabla IV.1). Sólo el potasio resultó ligeramente superior en las uvas tratadas con 200 ppm, lo que creemos que pudo deberse a la variabilidad intrínseca de la medición.

El tratamiento con BTH afectó al proceso de maduración. El contenido de azúcar en el día de vendimia fue menor en todas las muestras tratadas con BTH (0.3 y 1 mM) respecto a su control. Además, las muestras tratadas con 1 mM disminuyeron su concentración de antocianos totales y extraíbles (Tabla IV.1). Estos datos contrastan con

los resultados obtenidos por otros autores que describieron que la variedad Merlot duplicó su contenido en antocianos cuando fue tratada con 0.3mM de BTH (Iriti y col., 2004). En este ensayo, las muestras tratadas a esa concentración no experimentaron ninguna diferencia significativa en el contenido de antocianos (Tabla IV.1).

Tabla IV.1. Parámetros enológicos de los tratamientos precosecha y sus controles en el día de vendimia

	BOT			BTH			QUIT		UVC	
	CT	100 ppm	200 ppm	CT	0.3 mM	1 mM	CT	10 g/L	CT	UVC
Peso uva (g)	1.76	1.64	1.75	1.95	1.78	1.69	1.63	1.73	1.89	1.87
ºBrix	19.2	19.7	19.5	19.5[a]	18.8[ab]	17.2[b]	21.8	21.4	19.3	19.5
AT (g/L TH_2)	6.44	6.40	6.78	6.57	6.40	6.78	6.35	6.30	6.22	5.52
pH	3.42	3.45	3.42	3.36	3.42	3.38	3.41	3.34	3.45	3.51
Ác. Tartárico (g/L)	6.40	6.56	7.36	6.93	6.49	6.43	7.18	7.34	6.85	6.62
Potasio (mg/L)	1789[c]	1975[b]	2165[a]	1881	1874	1838	2182	1951	1898	1904
Antocianos tot. (mg/L)	645	577	628	625[a]	642[a]	445[b]	713	739	629	695
Antocianos ext. (mg/L)	338	316	288	305[a]	291[a]	244[b]	362	324	285	300
Extractabilidad (%)	47	45	54	51	54	45	49	56	55	57
Taninos (g/L)	1.92	1.94	1.97	1.91	1.87	1.78	2.29	1.99	1.85	1.78
IPT	13.70	13.86	14.08	13.68	13.37	12.74	16.39	14.22	13.2	12.7

BOT, botrydial; BTH, benzotiadiazol; QUIT, quitosano; AT, acidez total; Antocianos tot., antocianos totales; Antocianos ext., antocianos extraibles; IPT, índice de polifenoles totales. Las diferentes letras significan diferencias significativas entre tratamientos (nivel significación $p<0,05$).

El tratamiento con QUIT no produjo ningún cambio significativo en la maduración de la uva, coincidiendo con lo descrito por otros autores que lo estudiaron en condiciones precosecha. Al igual que nosotros concluyeron que el metabolismo de uva no se vio afectado (Meng y col., 2008; Duxbury y col., 2004).

Respecto al tratamiento postcosecha UVC, tras el periodo de conservación, se compararon los parámetros enológicos de las muestras tratadas con las muestras control. Como podemos observar en la Tabla IV.1 no existieron ninguna diferencia significativa entre el tratamiento y control.

3.1.2. Efecto de los tratamientos en el contenido de *trans*-resveratrol en uvas

Como podemos observar en la Figura IV.2 los tratamientos con BOT aumentaron ligeramente el contenido en *trans*-resveratrol de las uvas. Sin embargo, en el día de vendimia el contenido de *trans*-resveratrol fue muy similar en uvas tratadas (0.603 y 0.583 mg/Kg uvas para las concentraciones de 100 y 200 ppm respectivamente) que en las testigo (0.631mg/Kg uvas), no siéndolas diferencias significativas en este punto. Esto, sumado a la complejidad de la síntesis de las toxinas hace que este tratamiento se descarte en la vendimia 2010.

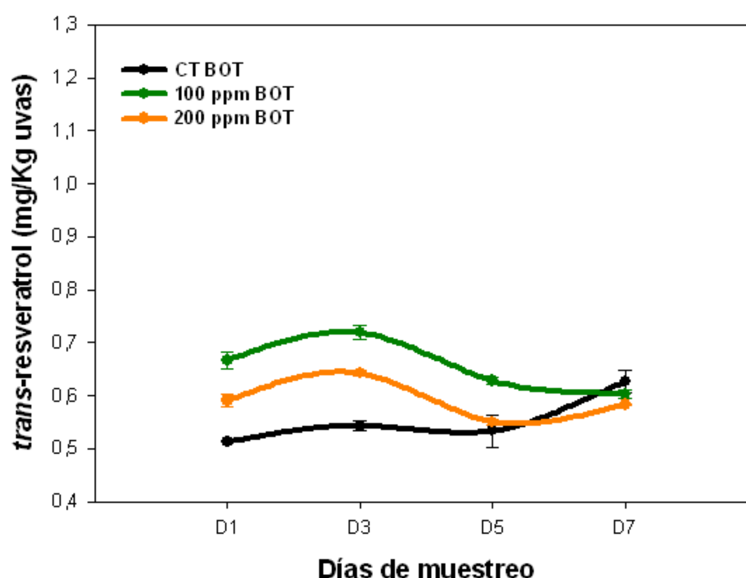


Figura IV.2. Evolución del contenido de *trans*-resveratrol tras la aplicación del tratamiento precosecha con Botrydial (BOT) hasta el día de vendimia.

Siguiendo el protocolo descrito en Materiales y Métodos, el tratamiento precosecha con BTH se realizó a dos concentraciones: 0.3mM y 1mM. En la Figura IV.3 se representa la evolución del contenido de *trans*-resveratrol en los diferentes días de muestreo.

El tratamiento produjo un incremento en la concentración de *trans*-resveratrol en ambas concentraciones ensayadas. Además, durante todo el periodo post-tratamiento el contenido en *trans*-resveratrol fue superior para la menor concentración ensayada (0.3 mM) excepto en el día de vendimia.

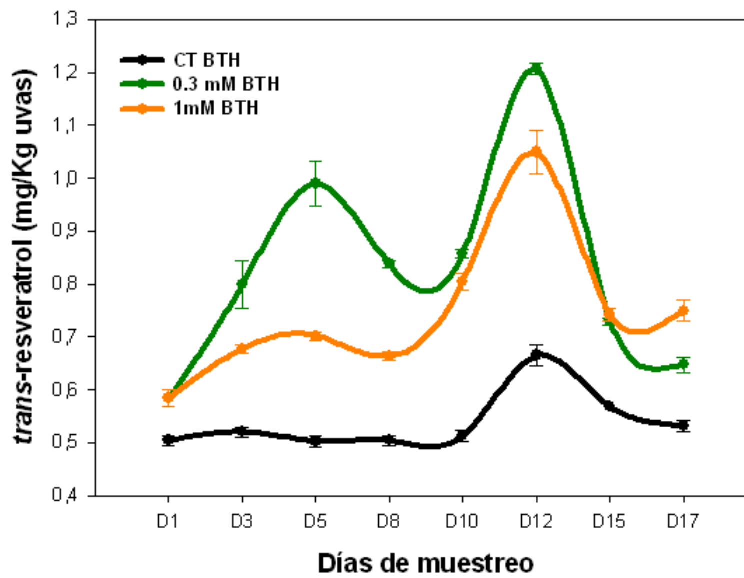


Figura IV.3. Evolución del contenido de *trans*-resveratrol tras la aplicación del tratamiento precosecha con benzotiadiazol (BTH) hasta el día de vendimia.

La máxima concentración de *trans*-resveratrol se obtuvo 12 días tras la aplicación del tratamiento. El resultado fue de 1.207 mg/Kg uvas para la concentración 0.3 mM BTH y de 1.049 mg/Kg uvas para la concentración 1 mM BTH. Sin embargo, ésta diferencia entre las muestras tratadas y las muestras control desapareció transcurridos cinco días, coincidiendo con la fecha de vendimia. En vendimia las concentraciones encontradas fueron las siguiente: 0.647 mg/Kg uvas para 0.3mM BTH, 0.749 mg/Kg uvas para 1mM BTH y 0.5310 mg/Kg uvas para CT. Teniendo en cuenta los resultados obtenidos, en la vendimia 2010 se repetirá el tratamiento pero únicamente a la concentración más baja (0.3mM). Además para la siguiente vendimia el primer tratamiento se aplicará tres días más tarde de lo que se aplicó en la vendimia 2009, buscando que la máxima concentración de *trans*-resveratrol se alcance en la fecha de vendimia.

Como observamos en la Figura IV.4, tras el tratamiento con QUIT no se encontraron diferencias significativas en el contenido de *trans*-resveratrol entre las muestras tratadas y las muestras control.

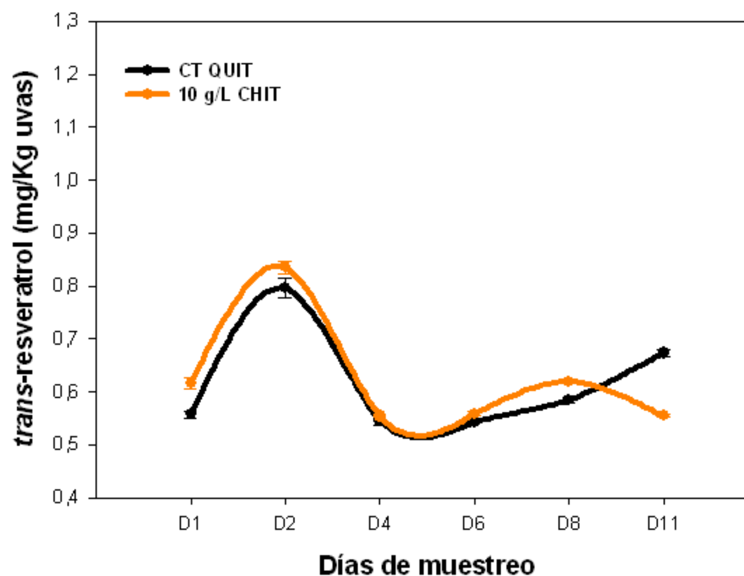


Figura IV.4. Evolución del contenido de *trans*-resveratrol tras la aplicación del tratamiento precosecha con quitosano (QUIT) hasta el día de vendimia.

Este resultado concuerda con datos obtenidos por otros autores (Duxbury y col., 2004) en los cuales se demostró que tras la aplicación de QUIT, la variedad Cabernet Sauvignon no modificó su contenido polifenólico. En otro estudio, la aplicación de 10 g/L de QUIT sobre uva de mesa no aumentó el contenido de *trans*-resveratrol aunque ayudó a controlar la infección por *Botrytis cinérea* (Romanazzi y col., 2006). En cuanto a la aplicación de QUIT en cultivos celulares se han encontrado resultados dispares, un estudio demostró que tras la aplicación de 50 mg/L de QUIT la concentración de resveratrol se vio casi duplicada (Ferri y col., 2009). En contraste, en otros ensayos con QUIT se observó que era menos efectivo para la inducción de estilbenos (Santamaría y col., 2010).

Finalmente, cuando se aplicó el tratamiento postcosecha UVC los resultados obtenidos fueron más llamativos. En la Figura IV.5 se puede observar la evolución del *trans*-resveratrol durante los días post-tratamiento y la comparación con su control. Se detectó una inducción (11.87 veces) del *trans*-resveratrol, alcanzando el máximo dos días tras el tratamiento UVC (14 mg/Kg bayas). Datos que corroboraban lo descrito en estudios anteriores (Guerrero y col., 2010b).

En este tratamiento, y a diferencia de los anteriores precosecha, se detectaron otros estilbenos: piceatanol, ε-viniferina, δ-viniferina. Sus concentraciones en el día de máxima inducción fueron 4.06, 0.96, 0.49 mg/Kg uvas respectivamente.

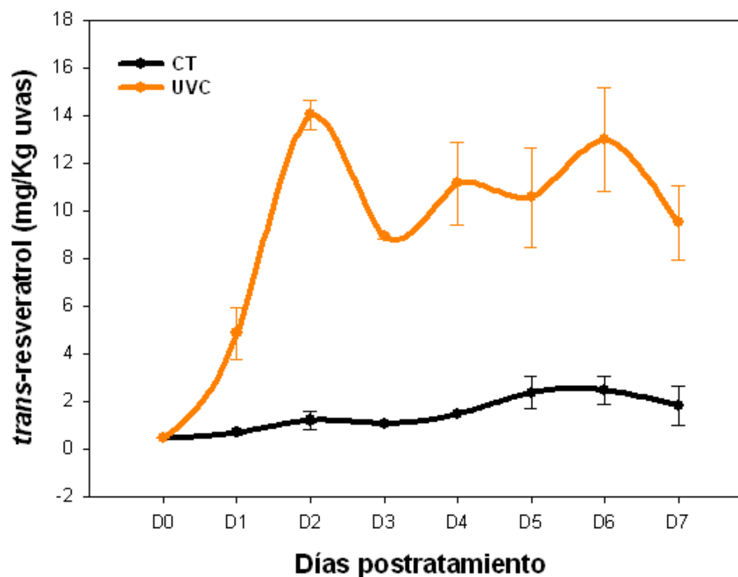


Figura IV.5. Evolución de la concentración de *trans*-resveratrol en la uva control (CT) y tratada (UVC) durante los días post-tratamiento.

Algunos autores han descrito efectos sinérgicos entre el QUIT y la irradiación UVC en uva de mesa (Romanazzi y col., 2006) y el metil jasmonato y la luz UV en cultivos celulares (Larronde y col., 2003, Zhang y col., 2002). Por lo tanto, en la segunda parte de este estudio (Vendimia 2010), i) se tuvieron en cuenta los resultados obtenidos para seleccionar concentraciones y modificar fechas de tratamiento ii) se investigó la combinación de tratamientos precosecha con el tratamiento postcosecha UVC.

3.2. Vendimia 2010

3.2.1. Efecto de los tratamientos en los parámetros enológicos de las uvas

Del mismo modo que en la campaña 2009, se estudiaron los parámetros enológicos en el día de vendimia para determinar cómo afectaban los diferentes tratamientos a la calidad de la uva, en este caso sobre la variedad Syrah (Tabla IV.2).

Tabla IV.2. Parámetros enológicos de los diferentes tratamientos en vendimia.

	BTH		MEJA		QUIT	
	CT	TR	CT	TR	CT	TR
Peso uva (g)	1.79	1.79	1.88	1.92	2.03	1.94
ºBrix	18.5[a]	16.4[b]	17.2	17.8	16.8	16.8
AT (g/L TH_2)	6.44[b]	6.80[a]	6.63	6.93	7.42	7.07
pH	3.37[a]	3.26[b]	3.32	3.26	3.28	3.26
Ác. Tartárico (g/L)	7.18	7.36	6.61	7.26	6.97	6.22
Potasio (mg/L)	1622[a]	1550[b]	1641	1619	1560	1668
Antocianos tot. (mg/L)	296	281	263	347	254	225
Antocianos ext. (mg/L)	106	86	106	117	82	86
Extractabilidad (%)	63	68	59	65	67	61
Taninos (g/L)	1.14	1.04	0.93	1.14	0.61	0.77
IPT	13.43	12.18	11.07	13.44	7.55	9.41
IM	27.64[a]	22.5[b]	24.59	24.53	21.43	22.49

BTH, benzotiadiazol; MEJA, Metil jasmonato; QUIT, quitosano; CT, control; TR, tratamiento; IPT, índice polifenoles totales; IM, índice de maduración. Las diferentes letras significan diferencias significativas entre tratamientos (nivel significación $p<0,05$).

Al igual que ocurrió en la vendimia 2009, el tratamiento precosecha con BTH afectó a los parámetros enológicos de la uva. Las uvas tratadas mostraron menor contenido en azúcar, pH y potasio, y mayor acidez comparadas con su testigo, apreciándose un retraso en la maduración (Tabla IV.2). Estos datos contrastan con los de otros autores que describieron ligeras diferencias cuando se realizó el mismo tratamiento a las uvas Monastrell (Ruiz-García y col., 2012).

Al suprimir el tratamiento con Botrydial, y tras una revisión bibliográfica, se decidió probar el tratamiento con metil jasmonato. El tratamiento con MEJA no modificó ninguno de los parámetros enológicos estudiados (Tabla IV.2), datos que concuerdan con otros autores (Ruiz-García y col., 2012). Sin embargo, estos autores observaron un aumento en los antocianos y flavonoles (medidos por HPLC). En otros estudios se describió un aumento de antocianos cuando se aplicó MEJA a cultivos celulares, especialmente cuando los medios de cultivo se combinaron con sacarosa (Belhadj y col., 2008).

El tratamiento precosecha con QUIT se repitió, pero en este caso usando una solución preparada. En este caso tampoco afectó a los parámetros enológicos de la uva (Tabla IV.2).

3.1.2. Efecto de los tratamientos precosecha en el contenido de *trans*-resveratrol en uvas

Tras los tratamientos precosecha se observaron pocas diferencias significativas en cuanto al contenido en *trans*-resveratrol. Sólo se estudió la evolución del *trans*-resveratrol ya que otros estilbenos como el piceatanol y las viniferinas se encontraban por debajo del límite de detección. Cuando las concentraciones de *trans*-resveratrol son bajas no se detectan otros estilbenos (Guerrero y col., 2010a).

El tratamiento con BTH aumentó significativamente el contenido en *trans*-resveratrol a partir del sexto día tras la aplicación (Tabla IV.3). En la vendimia el contenido de *trans*-resveratrol en las uvas tratadas fue 2.79 mayor respecto a su control, alcanzando la concentración máxima de 0.260 mg/Kg uvas.

Tabla IV.3. Concentración de *trans*-resveratrol tras los tratamientos precosecha BTH y MEJA

	BTH			MEJA		
	CT	**TR**	**nº veces inducción**	**CT**	**TR**	**nº veces inducción**
4 días tras TR	0.185	0.146	0.79	0.123	0.203	1.65
6 días tras TR	0.060[b]	0.125[a]	2.08	0.133	0.191	1.44
8 días tras TR	0.044[b]	0.109[a]	2.16	0.106	0.143	1.35
11 días tras TR	0.030[b]	0.125[a]	4.17	0.119[a]	0.065[b]	0.56
13 días tras TR vendimia	0.093[b]	0.260[a]	2.79	0.189[a]	0.079[b]	0.48

BTH, benzotiadiazol; MEJA, Metil jasmonato; CT, control; TR, tratamiento. Las diferentes letras significan diferencias significativas entre tratamientos (nivel significación $p<0,05$).

Respecto al tratamiento precosecha con MEJA no se obtuvieron diferencias significativas hasta el cuarto día de muestreo (11 días tras la aplicación, Tabla IV.3), en la que el contenido de *trans*-resveratrol fue superior en las uvas no tratadas. Observando los datos de la Tabla IV.3 podemos decir que la mayor concentración se alcanzó 4 días tras el tratamiento (0.203 mg/Kg uvas), siendo la concentración 1.65 veces respecto su control.Posteriormente, el *trans*-resveratrol disminuyó a lo largo de la maduración. Hecho que ha sido descrito también por otros autores. Larronde y col. (2003) describieron un aumento de la concentración de *trans*-resveratrol durante 15 días después del envero cuando fueron tratados con vapores de MEJA, seguida de una disminución marcada durante la maduración. Otros autores (Vezulli y col., 2007) describieron un aumento en el contenido de *trans*-resveratrol y ε-viniferina de forma acumulativa cuando aplicaron MEJA sobre la variedad Barbera. En otro estudio, las concentraciones de resveratrol y piceido aumentaron significativamente cuando se aplicó MEJA (18g/L), en presencia de azúcares, en cultivos celulares de vid (Belhadj y col., 2008). Otros autores describieron que el contenido de resveratrol aumentó notablemente (9 veces) cuando se aplicó MEJA (0.09 mg/L) en estado gaseoso en la variedad Cabernet Sauvignon. Además, MEJA se ha propuesto como una herramienta muy útil como inductor de resveratrol en cultivos celulares cuando se combina con ciclodextrinas (Lijavetzky y col., 2008).

Finalmente, el tratamiento precosecha con QUIT, a pesar de tener una formulación distinta, tampoco afectó al contenido de *trans*-resveratrol en ninguna de las muestras tomadas tras el tratamiento (Tabla IV.4).

Tabla IV.4. Concentración de *trans*-resveratrol (mg/kg uvas) tras el tratamiento precosecha QUIT

	QUIT		
	CT	**TR**	**nº veces inducción**
2 **días tras TR**	0.185	0.139	0.75
5 **días tras TR**	0.106	0.174	1.64
7 **días tras TR**	0.157	0.141	0.90
9 **días tras TR** vendimia	0.197	0.200	1.01

QUIT, quitosano; CT, control; TR, tratamiento. Las diferentes letras significan diferencias significativas entre tratamientos (nivel significación $p<0,05$).

3.1.3. Combinación de los tratamientos precosecha con el tratamiento postcosecha UVC

Tras la vendimia, los tres tratamientos precosecha se combinaron con el tratamiento postcosecha UVC (BTH-UVC, MEJA-UVC, QUIT-UVC) y se estudió la evolución de los estilbenos de cada uno de ellos durante siete días. Además, como testigo se realizó el tratamiento UVC en uvas sin tratar previamente en campo (UVC).

Como se observa en la Figura IV.6, todas las combinaciones aumentaron su concentración en *trans*-resveratrol tras el tratamiento postcosecha con UVC. Ahora bien, la concentración máxima alcanzada para cada combinación fue diferente. Los tratamientos UVC y BTH-UVC alcanzaron el máximo el día 7, siendo inferior la concentración de resveratrol alcanzada para éste último (7.97 mg/Kg uvas para UVC y 5.13 mg/Kg uvas para BTH-UVC). El tratamiento QUIT-UVC alcanzó su máximo el día 6 (6.73 mg/Kg uvas) y por último, el tratamiento MEJA-UVC mostró su máximo el día 4 (7.23 mg/Kg uvas).

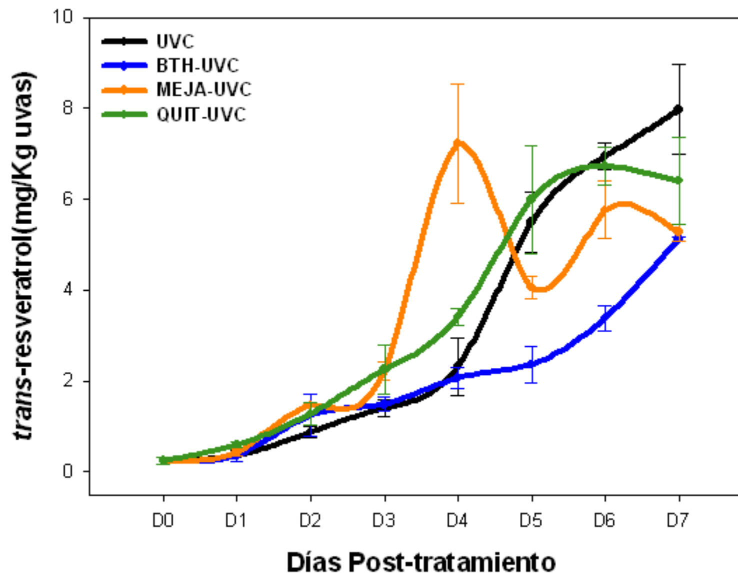


Figura IV.6. Evolución del *trans*-resveratrol tras el tratamiento UVC.

Además de *trans*-resveratrol, en las muestras se indujeron otros estilbenos como piceatanol, ε-viniferina y δ-viniferina (estas dos últimas expresadas como viniferinas).

De acuerdo con estos resultados, y para analizar los resultados de forma más sencilla, en la Figura IV.7 se muestra el contenido de estilbenos a día 4 y día 7. En el cuarto día de almacenamiento, el MEJA-UVC mostró una concentración total de estilbenos (10.03 mg/Kg uvas) significativamente más alta que el resto de tratamientos en el mismo día (Figura IV.7g), del mismo modo ocurrió con el piceatanol (1.95 mg/ Kg uvas, Figura IV.7c) y el *trans*-resveratrol (7.23 mg/Kg uvas, Figura IV.6, Figura IV.7a). Sin embargo, la concentración de viniferinas fue inferior a la alcanzada por el resto de los tratamientos (0.84 mg/Kg uvas, Figura IV.7e)

Por otro lado, el tratamiento UVC (sin combinar) alcanzó la máxima concentración de estilbenos totales el séptimo día de almacenamiento (13.43 mg/Kg uvas, Figura IV.7h), lo mismo ocurrió para cada estilbenos en particular: piceatanol (2.04 mg/Kg uvas, Figura IV.7h), *trans*-resveratrol (7.97 mg/Kg uvas, Figura IV.7b, Figura IV.6) y viniferinas (3.42 mg/Kg uvas, Figura IV.7f).

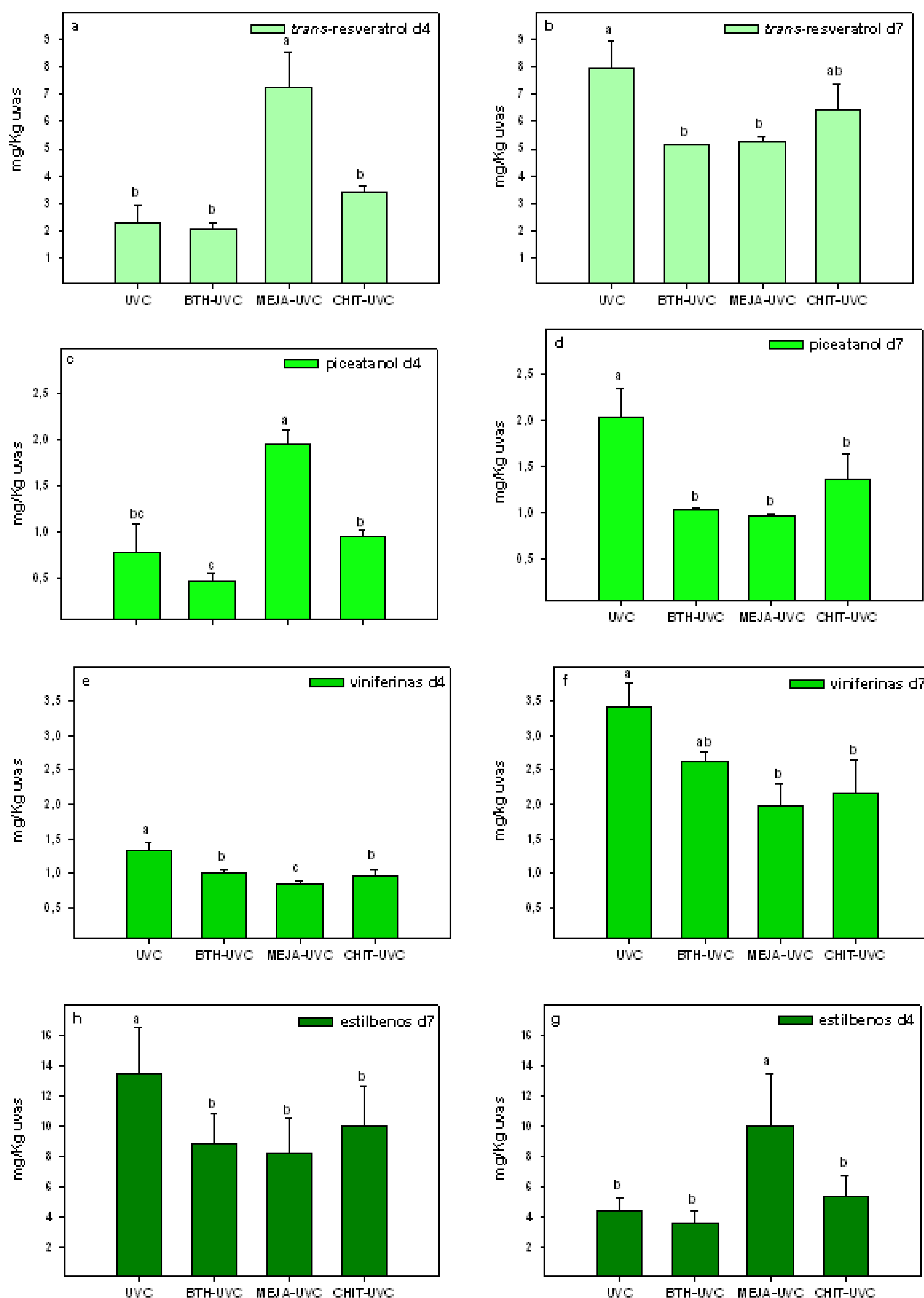


Figura IV.7. Concentraciones de *trans*-resveratrol, piceatanol, viniferinas y estilbenos totales en el cuarto (d4) y séptima (d7) días después del tratamiento post-cosecha UVC. Las diferentes letras muestran valores significativamente diferentes.

Finalmente, no se encontraron diferencias significativas en cuanto al contenido de *trans*-resveratrol entre los tratamientos CHIT-UVC y UVC, en el séptimo día de almacenamiento. Del mismo modo ocurrió en cuanto al contenido de viniferinas entre los tratamientos BTH-UVC y UVC en ese mismo día (Figura IV.7f).

A partir de los resultados obtenidos en esta vendimia, se puede concluir que, el tratamiento con BTH aumenta el contenido de *trans*-resveratrol pero este aumento va ligado con un retraso en la maduración de la uva.

Por otro lado, la combinación del tratamiento precosecha MEJA con el tratamiento postcosecha UVC (MEJA-UVC) mejoró la síntesis de estilbenos respecto al tratamiento UVC. A pesar de no superar la concentración de resveratrol obtenida con el tratamiento UVC (sin combinar), si se consigue reducir el periodo de almacenamiento de la uva tras el tratamiento UVC en tres días. Este hecho es un hallazgo importante, ya que el periodo de conservación de la uva es un factor limitante a la hora de aplicar el tratamiento UVC, debido a que la calidad de la uva se ve afectada.

La publicación correspondiente a este Capítulo se encuentra en el Anexo 4.

CAPÍTULO V

ISORHAPONTIGENIN: UN NUEVO ESTILBENO EN UVA DE VINIFICACIÓN

1. INTRODUCCIÓN

Se han descrito cerca de 800 estilbenos en fuentes de origen vegetal (Xiao y col., 2008). A pesar de encontrarse ampliamente distribuidos en el Reino Vegetal, su presencia en la dieta es escasa. La principal fuente de estilbenos en la dieta son las uvas y el vino (Zamora-Ros y col., 2008).

Los estilbenos más frecuentes encontrados en uvas son: *trans*- y *cis*-resveratrol (3,5,4'-trihidroxiestilbeno), *trans*- y *cis*-piceido (resveratrol-3-*O*-*β*-glucósido), piceatanol (3',4',3,5-tetrahidroxiestilbeno), pterostilbeno (3,5-dimetoxi-4'-hidroxiestilbeno) y ε-,δ-viniferinas (dímeros del resveratrol) (Guerrero y col., 2009). Sus concentraciones suelen ser muy bajas, sin embargo, pueden incrementarse debido a que son fitoalexinas y, por tanto, inducibles por distintos tipos de estrés. En particular, el tratamiento postcosecha con luz ultravioleta C (UVC) se ha propuesto como una de las herramientas más efectiva para obtener un aumento considerable en el contenido de estilbenos en uvas (Guerrero y col., 2010a).

El interés científico por estos compuestos se debe a que se les atribuyen numerosas propiedades beneficiosas para la salud. Han demostrado tener actividad antioxidante, anticancerígena, cardioprotectora, neuroprotectora y antiinflamatoria entre otras (Guerrero y col., 2009).

El objetivo de este estudio fue el aislamiento y la identificación de un estilbeno no descrito anteriormente en uva, que se detecta tras el tratamiento postcosecha con UVC en uva. Con este propósito se llevó a cabo su aislamiento en varias etapas mediante la cromatografía contracorriente (CCC) y HPLC semipreparativo y su identificación por UPLC- MS-MS y RMN.

2. MATERIALES Y MÉTODOS

2.1. Material vegetal

Para este trabajo se seleccionaron cuatro variedades de *Vitis vinifera sativa*, dos internacionales (Merlot y Syrah) y dos españolas (Graciano y Tempranillo). Las cuatro variedades se cultivaron bajo las mismas condiciones, durante la campaña 2009, en la viña experimental del IFAPA, Centro "Rancho de la Merced" ubicada en Jerez de la Frontera (Cádiz). Semanalmente se realizaron los controles de maduración para determinar la fecha óptima de la vendimia.

2.2. Tratamiento postcosecha con luz UVC y periodo de almacenamiento

Tras la vendimia manual se procedió al tratamiento con luz UVC descrito por la patente WO/2002/085137; ES 2177465) con algunas modificaciones descritas por Guerrero y col. (2010a).

Tras el tratamiento la muestra necesita de un periodo de conservación para que se produzca la síntesis de los estilbenos. En un estudio anterior se determinó el número de días de almacenamiento a temperatura ambiente necesarios para obtener la máxima concentración en uva que se denominó Dm (Guerrero y col., 2010a). Los racimos se almacenaron en tanques de acero inoxidable manteniéndose la temperatura a 20ºC y la humedad al 80%.

El Dm depende entre otros parámetros de la variedad. Este resultó ser para la Merlot y la Graciano fue de 5 días y para la Syrah y Tempranillo de 6 días.

Entre las cuatro variedades se vendimiaron un total de 30 Kg de uvas. Tras alcanzar el Dm, se pelaron y la piel se congeló a -80ºC para su posterior liofilización. Una vez liofilizadas, fueron pulverizadas con ayuda de un mortero para su posterior extracción.

2.3. Método de extracción

La extracción se realizó con dietil éter (4 ml por cada gramo de piel liofilizada), se homogenizó la muestra con ayuda de una Ultraturrax T-25 (Jankel and Kunkel, Ika-Labortechnick, Alemania). Posteriormente, se mantuvo en agitación durante una hora. Finalmente, se filtró la mezcla por papel de filtro para retirar el sólido. El extracto se llevó a sequedad en rotavapor y luego se conservó a –80ºC hasta su posterior análisis. La muestra en todo momento se protegió de la luz y se mantuvo en un baño con hielo para evitar posibles reacciones de oxidación y/o isomerización.

2.4. HPLC-DAD

El extracto disuelto en metanol (20 µl) fue analizado mediante un equipo Waters HPLC compuesto por bomba binaria modelo 1525, controlador de temperatura TCM y un detector de fotodiodo Waters 996. Para la separación realizada a 30ºC se utilizó como fase estacionaria una columna Mediterránea Sea_{18} (Teknokroma, España) (RP-18, 25x0.46 cm; 5 µm de tamaño de partícula) con precolumna del mismo material.

Las fases móviles consistieron en una mezcla agua:metanol:ácido acético de 88:10:2 para la fase A y 8:90:2 para la fase B. El programa de elución comenzó con un flujo de 1 ml/min, con 35% de B durante 3 min para llegar al 50% B a 10 min, al 70% B a 20 min y al 100% de B a 23 min para posteriormente mantenerse constante hasta 28 min (Guerrero y col., 2010a). El software usado fue Empower suministrado por Waters. Los estilbenos fueron cuantificados como *trans*-resveratrol a 306 nm.

2.5. UPLC-DAD-TQD

Una vez analizada la muestra por HPLC-DAD se procedió a su identificación utilizando el sistema UPLC-DAD-TQD.

El equipo Waters utilizado consistió en un sistema Acquity UPLC, un detector por fotodiodos alineados Acquity PDA (Mildford, EEUU), y un espectrómetro de masas de triple cuadrupolo Acquity TQD (Manchester, Reino Unido). Se utilizó el software MassLynx 4.0 para la adquisición de datos. La muestra se diluyó 1:1 en agua

acidificada con ácido fórmico al 0.01% (Panreac, Barcelona) de forma previa al análisis. La separación en fase reversa se realizó en una columna Acquity UPLC BEH C18 (2.1 x100 mm; 1.7 µm de tamaño de partícula) con un gradiente formado por 0.1% ácido fórmico-agua (fase A) y 0.1% ácido fórmico-metanol (grado HPLC, Panreac, Barcelona) (fase B) a un flujo de 0.5 ml/min a 40ºC.

La elución comenzó con un gradiente lineal de B al 5% hasta llegar en 5 min al 70%. El volumen inyectado fue de 10 µl. El detector PDA registró longitudes de onda entre 220-400 nm, siendo monitorizado a 320 nm. Las condiciones del espectrómetro de MS-MS en modo ESI^- fueron las siguientes: voltaje del capilar, 2.50 kV; voltaje de cono, 40.00 V; extractor, 3.00 V; flujo del gas portador (N_2), 50 L/h; flujo del gas de desolvatación (N_2), 650 L/h; flujo del gas de colisión (Ar), 0.15 ml/min.

2.6. Cromatografía contracorriente (CCC)

Para el aislamiento del estilbeno desconocido se utilizaron dos técnicas de cromatografía contracorriente (CCC).

El primer sistema utilizado fue el Spiral Coil-Low Speed Rotary CCC (Spiral Coil LSR-CCC), fabricado por Pharma-Tech Research Corp. (Baltimore, MD). La columna en espiral está formada por un tubo de Teflón enrollado de una sola pieza (Diámetro interior del tubo: 8.5 mm, volumen total: 5600 ml). El sistema disolvente utilizado se compone de una mezcla de acetonitrilo/n-hexano (1:1 v/v). El modo de elución fue cabeza-cola, usando la fase ligera (orgánica) como fase estacionaria y la fase densa (acuosa) como fase móvil. El caudal se fijó en 15 ml/min y la bomba que se utilizó fue HPLC (Knauer, Berlín).

Se inyectaron 5910 mg del extracto disueltos en 250 ml de la mezcla de los disolventes comentados anteriormente. Se recogieron 13 fracciones (F1-F13) con ayuda de un colector de fracciones de Pharmacia LKB súper Frac (Bromma, Suecia). La elución se controló con un UV-2501K/detector VIS (Knauer, Berlín, Alemania) a 280 nm y los cromatogramas resultantes fueron registrados por un plotter (ABB Goerz SE 120, Viena, Austria).

El segundo sistema de CCC utilizado fue el High Speed CCC (HSCCC). Las 4 primeras fracciones del sistema Spiral Coil LSR-CCC se combinaron para ser de nuevo fraccionados por el HSCCC modelo CCC-1000 fabricado por Pharma-Tech Research Corp. (Baltimore, MD). Está equipado con tres bobinas conectadas en serie (diámetro de la tubería interior de 2.5 mm y un volumen total de 800 ml). El sistema disolvente de dos fases se compone de hexano/acetato de etilo/metanol/agua (3:5:3:5; v/v/v/v) (Ito, 2005). El modo de elución fue cabeza-cola, usando la fase ligera (orgánica) como fase estacionaria y la fase densa (acuosa) como fase móvil. El caudal se fijó en 3 ml/min y la bomba utilizada fue una bomba HPLC modelo BT 3020 (Jasco, Gross-Umstadt, Alemania). La separación se realizó a 820 rpm.

En este sistema 442 mg de la muestra (F1-F4 como se explica en el apartado de resultados posteriormente) fueron disueltos en 25 ml de la mezcla de fase orgánica y fase acuosa (1:1). De esta nueva separación se obtuvieron ocho fracciones (FF1-FF8) de 12 ml que se recogieron con ayuda de un colector de fracciones (Pharmacia LKB Super Frac). La elución fue monitorizada por un detector de UV/Vis a 280 nm (K-2501, Berlín, Alemania).

Las fracciones recogidas de los CCC se observaron por cromatografía de capa fina sobre placas de gel de sílice 60 F254 (Merck) con cloroformo/acetato de etilo/metanol/agua (25:55:5:1; v/v/v/v) como sistema de disolventes. Los estilbenos y derivados fueron revelados pulverizando las placas con anisaldehido.

2.7. HPLC-ESI-MS/MS

Las fracciones de la CCC se secaron, se disolvieron en metanol y se analizaron mediante un sistema HPLC-EIS-MS/MS. El sistema de HPLC (bomba de la serie 1100, inyector automático serie 1200) de Agilent Technologies (Böblingen, Alemania) se conecta a un LC-ESI-MS/MS de Bruker Esquire (Bremen, Alemania). Los espectros de masas se registraron en modo negativo, voltaje del capilar a 1500 V, placa final en-500 V, salida capilar en -120.4 V, el gas seco a 330ºC, el flujo de gas a 11 L/min, nebulizador a 60 psi, la masa objetivo en m/z 500, rango de exploración m/z de 100 a 3000, el helio como el gas de colisión, y MS/MS fragmentación de la amplitud de 1,0

V. La resolución ESI-MS se registró en un espectrómetro de masas Thermo Ciencia LTQ Orbitrap. Se utilizó una columna analítica C18 (C18 Luna, 250x4.6 mm, 5 µm, Phenomenex, Aschaffenburg, Alemania), velocidad de flujo de 0.8 ml/min y como sistema disolvente 1% de ácido acético para la fase A y acetonitrilo para la fase B. El gradiente utilizado fue el siguiente: 0 min al 20%B, 5 min al 30%B, 15 min al 30%B, 18 min al 37%B, 29 min al 37%B, 35 min al 50%B, 57 min al 50%B, 58 min al 100%B, 61 min al 100%B.

2.8. HPLC-Semipreparativo

Después del HSCCC se requirieron 2 etapas de purificación de la muestra por HPLC semipreparativo, sistema Smartline de Knauer (Bomba 1000, manager 5000, detector UV-K2600, Berlín, Alemania).

La columna utilizada fue una Luna C18, 250x15.0mm, 5 micras, Phenomenex, (Aschaffenburg, Alemania), el sistema disolvente fue de agua/metanol/ác.acético (88:10:2) para la fase A; metanol/ agua / ácido acético (90:8:2) para la fase B y el flujo fue de 4 ml/min. Los dos gradientes aplicados fueron, 0 min a 20% B, 40 min a 35% B, 80 min a 45% B y 100 min a 100% de B para el primero y 0 min a 35% B, 30 min a 45 % B, 45 min a 55% y 120 min a 100% de B para el segundo. Los cromatogramas se registran a 280 y 306 nm.

2.9. RMN

Los espectros de RMN se registraron en un espectrómetro Bruker Avance 600 RMN (600 MHz para ^{1}H y 151 MHz para ^{13}C), en d_6-acetona a 300 K utilizando una sonda de 5 mm TXI with 90° proton pulse length of 7.5 µs a una potencia de transmisión de 0 dB y equipado con un sistema de generación de pulsos de gradiente de campo. La escala de desplazamientos químicos fue calibrado en el residuo de acetona deuterado en δ_H 2.05 ppm y δ_C 29.9 ppm. Se realizaron los siguientes experimentos de RMN: ^{1}H-RMN; (^{1}H-^{1}H) COSY; (^{1}H-^{1}H)-ROESY; (^{1}H-^{13}C)-HSQC y (^{1}H-^{13}C)-HMBC.

3. Resultados y discusión

3.1. Inducción de un estilbeno desconocido tras el tratamiento postcosecha con UVC

Para este trabajo se seleccionaron cuatro variedades (Merlot, Syrah, Graciano y Tempranillo) que en trabajos anteriores desarrollados en nuestras instalaciones mostraron un mayor contenido en estilbenos, tanto de basal como tras la aplicación del tratamiento postcosecha con luz UVC (Guerrero y col., 2010a).

Se identificaron los siguientes estilbenos, tras el análisis por HPLC-DAD y UPLC-DAD-TQD, en las muestras extraídas después del tratamiento postcosecha con luz UVC: piceatannol (1), *trans*-resveratrol (2), estilbeno desconocido (3), ε-viniferin (4) y δ-viniferin (5) (Figura V.1, Tabla V.1).

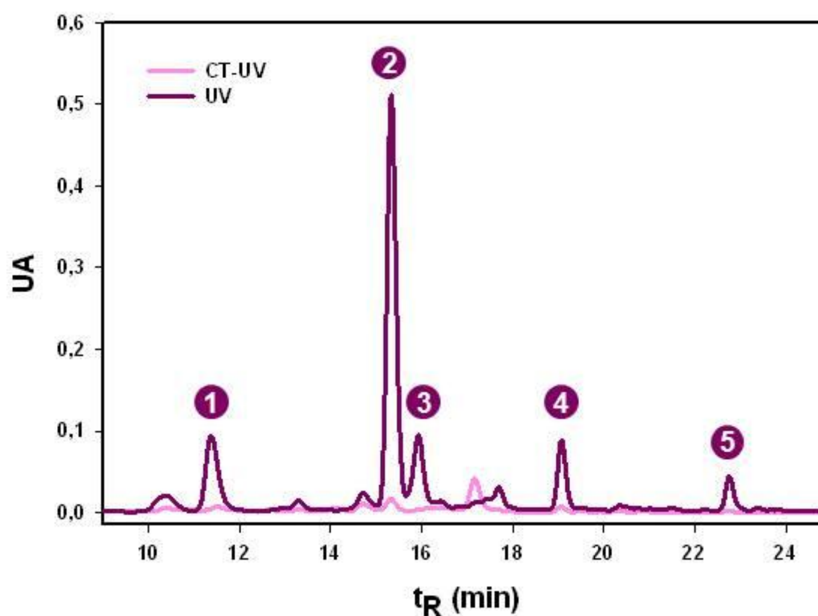


Figura V.1. Comparación cromatogramas HPLC-DAD entre el CT y el UVC a 306nm. (1) Piceatanol, (2) *trans*-resveratrol, (3) estilbeno desconocido, (4) ε-viniferina y (5) δ-viniferina.

Los espectros de masa coinciden con los descritos previamente en la bibliografía (Buiarelli y col., 2007; Püssa y col., 2006; Pezet y col., 2003).

Tabla V.1. Identificación de estilbenos por HPLC (espectro) y UPLC-DAD-TQD (datos de masas).

Compuesto	t_R (min)	Estilbeno	$[M^-]$ (m/z)	MS^2 (m/z)	λmax (nm)
1	11.2	Piceatanol	243	225/201/159/143	323.8
2	15.6	*trans*-Resveratrol	227	185/143/159	306.0
3	**16.2**	**Estilbeno desconocido**	**257**	**241/224**	**325.0**
4	18.9	ε-Viniferina	453	435/411/369/359/347	322.6
5	22.7	δ-Viniferina	453	435/411/369/359/347	321.4

En el cromatograma HPLC-DAD (Figura V.1) se observa que tras el tratamiento postcosecha con luz UVC se produce la inducción de un nuevo compuesto (compuesto 3) que anteriormente estaba por debajo del límite de detección. Su tiempo de retención (16.2 min) es muy similar al del *trans*-resveratrol por lo que se supone que posee una estructura similar. Su espectro DAD muestra un máximo a una longitud de onda de 325 nm y su masa 257. Estos datos no coinciden con ninguno de los espectros de estilbenos descritos en uva. El espectro de masas ESI^- muestra un ión molecular con una m/z de 257 y sus fragmentaciones principales tienen una m/z de 241 y 224 (Tabla V.1). Comparando estos datos con la bibliografía (Jerkovic y col., 2007) y la base de datos de masas (http://www.massbank.jp/), existen dos estructuras posibles, descritas en otras fuentes vegetales, para este estilbeno: 3,3',5'-trihidroxi-4-metoxiestilbeno, también llamado como rhapontigenin (RHA), o 4,3',5' trihidroxi-3-metoxiestilbeno, también llamado isorhapontigenin (ISOR) (Figura V.2). Dado que los espectros MS/MS y la fragmentación de ambos compuestos son idénticos no se pueden distinguir por análisis de espectrometría de masas (Jerkovic y col., 2007).

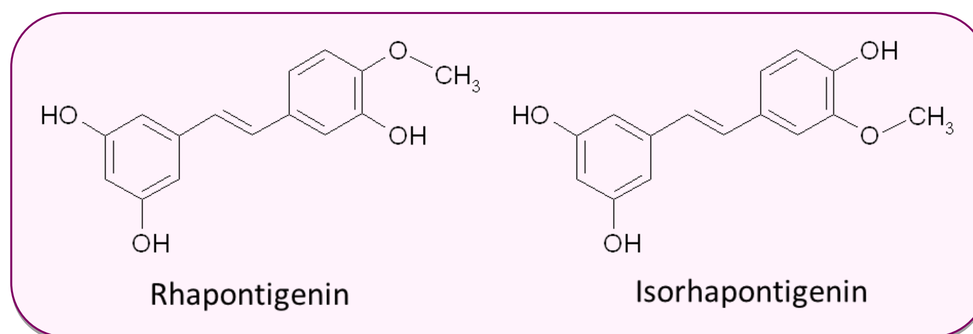


Figura V.2. Estructura química del rhapontigenin y del isorhapontigenin.

El RHA, es un estilbeno que se ha encontrado en plantas medicinales asiáticas como el ruibarbo (*Rhei undulatum*) (Ko y col., 1999). Recientemente también se ha encontrado en las bayas de *Vitis coignetiae* (Kim y col., 2009). Numerosas investigaciones han demostrado que el rhapontigenin es un compuesto con propiedades antioxidantes, antialérgicas, anticancerígenas, anticoagulantes y antiinflamatorias (Roupé y col., 2005; Roupé y col., 2006; Aburjai, 2000).

Por otro lado, el ISOR al igual que el rhapontigenin, se ha encontrado en el ruibarbo (Matsuda y col., 2001; Matsuda y col., 2004), así como en muchas especies de *Gnetum* (Ali y col., 2003; Li y col., 2004; Kato y col., 2009).

El ISOR también presenta numerosas actividades biológicas. Esto demuestra una potente actividad antioxidante *in vitro*, incluso más alta que la mostrada por la vitamina E (Wang y col., 2001; Iliya y col., 2003), actividad antialérgica (Matsuda y col., 2004), efecto cardioprotector (Li y col., 2005) y actividad antiviral (Liu y col., 2010). Ninguno de estos compuestos se ha descrito en uvas de *Vitis vinífera* (Pawlus y col., 2012).

Se realizó un diseño de aislamiento para poder identificar el compuesto desconocido. Tras el proceso de extracción, descrito previamente en el apartado 2.3, se obtuvo un extracto en el que se estimó entre 0.67-1.30 mg/Kg de bayas del compuesto desconocido, cuantificado como resveratrol, cantidad suficiente del extracto para someterse a las siguientes etapas de aislamiento.

3.2. Aislamiento del estilbeno desconocido

Como técnica de separación se utilizó la cromatografía a contracorriente (CCC). Es un tipo de cromatografía líquido-líquido, donde tanto la fase estacionaria como la fase móvil son líquidas. La CCC es una técnica que separa compuestos por sus diferentes coeficientes de distribución en dos solventes inmiscibles. Desde su nacimiento hace casi 60 años esta tecnología se ha desarrollado cada vez más. Una de las principales ventajas de esta técnica es que al eliminar los soportes sólidos, se evita la adsorción permanente de la sustancia analizada en la columna, y se puede recuperar casi el 100 % de la muestra.

Se utilizaron dos equipos de CCC. Primero se utilizó el Spiral Coil LSRCCC ya que en este equipo se puede inyectar mayor cantidad de muestra. El sistema de disolventes utilizado fue hexano-acetonitrilo (1:1). Este sistema disolvente hidrófobo se recomienda para la separación de compuestos no polares (Ito, 2005). 5.9 g del extracto de piel de uva se disolvió en 250 ml de hexano:acetonitrilo (1:1) y se inyectó una vez alcanzado el equilibrio hidrodinámico. De este sistema se obtuvieron 13 fracciones (F1-F13) que fueron analizadas por cromatografía en capa fina y por HPLC-EIS-MS/MS se observó que tanto el compuesto desconocido como otros estilbenos se encontraban en las 4 primeras fracciones (F1-F4). Estas cuatro fracciones (442 mg) se combinaron para una posterior separación por HSCCC. En este caso se utilizó una mezcla de hexano/acetato de etilo/metanol/agua (3:5:3:5 v/v/v/v) como disolventes. En esta nueva separación se obtuvieron 8 fracciones (FF1-FF8) (Figura V.3).

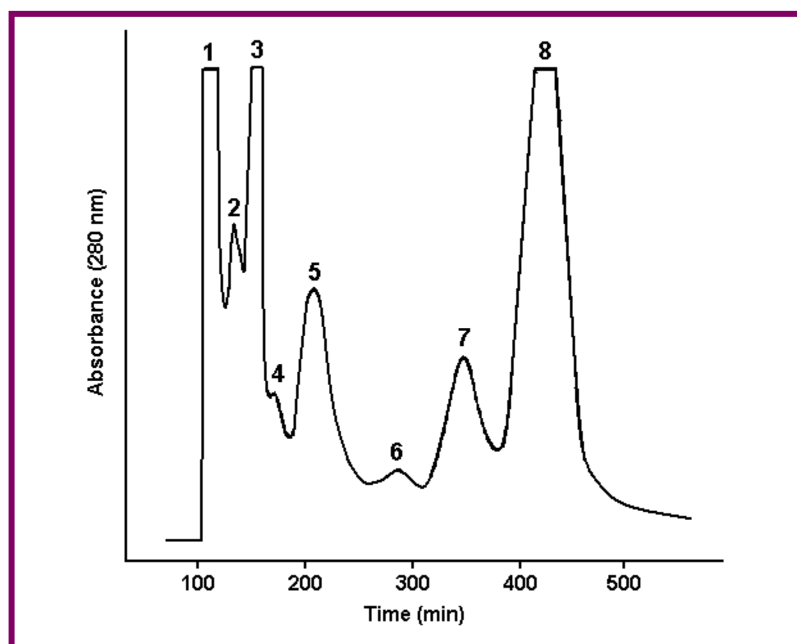


Figura V.3. Cromatograma del HSCCC del extracto de piel de uva (FF1-FF8).

Las fracciones resultantes se analizaron por TLC y HPLC-ESI-MS/MS (Tabla V.2).

Tabla V.2. Datos de masas y cuantificación de las fracciones obtenidas por HSCCC.

Fracción	Cantidad (mg)	$[M-H]^-$ (m/z)	Fragmentaciones	Compuesto principal
FF2	11.11	453	435/ 411/ 359/ 317/289	Palidol
FF3	12.51	453	435/ 411/ 359/ 317/289	Palidol
FF4	4.0	417 395	409/329/255 313/227	Compuestos desconocidos (Posibles dímeros)
FF5	34.8	243 469 679	225/201/ 159/ 143 451/375/ 363 661/637/573/555/479/ 273/239	Piceatanol Ampelopsin D α-Viniferina
FF6	5.27	453	435/411/369/359/347	δ-Viniferina
FF7	13.0	453	435/411/369/359/347	ε-Viniferina
FF8	22.31	227 257	185/143/159 241/224	Resveratrol **Estilbeno desconocido (Compuesto 3)**

La fracción 1 (FF1) resultó ser una montaña de numerosos compuestos de naturaleza no estilbénica mayoritariamente. En las fracciones 2 y 3 (FF2 y FF3) se detectó de forma mayoritaria un compuesto con m/z de 453. Podría ser una viniferina pero su patrón de fragmentación difiere de lo descrito en la bibliografía (Pezet y col., 2003, Püssa y col., 2006). Dicho compuesto podría ser tentativamente identificado como palidol, que muestra el mismo patrón de fragmentación de masas (Mulinacci y col., 2010) y una longitud de onda máxima a 280 nm de acuerdo con la bibliografía (Vitrac y col., 2002; Landrault y col., 2002).

La fracción 4 (FF4) presentó varios compuestos no identificados cuyos datos espectrales de masa podría estar relacionado con dímeros de estilbenos (Mulinacci y col., 2010). Debe tenerse en cuenta que el tratamiento UVC puede inducir otros estilbenos, así como reacciones de oxidación.

La fracción 5 (FF5) presenta mayoritariamente piceatanol, α-viniferina (Püssa y col., 2006) y un dímero de resveratrol que podría ser ampelosin D teniendo en cuenta sus datos de masa (Vergara y col., 2012). Ampelopsin D se ha descrito previamente en la familia *Vitaceae* (Jeandet y col., 2002; Mattivi y col., 2011; Pawlus y col., 2012) y se ha sugerido que su síntesis proviene de los precursores ε-viniferina y δ-viniferina (Püssa y col., 2006).

Las fracciones 6 y 7 (FF6 y FF7) contenían principalmente *δ*-viniferina y *ε*-viniferina respectivamente, de acuerdo con la Tabla V.1 y otros autores (Pezet y col., 2003; Püssa y col., 2006).

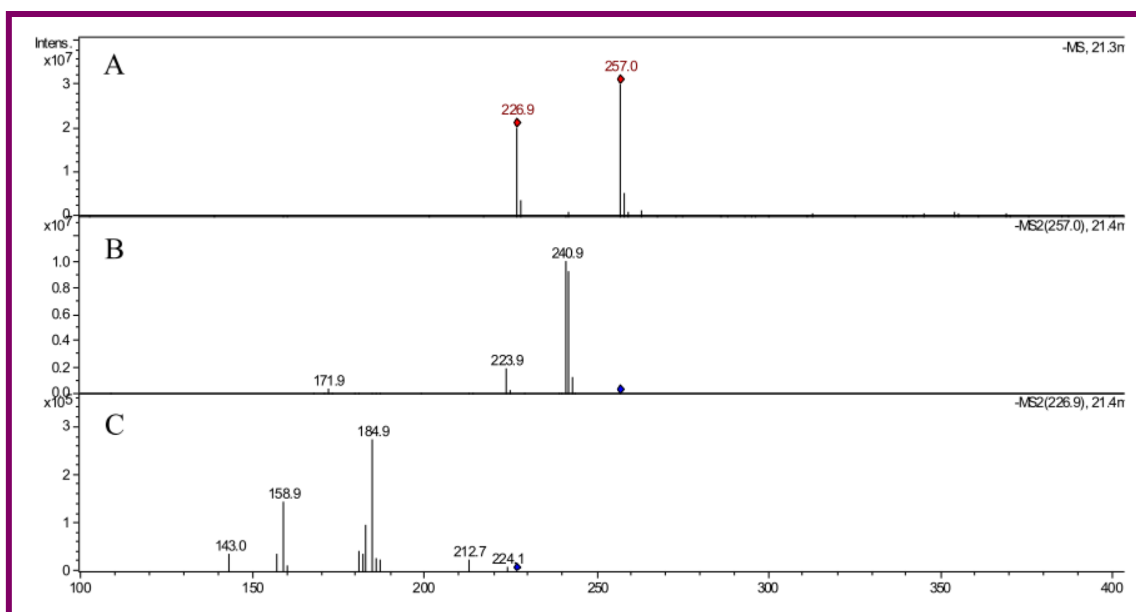


Figura V.4. Espectro MS/MS y fragmentaciones de la fracción 8 (FF8) del HSCCC. (A) ión molecular de m/z 227 y 257 (B) fragmentación de pico de m/z 257 (C) fragmentación de pico de m/z 227.

La fracción 8 (FF8) contenía *trans*-resveratrol y el estilbeno desconocido. La identificación de *trans*-resveratrol (Figura V.4C) se hizo por comparación con el estándar puro, su principal fragmentación tiene una m/z de 185 y representa la pérdida de una cetena. El estilbeno desconocido se caracteriza por tener un ión molecular de m/z 257 y su fragmentación principal con una m/z 241 (Figura V.4B). Este pico se dedujo a partir de la pérdida de radical metilo que produjo el ion orto-quinona después de reagrupamiento.

Para enriquecer la muestra en el compuesto desconocido la FF8 (22.31 mg) se inyecta en el sistema HPLC semipreparativo. Se obtuvieron 6 fracciones, tras su análisis por HPLC-DAD-MS se observó que sólo la primera fracción (12.7mg) contenía tanto el compuesto desconocido como el *trans*-resveratrol. El resto de las fracciones fueron descartadas. Esta nueva fracción se inyectó nuevamente por HPLC semipreparativo para purificarla obteniéndose finalmente una fracción de 7.9 mg que contenía un 97% de *trans*-resveratrol y un 3% del estilbeno desconocido. Finalmente esta fracción fue la que se usó para la identificación por RMN.

3.3 Identificación del estilbeno desconocido por RMN

Las estructuras fueron identificadas por RMN en 1D y 2D (1D y 2D (^{1}H-^{1}H) COSY, 2D (^{1}H-^{1}H) ROESY, 2D (^{1}H-^{13}C) HSQC y 2D (^{1}H-^{13}C) HMBC).

Los datos de ^{1}H-RMN y de ^{13}C-RMN para el compuesto desconocido se muestran en la Tabla V.3, Figura V.5). El compuesto desconocido resultó ser el isorhapontigenin. Los espectros de RMN de la fracción analizada mostraron la presencia de dos compuestos: *trans*-resveratrol e isorhapontigenin. El *trans*-resveratrol fue confirmado por comparación con las referencias en la bibliografía (Mattivi, Reniero y Korhammer, 1995). Debido a las señales solapadas, la presencia del isorhapontigenin fue confirmado a través de 2D-RMN (Figura V.6). Estos datos concuerdan con los descritos para el isorhapontigenin aislado a partir de bulbos de *Scilla nervosa* (Silayo y col., 1999).

Tabla V.3. Datos del espectro de RMN del isorhapontigenin en d_6-acetona.

Nº	δ_C	δ_H J(Hz)	COSY	HMBC
1	129.1	-	-	H-8
2	109.6	7.21 *d*(2.0)	H-6	H-6, H-7
3	148.5	-	-	H-5, OMe
4	147.1	-	-	H-2, H-6
5	115.9	6.82 *brd*(8.2)	H-6	-
6	120.8	6.97 *brd*(8.2)	H-2, H-5	H-2, H-7
7	128.6	7.00 *d*(16.5)	H-8	H-2, H-6, H-8
8	126.4	6.93 *d*(16.5)	H-7	H-7, H-10(14)
9	140.6	-	-	H-7, H-8
10(14)	105.1	6.61 *brs*	H-12	H-8, H-12
11(13)	159.1	-	-	H-12, H-10(14)
12	102.1	6.27 *brs*	H-10/14	H-10(14)
OMe	56.2	3.86 *s*	-	-

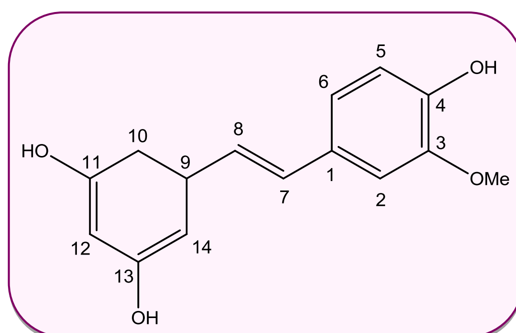


Figura V.5. Estructura química del isorhapontigenin.

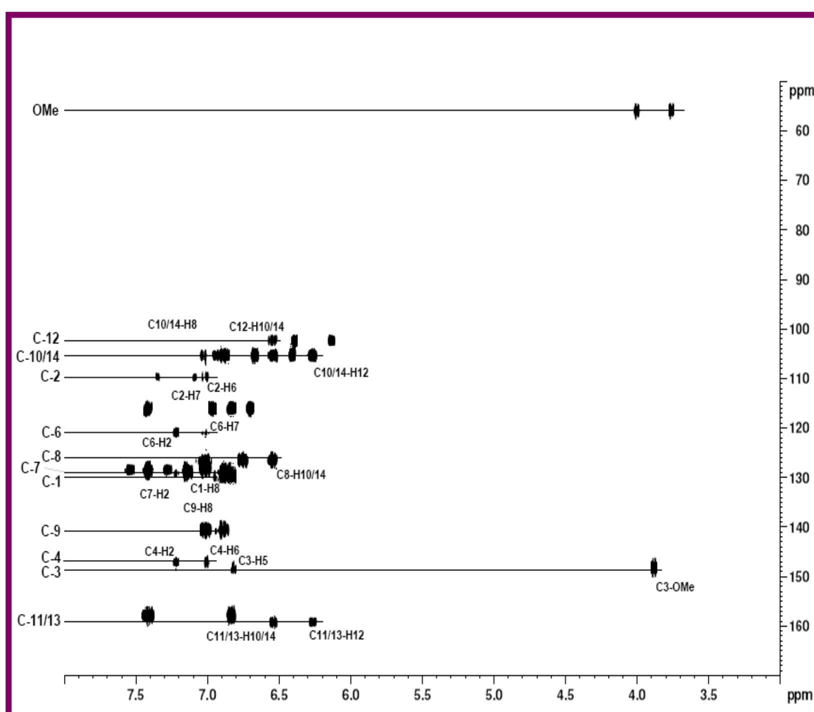


Figura V.6. Espectro HMBC del isorhapontigenin.

Los datos del espectro de ^{1}H-RMN del isorhapontigenin indicaron la presencia de ocho protones de resonancias arilo/vinilo. Los datos espectrales de ^{13}C-RMN mostraron trece señales arilo/vinilo, y no hay resonancias de carbonilo. Los datos espectrales de ^{1}H-RMN mostró un par de señales de protones olefínicos en δ_H 6.93 y 7.00 (*d*, J = 16.2 Hz).

La gran constante de acoplamiento indica una geometría *trans* (Mattivi y col., 1995), un conjunto de tres de protones de resonancia en δ_H 7.21, 6.82 y 6.97 asignado a un grupo fenilo 1,3,4-trisustituido, un conjunto de dos señales de protones a δ_H 6.27, 6.61 asignado a un segundo grupo fenilo 1,3,5-trisustituido y un grupo metoxilo aromático en δ_H 3.86. Los datos anteriores son compatibles con un esqueleto de estilbeno.

Los datos de ^{13}C-RMN del isorhapontigenin también concuerdan con la estructura asignada (Tabla V.3). En el 2D-(^{1}H-^{13}C)-HMBC, se observó una correlación de protones del *O*-metil (δ_H 3.86) con C-3 (δ_C 148.5) que sugieren la ubicación del grupo *O*-metilo en el C-3 (Figura V.6). El pico de cruce entre H-2 (δ_H 7.21) y el protón *O*-metilo (δ_H 3.86) en el espectro 2D (^{1}H-^{1}H) ROESY confirmó la localización de este grupo *O*-metilo (Figura V.6).

En el presente trabajo el isorhapontigenin (ISOR), estilbeno presente en muchas plantas medicinales tradicionales, es descrito por primera vez en uvas, siendo ésta la única fuente dietética conocida. La concentración en uvas puede ser incrementada notablemente a mediante tratamiento postcosecha con luz UVC.

Se ha demostrado que el ISOR, aislado de diferentes fuentes, presenta propiedades beneficiosas sobre la salud tales como antioxidantes y protectores. De forma que su presencia en uva podría aumentar las propiedades saludables de esta. En cualquier caso, se necesitan estudios más detallados sobre la importancia biológica de este compuesto en la dieta.

La publicación correspondiente a este Capítulo se encuentra en el Anexo 5.

CAPÍTULO VI

AUMENTO DE ESTILBENOS EN VINO POR APLICACIÓN DE DIFERENTES TRATAMIENTOS EN UVA

1. INTRODUCCIÓN

El consumo moderado de vino, en especial del tinto, tiene efectos beneficiosos para la salud. Científicamente, se han demostrado numerosas propiedades tales como antioxidantes, anticancerígenos o antihistamínicas, entre otras (Pignatelli y col., 2006). Estos efectos son debidos en gran parte a algunos de sus componentes, entre los que han destacado los estilbenos, especialmente el resveratrol.

La uva y el vino constituyen la principal fuente de resveratrol en la dieta. De hecho, existen estudios donde se demuestra que dosis relativamente baja de resveratrol adquirido con regularidad a partir de vino tinto u otras fuentes de la dieta podría ser terapéutico (Bertelli y col., 1998). Aparte de resveratrol, se han descrito la presencia de otros estilbenos en el vino, tales como piceído, astringina, piceatanol y viniferinas. Además, recientemente se ha descrito un nuevo estilbeno en uva: el isorhapongenin, que también podría encontrarse en vinos (Fernández-Marín y col., 2012). Todos estos estilbenos, al igual que el resveratrol, exhiben una pronunciada actividad antioxidante, sin embargo sus concentraciones en ambos, uva y vino, son bastante bajas (Guerrero y col., 2009).

La concentración de resveratrol en uva, y por tanto en vinos varía según distintos factores: variedad de uva, región geográfica, factores agronómicos, clima, estrés de la planta y prácticas enológicas (Bavaresco y col., 2009; Stervbo y col., 2006). Además, la concentración de estos compuestos se puede aumentar debido a que son fitoalexinas y, por lo tanto, se pueden inducir por diferentes tipos de estrés. De entre los diferentes tipos de estrés, el tratamiento precosecha con metil jasmonato (MEJA), el tratamiento postcosecha ultravioleta C (UVC) y su combinación (Capítulo IV) han sido sugeridos como métodos adecuados para aumentar el resveratrol en uvas (Larronde y col., 2003; Guerrero y col., 2010a).

Por otra parte, una vez que la uva ha sido enriquecida en estilbenos, el proceso de vinificación también puede ser optimizado en cada una de sus etapas para obtener vinos tintos enriquecidos en estilbenos (Guerrero y col., 2010b).

El objetivo de este Capítulo fue la combinación de todos los resultados previos acerca de cómo obtener uvas y vinos enriquecidos en estilbenos. Para ello se seleccionó la variedad Syrah y el terruño Andaluz de Jerez de la Frontera. El viñedo fue tratado previamente con metil jasmonato y después de la vendimia se trataron las uvas con luz UVC. Por último, la vinificación se desarrolló de forma óptima para maximizar la extracción de estos compuestos desde la uva hasta el vino, y de esta manera obtener vinos enriquecido en estilbenos.

2. MATERIALES Y MÉTODOS

2.1. Material vegetal.

Este trabajo se ha desarrollado sobre la variedad Syrah cultivada en la viña experimental del Centro IFAPA "Rancho de la Merced", ubicada en Jerez de la Frontera durante la vendimia 2011. Fue seleccionada debido a que mostró mayor capacidad de inducción en capítulos previos (Capítulo III).

La maduración de la uva fue monitorizada semanalmente desde el envero hasta la cosecha para determinar la fecha óptima de la vendimia.

2.2. Tratamiento precosecha con Metil Jasmonato.

El experimento se estableció utilizando un diseño en bloques al azar constando de tres repeticiones de 10 cepas por cada tratamiento (CT y MEJA).

El tratamiento con MEJA se realizó por pulverización, la primera aplicación 20 días antes de la fecha de vendimia y las siguientes, tres y cinco días después de esta primera aplicación siguiendo el protocolo propuesto por Vezzulli y col. (2007). Para ello, se preparó una concentración de 10 mM de MEJA disuelto en etanol absoluto que fue suministrado empleando 50 ml de dicha disolución por cepa en cada aplicación. Las cepas control se pulverizaron con etanol absoluto.

Una vez terminado el tratamiento se procedió a la toma de muestra para ver la evolución de estilbenos tras el tratamiento. La primera muestra se tomó transcurridas 24 h de la

última aplicación y el resto de las muestras se cogieron cada 48 horas hasta alcanzar el día óptimo de vendimia. En total se cogieron 4 muestras (Tabla VI.1).

2.3. Tratamiento postcosecha UVC y periodo de inducción.

Una vez alcanzado el grado de madurez óptimo se procedió a la vendimia. Se vendimiaron 88 Kg de uvas CT y 88 Kg de uvas MEJA. Cada uno de estos lotes se dividió en dos obteniendo un total de cuatro lotes, como se muestra en la Figura VI.1.

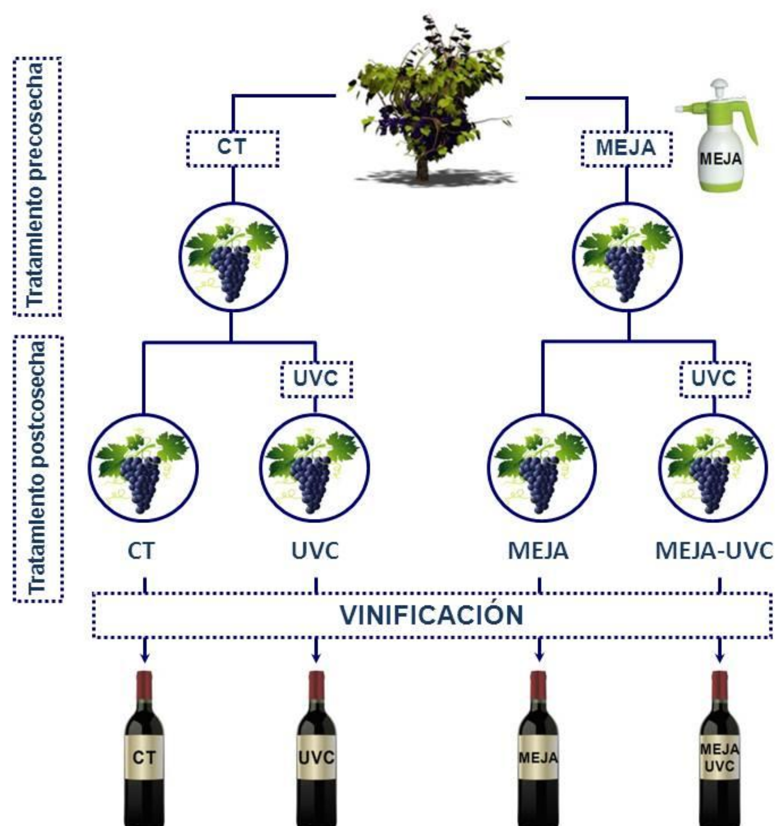


Figura VI.1. Esquema del ensayo realizado sobre la variedad Syrah.

El primer lote (CT) se formó con uvas que no fueron tratadas. El segundo lote (UVC) se formó con las uvas que no fueron tratadas con MEJA pero sí con el tratamiento postcosecha con UVC. El tercer lote (MEJA) fue el formado por las uvas tratadas sólo con MEJA. Por último, el cuarto lote (MEJA-UVC) se formó con uvas que recibieron ambos tratamientos.

El tratamiento UVC se realizó por triplicado y se llevó a cabo de acuerdo con el procedimiento descrito por la patente WO/2002/085137; ES 2177465. Tras el tratamiento la uva necesita de un periodo de almacenamiento para que ésta alcance la máxima concentración de *trans*-resveratrol (Guerrero y col., 2010a). En el Capítulo V de esta tesis se determinó que el periodo de almacenamiento óptimo para uvas tratadas con MEJA y UVC debería ser de cuatro días. Por esta razón, todos los lotes se almacenaron a 20 ºC hasta el cuarto día.

2.4. Proceso de vinificación

Después de cuatro días de almacenamiento, cada lote fue despalillado, estrujado y encubado en tanques de acero inoxidable de 10 L. El proceso de fermentación se optimizó para evitar la pérdida de resveratrol (Guerrero y col., 2010a).

La fermentación alcohólica se realizó a una temperatura controlada con inoculación de levadura seca activa (Actiflore F33, Laffort, Francia, 20 g/hl). Durante la fermentación se realizaron remontados y bazuqueos (uno por día) así como controles de densidad y temperatura diarios. Tan pronto como la fermentación tumultuosa había terminado el vino fue prensado (Willmes, Alemania).

Después de 24 horas, la fermentación maloláctica se indujo inoculando la bacteria láctica *Oenococcus oeni* (1 g/hl, Challenge EASY ML, Sepsa-Enartis, España) acompañada de nutrientes (20 g/hl Nutriferm ML, Sepsa-Enartis, España). Una vez terminada esta etapa (concentración de ac. málico menor de 0.3 mg/L), los vinos se trasegaron y se almacenaron en la cámara frigorífica (a 2ºC) hasta su embotellado.

Los parámetros enológicos básicos del mosto/vino obtenido se determinaron en las etapas de encubado, prensa, deslío y embotellado. Para el análisis de estilbenos se tomaron muestras de líquido en las mismas etapas descritas con anterioridad y durante toda la fermentación alcohólica y muestras de sólido sólo en prensa, deslío y embotellado.

La vinificación de cada lote se realizó por triplicado.

2.5. Extracción de estilbenos.

Para las muestras sólidas (piel, orujo, lías y tartratos) se siguió el protocolo descrito en el Capítulo III.2.5. En el caso de las muestras líquidas (mostos y vinos) la extracción se realizó siguiendo el mismo protocolo pero adaptado a muestras líquidas.

2.6. Identificación y cuantificación de estilbenos.

Los estilbenos se determinaron según el método detallado en el Capítulo III.2.6.

2.7. Parámetros enológicos en uva y vino

En cada una de las etapas se determinaron los siguientes parámetros enológicos en mosto como: Brix, Acidez Total, pH, ác. tartárico, ác. málico, potasio, antocianos, taninos, intensidad colorante, tonalidad, CIELAB. Al vino final se le determinó el grado alcohólico, acidez total y volátil, pH, extracto seco, glicerina, azúcar, taninos, antocianos, metales (Cu, Fe, Zn y K), ácidos orgánicos (ácidos cítrico, málico, tartárico, láctico, succínico y acético) y alcoholes superiores de acuerdo a los métodos oficiales (OIV, 1990; Saint-Criq y col., 1998).

2.8. Análisis sensorial

Para la descripción de las características organolépticas de los vinos se estableció una ficha de cata con los atributos más susceptibles de ser afectados por el proceso de elaboración del vino: apariencia, intensidad del color, intensidad aromática, afrutado, herbáceo, intensidad del sabor, persistencia, astringencia y amargor. El panel estuvo formado por 10 expertos catadores. Cada atributo se puntuó entre 1 (baja) y 10 (alta), dependiendo de la intensidad percibida por el panelista.

2.9. Análisis estadístico

El análisis de los datos se realizó utilizando el software estadístico Statistix versión 8.0. Los datos se sometieron al análisis de la varianza (ANOVA) seguido del método Tukey con un nivel de significación de $p \leq 0.05$.

3. RESULTADOS Y DISCUSIÓN

3.1. Tratamiento precosecha con Metil Jasmonato.

Una vez aplicado el tratamiento precosecha con MEJA se tomó muestra hasta el día de vendimia para ver la evolución de los estilbenos. Como se puede observar en la Tabla VI.1, piceatanol y *trans*-resveratrol fueron los únicos estilbenos que se pudieron cuantificar en las muestras.

Tabla VI.1. Concentración de estilbenos (mg/Kg uva) tras el tratamiento con MEJA

	Piceatanol		*trans*-Resveratrol	
	CT	**MEJA**	**CT**	**MEJA**
24h tras tr. MEJA	n.d.	0.298	0.943[b]	2.466[a]
72h tras tr. MEJA	n.d.	0.216	1.155[b]	1.842[a]
120h tras tr. MEJA	n.d.	0.197	0.776[b]	1.401[a]
148h tras tr. MEJA (vendimia)	n.d.	0.175	0.742[b]	1.494[a]

tr. tratamiento; nd, no detectado. Los superíndices a y b indican diferencias significativas entre tratamientos (nivel de significación $p < 0,05$).

El tratamiento precosecha con MEJA aumentó significativamente las concentraciones de piceatanol y *trans*-resveratrol respecto a su control (Tabla VI.1). Es destacable que el aumento en ambos compuestos se produce tras la aplicación del tratamiento y va disminuyendo progresivamente hasta la vendimia.

Las concentraciones obtenidas, en las uvas tratadas, en el día de la vendimia fueron 0.175 mg/Kg uvas para piceatanol y 1.494 mg/Kg uvas para *trans*-resveratrol. Dichas concentraciones fueron significativamente mayores que la de los controles, ya que se obtuvo 0.742 mg/Kg uva de *trans*-resveratrol y piceatanol no se detectó (Tabla VI.1). Estos resultados concuerdan con otros obtenidos por Larronde y col. (2003). Los autores describieron un aumento de la concentración de *trans*-resveratrol en uvas

durante 15 días después de ser tratadas con vapores de MEJA y posteriormente se produce un descenso en la concentración conforme avanza la maduración de la uva.

En cuanto a los parámetros enológicos de la uva realizados en el día de la vendimia, sólo se observó un ligero aumento en el contenido de azúcar (19.5 ºBrix para CT, 21.5 ºBrix para MEJA) y de antocianos (1332 mg/kg de uvas para la CT y 1449 mg/kg de uvas para MEJA) en las uvas tratadas con MEJA en comparación con las uvas control (datos no mostrados). Estos datos concuerdan con resultados obtenidos por otros autores (Ruiz-García y col., 2012).

3.2. Tratamiento Postcosecha UVC.

Una vez que se realizó la vendimia, las uvas se dividieron en cuatro lotes como se detalló en el apartado de materiales y métodos (Figura VI.1). Dos lotes recibieron el tratamiento postcosecha UVC y posteriormente los cuatro lotes (CT, MEJA, UVC, MEJA-UVC) se almacenaron a 20ºC y 80% h.r. durante cuatro días, como se explicó anteriormente, buscando la máxima concentración de *trans*-resveratrol.

Durante el periodo de almacenamiento (cuatro días) se siguió el contenido de estilbenos de todos los lotes (Figura VI.2).

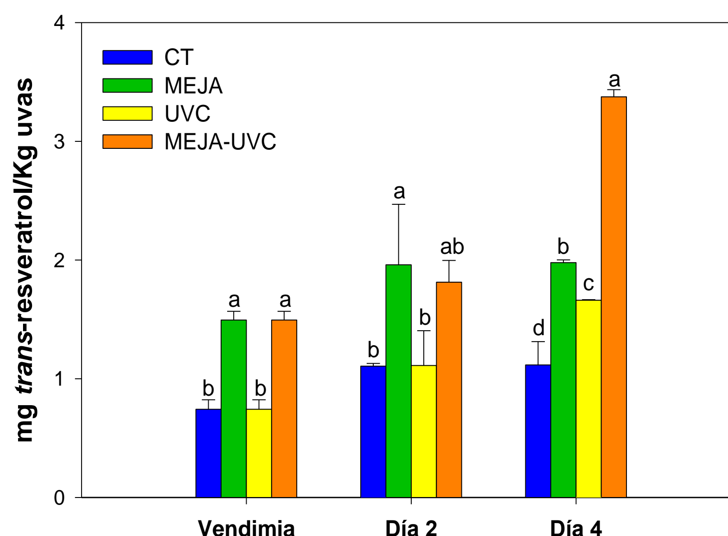


Figura VI.2. Contenido de *trans*-resveratrol tras el tratamiento postcosecha UVC durante el periodo de conservación. Las letras a, b, c, d indican diferencias significativas entre tratamientos (nivel de significación p <0,05).

Como era de esperar, en el día de la vendimia, las uvas tratadas con MEJA (MEJA y MEJA-UVC) mostraron un mayor contenido en *trans*-resveratrol respecto a sus controles (CT y UVC). Durante los cuatro días, todos los lotes aumentaron su contenido en *trans*-resveratrol, sin embargo, la velocidad de inducción no fue igual para todos. MEJA y MEJA-UVC tuvieron una velocidad de inducción mayor, por tanto, estos lotes alcanzaron mayores concentraciones de *trans*-resveratrol al final del periodo de almacenamiento (Figura VI.2).

El análisis de la varianza (ANOVA) realizado mostró diferencias significativas ($p<0.05$) en la concentración de *trans*-resveratrol en los cuatro lotes trascurridos los cuatro días. La concentración varió de la siguiente forma creciente: CT< UVC< MEJA< MEJA-UVC (Figura VI.2). La luz UVC provoca un estrés mayor que el que se obtiene con el tratamiento con MEJA, debiendo de alcanzar valores más altos en la concentración de los estilbenos. Sin embargo para ello el tratamiento con UVC necesita más tiempo de inducción (Capítulo IV.3.1.3).

Además del *trans*-resveratrol, durante dicho periodo de almacenamiento, se indujeron otros estilbenos (piceatanol, isorhapontigenin y ε-viniferina) (Tabla VI.2). En el día de la vendimia sólo se muestran los datos del CT y del MEJA ya que todavía no se había realizado el tratamiento postcosecha UVC. En ese mismo día sólo se encuentra piceatanol en las uvas tratadas con MEJA. Tras dos días de almacenamiento (día 2, Tabla VI.2) se encontró piceatanol en todos los lotes, mientras que isorhapontigenin sólo se encontró en las muestras tratadas con MEJA (MEJA y MEJA-UVC). En el último día de almacenamiento (d4, Tabla VI.2) se detectó piceatanol, isorhapontigenin y ε-viniferina en todos los lotes. La tendencia de estos compuestos entre los distintos lotes fue similar a la encontrada para el *trans*-resveratrol. El lote CT fue el que mostró concentraciones más bajas de estilbenos, en contraste con el MEJA-UVC que mostró concentraciones más altas. Los lotes MEJA y UVC alcanzaron concentraciones intermedias y semejantes.

Tabla VI.2. Concentración de estilbenos (mg/Kg uvas) durante el periodo de almacenamiento tras el tratamiento UVC.

	Piceatanol				Isorhapontigenin				ε–viniferina			
	CT	**MEJA**	**UVC**	**MEJA-UVC**	**CT**	**MEJA**	**UVC**	**MEJA-UVC**	**CT**	**MEJA**	**UVC**	**MEJA-UVC**
Vendimia	nd	0.175	-	-	nd	Nd	-	-	nd	nd	-	-
Día 2	0.147	0.209	0.140	0.192	nd	0.350	nd	0.225	nd	nd	nd	nd
Día 4	0.121[b]	0.222[ab]	0.173[b]	0.323[a]	0.145[c]	0.214[bc]	0.244[b]	0.402[a]	0.144[c]	0.288[b]	0.207[bc]	0.394[a]

nd, no detectado. Los superíndices indican diferencias significativas entre tratamientos (nivel de significación p <0,05)

Por otra parte, se determinaron los parámetros enológicos durante todos los tratamientos realizados a cada lote para asegurar la calidad de la uva y por tanto del vino. En la Tabla VI.3 se muestran los parámetros enológicos tras el cuarto día de almacenamiento. Se observó que no existieron diferencias significativas entre los lotes en cuanto al contenido en azúcares, pH y ácido tartárico. El contenido en potasio fue menor para las muestras tratadas con el tratamiento postcosecha UVC (UVC y MEJA-UVC) y la acidez total fue menor para las muestras tratadas con MEJA (MEJA y MEJA-UVC). Así, las muestras tratadas con MEJA (MEJA y MEJA-UVC) mostraron un mayor índice de maduración (relación azúcar/acidez total), que coincide con los valores obtenidos en el día de la vendimia y también coincide con resultados obtenidos por otros autores (Ruiz-García y col., 2012).

Tabla VI.3. Parámetros enológicos en el encubado.

	CT	MEJA	UVC	MEJA-UVC
ºBrix	21.13	21.57	21.57	21.63
Acidez total (g/L)	4.93[a]	4.53[b]	4.93[a]	4.63[b]
pH	3.57	3.57	3.57	3.57
Ácido Tartárico (g/L)	5.18	5.04	5.67	5.35
K^+ (mg/L)	2085[a]	2052[a]	1871[c]	1991[b]
Índice Maduración	42.60[b]	47.46[a]	43.65[b]	46.90[a]

Los superíndices indican diferencias significativas entre tratamientos (nivel de significación p <0,05)

3.3. Proceso de vinificación

El proceso de vinificación se realizó de acuerdo con estudios previos para evitar la pérdida y oxidación del contenido en estilbenos (Guerrero y col., 2010b). En cada paso del proceso se controlaron tanto los parámetros enológicos como el contenido en estilbenos. La fermentación alcohólica transcurrió durante seis días y su evolución fue similar en todos los lotes (datos no mostrados).

En la Figura VI.3 se muestra la evolución del contenido en *trans*-resveratrol de los cuatro lotes desde el encubado hasta el embotellado. Se observó que los cuatro tratamientos evolucionaron de forma similar, primero se produjo un gran incremento en el contenido de *trans*-resveratrol desde el encubado hasta el segundo día de la fermentación alcohólica. Posteriormente se observó un ligero descenso en la concentración conforme avanzaba la fermentación hasta el día del prensado y finalmente continuó con un descenso más acentuado en las etapas del deslío y del embotellado.

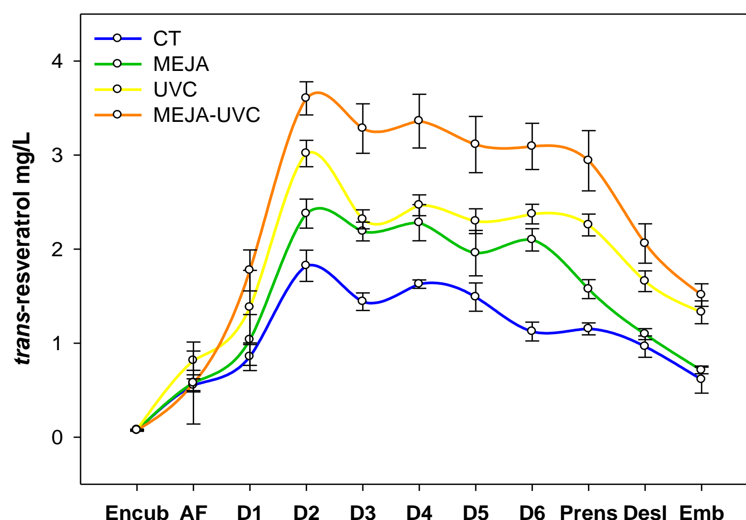


Figura VI.3. Evolución del *trans*-resveratrol (mg/L) desde el encubado hasta el embotellado (Encub=encubado; AF=antes de la fermentación alcohólica; D1, D2, D3, D4, D5, D6= días de la fermentación alcohólica; Prens=prensado; Desl=deslío; Emb= embotellado).

Respecto a las diferencias en cuanto al contenido en *trans*-resveratrol entre los diferentes lotes podemos decir que siguió el siguiente orden creciente: CT<MEJA< UVC<MEJA-UVC (Figura VI.3). En estas etapas del proceso el lote UVC obtuvo mayores concentraciones que el MEJA, no coincidiendo con los resultados obtenidos en el cuarto día de conservación (Figura VI.2). Nuestra hipótesis es que las uvas del lote UVC pudieron continuar la inducción de estilbenos unas cuantas horas más durante su encubado.

Durante la fermentación alcohólica, además del *trans*-resveratrol se detectó piceatanol a partir del tercer día de fermentación alcohólica pero sólo en las muestras tratadas con luz UVC (UVC, MEJA-UVC) (datos no mostrados).

En el prensado se encontraron las mayores diferencias en cuanto al contenido de estilbenos y a los parámetros enológicos, coincidiendo con trabajos realizados previamente (Guerrero y col., 2010b). Tras el prensado, en los vinos se encontró piceatanol, *trans*-resveratrol y ε-viniferina. Las diferencias en el contenido en *trans*-resveratrol fueron significativas entre los lotes excepto para el CT y el MEJA que tuvieron concentraciones similares. El piceatanol se detectó en todas las muestras aunque sólo se pudo cuantificar en las muestras tratadas con luz UVC (UVC y MEJA-UVC), siendo significativamente más alto en las muestras MEJA-UVC que en las UVC. En cuanto a la ε-viniferina, las concentraciones que se obtuvieron fueron bajas, variando desde 0.097 mg/L hasta 0.157 mg/L (Tabla VI.4).

Además, en esta etapa también se analizaron los orujos para ver si la totalidad de los estilbenos se habían trasferido al vino (Tabla VI.5). En las muestras sólidas detectamos pequeñas cantidades de isorhapontigenin (0,549 a 1,277 mg/kg orujo), compuesto que no se detectó el vino prensado. También nos encontramos que había *trans*-resveratrol y ε-viniferina. El contenido de ε-viniferina en los orujos fue mayor que en el vino ya que, debido a su carácter hidrófobo, su solubilidad en el vino es baja (Karacabey y Mazza, 2008; Guerrero y col., 2010b) (Tabla VI.5). Así, el orujo de los lotes estaban enriquecidos en estilbenos, especialmente las muestras del lote MEJA-UVC, y podrían ser una fuente de estilbenos para la fabricación de productos nutracéuticos.

En cuanto a los parámetros enológicos que se realizaron en el prensado, podemos decir que los parámetros básicos como el pH, ácido tartárico, ácido málico y potasio fueron similares entre los lotes (Tabla VI.4). Sólo la acidez total fue mayor para los lotes tratados con UVC (UVC y MEJA-UV). Sin embargo, los parámetros relacionados con el color mostraron algunas diferencias significativas. La concentración de antocianos siguió el siguiente orden creciente: CT <MEJA ≅ UVC <MEJA-UVC, datos que concuerdan con los obtenidos en la uva en la vendimia. Ésta tendencia también se observó para la intensidad del color, y lo contrario para el parámetro *L. Además, b* fue significativamente diferente entre los cuatro tratamientos. Como era de esperar, el aumento de los antocianos provocó un aumento en la intensidad del color, lo que disminuyo la luminosidad (valores menores de L*) y aumento el amarillo (valores mayores de b*) (González-Manzano y col., 2009). La concentración de taninos resultó mayor para las muestras MEJA-UVC. Por último, decir que no se encontraron diferencias significativas en el parámetro a* y en la tonalidad (Tabla VI.4).

Tabla VI.4. Contenido de estilbenos y parámetros enológicos en el vino prensa y en embotellado.

	Vino Prensa				Vino Embotellado			
	CT	**MEJA**	**UVC**	**MEJA-UVC**	**CT**	**MEJA**	**UVC**	**MEJA-UVC**
Piceatanol (mg/L)	trz	trz	0.794^{b}	1.089^{a}	0.336^{b}	0.486^{a}	0.489^{a}	0.676^{a}
***trans*-Resveratrol (mg/L)**	1.152^{c}	1.574^{c}	2.257^{b}	2.940^{a}	0.613^{b}	0.710^{b}	1.328^{a}	1.512^{a}
ε-Viniferina (mg/L)	0.097^{d}	0.145^{c}	0.116^{b}	0.157^{a}	0.132^{b}	0.149^{a}	0.129^{b}	0.131^{b}
Estilbenos totales (mg/L)	1.249^{d}	1.719^{c}	3.167^{b}	4.186^{a}	1.082^{d}	1.345^{c}	1.946^{b}	2.319^{a}
AT (g/L TH_2)	7.23^{b}	7.29^{b}	7.47^{a}	7.62^{a}	5,12	5,15	5,11	5,26
pH	3.56	3.59	3.56	3.54	3,64	3,66	3,65	3,63
Etanol (%vol.)					10.88	11.24	11.43	10.94
AV (g/L AcH)					0.36	0.37	0.35	0.36
Ác. tartárico (g/L)	3.69	3.47	3.64	3.84	1.40	1.28	1.45	1.40
Ác. málico(g/L)	1.93	1.92	2.18	1.95	0.19	0.20	0.19	0.22
Ác. acético (g/L)					0.35^{ab}	0.36^{ab}	0.30^{b}	0.47^{a}
Ác. cítrico (g/L)					0.12^{b}	0.14^{b}	0.36^{a}	0.07^{b}
Ác. láctico (g/L)					1.49	1.64	1.35	1.49
Ác. succínico (g/L)					1.44^{a}	1.56^{a}	1.43^{a}	0.78^{b}
K (mg/L)	1488	1517	1475	1502				
Antocianos (mg/L)	512^{c}	608^{b}	575^{b}	671^{a}	287^{c}	313^{bc}	335^{ab}	364^{a}
Taninos (g/L)	4.04^{b}	4.15^{b}	4.12^{b}	4.54^{a}	2,65^{c}	2,77^{bc}	2,83^{b}	3,02^{a}
IC	1.35^{c}	1.52^{bc}	1.57^{b}	1.81^{a}	0,88^{c}	0,95^{bc}	1,00^{ab}	1,10^{a}
Tonalidad	0.44	0.43	0.44	0.42	0,56	0,56	0,55	0,55
L	44.69^{a}	40.19^{b}	41.00^{b}	35.93^{c}	54,32^{a}	51,88^{ab}	51,47^{b}	47,65^{c}
a	64.40^{b}	64.84^{ab}	65.22^{a}	64.81^{ab}	47,36^{c}	49,09^{b}	50,32^{b}	52,63^{a}
b	-0.27^{d}	4.54^{b}	2.29^{c}	9.57^{a}	-0,02^{b}	1,99^{a}	-0,13^{b}	1,99^{a}
Glicerina (g/L)					8.04^{c}	7.96^{c}	8.50^{a}	8.24^{b}
Extracto seco (g/L)					19.38^{b}	20.53^{a}	20.82^{a}	21.09^{a}
Fe (mg/L)					0.41	0.39	0.30	0.36
Cu (mg/L)					0.03	0.02	0.01	0.01
Zn (mg/L)					0.60	0.58	0.47	0.51
Alcoh. Sup. (mg/L)					359	367	366	350

Las distintas letras indican diferencias significativas entre tratamientos (nivel de significación $p < 0,05$)
AT, acidez total; AV, acidez volátil; IC, intensidad colorante; Alcoh. Sup., Alcoholes superiores.

Tabla VI.5. Contenido de estilbenos de las muestras sólidas en las etapas de prensado, deslío y embotellado.

		piceatanol	*trans*-resveratrol	Isorhapontigenin	ε–viniferina	estilbenos
Orujos (mg/Kg orujo)	CT	---	1,359[c]	0,549[c]	1,875[b]	3,784[c]
	MEJA	---	1,927[b]	0,782[b]	2,897[a]	5,606[b]
	UVC	---	1,395[c]	0,836[b]	1,859[b]	4,091[c]
	MEJA-UVC	---	2,416[a]	1,277[a]	2,693[a]	6,386[a]
Lías (mg/Kg lías)	CT	3,074[c]	8,086[b]	---	1,176[b]	12,336[d]
	MEJA	4,197[b]	9,219[b]	---	1,521[a]	14,937[c]
	UVC	4,383[b]	13,693[a]	---	1,205[b]	19,281[b]
	MEJA-UVC	6,989[a]	13,896[a]	---	1,453[a]	22,338[a]
Embotellado (mg/Kg tartratos)	CT	4,133[c]	6,096[c]	---	1,047[c]	11,276[c]
	MEJA	4,378[c]	6,723[c]	---	1,391[b]	12,493[c]
	UVC	5,493[b]	10,558[b]	---	1,019[c]	17,070[b]
	MEJA-UVC	7,458[a]	15,327[a]	---	1,662[a]	24,447[a]

Las distintas letras indican diferencias significativas entre tratamientos (nivel de significación $p < 0,05$)

La fermentación maloláctica no afectó al contenido de *trans*-resveratrol. Sin embargo, en las etapas de deslío y estabilización se observaron pérdidas en el contenido de estilbenos. De hecho, fue detectado en lías (de 8 a 14 mg/Kg lías) y tartratos (de 6 to15 mg/Kg tartrato) de acuerdo con Guerrero y col. (2010b). El contenido de *trans*-resveratrol en los subproductos (lías y tartratos) de los lotes siguió el mismo orden que el descrito para el vino en la prensa: CT <MEJA <UVC <MEJA-UVC (Tabla VI.5).

A mayor concentración de *trans*-resveratrol presente en mosto/vino, mayor fue la concentración de éste en los subproductos. Debido a estas pérdidas, el contenido de estilbenos en los vinos embotellados fue menor de lo esperado. El piceatanol osciló desde 0,336 hasta 0,676 mg/L (Tabla VI.4), siendo significativamente menor en las uvas del lote CT en comparación con los otros tratamientos, destacando el tratamiento MEJA-UVC. *trans*-Resveratrol varió desde 0,613 hasta 1,512 mg/L, siendo las mayores concentraciones para las muestras tratadas con UVC (UVC y MEJA-UVC), datos que concuerdan con los obtenidos en la prensa. También se detectaron pequeñas cantidades de ε-viniferina (alrededor de 0,13 mg/L).

Para asegurar la calidad del producto final, también se analizaron los parámetros en los vinos embotellados (Tabla VI.4). No se encontraron diferencias en el contenido de etanol, acidez total y volátil, pH, ácidos tartárico, málico y láctico, tonalidad, metales (Fe, Cu y Zn) y alcoholes superiores. Se encontraron algunas diferencias en los ácidos acético, cítrico y succínico, especialmente en el lote MEJA-UVC. Tal vez pudo deberse a una pequeña desviación en la fermentación alcohólica. Sin embargo, todos los valores se encontraban bajo el rango habitual para los vinos producidos bajo condiciones experimentales. El extracto seco resultó mayor para los lotes tratados (MEJA, UVC, MEJA-UVC) que para el lote control (CT) y la glicerina fue ligeramente mayor en los lotes tratados con luz UVC.

Las diferencias más importantes se observaron en los parámetros relacionados con el color. Los antocianos y la intensidad colorante mostraron la misma tendencia que se produjo en los vinos prensa (CT<MEJA<UVC<MEJA-UVC) y en cuanto al parámetro L* ocurrió todo lo contrario. Sin embargo las diferencias fueron menores que en prensa. Se ha descrito que las diferencias de color se minimizan durante el proceso de

vinificación (Puertas y col., 2008). Por otra parte, las diferencias que presentaban el parámetro a* y los taninos en el vino prensa se mantuvieron en los vinos embotellados.

En resumen, las diferencias entre tratamientos de los vinos embotellados en cuanto al contenido total de estilbenos fueron significativas. Los vinos procedentes de la combinación de ambos tratamientos (MEJA-UVC) obtuvieron el doble de estilbenos totales que los vinos que se elaboraron como control (CT).
En cuanto a los parámetros enológicos, todos los lotes mostraron valores similares excepto en los parámetros relacionados con el color, donde los mejores resultados los obtuvo el lote MEJA-UVC.

3.4. Análisis sensorial

Por último, se realizaron análisis sensoriales de los vinos a fin de compararlos y poder establecer diferencias entre las técnicas empleadas.

A cada catador se le proporcionó una ficha de cata específica con la que se puntuaron los caracteres visuales (aspecto e intensidad del color), olfativos (intensidad aromática y afrutado) y gustativos (persistencia, herbáceo, amargor y astringencia). Cada uno de los caracteres se puntuó según la sensación percibida.

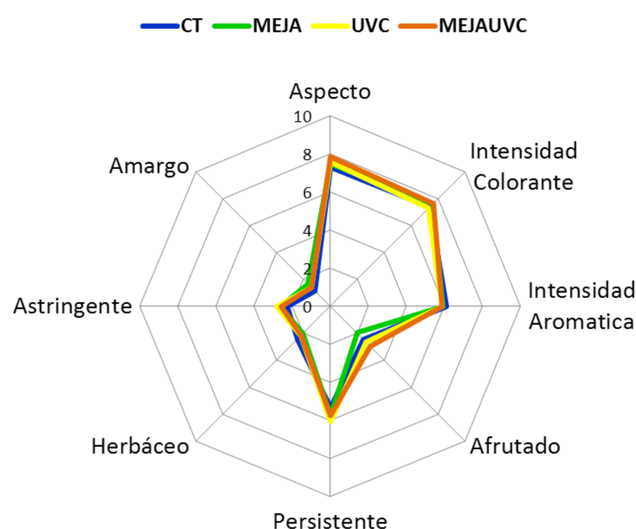


Figura VI.4. Características organolépticas de los vinos embotellados.

Todos los vinos fueron evaluados positivamente. Las puntuaciones para cada atributo fueron similares, por tanto, no se encontraron diferencias significativas entre los tratamientos. Los vinos obtenidos de las muestras MEJA-UVC obtuvieron puntuaciones más altas en el aspecto y en la intensidad colorante, datos que concuerdan con los obtenidos en los análisis de los parámetros enológicos.

En cuanto a la fase gustativa observamos que el lote CT, fue el vino menos afrutado, a pesar de que presentara una intensidad aromática ligeramente superior. Por otra parte, se observó que los valores de astringencia y persistencia fueron mayores para los vinos UVC.

CAPÍTULO VII

CONCLUSIONES

CONCLUSIONES

Las conclusiones a las que se ha llegado por los resultados obtenidos a lo largo de este trabajo de investigación son:

- Tanto la variedad como el *terroir* resultaron ser factores que afectaban a la concentración de estilbenos en uva. Ambos afectaron tanto a la concentración basal, como a la capacidad de inducción por tratamiento postcosecha con UVC. El *terroir* tuvo mayor influencia que la variedad sobre la concentración de estilbenos en uva. El análisis de componentes principales agrupa Cabra y Jerez como zonas con mayor concentración de estilbenos en uva, frente a Ronda y Cádiar como zonas con menor concentración de estilbenos en uva. En cuanto a las variedades, de forma general, Syrah fue la que destacó por su alta capacidad de inducción.

- Los tratamientos precosecha con benzotiadiazol, botrydial y quitosano, en las dosis y metodología aplicada, resultaron ser poco eficientes para aumentar el contenido de resveratrol en uva. El tratamiento con el benzotiadiazol aumentó de forma significativa la concentración de resveratrol en uva, de forma que en el momento de vendimia la uva tratada con benzotiadiazol tenía una concentración tres veces mayor que su testigo. Sin embargo, de forma paralela se observó un retraso en la maduración de la uva.

- La combinación del tratamiento precosecha MEJA junto con el postcosecha UVC se perfila como interesante para obtener uvas enriquecidas en estilbenos. A pesar de que el tratamiento no se consiguió aumentar la concentración de estilbenos respecto al tratamiento UVC, la velocidad de inducción de resveratrol aumentó de forma importante, disminuyendo el periodo de conservación necesario tras el tratamiento UVC en tres días. Este hecho es clave para conservar la calidad de la uva.

- Tras el tratamiento postcosecha UVC se observó la inducción de un compuesto desconocido. Dicho compuesto fue aislado mediante cromatografía contra corriente (CCC) y HPLC semipreparativo, e identificado mediante espectroscopia de

resonacia magenética nuclear (RMN) como isorhapontigenin. Este estilbeno ha sido identificado en muchas plantas medicinales tradicionales, siendo esta la primera vez que se describe en uva, única fuente dietética conocida.

- Tras la aplicación del tratamiento precosecha con metil jasmonato y postcosecha con UVC en uvas de la variedad Syrah cultivada en Jerez de la Frontera, y posterior vinificación de estas, se obtuvo un vino que duplicaba su concentración de estilbenos (resveratrol, piceatanol y ε-viniferina) respecto a su testigo. Además, el vino tratado presentó mejores parámetros de color, mayor concentración de antocianos y taninos, y mejor puntuación en el análisis sensorial

  Los subproductos obtenidos durante la vinificación, como son los orujos, lías y tartratos, presentaron altas concentraciones de estilbenos por lo que podrían ser usados como fuente de bioactivos para la elaboración de distintos productos cosméticos, nutraceuticos y/o agroalimentarios.

CONCLUSIONS

The conclusions that have been reached from the obtained results through this Thesis are:

- Both variety and *terroir* are key factors that affect stilbene concentration in grapes. They affected basal concentration as well as induction capacity when treated with UVC postharvest treatment. Terroir showed more influence in stilbenes in grapes than variety. Principal component analysis grouped Cabra and Jerez as area with higher stilbene concentration in grape and, Ronda and Cadiar as area with lower stilbene concentration in grape. Regarding varieties, in general terms, Syrah singled out due to its high induction capacity.Botrydial, chitosan and methyl jasmonate preharvest treatments, in doses and methodology applied, resulted in low effeciency to increase resveratrol grape content. Benzothyadiazole significantly increased resveratrol grape concentration. At harvest, benzothiadiazole treated grapes contained three-folds resveratrol concentration. However it also delayed grape ripeness.

- MEJA preharvest treatment and UVC postharvest treatment combination resulted an interesting tool for stilbene-enriched grape manufacturing. Despite this treatment did not reach higher stilbene concentration than UVC, the induction velocity significantly increased. It shortened the required storage period in three days after UVC. This fact is a keypointto preserve grape quality.

- A new grape stilbene was found after UVC postharvest treatment. It was isolated by counter current chromatography and semi-preparative HPLC. Subsequently it was identified by nuclear magnetic resonance spectroscopy as isorhapontigenin. This stilbene has been described in many traditional medicinal plants. However, it is the first time that it is described in grapes, the only known diet source.

- Syrah grapes cultivated in Jerez de la Frontera were treated as previously optimizated. MEJA-UVC treated grapes were used for wine production. Wine

achieved stilbene content (resveratrol, piceatannol and ε-viniferin) in two-folds. Moreover, color parameters were improved and, anthocyanin and tannin content were increased in wine improving marks in sensorial analysis.

Additionally, High stilbene concentration occurred in winemaking byproducts such as pomace, lees and tartrates. Therefore, they may be used as a valuable source of bioactive in the manufacture of cosmetic, nutraceutical and/or food products.

CAPÍTULO VIII

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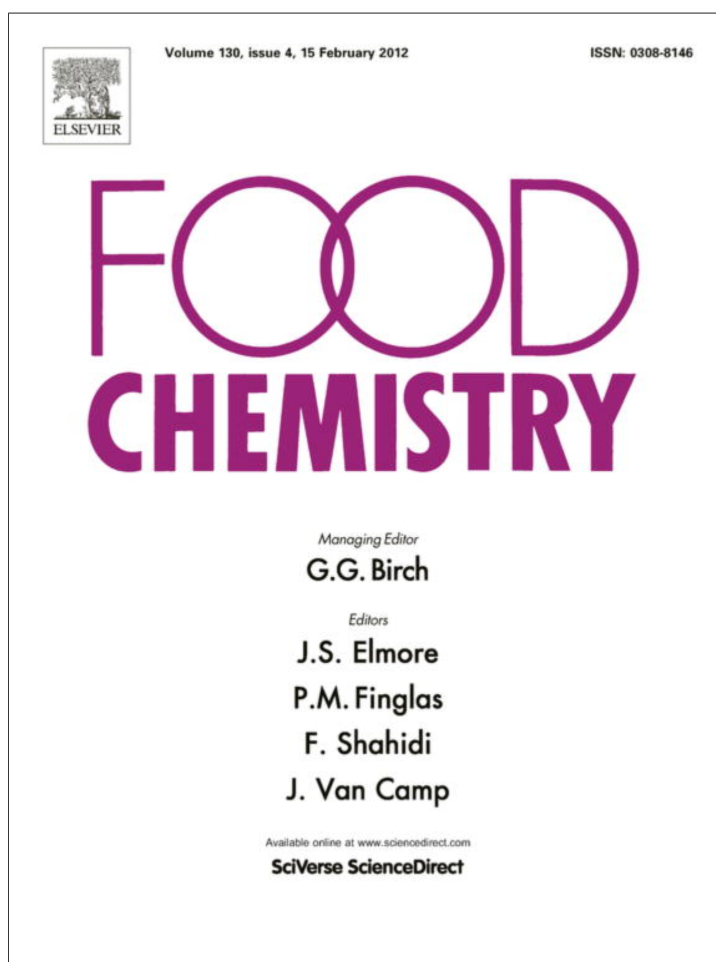

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Review

Bioactive compounds in wine: Resveratrol, hydroxytyrosol and melatonin: A review

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ABSTRACT

Regular moderate wine consumption is often associated with reduced morbidity and mortality from a variety of chronic diseases in which inflammation is the root cause. This review is focused on three of the numerous bioactive compounds present in wine: resveratrol, hydroxytyrosol and melatonin. Resveratrol and hydroxytyrosol are polyphenols. Melatonin, recently described in wine, is an indoleamine. Their structures, concentrations in wine, bioavailability, pharmacokinetic and health promoting properties are reviewed. Resveratrol seems to be one of the most promising compounds due to its bioactivity, with wine being the main source of resveratrol in diet. Hydroxytyrosol, which its main source in diet is olive oil has been also found in both red and white wine in considerable amounts. Melatonin has been found in wine in low amounts. However, both high bioactivity and bioavailability have been attributed to it. They show antioxidant, cardioprotective, anticancer, antidiabetic, neuroprotective and antiaging activities. However, human studies are still in the initial stages and therefore further studies are needed.

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1. Introduction

The relationship between diet and health has developed an intense research in bioactive compounds in foods. Wine seems to be an essential component and may be partially responsible for health-promoting properties observed among the Mediterranean population. The starting point for wine and health studies was the "French Paradox". Renaud and De Lorgeril (1992) published a study confirming the association between death by cardiovascular disease and dietary intake. Despite a diet being traditionally high in saturated fats, myocardial infarction rates in France are 40% lower than in the rest of Europe. If wine intake was considered, the French population perfectly fitted the regression model.

From all the studies that have been carried out in the health-and-wine field, it can be affirmed that supplementing the regular diet with red wine increases the total antioxidant capacity in plasma, HDL lipoprotein, fibrinolytic and antithrombin activity. Moreover, it reduces oxidative damage and platelet aggregation. Studies from different parts of the world with diverse population groups, suggested that moderate consumption (1–2 glasses per day) of wine drinks reduce cardiovascular risk (Avellone et al., 2006; Bertelli & Das, 2009; Leighton et al., 1999; Mezzano et al., 2001; Rimm et al., 1995).

Although less evident, wine could have an influence on cancer risk. Moderate consumption of wine reduces the risk of non-Hodgkin's lymphoma (Briggs et al., 2002), adenocarcinoma of the oesophagus, prostate cancer (Platz, Leitzmann, Rimm, Willett, & Giovannucci, 2004; Schoonen, Salinas, Kiemeney, & Stanford, 2005; Schurman, Goldbohm, & Van den Brandt, 1999) and gastric cardia (Gammon et al., 1997). However, other authors have not found any relationship (Bessaoud & Daures, 2008; Sutcliffe et al., 2007) and some of them even found a negative effect (Longnecker, Orza, Adams, Vioque, & Chalmers, 1990).

Among wines, red wine is considered to have a more protective effect due to its greater content in antioxidant substances released from the grape's skin and seeds (mainly polyphenols). A bottle of red wine contains a total of 1.8 g/l of polyphenols, whereas a bottle of white wine contains only 0.2–0.3 g/l of polyphenols (Bertelli & Das, 2009). In the making of white wine, skin and seeds are removed immediately from the must, which is left to ferment without them. As *in vitro* antioxidant capacity is strongly correlated with total polyphenol content *in vitro*, white wines present from five to ten times lesser antioxidant activity than red wines (Lugasi & Hovari, 2003). However, white wine additionally contains a high amount of hydroxycinnamic acids, tyrosol and hydroxytyrosol, which are also known to have some antioxidant properties.

The findings that red wine presented more health-promotion activity than beer or spirits caused research attention to focus on phenolic compounds. Several studies have been undertaken to differentiate the effects of phenolic and other non-alcohol components of wine from those due to alcohol. In animal models it has been demonstrated that a red wine polyphenolic extract prevents the development of cardiovascular problems and cancer. Al-Awwadi et al. (2004) compared blood pressure, heart weight and reactive oxygen species in rats whose feed had been supplemented with the polyphenolic extract, ethanol or both polyphenolic extract and ethanol together. They concluded that the polyphenolic extract was the most effective supplement for reducing cardiovascular risk. Clifford et al. (1996) demonstrated that the consumption of de-alcoholized red wine as a part of a defined complete diet delayed tumor onset in transgenic mice.

All these effects could be understood due to the synergistic effects that may occur among bioactive compounds. Synergy has been reported among the three phenols, resveratrol, caffeic acid and catechin (Norata et al., 2007; Pignatelli, Cao, & Zhu, 2006). Despite their relatively low plasma concentrations following moderate wine consumption, this synergy gives them useful biological activity, such as the inhibition of oxidative stress. Interaction between polyphenols may influence their kinetics and metabolism.

In this review we focus on three bioactive compounds present in wine resveratrol, hydroxytyrosol and melatonin. Resveratrol is one of the most promising compounds due to its bioactivity, with wine being the main source of resveratrol in diet. Hydroxytyrosol is a potent antioxidant mainly found in olive oil, but wine has been described as an additional source of hydroxytyrosol in the diet. Melatonin has been recently found in wine at low concentrations. However, its high bioactivity justifies its inclusion in the present review.

2. Resveratrol

2.1. Structure and concentration in wine

Resveratrol (3,5,4′-*trans*-trihydroxystilbene, Fig. 1a) is a member of the stilbene family of phenolic compounds. Langcake and Pryce (1976) detected it in *Vitis vinifera* grapevines. Resveratrol is synthesized by leaf tissues in response to fungal infection or exposure to ultraviolet light but, until 1992, it was not detected in wine (Siemann & Creasy, 1992).

Resveratrol and stilbenes in general are commonly found in many plants. However, their dietary sources are rather limited: peanut and its derivatives, pistachio, berries, dark chocolate, and grapes as well as their derivatives. Of all of them, grapes present

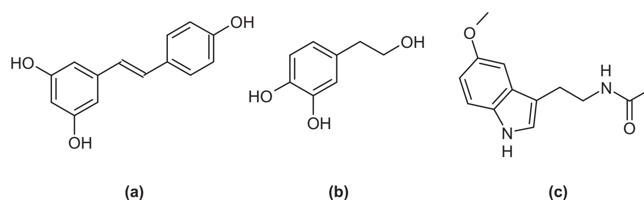

Fig. 1. Chemical structures: (a) *trans*-resveratrol, (b) hydroxytyrosol, (c) melatonin.

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Table 1
trans-Resveratrol content in varietal red wines from different regions.

Variety region	*trans*-Resveratrol (mg/l)		Samples average	Sample number	References
	Low	High			
Merlot					
Australia	Nq	Nq	1.0	1	Shao, Marriott, and Hugel (2003)
Brazil	3.1	5.1	4.0 ± 1.0	3	Souto et al. (2001)
Czech Republic	Nq	Nq	1.3	1	Melzoch, Hanzlíková, Filip, Buckiová, and Smidrkal (2001)
Hungary	1.3	14.3	3.9 ± 4.0	10	Mark, Nikfardjam, Avar, and Ohmacht (2005)
Italy	0.5	6.0	3.4 ± 2.3	4	Goldberg, Ng, Karumanchiri, Diamandis, and Soleas (1996a), Mattivi (1993)
Japan	0.6	2.1	1.5 ± 0.6	5	Sato, Suzuki, Okuda, and Yokotsuka (1997)
Spain	1.0	7.7	4.0 ± 2.9	4	Lamuela–Raventos, Romero–Perez, Waterhouse, and de la Torre–Boronat (1995)
USA	0.4	2.7	1.5 ± 1.0	4	Goldberg et al. (1996a), Gu et al. (1999)
All samples	0.3	14.3	2.8 ± 2.6	32	
Graciano					
Spain	0.8	2.8	1.9 ± 0.8	5	Abril, Negueruela, Perez, Juan, and Estopanan (2005), Lamuela–Raventos et al. (1995)
Syrah					
Australia	0.2	3.2	1.9 ± 0.9	8	Goldberg et al. (1996a, 1996b), Gu et al. (1999), Shao et al. (2003)
Greece	Nq	Nq	2.0	1	Sakkiadi, Stavrakakis, and Haroutounian (2001)
Hungary	1.2	1.8	1.5 ± 0.4	2	Mark et al., 2005
All samples	0.2	3.2	1.8 ± 0.9	11	
Cabernet Sauvignon					
Australia	0.2	1.5	0.9 ± 0.6	4	Goldberg et al. (1996a, 1996b), Gu et al. (1999), Shao et al. (2003)
Brazil	1.3	2.3	1.8 ± 0.5	4	Souto et al. (2001)
Czech Republic	Nq	Nq	1.0	1	Melzoch et al. (2001)
Greece	0.3	1.6	0.9 ± 0.4	15	Dourtoglou, Makris, Bois–Dounas, and Zonas (1999)
Hungary	1.2	9.3	2.9 ± 2.5	9	Mark et al. (2005)
Italy	1.3	7.2	4.0 ± 3.1	4	Goldberg et al. (1996b), Mattivi (1993)
Japan	Nq	Nq	0.9	1	Sato et al. (1997)
Spain	0.7	1.9	1.2 ± 0.4	8	Abril et al. (2005), Goldberg et al. (1996b), Lamuela–Raventos et al. (1995)
EEUU	n.d.	2.2	0.5 ± 0.6	11	Goldberg et al. (1996a, 1996b, 1999), Lamuela–Raventos and Waterhouse (1993)
All Samples	n.d	9.3	1.7 ± 1.7	43	
Tempranillo					
Spain	0.2	2.5	1.3 ± 0.7	12	Abril et al. (2005), Lamuela–Raventos et al. (1995), Martínez–Ortega, García–Parrilla, and Troncoso (2000)

Nq, no quantified.

the highest content but red wine is the most notable dietary source of resveratrol (Guerrero, García–Parrilla, Puertas, & Cantos–Villar, 2009).

Resveratrol is found in the seed and skin of grapes (not in flesh) and, hence, in grape juice and wine. Obviously its concentration in red wine is higher than in white wine, because in red winemaking the must, grape skin and often seeds are in contact during the whole fermentation process. For the same reason, the levels of resveratrol found in rose wines (0.41 mg/l) fall between the levels in red (1.90 mg/l) and white wines (0.13 mg/l) (Carando et al., 1999; Landrault et al., 2002; Romero–Pérez, Lamuela–Raventós, Waterhouse, & de la Torre–Boronat, 1996; Stervbo, Vang, & Bonnesen, 2006).

The amount of resveratrol in wine varies widely depending on many factors: grape variety, geographic region, agronomic factors, climatic factors, plant stress conditions and oenological practices. Regarding optimum oenological practices, all the processes that aim to maximize the extraction from skin are suggested (Soleas, Goldberg, Karumanchiri, Diamandis, & Ng, 1995; Vrhovsek, Wendelin, & Eder, 1997). It is difficult to predict the amount of resveratrol that a wine will contain because there are so many factors affecting resveratrol biosynthesis. Concentrations ranging from undetectable to 14.3 mg/l have been described (Frémont, 2002; Stervbo, Vang, & Bonnesen, 2007). As an example of this variability, *trans*-resveratrol content in wines from different varieties and region is shown in Table 1.

Resveratrol, as the majority constituent of phenolics, is synthesized from phenylalanine through the shikimic pathway. Three key enzymes are involved in this pathway: phenylalanine ammonium lyase, coenzyme A ligase and stilbene synthase (Fig. 2). The biosynthesis of the above enzymes can be induced by stress (Fritzemeier & Kindl, 1981). Therefore, resveratrol is a phytoalexin synthesized by grapes after exposure to biotic or abiotic stress. The presence of resveratrol in grapes depends on the degree of stress exposure. Pathogenic attack (Roldán, Palacios, Caro, & Pérez, 2003; Soleas, Diamandis, & Goldberg, 1997), preharvest chemical treatments such as BTH (benzothiadiazole) or chitosan (Iriti, Rossoni, Borgo, & Faoro, 2004; Romanazzi, Gabler, & Smilanick, 2006) and UVC (Guerrero, Puertas, Fernández, Palma, & Cantos, 2010) are potent factors that make resveratrol content in grapes increase and consequently in wines too. Another strategy proposed as responsible for increasing resveratrol in wine is the use of transgenic yeast (Becker et al., 2003; González–Candelas, Gil, Lamuela–Raventós, & Ramón, 2000). These treatments are now applied specifically with the aim of producing wines enriched in resveratrol and, what is commercially even more important, wines with a constant and predictable high level of resveratrol over the years or vintages. This would represent a proven added value for the product in nutritional and health terms, which can be exploited in the market (Barreiro–Hurlé, Colombo, & Cantos-Villar, 2008). In fact, commercial application has already been found (http://www.drnorrie.info/html/rew.html).

Apart from *trans*-resveratrol, the presence of other stilbenes has been described in grapevine. Poutaraud reported the main stilbenes found in grapevine leaf, which are *trans*-resveratrol and its derivatives: piceid, pterostilbene, ε-viniferin, and δ-viniferin

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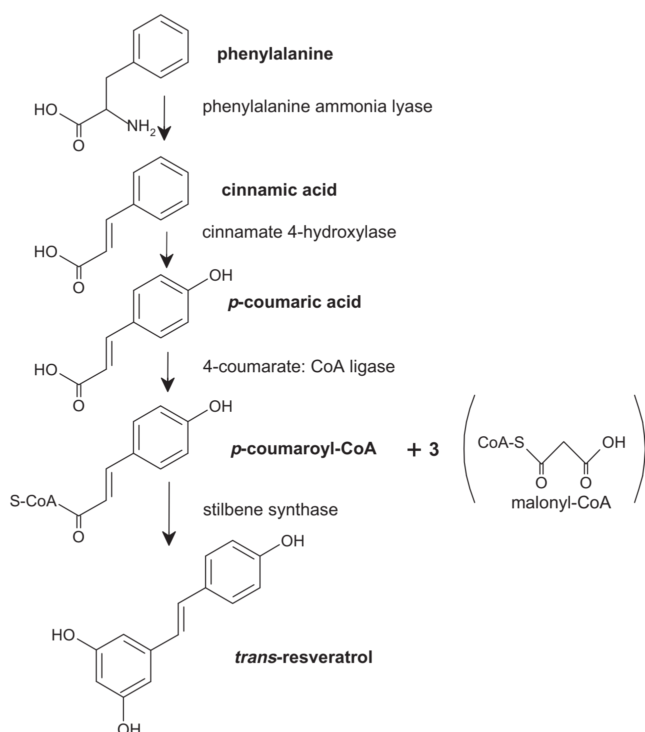

Fig. 2. Biosynthesis pathway of resveratrol (Ferrer, Austin, Steward, & Noel, 2008).

(Poutaraud et al., 2007); of course, many of these compounds, including resveratrol, piceid, astringin and stilbene oligomers (viniferins), are also found in finished wines (Buiarelli, Coccioli, Jasionowska, Merolle, & Terracciano, 2007; Güebailia et al., 2006; Ribeiro de Lima et al., 1999; Vitrac et al., 2005). However, the concentration of these compounds in wine is low, and therefore, their bioactivity has been investigated less than that of resveratrol.

Two different types of reaction lead to the formation of stilbene derivatives from *trans*-resveratrol (the initial compound in the stilbene pathway) in susceptible grapevines and resistant cultivars, respectively. In susceptible grapevines resveratrol is synthesized in large amounts early after an infection, but it is rapidly glycosylated into a non-toxic compound: piceid. In resistant varieties resveratrol is also synthesized in large amounts, but in this chemical environment it is rapidly oxidized into toxic viniferins (Pezet, Gindro, Viret, & Spring, 2004).

2.2. Bioavailability and pharmacokinetics

Numerous studies in animals and humans have shown that the bioavailability of unconjugated resveratrol is low. At least 70% of resveratrol ingested is absorbed, and readily metabolized to form mainly glucuronide and sulphate derivatives. The colon micro-flora can produce the metabolite dihydroresveratrol. Resveratrol metabolites reach their maximum concentration in plasma approximately 30 min after intake; the half-life of total metabolites is approximately 9.2 h (Walle, Hsieh, DeLegge, Oatis, & Walle, 2004). Plasma concentration of resveratrol and its metabolites depend on the dose administered (Marier et al., 2002). Five distinct metabolites are present in the urine after moderate consumption of red wine: resveratrol monosulphate, two isomeric forms of resveratrol monoglucuronide, dihydroresveratrol monosulphate and dihydroresveratrol (Vitaglione et al., 2005). In low density lipoprotein samples from volunteers who had ingested 250 ml of red wine containing a known quantity of resveratrol, up to six metabolites have been measured: *trans*-resveratrol-3-*O*-glucuronide, *cis*-resveratrol-3-*O*-glucuronide, *cis*-resveratrol-3-*O*-glucoside, free *trans*-resveratrol and, tentatively, resveratrol-4′-*O*-glucuronide and *trans*-resveratrol-4-*O*-glucoside (Urpi-Sarda et al., 2005). Since the *in vivo* concentration of individual metabolites from ingested resveratrol can be much higher than that of resveratrol itself, further studies of the activity of its metabolites are needed.

Bertelli, Giovannini, Stradi, Bertelli, and Tillement (1996) demonstrated that resveratrol present in red wine (6.5 mg/l as *cis* and *trans* forms) reached target tissues in rats. Plasma, urine and tissue levels of *trans*- and *cis*-reveratrol were measured in rats after the administration of a single dose of 4 ml of red wine containing 6.5 mg/l total resveratrol and after the administration of 2 ml red wine containing the same amount of the stilbene for 15 days. Resveratrol was found not only in the plasma and urine, but also in the heart, liver and kidneys. Resveratrol plasma levels within the same range were subsequently found also in a clinical trial in 10 healthy volunteers who drank 300 ml of red wine for 15 days, but not in another 10 healthy volunteers who drank 300 ml of white wine for 15 days. In a randomized, single-blind, crossover trial, in which 13 healthy volunteers drank wine, ethanol and water on three separate occasions, 2 weeks apart, the mean ± SE plasma resveratrol plasma level was 24.8 ± 5.8 μg/l after the ingestion of 155 ml of Australian Pinot Noir wine and 43.0 ± 9.4 μg/l after the ingestion of 310 ml. It is important to bear in mind that three servings (approx 450 ml) are more than sufficient to achieve plasma levels of free *trans*-resveratrol within the range of 100 nM–1 μM (Bertelli & Das, 2009).

Resveratrol proved *in vitro* anticarcinogenic activity at doses ranging from 5 to 100 mM, meanwhile the doses for the prevention of cardiovascular disease are between 100 nM and 1 mM (Bertelli, 2007), which means that at modest dosages, resveratrol was pharmacologically active both *in vitro* and *in vivo*. These authors suggested that an average drinker of wine could, particularly in the long term, absorb a sufficient quantity of resveratrol to explain the beneficial effect of red wine on human health. More importantly, this could help to explain how a relatively low dose of resveratrol obtained from red wine or other dietary sources could be therapeutic in some cases (Bertelli, Bertelli, Gozzini, & Giovannini, 1998). A dose-dependent response has been observed, and, at higher but pharmacologically achievable doses, protective effects of resveratrol are more frequently observed, and the results are more dramatic (Chen, Tseng, Lai, & Chen, 2004).

Resveratrol binds to albumin. It has been suggested that albumin could be a natural polyphenol reservoir in the *in vivo* context, where it might play a pivotal role in the distribution and bioavailability of circulating resveratrol (Jannin et al., 2004). The accumulation of resveratrol in other organs such as the heart, liver and lungs after chronic administration was described for the first time in 1996 (Bertelli et al., 1996) and has more recently been confirmed (El–Mohsen et al., 2006; Vitrac et al., 2003) and extended to the bile, stomach and kidneys (De Santi, Pietrabissa, Spisni, Mosca, & Pacifici, 2000).

It is also worth considering the potential interactions of resveratrol with other constituents of the diet. Resveratrol has been shown to synergize with both quercetin and ellagic acid in the induction of apoptosis in human leukemia cells (Mertens–Talcott & Percival, 2005), with ethanol in the inhibition of iNOS expression (Chan, Mattiacci, Hwang, Shah, & Fong, 2000), with vitamin E in the prevention of lipid peroxidation (Fang et al., 2002), with catechin in the protection of PC12 cells from β-amyloid toxicity (Conte, Pellegrini, & Tagliazucchi, 2003), with nucleoside analogues in the inhibition of HIV1 replication in cultured T lymphocytes (Heredia, Davis, & Redfield, 2000), and with tyrosol and β-sitoesterol in modulation of LDL oxidative stress and PGE2 synthesis (Vivancos & Moreno, 2008). The absorptive efficiency of *trans*-resveratrol, (+)-catechin and quercetin was investigated after oral application to

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healthy human subjects in three media (white wine, grape juice and vegetable homogenate). The absorption of these three polyphenols was equivalent in the different matrices but, at peak concentrations of 10–40 nmol/l, it was inadequate to permit circulating concentrations of 5–100 mmol/l consistent with *in vitro* biological activity (Goldberg, Yan, & Soleas, 2003). Moreover, one finding that has often been overlooked is that quercetin, which is also present in red wine, is a picomolar inhibitor of resveratrol sulphation in both the liver and duodenum, thus increasing the bioavailability of unconjugated resveratrol (De Santi et al., 2000).

As regards toxicity effects of resveratrol, it has been established than all these health benefits are not coupled with adverse side effects, unless extremely high doses are administered. Juan, Vinardell, and Planas (2002) found no adverse effects in rats after consumption for 28 days of the quantity of resveratrol equivalent to 1000-times the content of this compound in red wine. Recently similar results were found. A 28-day study was performed on rats, where Resvida™ (high purity resveratrol content) caused no adverse effects in rats at 50, 150 and 500 mg/kg body weight/day. Similarly, in a 90-day study, Resvida™ did not cause any adverse effects in rats at up to 700 mg/kg body weight/day, the highest dose tested (Williams, Burdock, Edwards, Beck, & Bausch, 2009).

2.3. Health-promoting properties

2.3.1. Antioxidant activity

Normal cellular metabolism generates reactive oxygen intermediates (ROI) such as superoxide, hydrogen peroxide and hydroxyl radicals, which are usually detoxified by intracellular enzymes such as glutathione, superoxide dismutase and catalase. However, an abnormal accumulation of ROIs can happen, which is commonly referred to as "oxidative stress". Exposure of macromolecules (lipids, protein, DNA) to ROIs results in their oxidative modifications with deleterious potential (Arthur, Niu, Rigby, Steer, & Jeffrey, 2008).

Resveratrol has an intrinsic antioxidant capacity that could be related to its chemopreventive effects. *In vitro*, the induction of detoxification enzymes has been shown after low doses of resveratrol (Li, Cao, & Zhu, 2006). *In vivo*, resveratrol has been shown to increase plasma antioxidant capacity and to decrease lipid peroxidation (Wenzel, Soldo, Erbersdobler, & Somoza, 2005; Whitehead, Robinson, Allaway, Syms, & Hale, 1995), which is strongly associated with the risk of coronary heart disease and myocardial infarction (Holvoet, 2004). Studies in rats, pigs and humans seem to indicate that resveratrol can suppress pathological increases in the peroxidation of lipids and other macromolecules *in vivo*, but whether the mechanism is direct, indirect or both, is not clear yet (Baur et al., 2006).

2.3.2. Cardioprotective capacity

Resveratrol protects the cardiovascular system in a multidimensional way (Hao & He, 2004). The most important point is that resveratrol, when at very low concentration, inhibits apoptotic cell death, thereby providing protection from various diseases including myocardial ischemic reperfusion injury, atherosclerosis and ventricular arrhythmias. In higher doses it facilitates apoptotic cell death and behaves as a chemo-preventive alternative (Das & Das, 2007).

Resveratrol modulates lipid and lipoprotein metabolism; it may suppress pathological increases of peroxidation in macromolecules such as lipids. In 1982 it was shown that resveratrol inhibits the deposition of cholesterol and triglycerides in the liver of rats, and decreases the rate of hepatic triglyceride synthesis (Arichi et al., 1982). Later, it was demonstrated that *trans*-resveratrol inhibits LDL peroxidation *in vitro* more than an extract of red wine (Frankel, Kanner, German, Parks, & Kinsella, 1993). Resveratrol has been detected in LDL particles from humans after consumption of red wine (Urpi-Sarda et al., 2005).

Platelet aggregation is one of the major contributors to the process of atherosclerosis. Resveratrol prevents platelet aggregation *in vitro* (Bertelli et al., 1995) and *in vivo* (Wang et al., 2002). Further research has shown that resveratrol reduces the formation of atherosclerotic plaques and restores flow-mediated dilation in rabbits fed a high-cholesterol diet (Wang et al., 2005).

Resveratrol also promotes vasodilatation through multiple mechanisms, mainly the stimulation of Ca^{2+}-activated K^+ channels and the enhancement of nitric oxide signalling in the endothelium, and therefore it can exert vaso-relaxant activity (Li, Chen, & Wu, 2000; Orallo et al., 2002). In guinea pigs, the addition of resveratrol to drinking water for 16 days (~14 mg per kg body weight) significantly increased its capacity to eliminate oxidants in cardiac muscle (Floreani, Napoli, Quintieri, & Palatini, 2003). The main mechanism seems to be an increase in nitric oxide concentrations by both increasing the expression of nitric oxide synthase and decreasing the inactivation of nitric oxide by free radicals.

2.3.3. Anticancer activity

Jang et al. (1997) reported the ability of resveratrol to inhibit carcinogenesis at multiple stages (initiation, promotion and progression). Their finding that topical application of resveratrol reduced the number of skin tumours per mouse by up to 98% triggered research on resveratrol all around the world. Resveratrol could slow tumour development through multiple complementary mechanisms. It inhibits the enzymatic activity of both forms of cyclooxygenase, which implies a risk reduction of developing many cancers. Another mechanism by which resveratrol could combat tumour formation is induction of cell cycle arrest and apoptosis. The anti-proliferative and pro-apoptotic effects of resveratrol in tumour cell lines have been extensively documented *in vitro* (Aggarwal et al., 2004). This is supported by down regulation of cell cycle proteins (Schneider et al., 2001) and increases in apoptosis (Garvin, Ollinger, & Dabrosin, 2006) in tumour models *in vivo*. However, in some *in vivo* experiments resveratrol failed to affect cancer, which suggests that other factors such as dosage, delivery method, tumour origin and other components of the diet could all contribute to the efficacy of resveratrol treatment. Overall, *in vivo* studies clearly show great promise for this molecule in the treatment of cancers, although studies of the association between red wine consumption and cancer in humans are still in their initial stages.

The efficacy of resveratrol in colorectal cancer has been extensively studied. In one study treatment of the CaCo-2 cells with 25 μM of resveratrol caused a 70% growth inhibition. Oral administration of high resveratrol doses in drinking water and diet has been demonstrated to reduce tumour incidence in mice (Athar et al., 2007). The promising results in studies of the effect of resveratrol on colon cancer have led to a clinical trial in which patients with colon cancer receive treatment with resveratrol and correlative laboratory studies will examine its effects directly on colon cancer and normal colonic mucosa. These studies will provide data on the mechanisms of resveratrol action and provide a foundation for future prevention trials, correlative studies and therapeutic clinical research with resveratrol.

In mice, resveratrol supplementation delays spontaneous mammary tumour development and reduced metastasis (Provinciali et al., 2005). In a population study conducted in Italy, an inverse relationship was observed between resveratrol from grape consumption and breast cancer, but not for resveratrol ingested in wine (La Vecchia & Bosetti, 2006).

Research studies show that drinking a glass of red wine a day may cut a man's risk of prostate cancer by half, and that the protective effect appears to be strongest against the most aggressive forms of the

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disease. It was also seen that men who consumed four or more 4-oz glasses of red wine per week have a 60% lower incidence of the more aggressive types of prostate cancer (http://www.cancer.gov/cancer-topics/factsheet/red-wine-and-cancer-prevention).

However, it is impossible to make definite statements or conclusions on the clinical efficacy in cancer patients because of the great variability and differences of the study designs, small patient numbers, short treatment duration and lack of a standardized drug formulation. Although some results from these clinical studies seem encouraging, reliable or long-term data on tumour recurrence, disease progression and survival are unknown. At present, there is no convincing clinical proof or evidence that resveratrol might be used in an attempt to cure cancer. Clinical trials in phase I are being conducted in healthy people in order first to determine the concentration of resveratrol and its metabolites in the plasma, urine, and feces of healthy participants; second, to correlate dose with systemic concentration of this drug and its metabolites in these participants; and thirdly, to determine the safety of this drug in these participants (http://www.cancer.gov/clinicaltrials/CCUM-2004-0535).

2.3.4. Antidiabetic activity

Data in the literature indicate that resveratrol may play a role in the prevention of diabetes and diabetic complications (Harikumar & Aggarwal, 2008). An *in vivo* experiment revealed that resveratrol, administered to normal rats at the dose of 50 mg/kg body weight, diminished blood insulin concentrations at 30 min, without concomitant changes in glycemia. These findings suggest the direct insulin-suppressive action of resveratrol in the rat (Szkudelski, 2008).

2.3.5. Neuroprotective activity

Neural dysfunction and metabolic imbalances underlie many progressive neurodegenerative conditions such as Alzheimer's, Huntington's and Parkinson's diseases (Sinclair, 2005). Resveratrol is capable of penetrating the blood–brain barrier and exerts strong neuroprotective effects, even at low doses. Resveratrol has been shown to combat the neuronal dysfunction caused in Huntington's and Alzheimer's diseases, through the SIRT1 pathway (Parker et al., 2005). The same authors showed that only 500 nM per day, an amount which is provided in one glass of red wine, is needed to protect neurones. The prevention of Parkinson's disease is based on the scavenging mechanism performed by resveratrol (Karlsson, Emgard, Brundin, & Burkitt, 2000). The efficacy of resveratrol against various different mechanisms has recently been confirmed, and resveratrol has been shown to be potentially useful in protecting against brain damage following cerebral ischemia (Dong et al., 2008).

It is worth mentioning an interesting study developed by Karuppagounder et al. (2009). They fed mice with clinically feasible dosages of resveratrol for 45 days. Neither resveratrol nor its conjugated metabolites were detectable in the brain. Nevertheless, resveratrol diminished plaque formation in a region specific manner. The largest reductions in the percent area occupied by plaques were observed in medial cortex (−48%), striatum (−89%) and hypothalamus (−90%). The changes occurred without detectable activation of SIRT-1 or alterations in APP processing. However, brain glutathione declined 21% and brain cysteine increased 54%, which may be linked to the diminished plaque formation. This study supports the concept that onset of neurodegenerative disease may be delayed or mitigated with the use of dietary chemo-preventive agents that protect against β-amyloid plaque formation and oxidative damage.

2.3.6. Anti-aging activity

Resveratrol extends the lifespan of *S. cerevisiae, Caenorhabditis elegans* and *Drosophila melanogaster*, as well as species of short-lived fish through activation of the sirtuin pathways (Howitz et al., 2003; Valenzano et al., 2006; Wood et al., 2004). More recently, Baur et al. (2006) have shown that resveratrol shifts the physiology of middle-aged mice on high-calorie diet towards that of mice on standard diet and significantly increases their survival. Specifically, studies in mice have shown that obese animals whose diet was supplemented with resveratrol not only lived longer, but were more active and produced fewer cases of the negative effects of a high-calorie diet; this diet also reduced insulin-like growth factor-1 levels, increased the number of mitochondria, and improved motor function.

3. Hydroxytyrosol

3.1. Structure and concentration in wine

Hydroxytyrosol is a phenyl ethyl alcohol, 2-(3,4-dihydroxyphenyl) ethanol (3,4-DHPEA) (Fig. 1b). The main source of hydroxytyrosol in the diet is virgin olive oil, being present, mainly as secoiridoid derivatives or as acetate and free form (Mateos et al., 2001). Hydroxytyrosol and its derivatives arise from oleuropein

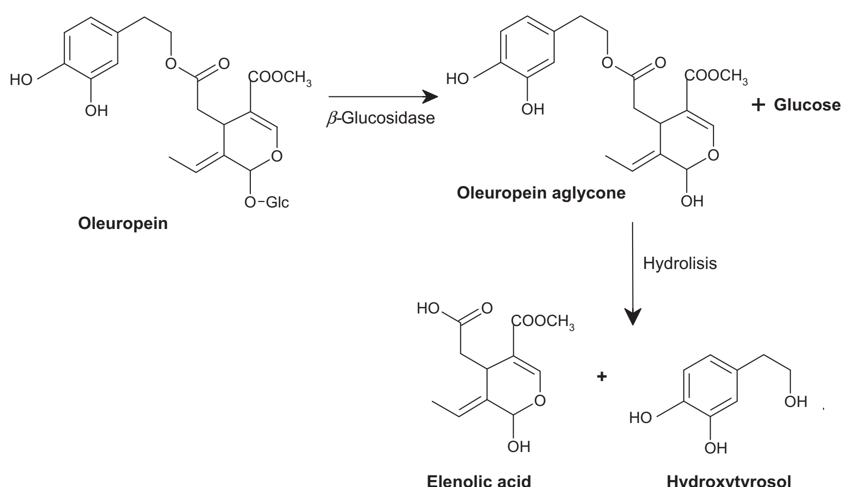

Fig. 3. Biosynthesis of hydroxytyrosol (Vissers et al., 2002).

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(ester of hydroxytyrosol and elenolic acid) present in olives during extraction of olive oil (Fig. 3).

Although factors such as variety, olive fruit maturity, olive oil processing or even agronomic factors strongly determine the final amount of phenolic compounds detected in virgin olive oil, concentrations between 100 and 600 mg/kg have been quantified, of which approximately half of this amount correspond to hydroxytyrosol and its derivatives (Brenes, García, García, Rios, & Garrido, 1999; Tripoli et al., 2005).

Wine seems to be another important source of hydroxytyrosol in our diet. It was firstly detected in Italian wines (Di Tommaso, Calabrese, & Rotillo, 1998). Some authors described higher concentrations in red wines (3.66–4.20 mg/l) than white ones (1.72–1.92 mg/l). They hypothesized the formation of hydroxytyrosol from tyrosol during alcoholic fermentation. Later, hydroxytyrosol was detected in Greek wines (Proestos et al., 2005), while simultaneously new Italian findings also confirmed the presence of hydroxytyrosol in their wines (Boselli, Minardi, Giomo, & Frega, 2006; Dudley et al., 2008).

Minuti, Pellegrino, and Teseo (2006) evaluated different extraction process of hydroxytyrosol from wines, quantifying concentrations ranged from 1.8 to 3.1 mg/l in red wine (Table 2). All of the above described evidences render wines as an important source of hydroxytyrosol in diet.

3.2. Bioavailability and pharmacokinetics

Regarding the bioavailability and pharmacokinetic of this bioactive compound, most studies are focused on hydroxytyrosol containing olive oil. As far as we know, very few researches have evaluated the uptake of hydroxytyrosol containing wine (De la Torre, Covas, Pujadas–Bastardes, Fitó, & Farré, 2006; Schröder et al., 2009).

Several human (Caruso, Visioli, Patelli, Galli, & Galli, 2001; Garcia–Villalba et al., 2010; Miró–Casas et al., 2001, 2003a, 2003b; Visioli et al., 2000; Visioli et al., 2003; Vissers, Zock, Roodenburg, Leenen, & Katan, 2002) and animal (D'Angelo et al., 2001; Tuck, Freeman, Hayball, Stretch, & Stupans, 2001; Tuck & Hayball, 2002) studies have shown that olive oil phenols, and specifically hydroxytyrosol and its derivatives, are bioavailability in a dose-dependent manner. In a human ileostomy study it was shown that up to 66% of the ingested olive oil phenols were absorbed in the small intestine (Vissers et al., 2002). The absorption of hydroxytyrosol is rapid, reaching the maximum plasma concentration after 5–10 min of its ingestion followed by a rapid decline (D'Angelo et al., 2001). Therefore, absorption of this compound depends on the vehicle of administration. Tuck et al. (2001) demonstrated that rats absorbed 75% and 99% of the hydroxytyrosol when it was administered in an aqueous solution and an oily vehicle, respectively. In line with these results, Visioli et al. (2003) observed a higher enhanced excretion of hydroxytyrosol in urinary humans than in rats, detected a higher human hydroxytyrosol excretion after its administration as a natural component of olive oil (44.2% of hydroxytyrosol administered) than after its addition to refined olive oil (23%) or yogurt (13%).

Table 2
Hydroxytyrosol and tyrosol contents in wine.

	Hydroxytyrosol	Tyrosol	References
White wine (mg/l)	1.85	1.75	Di Tommaso et al. (1998)
	1.50	45	Boselli et al. (2006)
	2.69	17.06	Dudley et al. (2008)
Red wine (mg/l)	3.89	4.25	Di Tommaso et al. (1998)
	1.98	31.62	Minuti et al. (2006)
Aged red wine (mg/l)	25	Nq	Proestos et al. (2005)

Nq, no quantified.

Urinary recoveries as high as 80% of the ingested amounts of hydroxytyrosol have been reported (Miró–Casas et al., 2003a, 2003b). Over 90% of the urinary metabolites were conjugated (Caruso et al., 2001; Miró–Casas et al., 2001, 2003a, 2003b; Visioli et al., 2000; Vissers et al., 2002). Mainly glucuronidated metabolites, yet free phenols and methylconjugates, with or without glucuronidation, were also excreted in human urine. Sulfoconjugates of hydroxytyrosol, and other metabolites such as 3,4-dihydroxyphenylacetaldehyde and 3,4-dihydroxyphenylacetic acid were also identified after oral or intravenous dosing of hydroxytyrosol in animal experiments (D'Angelo et al., 2001; Tuck & Hayball, 2002). Analyses of urinary samples from ten healthy volunteers have been evaluated by LC-ESI-TOF-MS after a high intake of extra-virgin olive oil was carried out. More than 50 metabolites were tentatively identified, where methylation and glucuronidation were the most common metabolic reactions. Additionally, kinetic studies were conducted on the metabolites identified, and the highest level of the most compounds was detected after 2 h of olive oil administration (Garcia–Villalba et al., 2010).

To identify associations between polyphenol intake and health and disease outcomes in cohort studies, it is important to identify biomarkers of intake for the various compounds commonly consumed as part of the diet. In this sense, hydroxytyrosol could be considered as a useful biomarker of intake since its recovery yield in urine showed a high correlation with the dose ingested (Pérez–Jiménez et al., 2010).

The knowledge of structural form of olive oil polyphenols within the peripheral circulation (and other target tissues) is essential in order to obtain additional information about their mechanism of action *in vivo* (Kroon et al., 2004). In this sense, the radical scavenging potencies of the conjugates detected after hydroxytyrosol metabolism have also been investigated using the DPPH assay. The results showed out more activity for the monoglucuronide conjugate in comparison with its precursor, hydroxytyrosol; while the monosulphate conjugate did not show significant radical scavenging activity (Tuck & Hayball, 2002). In contrast with these results, the antioxidant activity of monoglucuronide conjugates was recently evalutated by DPPH assay and inhibition of Cu-mediated LDL oxidation at physiological concentrations (0.01–10 μM), without observing significant antioxidant activity at the concentration tested (Khymenets et al., 2010).

Although the conjugation suffered by hydroxytyrosol after its absorption changes the *in vitro* antioxidant activity in comparison with the unmodified compound, it is not possible to extrapolate these results to an *in vivo* situation. Therefore, it is important to identify target tissues of hydroxytyrosol. A study of the tissue distribution after intravenous administration of radioactive hydroxytyrosol in rats demonstrated a fast and extensive uptake of this molecule by the organs and tissues investigated, such as skeletal muscle, liver, lungs or heart, with a preferential renal uptake (D'Angelo et al., 2001). Moreover, 90% of the administered radioactivity was detected in urine, collected up to 5 h after injection mainly in its conjugated forms, while about 5% was found in feces and gastrointestinal content.

Regarding bioavailability of hydroxytyrosol containing wine, a recent study compared the hydroxytyrosol pharmacokinetics after moderate doses of wine and olive oil in healthy volunteers (De la Torre et al., 2006). It was observed that the daily ingestion of 250 ml of wine leads to plasma concentrations of about 8 ng/ml of hydroxytyrosol. Likewise, the results showed a higher urinary recovery of hydroxytyrosol after red wine administration in spite of the fivefold differences in the doses administered (0.35 mg for red wine and 1.7 mg for olive oil). These authors hypothesized the interaction between ethanol and dopamine after red wine ingestion, leading to the formation of hydroxytyrosol. In agreement with these findings, Schröder et al. (2009) confirmed that

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wine is an important source of hydroxytyrosol and they suggested alcohol as an indirect promoter of endogenous hydroxytyrosol generation.

Finally, as regards toxic effects of hydroxytyrosol, few studies have been carried out to evaluate its toxicity. D'Angelo et al. (2001) found that the oral administration of hydroxytyrosol to rats did not show any sign of toxicity up to 2 g/kg body weight. Recently Soni, Burdock, Christian, Bitler, and Crea (2006) performed toxicity studies with an extract rich in hydroxytyrosol (50–70% of weight extract). It did not cause toxicity at levels up to 2000 mg/kg/day. In the *in vivo* micronucleus assay, oral exposure of rats to hydroxytyrosol at dose levels up to 5000 mg/kg/day for 29 days did not induce increasement in polychromatic erythrocytes in bone marrow. The consumption of hydroxytyrosol is considered to be safe at levels up to 20 mg/kg/day according to the available studies of the extract and polyphenols.

3.3. Health-promoting properties

3.3.1. General

In this section the health benefits of hydroxytyrosol have been reviewed. As previously commented, few studies have been developed on the wine matrix. The majorities of these studies are actually performed on olive oil matrix since olive oil is the main source of hydroxytyrosol in diet.

3.3.2. Antioxidant activity

The phenolic antioxidant activity of hydroxytyrosol has been studied exhaustively using many different techniques in abiotic model systems where reactive oxygen species (ROS) and other radicals are generated by a variety of agents. When compared with other phenolic compounds, including tyrosol, hydroxytyrosol showed a much more effective antioxidant character. Evidently, antioxidant activity does not always correlate in different assay methods, and the catechol (*o*-dihydroxyl) structure is not always required for antioxidant activity. However, as a general rule, the ortho-dihydroxyl structure occurring in hydroxytyrosol and oleuropein have the highest antioxidant activity, followed by 4-*O*-monohydroxy compounds (ligstroside and tyrosol), and 3-*O*-hydroxy- substituted catechols; and all of these compounds are stronger antioxidants than either ascorbic acid or alpha-tocopherol (De Pinedo, Peñalver, & Morales, 2007; Mateos, Domínguez, Espartero, & Cert, 2003; Tuck & Hayball, 2002).

Hydroxytyrosol has been proven to have antioxidant activity *in vitro*, scavenging peroxyl, hydroxyl and other free radicals, reactive nitrogen species, and superoxide anions, breaking peroxidative chain reactions and preventing metal ion catalyzed production of reactive oxygen species (ROS) (Cornwell & Jiyan, 2008; Tripoli et al., 2005).

Although biological actions of phenolic compounds have been commonly related to its free radical scavenging activity (Goya, Mateos, & Bravo, 2007), current evidences strongly support that hydroxytyrosol may also offer an indirect protection by increasing the endogenous defence systems (Jemai, Bouaziz, Fki, El Feki, & Sayadi, 2008; Oliveras–López et al., 2008). In fact, Martín et al. (2010) confirmed this additional mechanism of action to prevent oxidative stress damage by inducing antioxidant enzymes, which act as critically important regulators in cell protection from oxidative stress and chemical-induced damage by controlling the intracellular redox status.

Considering the antioxidant inherent activity of hydroxytyrosol, it could be involved in the prevention of pathologies, such as cancer, cardiovascular disease, neurodegenerative disorders, diabetes, inflammation, etc., where etiology and progression have been related to ROS-mediated tissue injury. Biomarkers of oxidative damage based on the measurement of various relatively stable oxidation products, arising from DNA damage, LDL oxidation or reduced gluthathione (GSH) among others, have permitted to confirm the extensive biological activity detailed for this compound (Mateos & Bravo, 2007).

3.3.3. Cardioprotective capacity

Health effects of virgin olive oil intake in cardiovascular disease are greatly attributed to its high content in monounsaturated fatty acid (MUFA), but also to phenolic compounds such as hydroxytyrosol. In this sense, the EUROLIVE study held in 200 healthy male volunteers, demonstrated that the intake of three kinds of virgin olive oil with different phenolic content increased HDL-cholesterol and reduced lipid oxidative damage in a dose-dependent manner (Covas et al., 2006). These results are in agreement with those previously reported by Marrugat et al. (2004), who observed that the intake of high-phenolic content virgin olive oil decreased lipid oxidative damage. Furthermore, Visioli, Wolfram, Richard, Abdullah, and Crea (2009) recently showed that olive oil phenolic compounds obtained from olive mill waste water, in which hydroxytyrosol is the most bioactive component, increased total plasma glutathione levels when administered to 98 healthy volunteers. These results pointed out the capacity of hydroxytyrosol to prevent LDL oxidation and improve the lipid profile after its continuous consumption.

In addition to the ability of hydroxytyrosol to prevent LDL oxidation, hydroxytyrosol showed a beneficial effect on platelet function. The influence of phenolic compounds from virgin olive oil on cell adhesion molecules is uncertain. Pacheco et al. (2007) observed a postprandial decrease in molecular cell adhesion after intake of virgin olive oil compared with refined olive oil. However, consumption of virgin olive oil rich in phenolic compounds by stable coronary disease patients did not significantly influenced VCAM-1 and ICAM-1 plasma concentration (Fitó et al., 2008). Related to the benefits of hydroxytyrosol on platelet function, Dell'Agli et al. (2008) confirmed its ability to inhibit platelet aggregation *in vitro*. Otherwise, the intake of high-phenolic content virgin olive oil by 21 hypercholesterolemic volunteers, decreased plasminogen activator inhibitor-1 and factor VII, associated with changes in the postprandial hemostatic profile, leading to a less trombogenic state.

Concerning the development of atherosclerotic lesions, a study carried out in hyperlipidemic rabbits fed with a diet supplemented with hydroxytyrosol, showed an improvement of the antioxidant status and reduction of the size of atherosclerotic lesions when compared with control animals (González–Santiago et al., 2006).

It should be mentioned an interesting study in which the cardioprotective effects of hydroxytyrosol have been compared with others potent antioxidants, in addition to white and red wine. Rats treated for 14 days with hydroxytyrosol (2.5 mg/kg), tyrosol (2.5 mg/kg), resveratrol (2.5 mg/kg), as well as white wine and red wine, were sacrificed to isolate cardiomyocytes cells. The most surprising finding is the ability of white wine to induce the longevity proteins that in comparison with red wine and the rest of antioxidants showed the following order of activity: white wine > resveratrol > tyrosol > hydroxytyrosol > red wine. However, the cardioprotection exerted by reduction of infarct size and cardiomyocytes apoptosis followed a different pattern: resveratrol > red wine > hydroxytyrosol > white wine > tyrosol, suggesting the existence of different signaling mechanisms for the induction of longevity and survival (Mukherjee, Lekli, Gurusamy, Bertelli, & Das, 2009).

Finally, elevated concentrations of inflammation markers are associated with increased cardiovascular risk. Thus, reduction of tromboxane B_2 and leukotriene B_4 levels, as proinflammatory agents, has been repeatedly reported in intervention studies (Bogani, Galli, Villa, & Visioli, 2007; Oubiña, Sánchez–Muniz, Ró

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denas, & Cuesta, 2001; Visioli et al., 2005). Concerning pro-inflammatory cytokine and C-reactive protein, they significantly improved after the consumption of virgin olive oil rich in phenolic compounds in stable coronary disease patients (Fitó et al., 2008). Recently the activity of hydroxytyrosol prepared in a mixture of about 20% of this polyphenol (HT-20) in carrageenan-induced acute inflammation rats was evaluated. The rodents received different dosages (100, 250 and 500 mg/kg of body weight) by gavage of HT-20. This product significantly inhibited both the acute inflammation and the pain associated with carrageenan administration. The analgesic action of HT-20 was not in a dose-dependant manner and it was able to decrease pro-inflammatory cytokines IL-1beta and TNF-alpha, but not to increase the antiinflammatory cytokine mRNA expression of IL-10 (Gong et al., 2008).

3.3.4. Anticancer activity

The epidemiological studies carried out so far show evidence that olive oil consumption may reduce the risk of breast cancer (Trichopoulou & Dilis, 2007). Moreover, some intervention studies showed a significant improvement in makers of oxidative DNA damage after the intake of virgin olive oil, suggesting its ability to prevent some types of cancer. In the EUROLIVE substudy (Machowetz et al., 2007) the intake of olive oil reduced urinary 8-oxodeoxyguanosine levels, regardless of the phenolic content. In addition, the consumption of phenolic rich virgin olive oil could be responsible for the reduction of DNA damage in peripheral blood lymphocytes in postmenopausal women (Salvini et al., 2006).

The connection between chronic inflammation and tumour growth has received much attention and it is estimated that inflammation contributes to 15–20% of all cancers (Marx, 2004). In this sense, the described antiinflammatory activity in the cardioprotective capacity section for hydroxytyrosol implicitly demonstrates its potential anticarcinogenic activity. Indeed, hydroxytyrosol blocks the transcription of the enzymes COX-2 and 5-lipooxygenase, reducing the prostaglandin E2 synthesis and, thus, the chronic influence associated with diseases such as cancer (Cornwell & Jiyan, 2008). Likewise, Caco-2 cells treatment with olive oil polyphenols exerted anticancer effect by inhibition of COX-2 expression (Corona et al., 2007).

In addition, hydroxytyrosol alters tumour eicosanoid biosynthesis and shows a wide range of antitumour effects, inhibiting proliferation and promoting apoptosis in several human tumour-cell lines through several mechanisms (Corona et al., 2009; Fabiani, Fuccelli, Pieravanti, De Bartolomeo, & Morozzi, 2009; Han, Talorete, Yamada, & Isoda, 2009; Sirianini et al., 2010).

Finally, oxidative DNA damage is prevented by hydroxytyrosol in human blood mononuclear cells and HL60 cells (Fabiani et al., 2008).

3.3.5. Antimicrobial activity

Hydroxytyrosol is able to inhibit or delay the rate of growth of a range of bacteria, microfungi and pathogenic bacteria in humans, the antimicrobial activity of polyphenols containing olive fruit (Fleming, Walter, & Etchells, 1973), olive oil mill waste waters (Capasso et al., 1995), olive leaves (Markin, Duek, & Berdicevsky, 2003) and olive oil (Keceli & Robinson, 2002; Radford, Tassou, Nychas, & Board, 1991) has been reported. Medina, de Castro, Romero, and Brenes (2006) showed a strong bactericidal action of hydroxytyrosol against a broad spectrum of microorganisms, and was higher in general against Gram-positive than Gram-negative bacteria. Moreover, it showed bactericidal activity, not only against harmful bacteria of the intestinal microbiota (*Clostridium perfringens* and *Escherichia coli*), but also against beneficial microorganisms such as *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. Likewise, most of the foodborne pathogens tested (*Listeria monocytogenes, Staphylococcus aureus, Salmonella enterica, Yersinia sp.*, and *Shigella sonnei*) did not survive after 1 h of contact with this biophenol.

Recently, Brenes, Medina, Romero, and de Castro (2007) compared the antimicrobial activity of olive oil with that reported for foods such as tea, coffee and wine, among others. Results indicated a higher capacity of virgin olive oil than wine to inhibit the growth of pathogenic bacteria. Medina, Brenes, Romero, García, and de Castro (2007) evaluated comparatively the antimicrobial activity of olive oil, vinegar and various beverages, such as wine, against foodborne pathogens. Vinegar and aqueous extracts of virgin olive oil showed the strongest bactericidal activity against all strains tested, closely followed by red and white wines.

On the other hand, extracts from the byproducts of olive oil and wine production, showed high antimicrobial activity against *Escherichia coli, Candida albicans, Saccharomices cerevisise*, and *Bacillus cereus* (Serra et al., 2008). The authors suggest that the natural extracts may have important applications in the future as natural antimicrobial agents for the food industry, as well as for medical use. Indeed, this type of preservative has been tested in fish fillets with promising results (Pazos, Alonso, Fernandez–Bolanos, Torres, & Medina, 2006).

In addition, Bisignano et al. (1999) found that hydroxytyrosol has antimicrobial properties against several bacterial strains that are causal agents of intestinal or respiratory tract infections in humans. Likewise, in a more recent *in vivo* study Glatzle et al. (2007) demonstrated that virgin olive oil is more potent than fish oil to reduce septic pulmonary dysfunctions in rats. Moreover, Brenes et al. (2007) showed that hydroxytyrosol has a strong bactericidal activity *in vitro* against *Helicobacter pylory* that suggests its potential as a chemopreventive agent for peptic ulcers or gastric cancer.

Finally, hydroxytyrosol, not only shows antibacterial activity, but also antifungal activity against *Fusarium sambucinum, Verticillium dahliae* and *Alternaria solani*, as has recently been tested with enriched-hydroxytyrosol extracts (Yangui, Dhouib, Rhouma, & Sayadi, 2009).

3.3.6. Antidiabetic activity

Oxidative stress also plays a role in the pathogenesis of insulin resistance and it has been hypothesized that dietary antioxidants could diminish the risk of diabetes. Therefore, specific dietary strategies may contribute to improve glucose homeostasis and help in the prevention of this disease. In this sense, prospective observational studies and intervention studies support an inverse relationship between Mediterranean diet and insulin resistance (Pauwels, 2009).

Considering that hydroxytyrosol is involved in the prevention of stress oxidative, its effect in alloxan-induced diabetic rats after the consumption of purified compound was evaluated. Results confirmed the ability of hydroxytyrosol to inhibit oxidative stress (Hamden, Allouche, Damak, & Elfeki, 2009; Jemai, El Feki, & Sayadi, 2009) and hyperglycemia (Hamden et al., 2009).

Likewise, polyphenols containing olive leaf extract, such as oleuropein and hydroxytyrosol, reverted the chronic inflammation and oxidative stress that induces the cardiovascular, hepatic, and metabolic symptoms in this rat model of diet-induced obesity and diabetes, without changing blood pressure (Poudyal, Campbell, & Brown, 2010).

Hamden et al. (2010) recently found in diabetic rats an inhibitory action of hydroxytyrosol on pancreatic toxicity after consuming hydroxytyrosol-supplemented diets.

3.3.7. Neuroprotective activity

The importance of olive oil as a major component of the Mediterranean diet to counteract neurodegenerative age-related diseases such as Alzheimer's and Parkinson's diseases has been suggested (Berr et al., 2009; Scarmeas, Stern, Tang, Mayeux, &

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Luchsinger, 2006). On the one hand, results from the Three-City Study (Berr et al., 2009) revealed that participants with moderate or intensive use of olive oil compared to those who never consume olive oil showed lower odds of cognitive deficit for verbal fluency and visual memory during a 4-year follow-up of 6947 subjects. On the other hand, results published by Scarmeas et al. (2006) from a follow-up study carried out with 2258 subjects showed that higher adherence to the Mediterranean diet is associated with a reduction in the risk of Alzheimer's dysfunction.

Recent findings about the benefits of hydroxytyrosol to prevent neuronal diseases have proliferated. This hypothesis is supported considering that hydroxytyrosol could cross the blood–brain barrier to appear in the brain. Thus, the measurement of free hydroxytyrosol by liquid chromatography with fluorescence in microdialysates from blood and brain of anesthetized rats permitted to determine the rapid elimination of hydroxytyrosol and its uptake by brain (Wu, Lin, & Tsai, 2009).

The extract enriched in oleuropein has shown neuroprotective activity by forming a non-covalent complex with the Aβ peptide, which is a key hallmark of several neurodegenerative diseases like Alzheimer and Parkinson. Thus, hydroxytyrosol, which is the principal degradation product of oleuropein, has been suggested as the potential neuroprotective compound (Bazoti, Bergquist, Markides, & Tsarbopoulos, 2006). The neuroprotective effect of hydroxytyrosol was recently tested in a model of hypoxia-reoxygenation in rat brain slices, *in vitro* and *in vivo*. Hydroxytyrosol significantly inhibited LDL efflux in a dose-dependant manner, providing a preliminary basis for further study as potential neuroprotective compounds (González–Correa et al., 2008).

4. Melatonin

4.1. Structure and concentration in wine

Melatonin is an indolamine (*N*-acetyl-5-methoxytryptamine) (Fig. 1c). This neurohormone was discovered in the pineal gland and it is also produced as secondary metabolite in plants.

Melatonin has been shown to be synthesized from tryptophan *via* 5-hydroxytryptophan, serotonin and *N*-acetylserotonin and to be metabolized by deacetylation to 5-methoxytryptamine (Fig. 4). Moreover, melatonin can also be formed by *O*-methylation of serotonin followed by *N*-acetylation of 5-methoxytryptamine in yeast (Hardeland, Reiter, Poeggeler, & Tan, 1993; Sprenger, Hardeland, Fuhrberg, & Han, 1999).

Medicinal herbs such as *Tanacetum parthenium* or *Hypericum perforatum* are rich in melatonin (Murch, Simmons, & Saxena, 1997), but they cannot be considered as normal dietary sources. Melatonin has been reported in edible seeds, such as rice and sweet corn (Hattori et al., 1995; Manchester et al., 2000), roots, leaves and fruits of a considerable variety of plants. Indeed, melatonin is present in strawberries, kiwis, pineapples, bananas and apples (Paredes, Korkmaz, Manchester, Tan, & Reiter, 2009). In fact, the occurrence of melatonin in different varieties of strawberries and tomatoes has recently been reported (Stürtz, Cerezo, Cantos, & García–Parrilla, in press). The amounts range from 1.38 to 11.26 ng/g in strawberry and from 4.11 to 114.52 ng/g in tomatoes. The consumption of fresh fruits containing ascorbic acid, which protects melatonin from oxidation, was a positive factor in overall melatonin dietetic intake.

Melatonin has also been found in olive oil at higher levels in extra virgin olive oil than in refined olive or sunflower oil samples (De la Puerta et al., 2007). In addition, melatonin has recently been reported in grapes and wines. Iriti, Rossoni, and Faoro (2006) found melatonin in different grape varieties: Nebbiolo, Croatina, Sangiovese, Merlot, Marzemino, Cabernet franc, Cabernet sauvignon and

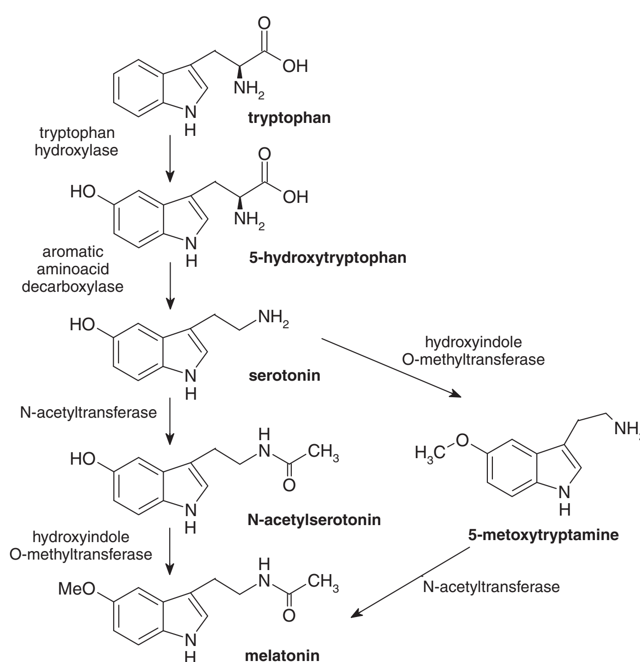

Fig. 4. Biosynthesis pathway of melatonin (Iriti et al., 2006).

Barbera. The melatonin concentration ranged from 0.005 to 0.9 ng/g. Melatonin was detected by mass spectrometry in Merlot ripening grapes (Murch, Hall, Le, & Saxena, 2010). The increase of indolamines as melatonin and serotonin during veraison of grapes from different vineyards reveals a role in plant physiology. Melatonin has also been reported in wine (0.4–0.5 ng/ml) as determined by HPLC-Fluorescence analysis (Mercolini et al., 2008). Melatonin was assessed in Malbec (0.24 ng/ml), Cabernet Sauvigon (0.32 ng/ml) and Chardonnay (0.16 ng/ml) by means of capillary electrochromatography (Stege, Sombra, Messina, Martinez, & Silva, 2010). Melatonin has recently been found, from 245 to 423 ng/ml, in ten monovarietal wines: Cabernet Sauvignon, Petit verdot, Prieto picudo, Syrah, Tempranillo (Rodriguez–Naranjo, Gil–Izquierdo, Troncoso, Cantos–Villar, & García–Parrilla, 2011a). Additionally, another compound with an identical MS fragmentation pattern, tentatively assigned to a melatonin isomer, was identified in Cabernet Sauvignon, Merlot, Palomino Negro, Prieto picudo and Tempranillo (Rodriguez–Naranjo, Gil–Izquierdo, Troncoso, Cantos, & García-Parrilla, 2011b). However, these authors did not find melatonin in any part of the different varieties of grape (pulp, skins or seeds). Despite melatonin not being present in the initial musts of six grapes varieties, the compound and its possible isomer were detected in finished wines. Experimental winemaking processes revealed that it is formed after the inoculation with yeasts and the role of *Saccharomyces cerevesiae* is crucial (Rodriguez–Naranjo et al., 2011a). Melatonin content in wine is shown in Table 3.

Even though the concentration of melatonin is lower than other compounds, it could be said that, depending on dietary habits, melatonin may account for a significant contribution to the diet (Dubbels et al., 1995).

4.2. Bioavailability and pharmacokinetic

The European Food Safety Authority has recently accepted the scientific evidence of health claims in relation to melatonin and alleviation of subjective feelings of jet lag, reduction of sleep onset latency, and contribution to sleep quality. The melatonin dose should be between 0.5 and 5 mg and should be taken close to be

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Table 3
Melatonin content in wines.

Bioactive compound (ng/ml)	White wine	Red wine	References
Melatonin	0.4	0.5	Mercolini et al. (2008)
	0.16	0.3	Stege et al. (2010)
	391	35	Rodríguez–Naranjo et al. (2011a, 2011b)

dtime on the first day (and any subsequent day) of travel and on the following few days after arrival at the destination. The target population is the general population (EFSA, 2010).

It has already been demonstrated that melatonin is well absorbed after oral administration. After an oral intake of 250 µg plasmatic concentration varies from 155 pg/ml to 720 pg/ml, depending on the sex of the volunteer. Absorption is affected by parameters that can vary widely from one subject to another. However, other pharmacokinetic variables such as elimination and distribution half-life seem to be constant from one subject to another. Bioavailability reported values vary from 22% (Waldhauser et al., 1984) to 8.7% (Fourtillan et al., 2000), or to 33.0% (Di, Kadva, Johnston, & Silman, 1997).

The effect of food containing melatonin on melatonin levels was tested (Hattori et al., 1995). Chickens were fed with corn, rice, beans and milo after the animals had been fasting for 48 h. Doubled daytime melatonin levels were detected. Despite the fact that melatonin content in foods is low, melatonin plasmatic concentration increased after animals were fed with nuts (*Juglans regia*) in a reasonable dose (3 g). The dietary intake was 10.5 ng and the plasmatic concentration increased from 11.5 ± 1.9 to 38.0 ± 4.3 pg/ml (Reiter, Tan, & Maldonado, 2005), and plasma antioxidant capacity determined with the FRAP method simultaneously increased.

In addition to plasmatic levels of melatonin, the melatonin metabolite 6-sulfatoxymelatonin, excreted in urine, is easy to determine. Indeed, a higher excretion of this metabolite in the first morning urine was 16% higher in women with the highest quartile vegetable intake in comparison to the lowest quartile intake (Nagata, Nagao, Shibuya, Kashiki, & Shimizu, 2005). Moreover, nutritional and lifestyle have also been correlated with circulating melatonin levels (Dopfel, Schulmeister, & Schernhammer, 2007). An statistical inverse relation among age, smoking and body mass index with urinary 6-sulfatoxymelatonin was found (Schernhmmer & Hankinson, 2005). In addition to these statistical associations, a study carried out with rats established a relationship between melatonin and body weight. Animals were put on a diet to induce obesity. Afterwards, they were treated with melatonin for 3 weeks (30 mg/kg) or pinealectomized. Adipose tissue, weight, insulinemia and glycemia increased in the pinealectomized rats, while treatment with melatonin partially prevented these effects (Prunet–Marcassus et al., 2003). In ovarectomized rats melatonin reduced food intake and partially prevented the increase of body weight and cholesterol without changing leptin levels (Sánchez–Mateos et al., 2007). Melatonin administered in high quantities significantly decreased food intake in two different fish species: goldfish (diurnal, 16.7%) and tench (nocturnal, 37%) (Lopez–Olmeda, Madrid, & Sanchez–Vazquez, 2006). Melatonin peripherally administered to goldfish inhibited food intake (Pinillos, De Pedro, Alonso–Gómez, Alonso-Bedate, & Delgado, 2001). The authors suggested that melatonin is involved in the peripheral satiety mechanisms in goldfish.

Another interesting aspect is the influence of plasma level of melatonin and the preference for a kind of food. An experiment was performed with rats after intraperitoneal administration of four different doses of melatonin. Records of food intake show that four hours post injection animals present a short term increase in total food intake due to an increase in carbohydrate-rich diets. Data reported were consistent for the different melatonin doses employed. However, the authors could not find a consistent pattern for protein-rich diets (Angers, Haddad, Selmaoui, & Thibault, 2003). The hypothesis to explain the obtained results considers the effect of melatonin as a circadian marker. If melatonin marks the night period, rodents involved in the study will eat more. However, the opposite effect is expected in humans (eating less as night starts). Indeed, higher melatonin concentration was found in anorexic people (Arendt, Bhanji, Franey, & Mattingly, 1992). The work by Angers et al. (2003) reveals the need to study the effect of melatonin on food intake as being dependent on the dose and time when supplements are taken.

Mustonen, Nieminen, and Hyvarinen (2002) investigated subacute effects of persistent melatonin treatment and continuous light on carbohydrate and fat metabolism of rat liver and kidney. Exogenous melatonin enhanced utilization of liver carbohydrates, but suppressed hepatic lipolysis.

Finally, as regards toxic effects of melatonin few studies have been carried out to evaluate the melatonin toxicity. Melatonin itself turned out to be neither toxic nor mutagenic in high concentration assays (Anisimov, 2003). However, further studies and clinical trials are needed to verify that melatonin is safe.

4.3. Health-promoting properties

4.3.1. Antioxidant capacity

Melatonin is a significant free radical scavenger and antioxidant at both physiological and pharmacological concentrations *in vivo*. Like other secondary metabolites, melatonin has antioxidant properties as a direct free radical scavenger and by stimulating antioxidant enzymes (Hardeland & Pandi–Perumal, 2005; Reiter et al., 2005).

Indeed, melatonin is able to scavenge H_2O_2 in a dose-dependent manner. As a result, *N*(1)-acetyl-*N*(2)-formyl-5-methoxykynuramine (AFMK) is formed (Tan et al., 2000). Moreover, the biological activities of melatonin metabolites AFMK and N^1-acetyl-5-methoxykynuramine (AMK) have been described. AFMK is a potent antioxidant providing protection to DNA and lipids through several mechanisms. AMK is also a potent antioxidant and it is able to inhibit prostagladin biosynthesis and to bind diazepan receptors (Guenther et al., 2005; Schaefer & Hardeland, 2009; Than, Heer, Laatsch, & Hardeland, 2006). AFMK and AMK are major melatonin metabolites.

Melatonin can scavenge other reactive oxygen species such as $ONOO^-$, $NO^{\cdot}$ and H_2O_2 (Hardeland et al., 2005). Melatonin and other indoles present in the diet also scavenge the $ABTS^{\cdot+}$ in a common test to assess antioxidant activity of foods (Herraiz & Galisteo, 2004). The amphipathic character of the molecule enables it to trespass physiological barriers. Accordingly, it has been found in cytosol, nucleus, membranes and mitochondria (Karbownk, Lewinski, & Reiter, 2001). This fact is quite relevant because it means that the molecule can be at the places where radicals are formed, providing antioxidant defences where they are needed; other dietetic antioxidants cannot fulfil this requirement.

In addition to these actions, melatonin stimulates cellular antioxidant defence by increasing messenger RNA (mRNA) and protein levels of the major antioxidant enzymes (Mayo et al., 2003) and reducing the activity of a pro-oxidative enzyme, i.e. iNOS (Pozo, Reiter, Calvo, & Guerrero, 1994). Numerous reports have already shown that melatonin protects lipids, proteins and DNA from the harmful effects of free radicals. These protective actions of melatonin are typically associated with preservation of cell viability.

When administered to mice, it was able to reduce the chronic oxidative stress related to aging (Nogues et al., 2006) and it might

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even reduce blood pressure in males with chronic hypertension (Scheer, Van Montfrans, Van Someren, Mairuhu, & Buijs, 2004).

As an antioxidant melatonin has been found to be particularly effective in reducing free-radical-mediated damage to DNA. Damaged DNA, if it goes unrepaired, may mutate and initiate a tumour.

4.3.2. Anticancer activity

Since 1994, when the first paper was published documenting the role of melatonin in apoptosis, the number of reports in this area has rapidly increased (Maestroni, Covacci, & Conti, 1994; Sainz et al., 2003).

Melatonin may play an important role in carcinogenesis, as suggested by substantial laboratory and less direct epidemiologic evidence. Particularly, in experimental animals cancer growth is exaggerated when the animals are repeatedly phase advanced (as occurs during easterly flights) or exposed to light at night (Reiter et al., 2007). If, in fact, physiological levels of melatonin normally restrain tumour growth, the age-associated reduction in melatonin production may be contributory to the increased frequency of cancer in the elderly. There is also some evidence to indicate that the efficacy of melatonin in limiting tumour cell proliferation depends on the time of day of its administration, with melatonin given late in the light phase being more effective (Sauer, Dauchy, & Blask, 2001).

Mechanistically, how melatonin inhibits tumour cell proliferation has partially been defined and it apparently involves a number of mechanisms. Apart from those, it is remarkable that there are also some other actions implied. In oestrogen-receptor-positive human breast cancer cells melatonin is thought to modulate oestrogen receptor expression and transactivation. Other potential mechanisms still include melatonin's ability to reduce angiogenesis in tumours, to delay the G1 to S phase transition in the cell cycle, to improve cellular communication between normal and cancer cells, and to alter the intracellular redox state. Besides inhibiting established tumours, melatonin may also decrease their initiation (Reiter, 2003).

The use of melatonin in humans reduced tumour growth in some cases and prolonged survival of cancer patients compared with individuals given conventional cancer therapy (Lissoni, 2002). And, more importantly, melatonin administration, when combined with standard chemotherapies, often improves the quality of life. This is probably related to melatonin's ability to reduce the toxicity of chemotherapeutic agents and melatonin action synergistically. Apart from the objective benefits achieved in the patients, researchers also commented on probable subjective advantages of using melatonin. Many of these effects include amelioration of hypotension, myelotoxicity, and lymphocytopenia associated with concomitantly prescribed toxic therapeutic regimens. Perhaps the most clinically relevant feature is that patients receiving melatonin achieve and maintain a better performance status and also have less anxiety than those treated without melatonin. Lissoni (2000) have also reported substantial beneficial effects of treatment of cancer patients with melatonin analogues as well. The observations reported in all these clinical investigations are encouraging and indicate that melatonin administration was generally deemed to improve patients suffering from a variety of cancers (Lissoni, 2000; Vijayalaxmi, Thomas, Reiter, & Herman, 2002).

4.3.3. Immunomodulatory agent

Melatonin exhibits immunomodulatory properties which are mediated via membrane and nuclear receptors (Guerrero & Reiter, 2002). Data were reported on activation of T, B, NK cells and monocytes, thymocyte proliferation, release of cytokines (IL-1, IL-2, IL-6, IL-12, and IFN), met-enkephalin, other immunoopioids, and antiapoptotic effects, including glucocorticoid antagonism. Signalling mechanisms are only partially understood and some findings are still contradictory (Liu, Ng, & Fung, 2001).

Daily oral melatonin administration in humans increases natural killer (NK) cell activity (Guerrero et al., 2000). Additionally, melatonin reportedly regulates gene expression of several immunomodulatory cytokines including tumour necrosis factor-a (TNFa), transforming growth factor beta (TGFb) and stem cell factor (SCF) by peritoneal macrophages as well as the levels of interleukin-1beta (IL-1b), interferon gamma (INFg), TNFa and SCF by splenocytes (Liu et al., 2001). Melatonin is also a potent inhibitor of apoptosis in the immune cell (Reiter, 2003).

The fact that melatonin is generally considered to be immunostimulatory raises the question that whether it should be taken by individuals suffering from an autoimmune disease. To date, the information is meagre regarding this issue, although in one case of Crohn's disease the condition of excessive immuno reactivity of the gut wall was aggravated by melatonin. Whether this will be a general finding in autoimmune diseases, however, remains to be established (Reiter, 2003).

4.3.4. Neuroprotective activity

A special, but important, aspect is melatonin's role in neuroprotection. Melatonin has been tested in sleep disorders. It generally reduces sleep latency and improves sleep especially when circadian phasing is disturbed. In the latter case, this was particularly effective in patients with neurodegenerative diseases (Srinivasan et al., 2005). Numerous attempts have been made or are under current investigation to mitigate neurodegenerative diseases, such as Alzheimer's, Parkinson's, and Huntington's diseases and amyotrophic lateral sclerosis. Melatonin was shown to inhibit Aβ fibrillogenesis (Asayama et al., 2003; Harderland, Pandi–Perumal, & Cardinali, 2006). It has recently been shown that melatonin (200 mg/kg) reduced edema in impacted striatum versus traumatic brain injury (Kabadi & Maher, 2010).

5. Conclusions

The Mediterranean diet has become recognised as a model diet for preventing several serious diseases and cardiac disease in particular. Wine seems to be a key component in this diet and a moderate, regular consumption of wine (two glasses of red wine per day) is recommended.

Resveratrol, hydroxytyrosol and melatonin are three compounds naturally present in wine. They could act synergically to ensure a higher cytoprotective effect against oxidative stress, thus further supporting the hypothesis that health benefits of Mediterranean diet are partly due to wine.

Wine comes in a wide variety of styles (varieties, winemaking, storage conditions, etc.), and therefore they contain quite different bioactive compounds. On average a service of red wine (200 ml) could provide 0.38 mg of resveratrol, 0.45 mg of hydroxytyrosol and 61.4 μg of melatonin, apart from other important bioactive compounds.

It is impossible to make a definitive statement or conclusion about the real effect of these molecules on health since human studies are still in initial stages and also because there is too much variability in the study designs. What can be said is that these molecules look promising in the field of medicine.

However, there are still some issues that need to be addressed. How much resveratrol, hydroxytyrosol and melatonin can be taken and recovered from the organism? How active are the metabolites derived from them? How does the type of meal consumed in association with the ingestion of red wine influence the bioavailability in humans? These are all questions still to be answered.

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Abstract	Grape is one of the earliest cultivated plants all around the world. Health-benefiting grape properties have been widely studied in vitro, ex vivo, and in vivo. These properties are mainly attributed to phenolic composition, which is also responsible for many quality properties. Grape contains anthocyanins, flavonols, flavanols, hydroxycinnamic acid derivatives, hydroxybenzoic acids, and stilbenes. All these show bioactivity and, therefore, antioxidant, cardioprotective, anticarcinogenic, neuroprotective, and other activities are nowadays associated with grape consumption. Clinical studies on the intake of grape or grape derivative products report positive results. For this reason, numerous food products are enriched with different types of grape extracts. Grape extracts are added to meat, fish, dairy products, bread, and beverages so as to increase their nutritional value. The functional product market is an emerging market, and this type of products can be expected to increase in the near future. In this sense, further research is required.

Keywords (separated by "-")	Anticarcinogenic - antioxidant - bioactive - cardioprotective - functional - grape - neuroprotective - phenolic compounds

Functional Grapes

69

Maria Isabel Fernández-Marín, Raúl F. Guerrero, Belén Puertas, María Carmen García-Parrilla, and Emma Cantos-Villar

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Abstract

Grape is one of the earliest cultivated plants all around the world. Health-benefiting grape properties have been widely studied in vitro, ex vivo, and in vivo. These properties are mainly attributed to phenolic composition, which

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is also responsible for many quality properties. Grape contains anthocyanins, flavonols, flavanols, hydroxycinnamic acid derivatives, hydroxybenzoic acids, and stilbenes. All these show bioactivity and, therefore, antioxidant, cardioprotective, anticarcinogenic, neuroprotective, and other activities are nowadays associated with grape consumption. Clinical studies on the intake of grape or grape derivative products report positive results. For this reason, numerous food products are enriched with different types of grape extracts. Grape extracts are added to meat, fish, dairy products, bread, and beverages so as to increase their nutritional value. The functional product market is an emerging market, and this type of products can be expected to increase in the near future. In this sense, further research is required.

Keywords
Anticarcinogenic • antioxidant • bioactive • cardioprotective • functional • grape • neuroprotective • phenolic compounds

1 Introduction

The Vitaceae family consists of almost 1,000 species, grouped into 17 genera. Grapevines are classified in the *Vitis* genus, and the most widely cultivated *Vitis vinifera* is generally accepted to comprise up to 5,000 true cultivars used in wine, table (fresh fruit), and dried grape manufacture around the world. The *Vitis vinifera* grape is one of the earliest cultivated plants and is thought to have originated in the region between the Mediterranean and the Caspian Seas. Vine cultivars are thought to have slowly spread eastward across southern Asia and westward around the Mediterranean Sea. The Germplasm Resources Information Network (www.ars-grin.gov) of the United States Department of Agriculture describes the genera and 43 species, 5 natural hybrids, and 15 varieties of species in *Vitis*. *V. Vinifera* as the most successfully used grape species, with thousands of wine, table, and raisin grape cultivars grown throughout the world's temperate zones.

The numerous uses of the grapevine fruit, especially for wine and beverages, have made it one of the most important plants worldwide. Grapes are grown in more than 90 countries and are the world's largest fruit crop with a total production of 69 million tons. The countries with the greatest acreage are Spain, France, Italy, Turkey, China, and the United States [1].

Grapes have been praised for thousands of years for their medicinal and nutritional values. Since the ancient age of human civilization, grapes have been considered as a fruit with "healing power." Egyptians loved grapes, and ancient Greek and Roman philosophers and physicians heralded their healing powers, particularly as fermented grape juice or wine. This health character of grapes has been supported scientifically in recent years.

Chronic diseases are the most prevalent cause of death in the world, led by cardiovascular diseases and followed by cancer, chronic lung diseases, and diabetes mellitus. Epidemiological studies indicate that high serum cholesterol might have

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a strong correlation with increased risk of coronary heart disease (CHD). These findings led to the classical diet – heart hypothesis – which postulated the primary role of saturated fat and cholesterol in the development of atherosclerosis and CHD. In addition, initial studies suggested a direct relationship between high dietary fat intake and increased risk of breast and colon cancer. However, large prospective studies have not only addressed the effects of high dietary fat intake but also indicated the prevention of certain chronic conditions with a vegetable/fruit-rich diet. In recent years, the relationship between the consumption of specific foods and/or overall dietary patterns and the risk of CHD has been examined. Accumulating evidence from epidemiological, case control, and cohort studies suggests that a vegetable/fruit-rich diet may offer protection against chronic diseases. A study published in JAMA in 1999 by Joshipura et al. [2] clearly proved the need for higher fruit and vegetable consumption to reduce the risk of certain diseases such as ischemic stroke. However, no apparent further reduction in risk was observed beyond six servings per day. Another study has shown reduced risk of myocardial infarction in women consuming five fruit and vegetable servings [3]. The abovementioned literature suggests that fruits and vegetables may have an important role in keeping a healthy lifestyle. This observation leads to a million dollar question: which components of fruits and vegetables may be responsible for this protective effect? In this sense, many clinical trials have used different bioactive compounds for chronic disease prevention. Polyphenols are thoroughly researched for their human health-benefiting properties at different stages and have reached clinical studies, which confirm the potency of these bioactive compounds.

This chapter deals with grapevine products as functional foods and sources of nutraceuticals. Complex grape polyphenol chemistry is reviewed, and emphasis is laid on health benefits arising from the consumption of grape products. Finally, functional food from grapes is examined.

2 Chemical Composition

Grape contains (per 100 g): Water (80.5 g), carbohydrates (18.1 g), fat (0.16 g), protein (0.72 g), fiber (0.9 g), potassium (191 mg), sodium (2 mg), phosphorous (20 mg), calcium (10 mg), magnesium (7 mg), iron (0.36 mg), zinc (0.07 mg), vitamin C (10.8 mg), vitamin B1 (0.07 mg), vitamin B6 (0.09 mg), vitamin B2 (0.07 mg), vitamin A (66 IU), and vitamin E (0.19 mg). All above provide around 69 Kcal per 100 g of intake (from USDA Nutrient Database for Standard Reference, Release 24; http://ndb.nal.usda.gov/ndb/foods/list).

Moreover, apart from these main components, grapes contain secondary metabolites such as flavonoid and non-flavonoid phenolic compounds, sesquiterpenes, and melatonin. Most of the grape's medicinal value can be attributed to its seed and skin, which researchers have found to be rich in nutritional value due to the presence of polyphenolic antioxidants. At least 500 different types of antioxidants have been found in various parts of this fruit [4].

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2.1 Phenolic Compounds

Grapevine quality, as with most plants, mainly depends on its metabolites. Metabolite production is especially sensitive to external conditions. In particular, the chemical diversity of grapevine is mostly affected by secondary metabolites.

These secondary metabolites consist of a wide array of species-specific chemicals and belong to different phytochemical groups such as alkaloids, terpenes, antibiotics, volatile oils, resins, cardiac glycosides, tannins, sterols, saponins, and phenolics, many of which have proven highly valuable for the pharmaceutical, agrochemical, food, and fragrance industries [5]. In general, secondary metabolites are known to play key physiological functions in plants, including their adaptation to the environment [6], acquired resistance to pests and diseases, pollinator attractant capacity, and the building of symbiotic relations with microorganisms [7]. They are also very often crucial in quality determination in food attributes (color, taste, and aroma) and colors and pigments in ornamental plants [8].

We have focused on phenolics because they are a large and complex group of metabolites that particularly contribute to the (sensorial and bioactivity) features of grapes.

There are a large number of phenolic compound structures and, therefore, a large number of properties as well. Grapevine phenolics may either arise from the fruit (mainly skins and seeds) and vine stems or be products of yeast metabolism. Their schematic biosynthesis is shown in Fig. 69.1. Briefly, the phenylpropanoid pathway generates most phenolic compounds found in nature, including flavonoids and stilbenoids. Biosynthesis of phenylpropanoid compounds is not only developmentally activated in specific tissues and cell types but can also be activated in other tissues in response to environmental stresses such as wounding, pathogen infection, or UV irradiation. Phenylalanine is an end product of the shikimate pathway. The structural diversity of phenylpropanoids derived from phenylalanine and the key phenylpropanoid intermediate *p*-coumarate is due to the action of enzymes and enzyme complexes that bring about region-specific condensation, cyclization, aromatization, hydroxylation, glycosylation, acylation, prenylation, sulfation, and methylation reactions [9].

2.1.1 Anthocyanins

Anthocyanins are the most abundant pigments in red grape skins (Fig. 69.2a). These water-soluble pigments are responsible for blue, red, and purple color in red grape skin and red wine. Anthocyanins of *Vitis* are monoglucosides of five anthocyanidins, namely, delphinidin, cyanidin, petunidin, peonidin, and malvidin. Acylated anthocyanins are esters of the glucose moiety of the free anthocyanins with acetic, *p*-coumaric, or caffeic acids (Fig. 69.2a).

Malvidin 3-glucoside is the main anthocyanin in all the varieties of *Vitis vinifera*. The amount of anthocyanins in grapes, as well as in all phenolics, depends on the grapevine variety and is highly influenced by viticultural and environmental factors such as light, temperature, altitude, soil type, water, nutritional status, pathogenesis, and various developmental processes [10]. Temperature has a great

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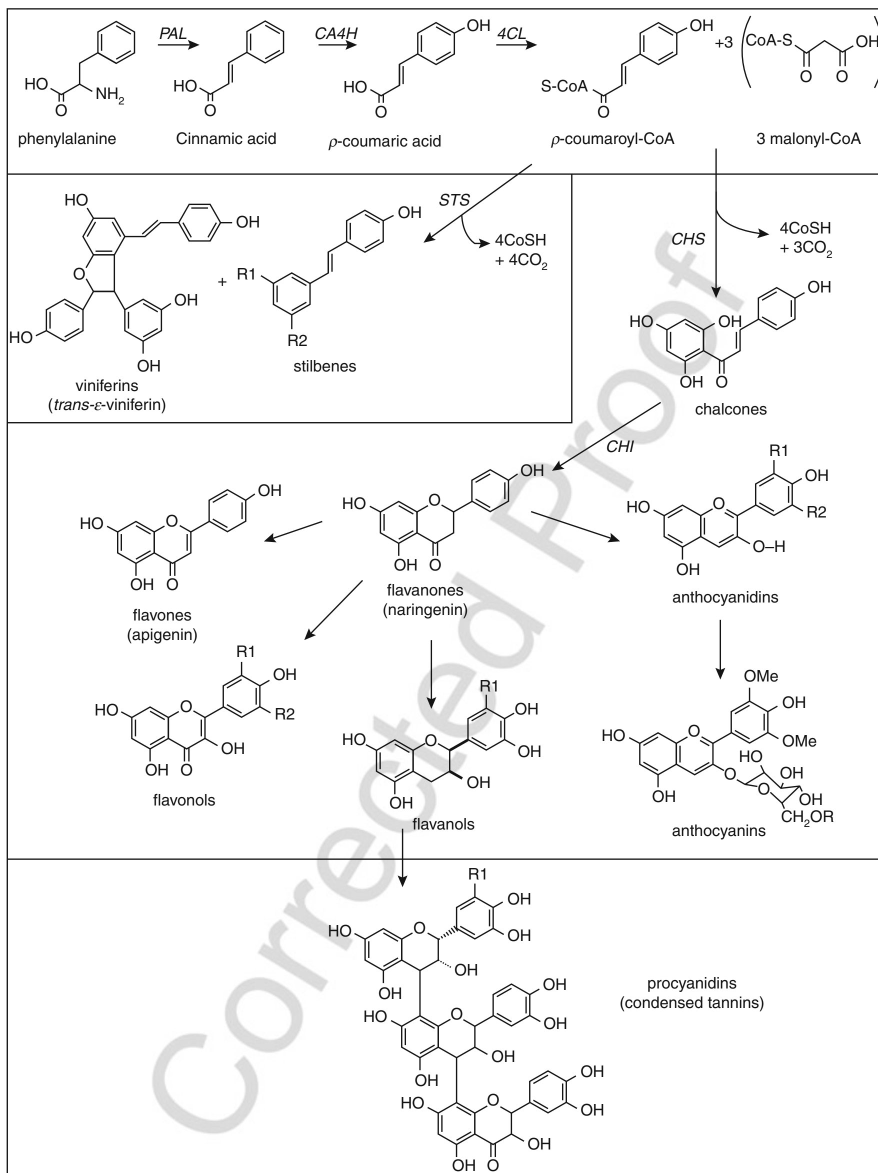

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Fig. 69.1 Biosynthesis pathway of grape phenolics

154 influence on anthocyanin biosynthesis. Anthocyanin levels in Cabernet Sauvignon
155 grapes are higher when day temperatures remain constant at around 20°C rather
156 than 30°C. Therefore, increased anthocyanin content is associated with grapes
157 grown at higher altitudes. However, this relationship is complicated by the effect
158 of diurnal differences in temperature: lower night temperatures result in greater

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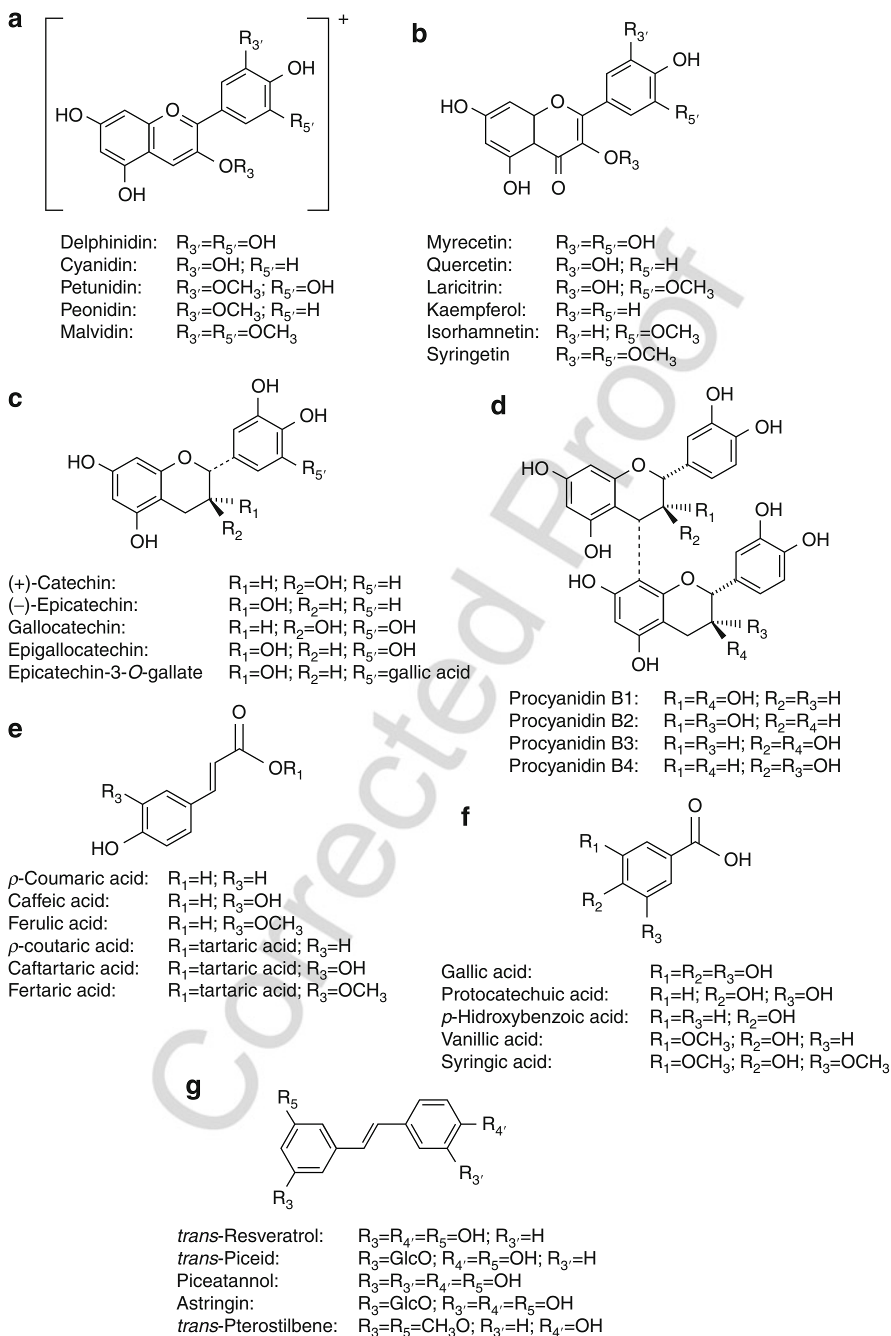

Fig. 69.2 (**a–g**) Chemical structure of the main bioactive compounds in grape

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t1.1 **Table 69.1** Phenolic compounds in red and white grapes

	Family	Phenolic compound	Red grape (mg/kg fw)	White grape (mg/kg fw)	References
t1.3	Anthocyanins	Dp-3-glc	500–5,000	–	[13–16]
t1.4		Cy-3-glc			
t1.5		Pt-3-glc			
t1.6		Pn-3-glc			
t1.7		Mv-3-glc			
t1.8		Acetyl-derivatives			
t1.9		*p*-Coumaroyl-derivatives			
t1.10	Flavonols	Q-3-glc/glu/gal/rut/glugal/gluxyl	3–300	1–200	[13, 16–19]
t1.11		K-3-glc/glu/gal			
t1.12		M-3-glc/glu			
t1.13		I-3-glc/glu/gal			
t1.14		L-3-glc/gal			
t1.15		Syr-3-glc			
t1.16	Total flavan-3-ols	Catechin	2,500–11,000	2,500–11,000	[17, 20, 21]
t1.17		Epicatechin			
t1.18		Gallocatechin			
t1.19		Epigallocatechin			
t1.20		Epicatechin-3-*O*-galate Procyanidin B1, B2, B4, C1			
t1.21	Hydroxycinnamic acid derivatives	Caffeoyl tartaric	1.5–50	4–45	[15–17, 22]
t1.22		Coumaroyl tartaric			
t1.23		Feruloyl tartaric			
t1.24	Hydroxybenzoic acids	Gallic acid	2–5	2–5	[22]
t1.25		Protocatechuic acid			
t1.26		p-Hydroxybenzoic acid			
t1.27		Vanillic acid			
t1.28		Syringic acid			
t1.29	Stilbenes	*trans-/cis*-Resveratrol	0–22	0–8.5	[23–25]
t1.30		*trans-/cis*-Piceid			
t1.31		Piceatannol			
t1.32		Astringin			
t1.33		Pterostilbene			
t1.34		ε- and δ-Viniferin			

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159 accumulation of anthocyanins [11]. The accumulation of anthocyanins starts at the
160 veraison stage with occasional decreases toward the end of ripe stage, especially in
161 hot climates [12]. In fact, reduced grape berry color has been observed in very hot
162 seasons. Whether this decrease occurs through degradation of existing anthocya-
163 nins or reduced anthocyanin biosynthesis is not known. Hence, the range of
164 concentration varies from 500 to 5,000 mg/kg fw (Table 69.1).

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2.1.2 Flavonols

Flavonols are found in grape only as glycosides of myricetin, quercetin, laricitrin, kaempferol, isorhamnetin, and syringetin (Fig. 69.2b). The corresponding aglycones can be found in wine, together with 3-glycosides. Glucose is the common sugar attached to the C-3 position of kaempferol, quercetin, myricetin, and isorhamnetin, but glucuronic acid has also been found as glycosylation of quercetin, kaempferol, myricetin and isorhamnetin, and laricitrin. Furthermore, quercetin has been found in grapes as 3-galactoside, 3-rhamnosylglucoside (also called rutin), 3-glucosylgalactose, and 3-glucosylxyloside. Kaempferol, laricitrin, and isorhamnetin have been described as galactoside derivatives (Table 69.1).

As described for anthocyanin, climate impacts on the amount of flavonol in grape. In contrast to anthocyanin and tannin synthesis, which are scarcely affected by shading treatments, a positive relationship was observed between sunlight exposure and increased flavonol accumulation [26, 27].

Flavonols are exclusively found in red and white grape skins, and its concentration ranges from traces to 300 mg/kg fw (Table 69.1).

2.1.3 Flavan-3-ol Monomers and Procyanidins

Flavan-3-ols are another large family of polyphenolic compounds comprising mainly catechin, epicatechin, epicatechin 3-*O*-gallate, gallocatechin, epigallocatechin, and epigallocatechin 3-*O*-gallate (Fig. 69.2c). Procyanidins, also known as condensed tannins, are both oligomeric and polymeric compounds arising from polyhydroxy flavan-3-ol and flavan-3,4-diol units and their epimers through C4 → C8 or C4 → C6 bonds. In grape seeds and skin, about 20 procyanidin dimers and trimers have been identified [28], B1, B2, B3, and B4 being the main ones (Fig. 69.2d). In *Vitis,* procyanidins are mainly present in grape seed, skin, and stem tissues. In the grape seed, they represent the major fraction of total polyphenols, characterized by a lower polymerization degree than those in grape skin. However, skin procyanidins are more easily extracted during winemaking, thus conferring organoleptic properties on wine such as astringency and bitterness [29]. Moreover, the grape seed contains procyanidin of catechins, epicatechin, and epicatechin gallate units, while grape skin and wine show procyanidins mostly based on epicatechin and epigallocatechin units [30].

Unlike anthocyanins and flavonols, climatic conditions have little effect on flavan-3-ols, as these compounds mainly occur in the seed. In fact, climate seems to have greater effect on composition than on quantity. Low-vigor vines show grapes with higher procyanidin content, increased proportion of epigallocatechin subunits in procyanidins, and increased polymer size, and, therefore, these compounds show decreased astringency [10]. Besides, considerable differences in types and concentrations have been observed among cultivars [31].

Seed flavanol monomers and polymers ranged from 240 to 730 and from 330 to 790 mg/Kg fw, respectively [17, 20]. Skin flavonol monomers and polymers ranged from 10 to 40 and from 40 to 100 mg/kg fw, respectively. The total amount of procyanidins is reported to vary from 1.7 to 4.4 g/kg of berries in skin, 1.1–6.4 g/kg in seeds, and 0.2–1 g/kg in pulp [21] (Table 69.1).

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2.1.4 Hydroxycinnamic Acid Derivatives

Hydroxycinnamics are the third most abundant group of phenolic compounds in grapes and mainly comprise caffeic, coumaric, ferulic acids, and their corresponding tartaric esters (Fig. 69.2e). The hydroxycinnamic esters are more concentrated (2- to 100-fold) in grape skin than in pulp. Differences in total amount and proportion have been reported according to grape varieties [32].

These compounds are major phenols in white wine, and their concentration in grape ranges from traces to 50 mg/kg fw (Table 69.1). In addition, these compounds are important constituents of acylated anthocyanins.

2.1.5 Hydroxybenzoic Acids

Hydroxybenzoic acids are a minor group of phenolic compounds in grapes (Fig. 69.2f). The commonest ones are gallic acid, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, and syringic acid, which are mainly found in free form [33, 34]. Hydroxybenzoic acids are mostly represented by gallic acid, which is found both free and acyl substituent in flavan-3-ols. Two forms of gallic acid (i.e., 3-*O*-b-glucopyranoside and 4-*O*-b-glucopyranoside) have been reported in grape [35]. They are found in grape skin, and their total amount ranges from 2 to 5 mg/kg fw (Table 69.1).

2.1.6 Stilbenes

Stilbenes are minor compounds in grapes. However, their importance is due to the fact that grape is the main source of stilbenes in diet. Stilbenes are essentially located in grape skin [36, 37] but have also been reported in grape seeds [38] and grape stem [39] (Fig. 69.2g). The main stilbenes in grape are resveratrol (*trans* and *cis*) and piceid (*trans* and *cis*), but some others have also been described: piceatannol, astringin, pterostilbene, ε-viniferin, and δ-viniferin [40].

As described for the other polyphenols, the amount of resveratrol in grape depends heavily on many factors such as grape variety, geographic region, and agronomic and climatic factors. Furthermore, as resveratrol is a grape-synthesized phytoalexin after grape exposure to biotic or abiotic stress, the presence of resveratrol in grapes depends on the degree of stress exposure.

Total stilbenoid concentration was calculated taking into account just piceids and resveratrol (the major ones) and resulted higher for red than for white grapes [23]. Total stilbenoid concentration can reach 8.5 and 22 mg/kg fw in white and red grapes, respectively (Table 69.1).

2.2 Other Bioactive Compounds in Grape

Sesquiterpenoids are compounds that show woody, spicy, sweet, floral, clove, and fresh-like flavors. More than 40 sesquiterpenoids have been identified in grapes. However, quantification is a difficult task due to the lack of standards. Sesquiterpenoids have been described to contribute to the varietal volatile composition between 56% and 80% [41].

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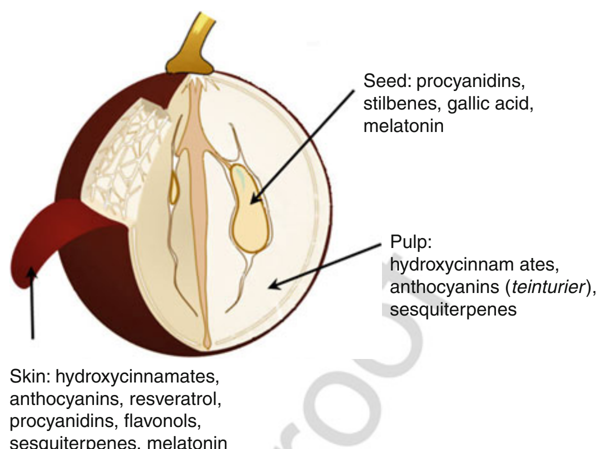

Fig. 69.3 Tissue localization of phenolic compounds in grape berry

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254 Another bioactive compound described in grape and wine is melatonin. As
255 reported for phenolic compounds, differences in genotypes and agrometeor-
256 ological conditions contribute to the differing melatonin content reported in
257 grape. Melatonin in grape ranged from traces to 0.9 ng/g fw [42, 43]. Some
258 authors suggest that the synthesis of melatonin in wine is directly related to
259 yeast metabolism [44].
260 Figure 69.3 shows the distribution/location of all abovementioned compounds in
261 grape seed, skin, and pulp.

262 3 Biological Activities

263 From a medical point of view, the bioactive compounds of plant food are not
264 considered pharmaceuticals, though they can improve life quality and expectancy.
265 These compounds are named nutraceuticals, since they act as dietary therapeutic or
266 pharmaconutrients (i.e., compounds that, if regularly included in diet, can prevent,
267 block, or delay the onset of major chronic diseases).
268 First of all, it is worth mentioning the most remarkable bioactivities and bio-
269 availability by phenolic family compounds separately. Secondly, we shall describe
270 the different bioactivities attributed to the consumption of grape derivatives.

271 3.1 Bioactivity and Bioavailability of Phenolic Compounds

272 *Anthocyanins* have been reported to be strong antioxidants. They inhibit the
273 growth of cancerous cells and inflammation, act as vasoprotectors, and have
274 antiobesity effects [45, 46]. It has been described that delphinidin may preserve

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endothelium integrity, as endothelium alteration leads to several pathologies, including cardiovascular diseases such as atherosclerosis, and is often associated with cancer [47].

Anthocyanins, as a supplement, have also shown beneficial results on HDL- and LDL-cholesterol concentrations. Increased HDL-cholesterol and decreased LDL-cholesterol were reported in a recent study on dyslipidemic patients who were given 160 mg anthocyanins twice daily throughout a 12-week trial. Furthermore, the anthocyanin supplementation led to decreased mass and activity of plasma cholesteryl ester transfer protein and increased cellular cholesterol efflux to serum [48].

Anthocyanins seem to be absorbed very rapidly but rather inefficiently [49]. However, the bioavailability of anthocyanins may have been underestimated due to either methodological inaccuracy or the different chemical forms that anthocyanins can take depending on pH. This may explain why all anthocyanins' effects are contingent on sufficient bioavailability in terms of exposure at both cell and organism level through the diet [50].

Flavonols comprise some of the most prominent dietary antioxidants. Among the flavonols, quercetin can be singled out, for it presents a wide variety of pharmacological activities that provide protection not only against osteoporosis, certain forms of cancer, and pulmonary and cardiovascular diseases but also against aging [51]. Even more significantly, quercetin plays a pivotal role in reducing blood pressure by reducing oxidative stress in a dose-dependent way [52, 53]. In the 1970s, quercetin was reported to be mutagenic. However, more recent studies in vivo indicate that quercetin is not carcinogenic. In fact, in the USA and Europe, quercetin supplements are commercially available, and their beneficial effects have been reported in clinical trials [54]. With respect to its bioavailability, quercetin is absorbed in humans and can reach high concentrations that are sufficient to increase plasma antioxidant capacity [55]. Moreover, quercetin glucosides, one of the main flavonols present in grape, are among the polyphenols most readily absorbed in humans [49].

Flavan-3-ols with various types of structure act as antioxidants, free radical scavengers, and anticarcinogenic. They have cardiopreventive, antimicrobial, and antiviral properties and may also play a significant role in maintaining neurological health [56]. Relationships have been established between the structure of flavan-3-ols and their strong antioxidant and free radical scavenging properties. Their antioxidant function depends on the ring structure and number of catechol groups [57]. However, some evidence points out that the opposite must also be considered, as flavan-3-ols may behave as antinutrients, procarcinogens, pro-oxidants, hemorrhage inducers, mutagens, or hepatotoxins depending on the source, type, amount, and existence of other dietary factors [58].

Bioavailability of flavan-3-ol monomers is generally good, although it differs markedly among different compounds. Catechin, present in high concentrations in the plasma of Mediterranean diet consumers, is able to reduce the progression of atherosclerosis in vivo [59]. The fact that grape is one of the main sources of catechin in diet supports the finding that grape has anti-atherosclerotic effect [60].

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Procyanidins are considered to be among the most effective antioxidants present in grapes. When rabbits were fed with procyanidins from grape seed extract, the compounds were active in preventing lipid oxidation while in the digestive tract [61]. The consumption of proanthocyanidin-rich foods has been shown to (1) increase plasma antioxidant capacity, (2) have positive effects on vascular function, and (3) reduce platelet activity in humans [62]. Unlike flavan-3-ol monomers, procyanidins are less permeable through cell walls and are therefore absorbed less readily. In fact, their polymerization impairs intestinal absorption [49, 56]. However, the health effects of proanthocyanidins may not require efficient absorption through the gut. These compounds may have direct effects on the intestinal mucosa and protect it against oxidative stress and carcinogens. Procyanidins bioactivity is not only due to their activity itself but also to their metabolites bioactivities and also to the modulation of intestinal bacterial population [63].

Hydroxycinnamic acid derivatives have shown antioxidant and anti-inflammatory properties in vivo and in vitro. They contribute to DNA protection and help to prevent Alzheimer's disease [64, 65]. However, data are still too limited for an informed assessment of hydroxycinnamics [49].

Stilbenes in general, and *trans*-resveratrol in particular, have been reported to be responsible for various beneficial effects. Resveratrol's biological properties include antibacterial and antifungal effects, as well as cardioprotective, neuroprotective, and anticancer action [40]. Anticancer resveratrol activity is one of the most promising bioactivities of resveratrol. In 1997, Jang et al. [66] reported the ability of resveratrol to inhibit carcinogenesis at multiple stages (initiation, promotion, and progression). Their finding that topical application of resveratrol reduces the number of skin tumors per mouse by up to 98% triggered research on resveratrol all around the world. Resveratrol could slow down tumor development through multiple complementary mechanisms. It inhibits the enzymatic activity of both forms of cyclooxygenase, which implies a reduction in the risk of developing many cancers. Another mechanism by which resveratrol could combat tumor formation is induction of cell cycle arrest and apoptosis. Its antiproliferative and pro-apoptotic effects in tumor cell lines have been extensively documented in vitro [67] and are supported by down regulation of cell cycle proteins [68] and increased apoptosis [69] in tumor models in vivo. However, in some in vivo experiments, resveratrol failed to impact cancer, which suggests that other factors such as dosage, delivery method, tumor origin, and other diet components could all contribute to the efficacy of resveratrol treatment. Overall, in vivo studies clearly show a promising use of this molecule in cancer treatment. Other remarkable activity of resveratrol is its neuroprotective character. It is able to penetrate the blood-brain barrier and exerts strong neuroprotective effect. Moreover, it has been shown to combat neuronal dysfunction in Huntington's, Alzheimer's, and Parkinson's diseases [70]. Resveratrol also has positive effects on longevity and age-related deterioration [71–73]. Other stilbenes such as piceatannol and viniferins are usually found in grape in lower concentrations than resveratrol, and, as a result, their bioactivity has been studied less than that of resveratrol. Nevertheless, some of their health-benefiting properties have also been researched [40, 74, 75].

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Numerous studies on animals and humans have shown resveratrol's low bioavailability. Once it is absorbed, at least 70% of the ingested resveratrol is readily metabolized to form mainly glucuronide and sulfate derivatives. Since the in vivo concentration of individual metabolites from ingested resveratrol can be much higher than that of resveratrol itself, further studies on the activity of its metabolites become necessary.

Resveratrol binds to albumin, and albumin has been suggested a natural polyphenol reservoir in in vivo context, where it might play a pivotal role in the distribution and bioavailability of circulating resveratrol [76]. The accumulation of resveratrol in other organs such as heart, liver, and lung after chronic administration was described for the first time in 1996 [77] and has more recently been confirmed [78, 79] and extended to bile, stomach, and kidneys [80]. It is also worth considering the potential interactions between polyphenols. For example, resveratrol has been shown to synergize with both quercetin and ellagic acid in the induction of apoptosis in human leukemia cells [81], with ethanol in the inhibition of iNOS expression [82], with vitamin E in the prevention of lipid peroxidation [83], with catechin in the protection of PC12 cells from β-amyloid toxicity [84], with nucleoside analogues in the inhibition of HIV1 replication in cultured T lymphocytes [85], and with tyrosol and β-sitoesterol in modulation of LDL oxidative stress and PGE2 synthesis [86].

Sesquiterpenoids have been related with medicinal plants with different health applications, mainly anti-inflammatory [87], anti-HIV [88], antibacterial [89], and antitumor activity [88]. Up to date, no study has covered the biological activity of sesquiterpenoids from *Vitis vinifera L* grapes. Sesquiterpenoids such as farnesol and nerolidol have been reported to have the ability to enhance bacterial permeability and susceptibility to exogenous antimicrobial compounds. These compounds increase the susceptibility of *Staphylococcus aureus* and *Escherichia coli* to antibiotics by disrupting the normal barrier function of the bacterial cell membrane, allowing permeation into the cell of exogenous solutes such as antibiotics [90].

Melatonin (*N*-acetyl-5-methoxytryptamine) is a neurohormone produced in the pineal gland. Its biological properties have been studied in depth, particularly those concerning the circadian rhythm. Melatonin shows antioxidant properties as a direct free radical scavenger and a stimulator of antioxidant enzymes [91–94]. Melatonin's role in neuroprotection is an important issue. Melatonin has been tested in sleep disorders. It generally reduces sleep latency and improves sleep especially in case of disturbed circadian phasing. In this case, melatonin was found particularly effective in patients with neurodegenerative diseases [95]. The European Food Safety Authority (EFSA) accepted the health claims related to melatonin and alleviation of subjective feelings of jet lag. Melatonin dosage must be within 0.5 and 5 mg per day [96]. Numerous research attempts have been made or are currently being developed to mitigate neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases and amyotrophic lateral sclerosis. Melatonin was shown to inhibit Aβ fibrillogenesis [97, 98]. Melatonin (200 mg/Kg) has recently been shown to reduce edema in impacted striatum versus traumatic brain injury [99].

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t2.1 **Table 69.2** Antioxidant compounds from grapes and main bioactivity

	Family	Most prominent bioactivity against disease	References
t2.3	Anthocyanins	Decrease cardiovascular and cancer risk	[45–48]
t2.4	Flavonols	Provide protection against osteoporosis, cancer, pulmonary and cardiovascular diseases	[51–53]
t2.5	Total flavan-3-ols	Anticarcinogenic, cardiopreventive, antimicrobial, and antiviral properties	[56, 57]
t2.6	Hydroxycinnamic acid derivatives	DNA protection and help prevent Alzheimer's disease	[64, 65]
t2.7	Stilbenes (resveratrol)	Cardioprotective, neuroprotective, antiaging, and anticancer actions	[66, 70, 71]
t2.8	Sesquiterpenes	Anti-inflammatory and antibacterial actions	[87, 89]
t2.9	Melatonin	Neuroprotection action	[70]

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410 In addition to plasma melatonin levels, urine-excreted melatonin metabolite
411 6-sulfatoxymelatonin is easy to determine. Indeed, higher melatonin excretion in
412 the first morning urine was 16% higher in women with higher quartile vegetable
413 intake in comparison to those with lower quartile intake [100]. Moreover, nutrition
414 habits and lifestyle have also been correlated with melatonin [101]. A statistically
415 inverse relation was found between age, smoking, and body mass index, on one
416 hand, and urinary 6-sulfatoxymelatonin [102].
417 The most important bioactivities of these compounds in diseases are summa-
418 rized in Table 69.2.
419 Moreover, many of the described health properties may not be attributed to
420 single polyphenols present in grape but rather to synergism among polyphenols
421 themselves and/or between them and other types of bioactive compounds. For
422 instance, some studies on grape or grape juice are detailed next.

423 3.2 Antioxidant Activities of Grape and Grape Derivative 424 Products

425 The most widely researched biological activity of polyphenols is their antioxidant
426 capacity, though they also have a plethora of more or less correlated properties such
427 as antimutagenic, anti-inflammatory, antitumoral, antineurodegenerative, antihy-
428 pertensive, and cardioprotective activities.
429 Oxidative stress is broadly defined as a perturbation of cellular homeostasis, so
430 that the production rate of reactive oxygen species (ROS) exceeds their neutraliza-
431 tion rate. If homeostasis is not reestablished, oxidative stress may progress toward
432 the onset of apoptotic cell death and tissue degeneration [103]. In order to cope with
433 excessive production of free radicals, human bodies have developed sophisticated
434 mechanisms for maintaining redox homeostasis. These protective mechanisms
435 include ROS scavenging or detoxification, ROS production blockage, and seques-
436 tration of transition metals, as well as enzymatic and nonenzymatic antioxidant

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defenses both endogenous (body-produced) and exogenous (diet-supplied). Among them, dietary polyphenols have been widely studied for their strong antioxidant capacities and other properties that regulate cell functions [104].

Flavonoids act as antioxidants by donating electrons and stopping radical chains. This activity is attributed to phenolic hydroxyls, especially in 3′,4′ positions in the B-ring and to the 2,3-double bond in the C-ring, thus increasing with the number of OH groups in A and B rings [105]. In fact, most epidemiological and intervention studies on flavonoids' beneficial effects have been focused on their antioxidant capacity.

The antioxidant capacity of red grapes was evaluated in HepG2 (human hepatocellular liver carcinoma) cells and positively correlated with the total phenolic content and the oxygen radical absorbance capacity (ORAC) values of grape extracts. Results suggest that increasing fruit consumption is a suitable strategy to counteract oxidative stress [106].

A similar conclusion is drawn in a study developed on oxidative stress markers in 32 healthy subjects. Daily consumption of grape juice (10 mL/kg body weight) for 2 weeks resulted in increased resistance of LDL to ex vivo oxidation, comparable to the value obtained after α-tocopherol. Furthermore, decreased protein-carbonyl concentration was observed at the same time [107]. These results agree with those contributed by other authors, who showed that the daily intake of grape juice (125 mL) for 1 week in a group of six men and six women led to significantly reduced LDL oxidation [108]. In a short-term study, the acute intake of a phenolic-rich juice (400 mL), with grapes as a major ingredient, improved the antioxidant status in healthy subjects according to their plasma thiobarbituric levels [109]. In a group of 27 hemodialysis patients, regular ingestion of concentrated red grape juice (100 ml) for 2 weeks reduced inflammatory biomarkers to a greater extent than vitamin E [110].

High daily intakes of grape juice are not feasible in most population. To overcome this difficulty, new derivative products such as power extracts are proposed. The daily supplementation of lyophilized grape powder (36 g) for 4 weeks reduced urinary F2-isoprostanes (in women), which are biomarkers of oxidative stress, in pre- and postmenopausal women [111]. The administration of grape seed extract (600 mg/day) for 4 weeks was reported to have led to significant improvement in insulin resistance and plasma CRP markers in a group of 32 type 2 diabetic patients [112]. The consumption of black grape (1 g/kg body weight) has also been described to exert similar effects to juice and powder: significantly increased antioxidant potential was observed in healthy volunteers 4 h after ingestion [113].

Furthermore, this antioxidant capacity of grape is also able to protect DNA oxidation and therefore mutagenesis-, carcinogenesis-, and aging-related DNA damage. Daily grape juice supplementation (480 mL) for 8 weeks led to reduced DNA strand breaks in peripheral lymphocytes, apart from decreasing the amount of released ROS [114]. Similarly, treatment of human lymphocytes with grape seed extract reduced the frequency of micronuclei by 40% and the production of malonyldialdehyde, a biomarker of lipid peroxidation, by 30%, while it increased the activity of antioxidant enzymes catalase and glutathione S-transferase by 10% and 15%, respectively [115].

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3.3 Cardioprotective Activity of Grape and Grape Derivative Products

Vessel injury and thrombus formation are the cause of most ischemic coronary syndromes, and in this setting, activated platelets stimulate platelet recruitment up to the growing thrombus. Hypertension is one of the major risk factors for cardiovascular disease, with an impact on global health. Multiple studies have suggested that various dietary factors are associated with blood pressure and hypertension. However, the effects of fruit and vegetable consumption on plasma lipid levels, diabetes, and body weight have not yet been thoroughly explored [116]. Evidence suggests an inverse relationship between grape product consumption and cardiovascular disease. In studies conducted with grapes and grape juice, clinical trials demonstrated improved endothelial function, reduced platelet aggregation, and a positive influence on biomarkers such as LDL and HDL. In a study on 15 patients with coronary artery disease, the consumption of 8 mL/kg/day of red grape juice for 2 weeks improved the endothelial function and reduced the susceptibility of LDL-cholesterol to oxidation [117]. In fact, Vison et al. found that red grape juice, in contrast with orange juice, enriched LDL and VLDL and reduced their oxidation susceptibility in vitro, ex vivo, and in vivo [118]. Regarding platelet aggregation, many promising results have been reported. Platelet incubation with red grape juice led to inhibited aggregation, enhanced the release of platelet-derived NO, and reduced superoxide production. Oral consumption of standardized grape extract (100 and 200 mg/kg) provided significant cardioprotection by improving postischemic ventricular recovery and reducing myocardial infarction in rats [119].

In other study, 20 healthy subjects were supplemented with grape juice (7 mL/kg) for 14 days. Significantly decreased platelet aggregation, increased platelet-derived NO release, and decreased superoxide production were observed. The suppression of platelet-mediated thrombosis represents a potential mechanism for the beneficial effects of purple grape products in cardiovascular disease [120]. Similarly, proanthocyanidin-rich extract of grape seed (50 and 100 mg/Kg for 3 weeks) had cardioprotective effects against reperfusion-induced injury in isolated rat hearts [121]. A lower dose (36 g of lyophilized power grape/day for 4 weeks) was tested in pre- and postmenopausal women. Lipoprotein metabolism, oxidative stress, and inflammatory markers were achieved, and, therefore, CHD risk factors were reduced [122].

Moreover, numerous studies have been developed on CHD patients. The intake of red grape polyphenol extract – which contains epicatechin, catechin, gallic acid, *trans*-resveratrol, ε-viniferin, rutin, quercetin, p-coumaric, and ferulic acid – improved the endothelial function in CHD patients. Similar results have been found for red grape juice [123]. Hypercholesterolemic patients were asked to consume red grape juice (500 mL/day) and red wine (250 mL/day) for 14 days. Results showed increased brachial artery flow-mediated dilation in both cases. However, increased endothelium-independent vasodilation was observed only in red wine-drinking patients [124]. Similar results and the same conclusions were drawn from a similar study on red grape juice. The daily ingestion of moderate

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amounts of red grape juice improves endothelial function in patients with atherosclerotic vascular disease, has no adverse affects on lipid and glucose metabolism, and reduces LDL susceptibility to oxidation [117, 125]. The consumption of purple grape juice offered protection against the oxidation of LDL-cholesterol, as shown by an in vivo study with dogs, monkeys, and humans from which the flavonoids of purple grape juice and red wine could be inferred to be able to inhibit the initiation of atherosclerosis [126].

3.4 Anticarcinogenic Activity of Grape and Grape Derivative Products

Cancer is a term commonly used for diseases in which abnormal cells divide uncontrollably and invade other tissues. Cancer cells can spread to other parts of the body through the blood and lymph systems. Cancer is not just one disease but many diseases. There are more than 100 different types of cancer, hence, the complexity of cancer treatment. It is the second leading cause of death worldwide after heart disease, and its risk and incidence increase with patient age (www.cancer.gov). In addition to genetic factors, environmental and nutritional factors play a main role in cancer etiology. In westernized countries, breast, prostate, and colon-rectum cancers predominate because diets are usually rich in animal-source foods and refined carbohydrates and deficient in plant foods. Conversely, in developing countries, where diets are largely based on cereal/starchy foods, esophageal, stomach, and liver cancers are more incident [127].

Fruit and vegetable consumption may reduce the risk of oropharynx, esophagus, lung, stomach, and colon-rectum cancers. A recent study has evaluated the potential effect of interventions aimed at increasing the intake of fruits and vegetables up to the recommended level (500 g/day) on future cancer incidence in Europe. Data on cancer incidence and daily intake of fruit and vegetables were collected for France, Germany, the Netherlands, Spain, and Sweden. The results predicted 212,000 fruit- and vegetable-related cancer cases in these countries in 2050, out of which 398 (0.19%) might be prevented if the 500 g/day fruit and vegetable intake was achieved in the aforementioned countries. The largest absolute impact was observed for lung cancer with 257 (out of 136,517) preventable cases if the intervention was successfully implemented. Increasing fruit and vegetable consumption has a small impact on reducing the burden of cancer in Europe. Nevertheless, although health impact is rather limited, it can be used as a tool in chronic disease prevention [128]. In another previous study, quantitative conclusions regarding the contribution of fruit and vegetables intake to the occurrence of oral cancer were drawn from a meta-analysis. Promising results were contributed in this study. The authors conclude that each portion of fruit consumed per day significantly reduces the risk of oral cancer by 49%. Moreover, the multivariable meta-regression showed that the lower risk of oral cancer associated with fruit consumption was significantly influenced by both the type of fruit consumed and the time interval of dietary recall [129].

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Many of the studies on the cancer-preventive mechanisms of phenolic compounds have been focused on individual compounds (mainly on resveratrol, procyanidins, and melatonin at too high concentrations to be achieved via dietary consumption). In contrast, few intervention studies have been found in the database. Some of them, related with their antioxidant activity, are cited in Sect. 3.2 in this chapter. Some others are detailed below. The effects of black grape extract, including seeds, on the activity of DNA turnover enzymes in cancerous human colon tissues have been tested. Results showed that the extract inhibited the activity of the enzymes involved in rapid DNA synthesis. Thus, extract intake may have beneficial effects on colon human cancer [130]. In vitro studies on grape seed extract (usually on grape seed extract) also showed promising efficacy against both angiogenesis and metastasis, which are involved in cancer progression in mammary, colon, prostate, and breast carcinoma [131–134]. Skin carcinogenesis in mice was reduced by combining topical and dietary treatment with freeze-dried grape powder [135].

Literature review for this chapter found only one human study that had examined the relationship between grape products and cancer. In an intervention study involving smoking and nonsmoking humans, Park et al. found that supplementation with 480 mL/day of purple grape juice for 8 weeks decreased lymphocyte DNA damage, reduced the release of reactive oxygen species by 15%, and reduced DNA damage to a greater extent in smokers (25% vs. 18% decrease). While these results suggest grape juice's potential anticarcinogenic role, further well-designed studies in humans are needed to corroborate these findings.

3.5 Neuroprotective Activity of Grape and Grape Derivative Products

Neural dysfunction and metabolic imbalances underlie many progressive neurodegenerative conditions such as Alzheimer's, Huntington's, and Parkinson's diseases. As commented before, resveratrol and melatonin can penetrate the blood-brain barrier and exerts strong neuroprotective effects, even at low doses. Several studies support that the onset of the neurodegenerative disease may be delayed or mitigated with the use of dietary chemopreventive agents that provide protection against β-amyloid plate formation and oxidative damage [136, 137].

The effect of grape seed extract, rich in procyanidin, on the stress-induced neuronal cell death model has been studied in vitro using a hippocampal neuron-rich culture. The extract led to increased interleukin-6, which protected neuronal cells from death by oxidative stress [138]. Ono et al. [139] showed that a commercially available grape seed polyphenolic extract (MegaNatural-AZ) significantly attenuated Alzheimer´s disease-type cognitive deterioration and reduced cerebral amyloid deposition. Similarly, Wang et al. [140, 141] found that a naturally derived grape seed polyphenolic extract can significantly inhibit amyloid β-protein aggregation into high-molecular-weight oligomers in vitro.

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Regarding in vivo studies, when orally administered to Tg2576 mice, this polyphenolic preparation significantly attenuated Alzheimer's disease-type cognitive deterioration, coincidentally with reduced HMW soluble oligomeric Aβ in the brain. Grape seed-derived polyphenolics were suggested to be useful agents to prevent or treat Alzheimer's disease. Moderate consumption of two unrelated red wines made from different grape species (Cabernet Sauvignon and Muscadine) and characterized by different component composition of polyphenolic compounds significantly attenuated the development of the Alzheimer's disease-type brain pathology and memory deterioration in a transgenic Alzheimer's disease mouse model [142]. Treatment with Cabernet Sauvignon was found to reduce the generation of Alzheimer's disease-type Aβ peptides, while the Muscadine treatment was found to attenuate Aβ neuropathology and Aβ-related cognitive deterioration in Tg2576 mice by interfering with oligomerization of Aβ molecules to soluble HMW Aβ oligomer species, which are responsible for initiating a cascade of cellular events resulting in cognitive decline. These authors suggested the possibility of developing a "combination" of dietary polyphenolic compounds for Alzheimer's disease prevention and/or therapy by modulating multiple Aβ-related mechanisms.

Concord grape juice supplementation has been shown to reduce inflammation, blood pressure, and vascular pathology in patients with cardiovascular disease. Besides, the consumption of such flavonoid-containing foods is associated with reduced risk of dementia. In addition, preliminary animal data have indicated improved memory and motor function with grape juice supplementation, suggesting its potential for cognitive benefits in aging humans. In this initial research on neurocognitive effects, 12 older adults with memory decline but not dementia took part in a randomized, placebo-controlled, double-blind trial with Concord grape juice supplementation for 12 weeks. Significant improvement was observed in verbal learning measurements, as well as nonsignificant enhancement of verbal and spatial memory. The intervention was observed to have no appreciable effect on both depressive symptoms and weight or waist circumference. These preliminary findings suggest that Concord grape juice supplementation may enhance cognitive function in older adults with early memory decline. In addition, they set a basis for further comprehensive research on its potential benefits and assessment of its mechanisms of action.

Prospective cohort studies on flavonoid intake and risk of developing dementia have led to inconsistent results [143–148], since high blood levels of homocysteine may be increased in Alzheimer's disease and hyperhomocysteinemia may contribute to disease pathophysiology by vascular and direct neurotoxic mechanism [149]. The effect of a polyphenol-rich antioxidant beverage on plasma homocysteine levels in Alzheimer patients has been recently evaluated. With this purpose, Morillas-Ruiz et al. performed a multicenter, randomized, double-blind controlled clinical trial with polyphenol supplementation in 100 subjects [150]. Twenty-four patients with initial Alzheimer's disease, 24 patients with moderate AS, and 52 controls were randomly assigned to assume either a polyphenol-rich antioxidant beverage or an identical placebo beverage (200 ml/day) for 8 months. The fasting plasma homocysteine concentration levels measured before and after the ingestion

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655 of the beverage showed higher baseline levels in Alzheimer patients than in both
656 mild-Alzheimer and control patients. The antioxidant beverage versus placebo
657 attenuated homocysteine increase in the control and Alzheimer groups, especially
658 in the mild one, yet no other effects were achieved.

659 4 Functional Food from Grapes

660 Functional foods were first described by Roberfroid [151] as "Food similar in
661 appearance to conventional food that is intended to be consumed as part of
662 a normal diet, but has been modified to subserve physiological roles beyond the
663 provision of simple nutrient requirements." More simply put, functional foods are
664 foods that may provide health benefits beyond basic nutrition. Functional foods can
665 arise from a desire to provide additional benefits to consumers in the way of
666 enhanced nutrition. They can also be useful in making nutrients more available
667 by providing particular dietary components in foods, thus increasing their usual
668 availability and palatability [152].
669 Enrichment of foods with phenolics can be a promising strategy to produce
670 functional foods with higher antioxidant activity. A number of products such as
671 "grape seed extract," "grape extract," "red wine powders," "anthocyanin extracts,"
672 and even "leaf extracts" are currently being marketed and employed in the formu-
673 lation of dietary supplements. Among these, grape seed extract (GSE) seems the
674 most widely extended. In addition, plant callus/cell cultures have proven potentially
675 promising for the production of secondary metabolites [153]: mainly anthocyanins
676 and other phenolics in grapes [154–156]. These in vitro cultures involve some
677 advantages over fresh fruit extracts such as the possibility of continuous production
678 of natural compounds, large scale production depending on specific needs, lower
679 cost and opportunity for the manipulation of the direction of the biosynthesis of
680 anthocyanins, or other phenolics [155].
681 Finally, other grape derivative, phenolic-rich products are by-products: mainly
682 pomace, which is made of solid residues; 80% of grape production is used in
683 winemaking, and therefore millions of tons of grape pomace are produced within
684 a few weeks after harvest. Pomace's phenolic extracts show high antioxidant and
685 antimicrobial properties [157].
686 The following section contributes a revision of different grape extract-enriched
687 food products.

Corrected Proof

688 4.1 Enrichment of Dairy Products

689 In cheese-making, the curd is generally made by coagulating milk casein with an
690 enzyme, an acid, and either with or without further curd treatments by heat,
691 pressure, salt, and fermentation with selected microorganisms. The retention coef-
692 ficient is an important parameter in predicting the recuperation rate of value-added
693 functional ingredients such as polyphenols. Indeed, low-molecular-weight soluble

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compounds are often lost to a great extent in the cheese whey. A high retention coefficient is considered desirable in the cheese-making process, as higher retention coefficients indicate reduced loss of functional ingredients. The retention coefficients of phenolic compounds in cheese are attributable to the interactions between phenolic compounds and proteins. Therefore, there is growing interest in determining the interactions between bioactive ingredients and milk proteins to enhance their recovery in cheese [158]. In this sense, grape extracts including skin, pulp, and seed (among other extracts) were added as functional components to prepared cheese. The nutritional value of cheese product was improved, since the retention coefficient of bioactive compounds and antiradical activity in cheese curd increased 1.5- and 3.5-fold, respectively [158]. However, it affected milk's gel-formatting kinetics, mainly because of pH and curd moisture content, and textural properties were therefore affected too [159]. Further research is needed before applying this technology in the food industry.

In fact, many aspects beyond the functional properties must be considered for the addition of polyphenols to dairy products. Apart from their antioxidant capacity, their sensorial attributes such as bitterness or the astringency of procyanidins can be easily translated into the dairy product [160]. When grape seed extract was added to low-fat ultra-high temperature (UHT) milk, the flavor characteristics of the milk were suppressed. Moreover, astringency and bitterness were also detected in fortified milks. The authors suggest joining this extract to cyclodextrins, thus forming complex polyphenols that result in reduced perception of sensory attributes [160, 161].

Yogurt is another fermented dairy product; beyond its nutritional characteristics and importance for the human diet, yogurt is not currently considered a significant source of phenolic compounds. Therefore, plant-based additives have been used to enhance phenolic content in yogurt [155, 162].

In recent research, high anthocyanin and phenolic levels (as much as 17.7 and 78.46 mg/kg, respectively) were reported in yogurts inoculated with extracts obtained from red grape varieties or grape callus cultures. Thus, high level of free radical scavenging capacity was also observed. Phenolic acids such as gallic acid, caffeic acid, p-coumaric acid, vanillic acid, gentisic acid, vanillin, catechin, epicatechin, *trans*-resveratrol, hesperidin, and quercetin were identified in fortified yogurts. These compounds were concluded the main constituents of the antioxidant power of yogurts enriched with functional ingredients. However, decreased total phenolic content, anthocyanin content, and antioxidant activity were observed in all assayed yogurt samples along time (2 weeks of storage). Finally, the authors state that grape callus/cell cultures can be a valuable alternative for the biomanufacturation of chemopreventive and nutraceutical fortified yogurt [155].

4.2 Enrichment of Meat Products

Grape seed extract (GSE) has proven antioxidant activities both in vivo and in vitro in various meat products [163–167]. In the meat system, GSE proves its antioxidant

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736 activity by reducing the amount of primary (e.g., lipid hydroperoxides and hexanal)
737 and secondary (e.g., thiobarbituric acid reactive substances – TBARS) lipid oxida-
738 tion products [163]. GSE has reduced rancid flavor development and antioxidant
739 activities in various meat products like raw beef, cooked beef, raw and cooked pork
740 patties, turkey, and ground chicken breast and thigh meat [168–173]. The antiox-
741 idant activity of GSE is concentration-dependant between 0.02% and 0.1% [168].
742 Grape seed extract at 0.1% (w/w) is an effective radical scavenger in muscle tissues
743 and has been shown to reduce secondary oxidation products in beef, chicken, and
744 turkey during refrigerated storage [168, 173, 174]. At this level (0.1% w/w), GSE
745 can be used as an effective antioxidant in both raw and cooked meat systems.
746 Addition of GSE ($\geq$1,000 μg/g) results in minor increase ("a" – redness values) in
747 the surface color of raw meat and retention (due to the anthocyanins present in
748 GSE) in cooked meat, which may have a negative impact on consumer preference
749 based on meat product color without affecting meat eating quality [168, 171, 175].
750 The addition of GSE (6,000 ppm) does not change flavor scores in irradiated and
751 nonirradiated whole chicken breasts [176]. Furthermore, GSE (0.1% w/w) was
752 observed to have no effect on pH, yield, and water activity in ground chicken breast
753 samples [170].

754 Other authors have researched the usefulness of grape seed flour from grape
755 by-products [177]. Grape seed flour can beat alternative materials used in various
756 food products due to its high antioxidant activity and high dietary fiber content
757 (http://www.vitis-vital.de/). Grape seed flour was incorporated into frankfurters
758 at seven different concentrations (from 0% to 5%), and its effects were
759 observed on the researched products' physical, nutritional, and sensory fea-
760 tures. Oxidation in frankfurters was minimized with increasing levels of grape
761 seed flour in their formulation as a result of the strong antioxidant properties
762 of grape seed flour. Moreover, increased levels of grape seed flour led to
763 frankfurters' increased total dietary fiber and water-holding capacity, thus
764 increasing the products' value. However, the products' sensorial properties
765 were significantly modified, and further research therefore becomes necessary
766 to improve product palatability [177].

Corrected Proof

767 4.3 Enrichment of Bread

768 Bakery products, particularly bread, have a significant share in the food guide
769 pyramid for daily food choices recommended by the US Department of Health
770 and Human Services. Therefore, the development of polyphenol-enriched bread is
771 an efficient way to increase polyphenol intake.

772 In a recent study, different amounts of grape seed extract (300, 600, and
773 1,000 mg), source of catechin and procyanidin, were added to bread ingredients
774 before the bread-making process. Although thermal processing decreased the
775 antioxidant activity of the extract, fortified bread showed significantly higher
776 antioxidant capacity. Moreover, the antioxidant capacity of the extract contributed
777 to the reduction of N^{ε}-carboxymethyllysine, a potential toxicant in food, in enriched

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bread. Regarding bread sensory properties, only a favorable change in bread color was observed, with no significant alteration in quality parameters (sweetness, porosity, astringency, and stickiness) [178].

The study evaluated the effect of grape by-products (GP) on the chemical composition, soluble and insoluble dietary fiber, phenolic compounds and antioxidant activity, and organoleptic characteristics of sourdough mixed rye bread. The following samples of sourdough mixed rye bread were prepared: control bread and breads with GP at four different levels, 4%, 6%, 8%, and 10%. The addition of GP significantly improved dietary fraction contents, as bread with 10% added GP accounted for 39% and 37% higher contents of soluble and insoluble dietary fiber than control bread. The assay of radical scavenging activity and reducing ability showed that GP addition greatly enhanced the antioxidant properties of mixed rye breads. The profiles of phenolic compounds of supplemented breads were dominated by procyanidin B1 and B2, catechin, epicatechin, caffeic acid, and myricetin. Increased GP levels led to significantly increased bread hardness and gumminess. Although both control bread and supplemented breads showed common volatile compound profiles, slight differences were observed in the concentration of these components. Sensory evaluation of GP-enhanced breads revealed that a maximum of 6% GP could be added to prepare acceptable products.

4.4 Enrichment of Seafood

Lipid oxidation is still today a problem in the food industry, especially for products that contain marine lipids. The high content of polyunsaturated fatty acids (PUFA), highly beneficial for human health particularly in preventing cardiovascular diseases, in fish oil makes it very attractive to the rising market of functional products. However, at the same time, this high PUFA content makes marine lipids highly susceptible to oxidation, consequently affecting fish oil quality during storage through flavor, odor, color, and texture deterioration and even producing toxic compounds [179].

Antioxidant phenolics are often added to food to inhibit the initiation and propagation of oxidation's radical chain reactions, thus delaying the oxidation process. New antioxidants capable of retarding oxidation in fish oil-enriched foods would be desirable, especially if these new antioxidants had relevant biological properties. In this sense, grape procyanidins and resveratrol derivatives have been examined as inhibitors of oxidation in fresh Atlantic horse mackerel [180, 181]. Procyanidins were added at 50 and 100 ppm (W/W) to minced muscle fish. Procyanidin supplementation stabilized the fish product and maintained its functionality associated with the presence of PUFA and α-tocopherol. Regarding the stability and biological activity of grape procyanidins during chilled storage, their antioxidant activity has been described to remain stable for more than one year at 4 °C [180]. On the other hand, resveratrol, piceid, and some resveratrol derivatives were tested at similar concentration (100 ppm) on minced muscle fish. Resveratrol and piceid showed notable antioxidant activity in fish oil-in-water emulsions.

820 Neither lipophilization nor glycosylation of resveratrol led to improved antioxidant
821 efficiency [181]. The authors conclude that these biologically relevant phenols
822 could be used as natural antioxidant in this type of food matrix.

823 ## 4.5 Enrichment of Grape Derivative Beverages

824 As the fastest growing segment in the food industry, functional beverages represent
825 an attractive alternative to conventional food products for health conscious indi-
826 viduals. Through the incorporation of fortifying agents and antioxidant-eliciting
827 ingredients, ordinary beverages are being transformed into "superfoods" [182, 183].
828 Functional beverages and plant-based fortified products are especially attractive to
829 consumers [153] and represent new opportunities for beverage producers.
830 Newly developed foods and beverages have incorporated grape-derived extracts
831 to gain health appeal [184]. A concentrated food ingredient comprising green tea
832 and grape skin and seed extracts has been developed. A mix of green tea extract
833 (3.0 g/L)+grape skin extract (12.0 g/L)+grape seed extract (0.5 g/L)+fungus extract
834 (0.1 g/L)+vitamin C (0.3 g/L) yielded 1,155 mg/L of polyphenol content [185].
835 Other authors have formulated new grape extracts rich in procyanidins: 15% mono-
836 mers and 20% of dimers and up to 30% of trimers, tetramers, and pentamers by
837 weight [186]. The product was tested in the treatment of prehypertensive patients
838 and was successful in reducing both systolic and diastolic blood pressure by 8% on
839 a dose of 300 mg/day for 8 weeks.
840 In 2008, a patent application by Perlman et al. [187] presented an interesting use
841 of the "rich" by-product of the grape juice industry. Based on the fact that grape
842 pomace solids contain at least ten times greater amounts of polyphenols than
843 pressed grape juice, the authors proposed the fortification of such juice with pomace
844 polyphenol extract. The beverage was tested at different extract concentrations for
845 sensorial acceptance and antioxidant activity. Unacceptable astringency was
846 observed together with significantly increased antioxidant activity. As described
847 for other food products, cyclodextrin was proposed for successful reduction of the
848 astringent sensation. Draijer et al. [188] described the invention of a beverage
849 containing 550 mg of red wine polyphenols + 250 mg of red grape polyphenols
850 added to 200 mL of a soy-based drink. Results of daily doses for breakfast suggest
851 a positive impact on blood pressure.
852 Wine has traditionally been identified as a health-benefiting product due to its
853 effects on coronary heart disease after the so-called French paradox [189] delaying
854 tumor onset [190] and its high antioxidant activity [191]. These benefits have been
855 ascribed to phenolic compounds abundant in red wine. As previously commented,
856 resveratrol is one of the main bioactive compounds found in wine. Wine with higher
857 resveratrol content may therefore be regarded as "functional wine" due to
858 resveratrol's positive health effects. Some research is currently being developed
859 in this sense. Metabolic engineering has been used to obtain transgenic yeasts that
860 enhance resveratrol content in wine [192, 193]. In some cases, the conversion rate
861 was higher than 20-fold [194]. Moreover, as resveratrol is a phytoalexin

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862 synthesized by grapes after exposure to biotic or abiotic stress, the presence of
863 resveratrol in grapes depends on the degree of stress exposure. Preharvest chemical
864 treatments such as BTH, chitosan, methyl jasmonate, jasmonic acid, salicylic acid,
865 beta-aminobutyric acid, ozone, aluminum chloride, and UVC can be used to
866 enhance nutraceutical grape properties [40]. Guerrero et al. obtained resveratrol-
867 enriched red wines by using UVC [195], while Gaudette et al. did it by adding
868 resveratrol directly to the wine [183]. Both studies concluded that increased res-
869 veratrol content does not alter wine's quality properties. In addition, enriched wines
870 showed significantly higher antioxidant capacity compared to control wines [183].

871 Market studies suggest that the functional attribute in wine positively and
872 significantly affects the probability of selecting a particular red wine and consumer
873 willingness to pay for this attribute [196]. However, it should be mentioned that the
874 term "functional" cannot be used for beverages with over 1.2% alcohol content in
875 Europe according to the EU Regulation 1924/2007. Remarkably, a winery is
876 already commercializing this type of wines in the Australian market (www.
877 winedoctor.com.au). They extract resveratrol from grape and add it to wine, thus
878 achieving resveratrol wine content up to 100 g/L – an outstanding achievement
879 regarding usual resveratrol content in wine.

Corrected Proof

880 5 Conclusion and Perspectives

881 From an economic viewpoint, grapevine is one of the most important crops in the
882 world and has a deeply rooted significance in human culture. The functional
883 ingredients of grapevine include several flavonoids that have been reported to
884 show different activities including antioxidant, anticancer, and prevention of car-
885 diovascular disease, as well as the treatment of several neurological disorders.
886 Grape seed extract and its active components such as proanthocyanidins, resvera-
887 trol, and quercetin seem to be potent antioxidants. The consumption of grapes and
888 grape juice is likely to have positive effects on human health.

889 In this sense, numerous food products are enriched with grape polyphenols, thus
890 entering the market of functional foods. Functional foods (and nutraceuticals)
891 constitute a promising field to improve health and prevent age-related chronic
892 diseases. There has been a growing interest in researching, developing, and com-
893 mercializing functional food. Moreover, recent trends have shown consumers'
894 growing interest in many health-promoting food and supplements. The addition
895 of fruit polyphenols to food products is increasing with the currently emerging
896 popularity of functional food (ISI Web of Knowledge; 1,585 entries in the last
897 11 years). For that reason, further research is needed, since the effectiveness of
898 functional products in vivo is a really complex topic on which there is still too little
899 knowledge. In this way, both synergistic effects among phenolics and the effect of
900 food matrices on bioavailability will be taken into account.

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Corrected Proof

ANEXO 3

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Title: Terroir and variety: two key factors for obtaining stilbene-enriched grapes.

Article Type: Original Research Article

Keywords: Terroir; climate; red grape variety; stilbenes; UV-C postharvest treatment; oenological parameters.

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Abstract: Grape and wine are the main source of bioactive stilbene in the diet. The stilbene composition of grapes depends on both variety and terroir. The effect of terroir on grape stilbene has not been widely studied. In this research the study of the stilbene content (resveratrol, piceatannol and viniferins) in four different red grape varieties (Syrah, Merlot, Cabernet sauvignon and Pinot noir) and from four different Andalusian terroirs (Jerez, Cabra, Cadiar and Ronda) was carried out during vintage 2009. Moreover, the stilbene induction capacity was studied in each terroir and variety after UVC postharvest treatment. Data were analysed by multivariate statistical methods. Principal component analysis applied on the oenological parameters and stilbene data classified satisfactorily varieties into two terroir groups: Jerez and Cabra on the one hand, and Ronda y Cadiar on the other hand. Terroir effect is stronger than variety on stilbene induction capacity after UVC treatment. Syrah from Cabra standed out due to both high basal and induced stilbene concentration that was reached. Therefore, Cabra and Syrah are claimed to be the most suitable terroir and variety, respectively, for obtaining a stilbene-enriched wine.

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Jerez de la Frontera (Spain) January 15th, 2012

Dear Editor,

Please find enclosed a copy of the manuscript entitled "***TERROIR AND VARIETY: TWO KEY FACTORS FOR OBTAINING STILBENE-ENRICHED GRAPES"*** for its consideration and possible publication in "*Journal of Food Composition and Analysis*".

Many health-properties have been attributed to stilbenes (mainly resveratrol). Grape (and wine) are the main source of bioactive stilbene in the diet
The present manuscript is a survey of how both variety and terroir affect bioactive stilbene concentration in grapes. There are several studies about the influence of the variety on stilbene content in grapes. However, the effect of terroir on stilbene content is lesser studied.
We have studied four of the most widely cultivated red grape varieties (Syrah, Merlot, Cabernet sauvignon and Pinot noir) in four important Andalusian terroirs (Jerez, Cabra, Ronda and Cadiar) and how stilbene content is affected by these variables. Statistical results allow us to establish that terroir effect is higher than variety effect.
Moreover, we propose the variety Syrah and the terroir Cabra for obtaining stilbene-enriched grape, as a raw material for stilbene-enriched wine.

I confirm that the present manuscript fulfils the following requirements:

- Data and text are original
- Manuscript has not been previously published and it is not under consideration for publication in any another journal

- All authors agree with publication in the journal in its current form

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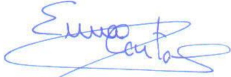

Emma Cantos Villar

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1 # Terroir and variety: two key factors for obtaining stilbene-enriched
2 grapes.

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15 **Abstract**
16 Grape and wine are the main source of bioactive stilbene in the diet. The stilbene
17 composition of grapes depends on both variety and terroir. The effect of terroir on grape
18 stilbene has not been widely studied. In this research the study of the stilbene content
19 (resveratrol, piceatannol and viniferins) in four different red grape varieties (Syrah,
20 Merlot, Cabernet sauvignon and Pinot noir) and from four different Andalusian terroirs
21 (Jerez, Cabra, Cadiar and Ronda) was carried out during vintage 2009. Moreover, the
22 stilbene induction capacity was studied in each terroir and variety after UVC
23 postharvest treatment. Data were analysed by multivariate statistical methods. Principal

component analysis applied on the oenological parameters and stilbene data classified satisfactorily varieties into two terroir groups: Jerez and Cabra on the one hand, and Ronda y Cadiar on the other hand. Terroir effect is stronger than variety on stilbene induction capacity after UVC treatment. Syrah from Cabra standed out due to both high basal and induced stilbene concentration that was reached. Therefore, Cabra and Syrah are claimed to be the most suitable terroir and variety, respectively, for obtaining a stilbene-enriched wine.

Keywords: Terroir; climate; red grape variety; stilbenes; UV-C postharvest treatment; oenological parameters.

1. Introduction

Phenolic compounds, which mainly consist of anthocyanins, flavan- 3-ols, flavonols, phenolic acids (including hydroxybenzoic acids and hydroxycinnamic acids) and stilbenes, play one of the most important roles in the quality of red grapes and wines. These compounds have been reported to have multiple biological health-promoting properties such as antioxidant and cancer protective effects among others (Soleas et al., 2006). In this sense stilbenes, and in particular (*Z)*-resveratrol, can be standed out. Resveratrol's biological properties include cardioprotective, neuroprotective and anticancer actions (Guerrero et al., 2009a). In fact, (*Z)*-resveratrol seems to be one of the most promising compounds due to its bioactivity, being wine the main source of resveratrol in diet. Piceatannol and viniferins are stilbenes usually found in lower concentrations than resveratrol in grape and wine. Consequently, their bioactivity has been less studied. Nevertheless, some of their health-promoting properties have been investigated (Guerrero et al., 2009a).

47 The amount of phenolics in grapes depends on the variety of grapevine (Katalinić et al.,
48 2010; Dimitrovska et al., 2011; Navarro et al., 2008; Yang et al., 2009; Guerrero et al.,
49 2009b) and is highly influenced by terroir. The French term ‘‘terroir” is used in
50 oenology to define a geographical and environmental origin where the grapes used for
51 the vintage were grown. This word includes such characteristics as soil type, climate
52 (sunlight, temperature and rainfall) and topography. The terroir impact on the phenolic
53 compounds in grape has been widely investigated (Gambelli & Santaroni, 2004;
54 Goldberg et al., 1998; Gonzalez-San et al., 1990; Mc-Donald et al., 1998; Rastija et al.,
55 2009). Previous researches showed that light, water deficits and higher temperature
56 differences between daytime and night-time could up-regulate the gene expression
57 related to flavonoid metabolism, and thus the contents of flavonoids increase
58 significantly (Mateus et al., 2001; Kennedy et al., 2002).
59 In the case of (*Z*)-resveratrol, its content in grapes is rather low and it highly depends on
60 the variety (Katalinić et al., 2010; Gatto et al., 2008; Guerrero et al., 2010; Lee &
61 Rennaker, 2007). Regards to terroir, few studies have been published on this subject.
62 However, for our far knowledge, the effect of both terroir and variety in stilbene
63 concentration in grapes has been even less studied. It has been described that the content
64 of stilbenes varies depending on factors such as climate, geographical area of cultivation
65 and growing conditions (Bavaresco, 2003; Bavaresco et al., 2008). Goldberg et al.
66 (1995) and Abril et al. (2005) stated that cold climatic conditions seem to promote
67 stilbene biosynthesis, but controversial data have also been found (Li et al., 2011).
68 Regarding soil effect on stilbene concentration, few data have been reported (Andres de
69 Prado et al., 2007). Finally, all the cultural practice, that somehow stresses to the

70 vineyard, make resveratrol content increase (Bavaresco, 2003; Bavaresco et al., 2001;
71 Fregoni et al., 2001; Dani et al., 2007; Deluc et al., 2011).
72 In Andalusia (South Spain) there are twenty two main wine-producing sub-regions and
73 terroirs vary among them. Four of the most representative terroirs with large differences
74 are Jerez, Cabra, Ronda and Cadiar. Jerez is placed ten kilometres away from the
75 Atlantic coast. Cabra is located in the foothills of the Subbetic range; Ronda is under
76 Mediterranean climate; and Cadiar is located at 1369 m above sea level.
77 On the other hand, the synthesis of stilbenes can be constitutive or induced by biotic and
78 abiotic elicitors. It is possible to deliberately manipulate environmental stress factors to
79 influence nutraceutical content within grapes. Among all the abiotic elicitors described,
80 UVC postharvest treatment has been considered one of the most effective stresses for
81 increasing stilbene content in grapes (Guerrero et al., 2010).
82 The aim of this study was to determine the contribution of both variety and terroir on
83 stilbene concentration and on its induction capacity after UV-C light postharvest
84 treatment in order to find the most suitable terroir and variety to obtain a stilbene-
85 enriched wine.
86 **2. Materials and methods**
87 *2.1. Reagents*
88 (*Z*)-Resveratrol (3,5,4′-trihydroxy-(*Z*)-stilbene) and piceatannol (3,3′,4,5′-
89 tetrahydroxystilbene) were purchased from Sigma-Aldrich (Madrid, Spain). Analytical
90 grades of acetic acid, methanol and ethyl acetate dissolvent were supplied by Panreac
91 (Barcelona, Spain). Ultrapure water from a Mili-Q system (Millipore Corp., Bedford,
92 MA) was used in this research.
93 *2.2. Grapevine*

94 Four red grape varieties (Syrah, Merlot, Cabernet sauvignon and Pinot noir) were
95 cultivated in four well-characterized terroirs in Andalusia (Table 1): Jerez de la Frontera
96 (Cadiz), Cabra (Cordoba), Cadiar (Granada) and Ronda (Malaga). All vineyards were
97 planted between 1995 and 2000. The planting density ranged between 3000 and 4000
98 vines/ha. Vines were grown using the espalier system and trained with the royal
99 bilateral pruning system, with four renewal spurs per bunch, and two buds by spurs.
100 The soil was different depending on the terroir. Soil data were provided by
101 collaborating wineries (Table 1).
102 Grape ripeness was monitorized weekly from veraison to harvest in each terroir to
103 determine the optimum harvest date (data not shown).

104 *2.3. Meteorological parameters*

105 Meteorological data (temperature, relative humidity, solar radiation, and rainfall) of the
106 harvest period were provided by the Weather Station Network Management
107 Consultancy Services of Junta de Andalusia.
108 http://www.juntadeandalucia.es/agriculturaypesca/ifapa/ria/servlet/FrontController).

109 *2.4. Oenological parameters of grapes*

110 Dry residue, weight grape, Brix degree, total acidity, pH, tartaric acid, malic acid,
111 potassium, Folin-Ciocalteau index (IFC), total polyphenols index (TPI), total
112 anthocyanins, extractable anthocyanins and tannins were determined in grapes at
113 harvest with the official methods (OIV, 1990).

114 *2.5. UVC treatment and storage period*

115 Harvested grapes were divided into two batches, the first batch (CT) was not treated and
116 the second batch (UV) was irradiated with UVC light after harvesting. Grape clusters
117 were irradiated following a system comprising 34 UV-C lamps, with 17 lamps (254 nm,

118 Silvana, G30T8) in each of two panels positioned above and below the grapes. A
119 theoretical power of 1020 W at 42 cm was applied for 60 s, according to the protocol
120 proposed by Cantos et al. (2001) (patent WO/2002/085137; ES 2177465).
121 Subsequently, grape clusters were stored in a stainless steel vessel at 20 °C and 80%
122 relative humidity for seven days (post-treatment period). During this period 100 g of
123 grapes were randomly and carefully sampled every day from different bunches, and
124 peeled. Grape skins were stored at −80 °C until extraction was performed.
125 The control grape clusters (CT) were stored under the same conditions as those
126 described above but without undergoing the UV-C treatment. The parameter "maximum
127 day" (Dm) was defined as the number of days elapsed after UVC treatment to achieve
128 the maximum (*Z*)-resveratrol concentration in grape. The induction velocity of (*Z*)-
129 resveratrol (IV_{resv}) was defined as the difference between resveratrol concentrations at
130 Dm (C_{Dm} resv) and on the day of harvesting (C_0 resv), divided by the number of days
131 needed to reach maximum content of resveratrol (More details are given in Guerrero et
132 al., 2010).
133 *2.6. Stilbene extraction method*
134 The extraction method used was described by Bavaresco et al. (2001) with some
135 modifications. 0.25g freeze-dried skin are extracted twice with 5ml of $NaHCO_3$ (5%)
136 and 5 ml ethyl acetate. The samples were ground using Ultraturrax T-25 equipment
137 (Janke & Kunkel, Ika-Labortechnick, Deutschland, Germany) and stirred at 1200 rpm
138 for 20 minutes. The organic phase was first dried with a vacuum centrifuge
139 concentrator, then re-dissolved in 2 ml of MeOH and, finally, filtered through a 0.22 µm
140 filter (PVDF Teknokroma, Barcelona, Spain). Extractions were performed in triplicate

141 at the dark and low temperature to avoid the oxidation and isomerization. The data are
142 expressed in mg/kg fresh weight (fw).

143 *2.7. Identification and quantification of stilbenes*

144 Stilbenes were quantified as described by Guerrero et al., (2010). Briefly, samples
145 (20μl) were analysed using a Waters HPLC system with a model 1525 pump and a
146 Waters 996 Photodiode Array Detector. Separations were performed on a Mediterranea
147 Sea18 column (Tecknokroma, Barcelona, Spain) (RP-18, 25×0.46 cm; 5 μm particle
148 size) and a guard column of the same material, at 30 °C. The mobile phases consisted of
149 a water:methanol:acetic acid mixture, solvent A 88:10:2 and solvent B 8:90:2 at a flow
150 rate of 1 ml/min. Stilbenes were quantified at 306 nm as (*Z*)-resveratrol.

151 *2.8. Statistical analysis*

152 All samples were analysed in triplicate. Data regarding to both terroir and variety were
153 included in the statistical analysis. First, the Z-score test was used to remove outliers,
154 i.e. for the same chemical determination, any result showing a $Z \geq |2|$ was not included in
155 the multivariate statistical analysis. Statistica 6.0 software was used in the analysis of
156 the resulting data. By means of Principal Component Analysis (PCA) redundant
157 variables providing similar information were removed. Variables contributing most to
158 the variance of data matrix were selected for further statistical analysis. Cluster analysis
159 using Ward's method allows to explore data trends. Finally, discriminant analysis was
160 conducted to build classification functions to discriminate samples according to the
161 terroirs.

162 **3. Results and discussion**

163 *3.1. Harvested grapes*

164 Oenological parameters were monitored weekly from veraison to harvest (data not

165 shown). Data at harvest are shown in Table 2. As expected, the date of harvest depended
166 on terroir. Cabra was the terroir with the earliest harvest date followed by Jerez, Ronda
167 and Cadiar.
168 Dry extract ranged from 18.30 to 24.55, neither the variety nor the terroir accounts for
169 an effect on these values. Similar values were observed for weight grape, although
170 Cadiar reached higher values for all the varieties. Sugar content determined the date of
171 harvest, being quite similar in all cases (terroirs and varieties). Only Pinot noir from
172 Jerez was harvested before of the optimum sugar content due to the dehydration process
173 observed in the vine. This variety is very sensitive to warm climate and grapes started to
174 lose weight instead of following the normal ripeness process. Merlot from Ronda and
175 Cadiar grapes achieved the highest sugar content; the harvest of Merlot grapes in these
176 terroirs was delayed to reduce total acidity values.
177 In contrast, some parameters seemed to be affected by both terroir and variety. Total
178 acidity varied widely among terroirs and varieties. Pinot noir variety reached the highest
179 total acidity values in all the terroirs, which could be due to its high content in both
180 tartaric and malic acids, being especially high malic acid content. Jerez singled out for
181 the lowest acidity values in all the varieties studied. The high solar radiation level
182 suffered by this terroir at harvest may explain this fact. Potassium concentration in
183 grape was also very influenced by terroir (Table 1) due to the different type of soil as it
184 has been described (Kodur, 2011; Gómez-Míguez et al., 2007). Potassium reached
185 higher values in Cabra and Jerez than in Ronda and Cadiar. pH data were in accordance
186 with acidity and potassium data. The IFC values were quite similar among different
187 terroirs regardless of the variety. Moreover, when terroirs were compared, the highest
188 anthocyanin concentration was reached in Cadiar but Pinot noir, which reached the

189 highest anthocyanin content in Cabra. In fact, Cadiar is the terroir with the highest
190 altitude (Table 1). Therefore, during ripeness period grapes suffered high differences of
191 temperatures between day and night which could justify the high anthocyanin content
192 (Mateus et al., 2001; Yamane et al., 2006). However, this terroir showed the lowest
193 percentage of extractable anthocyanins, and therefore higher extractability, if compared
194 to the others in all cases (Table 2). Tannin and TPI did not show any significant
195 difference neither in terroir nor in variety. In fact, tannins are more affected by climatic
196 effects in composition than in content (Mateus et al., 2001).
197 *3.2. Basal stilbene content in grapes: variety vs terroir*
198 Stilbene compounds have been determined in four varieties cultivated in four terroirs at
199 harvest (before UV-C postharvest treatment, basal content, C_0, Table 3). In agreement
200 with previous studies, the following stibenes were found in the grape samples:
201 piceatannol, (*Z)*-resveratrol, ε-viniferin and δ-viniferin (Figure 1). All above stilbenes
202 were identified by UPLC-DAD-TQD in our lab (Guerrero et al., 2010).
203 The basal content of resveratrol from highest to lowest was as follows: Cabra > Jerez>
204 Cadiar> Ronda, in all grape varieties studied (C_0 resv, Table 3).
205 At harvest, piceatannol was detected in all varieties cultivated in Cabra and Jerez
206 terroirs (C_0 pictnol, Table 3). Moreover, piceatannol was also detected in Syrah,
207 Cabernet sauvignon and Pinot noir from Cadiar. Ronda was the only terroir where basal
208 piceatannol was not detected in any variety. Viniferins were only detected in a few
209 samples: both Syrah and Pinot noir from Jerez, Cabra and Cadiar terroirs, and Cabernet
210 sauvignon from Cabra. Merlot did not content viniferins in any of the terroirs studied. In
211 fact, Merlot variety has been described as a variety with an innate low stilbene content
212 (*16*). Cabra presented the highest basal stilbene content in Syrah and Cabernet

213 sauvignon. The other varieties (Merlot and Pinot noir) did not reach the highest
214 concentration but also reached a high stilbene basal level. Cabra terroir showed the
215 highest temperature average and temperature difference range, joint to the lowest
216 relative humidity average during harvest period (Table 1). Moreover, the rainfall during
217 late berry development could also be an important stress factor in agreement with other
218 authors (Andres de Prado et al., 2007; Li et al., 2006). It is worth mentioning that apart
219 from the rainfall no disease symptom was observed in the vineyard.
220 On the contrary, Ronda terroir, that showed low rainfall, the lowest temperature
221 difference range and low relative humidity average (Table 1), presented low basal
222 stilbene content but for Merlot. Resveratrol was only found in low amounts in the four
223 varieties cultivated in this terroir. With regard to solar radiation and average
224 temperature values, Cabra and Jerez showed higher values in comparison with Ronda
225 and Cadiar, which could also be stressful for the vineyard (Table 1). Taking into
226 account all statements above, it could be established that high variations in climatic
227 conditions could stimulate the natural biosynthesis of stilbenes. However, the effect of
228 climate on stilbene biosynthesis cannot be isolated from the rest of the effects. In fact,
229 soil effect on stilbene amount has been proved to be as important as climate effect
230 (Andres de Prado et al., 2007). Cabra terroir presented a nutrient-poor soil. The soil
231 found in Jerez (called *albariza*) is similar to Cabra soil but with a difference in its
232 texture (Table 1). Cabra and Jerez terroirs showed high water holding capacity (Table
233 1). In fact, it has been described that soils with high water-holding capacity might
234 stimulate the stilbene biosynthesis in grape (Andres de Prado et al., 2007; Koundouras
235 et al., 2006; Bavaresco et al., 2009). Ronda and Cadiar showed very low active lime
236 soils due to its sandy texture. However, few studies have been developed in this sense.

237 The term terroir involves so many factors that it is extremely difficult to compare with
238 other studies. Li et al. (2011) studied the effect of terroir on stilbene concentration of
239 Cabernet sauvignon. In that study the terroir with sandy soil, low altitude, semi-humid
240 climate and slight temperature difference was the best for high basal stilbene
241 concentration. Conditions are so different for each terroir that it is difficult to make a
242 direct comparison.

243 *3.3. Induction capacity of stilbenes in grapes: variety vs terroir*

244 Stilbene compounds have been determined in four varieties cultivated in different
245 terroirs after UV-C postharvest treatment. Thus, the induced content of stilbenes was
246 examined (Table 3, maximum stilbene concentration reached, C_{Dm}). The induction of
247 stilbenes was characterised in order to select the most suitable variety and terroir to
248 obtain a stilbene-enriched wine.

249 After UVC postharvest treatment all the varieties in each terroir increased resveratrol,
250 piceatannol and viniferin contents. The amount of stibenes was different depending on
251 the terroir and the variety (Table 3). Cabra was the terroir where the varieties studied
252 showed the highest induction capacity, especially Syrah variety. Syrah from Cabra
253 reached the highest amount of resveratrol (23.55 mg/Kg fw), piceatannol (6.13 mg/Kg
254 fw) and viniferins (3.46 mg/Kg fw) and, therefore, the highest induction velocity (2.02),
255 which means that, on average, Syrah from Cabra terroir synthesized 2.02 mg/Kg per
256 day after the UVC postharvest treatment. This is in agreement with previous results in
257 which Syrah increased its stilbene content more than other thirteen varieties studied in
258 only one terroir (Guerrero et al., 2010). Cabernet sauvignon from Cabra was the variety
259 with the second highest stilbene content after UVC postharvest treatment. It achieved
260 13.85 mg/Kg fw of total stilbenes (Table 3). In addition, Syrah from Cadiar also

261 achieved a high concentration of total stilbenes (12.32 mg/Kg fw).

262 In Jerez terroir all varieties but Merlot reached about 7 mg/Kg and 10 mg/Kg of
263 resveratrol and total stilbenes respectively at Dm (Table 3), that may be considered a
264 high level in comparison with non-treated grapes.

265 Ronda and Cadiar, as occurred with basal content, were the terroirs where the
266 resveratrol concentration after UVC postharvest treatment was low but for Merlot from
267 Ronda (Table 3).

268 With regard to piceatannol and viniferins, higher concentrations were found in varieties
269 which achieved higher resveratrol levels, since resveratrol has been described as the
270 precursor of the other stilbenes (Coutos-Thévenot et al., 2001). Thus, resveratrol
271 determine the tendency of the other stilbenes and the amount of total stilbenes.

272 It can also be observed that the induction velocity was higher in Cabra and Jerez terroirs
273 in all varieties but Merlot from Ronda, in which IV reached 0.82 mg/kg.

274 Moreover, varieties reached the highest resveratrol induction in different periods of time
275 (Dm) depending on the terroir. Dm was constant for Cabernet sauvignon (Dm = 7).
276 Pinot noir showed the same Dm in all terroirs (Dm = 6) but in Cadiar, where it was
277 delayed one day (Dm = 7). However, in Syrah and Merlot varieties it changed
278 depending on the terroir, ranged between four and seven days (Table 3). This fact
279 complicates the standardization of the UVC treatment since Dm should be established
280 for every different terroir and variety.

281 If we focus on varieties, Syrah was the variety with the highest induction capacity in all
282 terroirs but in Ronda, where Merlot was the highest. This was surprising because, in
283 general, Merlot showed low induction capacity. Cabernet sauvignon and Pinot noir from
284 Jerez and Cabra terroirs (high stilbene production) showed different induction capacity

285 to Ronda and Cadiar terroirs (low stilbene production).

286 *3.4. PCA-Based Display Methods and Cluster Analysis*

287 Data matrix included a set of sixteen samples corresponding to the four varieties and
288 four terroirs. Twenty-four analytical determinations were performed in each sample in
289 triplicate, giving rise to twenty four variables (C_0 resv, C_{Dm} resv, Dm, induction velocity
290 of resveratrol, C_{Dm} resv−C_0 resv, capacity for the induction of resveratrol, harvest date,
291 dry residue, weight grape, Brix degree, total acidity, pH, tartaric acid, malic acid,
292 potassium, IFC, TPI, tannins, anthocyanins, extractable anthocyanins, extractability,
293 skin tannins, seed tannins, seed maturity).

294 When data matrix was subjected to PCA, two significant principal components (PCs)
295 arose according to both Kaiser's criterion and the assurance of suitable communalities
296 for variables (>0.5). With these factors, 52.38% of total variance is explained. The first
297 PC, PC1 (which explains 31.75% of total variance), mainly contains the descriptors C_0
298 resv, C_{Dm} resv, IV_{resv}, potassium, total anthocyanins, extractable anthocyanins, and
299 tannins. Indeed, potassium content is a variable significantly correlated ($p<0.05$) with
300 the resveratrol content and also with tannin and total and extractable anthocyanins.
301 These factors includes variables mainly related to the ability to induce resveratrol and
302 polyphenol content in grapes. The second PC, PC2 (which explains 20.63% of total
303 variance), is contributed by factors such as index of maturity, pH, total acidity and
304 tartaric acid. All factors above are mainly related with the ripeness of the grapes.

305 Plots of the two first principal components issued from PCA may be of interest to
306 visualize data trends. The corresponding score plot for the studied varieties is shown in
307 Figure 2. A linear separation of classes based on geographical origin was found in
308 accordance with other similar studios (Rastija et al., 2009). Two groups of samples were

309 observed. The first group was constituted by varieties from Cadiar (Cd) and Ronda (R)
310 terroirs, and the second one by varieties from Jerez (J) and Cabra (Cb) terroirs. It could
311 be observed that in the second group more dispersed data were found in comparison
312 with the first group.
313 These results demonstrate the general trends exposed in previous sections (Tables 2 and
314 3). As commented before, grouping was observed according to terroirs rather than to
315 varieties.
316 Anthocyanins and total acidity values were higher in the first group when compared to
317 the second one. On the contrary, low potassium concentrations were found in the first
318 group in comparison with the second one. Only Pinot noir from Cabra was not well
319 settled in the right group, which could be due to its high anthocyanin content and also
320 its high total acidity, which are characteristics of the varieties belonging to the second
321 group. Terroir characteristics are more similar between Cabra and Jerez, as well as
322 between Cadiar and Ronda, than among any others (Table 1). Climatic conditions were
323 similar in Cabra and Jerez on one hand and in Cadiar and Ronda on the other hand, as
324 already discussed. The same happens with soil type. Jerez and Cabra soils are found less
325 sandy and with higher active lime and higher soil water holding capacity than Cadiar
326 and Ronda soils.
327 In addition, cluster analysis explores natural trends between samples. Ward's method
328 was selected as grouping method. If variables contributing to Factor 1 are used in
329 cluster analysis, the resulting tree diagram is displayed in Figure 3. Samples are grouped
330 into two main clusters. Each one of them includes 50% of samples. Samples in the first
331 one are from Ronda (75%) and Cadiar (100%) whilst in the second samples are from
332 Jerez (100%) and Cabra (75%). It is remarkable that in accordance with PCA results,

333 samples were grouped by terroir rather than by variety, similar to Croatian wine terroirs
334 (Rastija et al., 2009). As commented before, Pinot noir from Cabra was not settled in
335 the right group. The same happened to Pinot noir from Ronda. Pinot noir is a peculiar
336 variety, since its anthocyanins content, and, therefore, its phenolic content, is quite low
337 (Mazza et al., 1999). Maybe this peculiarity makes it show a different behaviour. If
338 cluster analysis is applied with variables related to grape maturity which were selected
339 by Factor 2, no natural grouping related to terroir is observed. This could be due to the
340 low weight of Factor 2 (data not shown).
341 ANOVA proved statistical significant differences [$p<0.05$] among all varieties when all
342 variables are considered. Linear Discriminant Analysis (LDA) was applied to look for
343 the most useful variables to discriminate among classes. Applying the forward selection
344 approach, five variables were selected: C_0 resv, C_{Dm} resv, induction velocity of
345 resveratrol, potassium and total anthocyanins. Classification matrix shows a 93.7% of
346 correct classification samples. Of the 16 samples just one sample was not well
347 classified. Cabernet sauvignon from Cabra was classified as Jerez sample (data not
348 shown). Thus, discriminant analysis for terroirs with these variables showed a 94% of
349 samples correctly classified. However, when applied to varieties, correct classification
350 was just a 50%.

351 **4. Conclusion**

352 Both variety and terroir are key factors for stilbene concentration in grapes. They
353 affected the basal concentration as much as the concentration after UVC postharvest
354 treatment. Terroir seems to be more important than variety. Cabra terroir (with loamy
355 sandy and high water-holding capacity soil, continental climate, high temperature
356 oscillation and low relative humidity during the harvest period) was the terroir with the

357 highest basal stilbene concentration in comparison with the other Andalusian terroirs.
358 This was particularly striking in Syrah variety. Actually, Syrah was the variety with the
359 highest basal stilbene content in three out of four terroirs studied. As varieties with a
360 higher basal stilbene concentration were higher producers after they were stressed,
361 Syrah reached the highest concentration in all the zones except in Ronda.

362 On the other hand, the standardization of the UVC postharvest treatment to increase
363 stilbene content in wine seems to be difficult. The induction capacity of stilbenes is
364 strongly affected not only by variety (as already described) but also by terroir (described
365 here for the first time). Even the maximum day of induction (Dm) is variable depending
366 on variety and terroir. Nevertheless, general recommendation can be stated. Syrah can
367 be suggested as one of the most suitable varieties for obtaining stilbene-enriched wines,
368 in agreement with previous results (Guerrero et al., 2010), and Cabra-type terroir as
369 accurate terroir for its cultivation, in order to obtain enriched wines in stilbenes with
370 added-value.

371 **Figure captions**

372 **Figure 1**. Chromatogram at 306 nm of CT and UVC Syrah grape in Cabra terroir.

373 **Figure 2**. Principal component analyses. Abbreviations : JSy, Jerez Syrah; JMlt, Jerez
374 Merlot; JCS, Jerez Cabernet Sauvignon; JPn, Jerez Pinot noir; RSy, Ronda Syrah; RMlt,
375 Ronda Merlot; RCS, Ronda Cabernet Sauvignon; RPn, Ronda Pinot noir; CbSy, Cabra
376 Syrah; CbMlt, Cabra Merlot; CbCS, Cabra Cabernet Sauvignon; CbPn, Cabra Pinot
377 noir; CdSy, Cadiar Syrah; CdMlt, Cadiar Merlot; CdCS, Cadiar Cabernet Sauvignon;
378 CdPn, Cadiar Pinot noir.

379 **Figure 3.** Dendogram representing the grouping of the varieties analyzed according to
380 stilbenic variables. Abbreviations: JSy, Jerez Syrah; JMlt, Jerez Merlot; JCS, Jerez

381 Cabernet Sauvignon; JPn, Jerez Pinot noir; RSy, Ronda Syrah; RMlt, Ronda Merlot;
382 RCS, Ronda Cabernet Sauvignon; RPn, Ronda Pinot noir; CbSy, Cabra Syrah; CbMlt,
383 Cabra Merlot; CbCS, Cabra Cabernet Sauvignon; CbPn, Cabra Pinot noir; CdSy, Cadiar
384 Syrah; CdMlt, Cadiar Merlot; CdCS, Cadiar Cabernet Sauvignon; CdPn, Cadiar Pinot
385 noir

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Table(s)

Table 1.

Soil type, geographical coordinates and climatic parameters of the four studied terroirs.

	Jerez	Cabra	Ronda	Cadiar
Soil type	Limestone	Limestone	Limestone	Slate
Soil texture	sandy (19%) clay (38.5%) silty (42.5%) loamy clavey silty	sandy (45.5%) clay (27.8%) silty (26.8%) loamy clavey sandy	sandy (63%) clay (23%) silty (14%) loamy clavey sandy	sandy (67%) clay (11%) silty (22%) loamy sandy
Active lime (%)	20.5	11.7	1.4	0
Water holding capacity (mm)	131.6	107.0	90.5	77.4
Latitude (N)	36:45:29	37:29:58	36:46:47	36:55:27
Longitude (W)	06:00:58	04:25:46	04:53:24	03:10:57
Altitude a.s.l. (m)	35	560	540	1300
Tmax (ºC)	33.12	38.77	29.94	35.63
Tmin (ºC)	16.55	13.02	15.41	13.25
Tavg (ºC)	24.96	25.96	22.57	23.14
RHmax (%)	80.79	86.47	60.94	90.90
RHmin (%)	32.08	10.39	24.36	9.27
RHavg (%)	56.72	38.61	40.80	45.20
Solar Radiation (MJ/m^2)	28.98	27.41	23.58	22.43
Rainfall Jul-Sept (mm)	0.02	23.8*	0.62	13.40

*Only one storm in August

Tmax, maximum Temperature; Tmin, minumum Temperature; Tavg, average Temperature; RHmax, maximum Relative Humidity; RHmin, minimum Relative Humidity; RHavg, average Relative Humidity.

Table 2. Enological parameters of red grape varieties at harvest in the four studied terroirs.

	SYRAH				MERLOT				CABERNET SAUVIGNON				PINOT NOIR			
	Jerez	**Cabra**	**Ronda**	**Cadiar**	**Jerez**	**Cabra**	**Ronda**	**Cadiar**	**Jerez**	**Cabra**	**Ronda**	**Cadiar**	**Jerez**	**Cabra**	**Ronda**	**Cadiar**
Harvest date	20/08	13/08	10/09	17/09	20/08	13/08	31/08	17/09	27/08	27/08	31/08	25/09	04/08	27/07	21/08	17/09
Dry extract (%)	21.42	19.15	21.23	20.84	21.61	20.87	24.55	22.29	22.00	23.80	22.27	22.47	18.30	22.12	22.40	21.73
Weight grape (g)	1.65	1.64	1.44	1.91	1.39	1.21	1.07	1.64	1.04	1.03	1.08	1.30	1.10	1.03	0.98	1.58
Brix degree	23.2	21.5	23.0	22.3	22.7	22.5	26.0	24.8	22.8	24.2	24.0	24.7	18.5	23.4	21.8	22.2
Total Acidity (g/L TH_2)	5.01	8.49	8.05	9.00	4.98	7.71	9.60	9.56	7.56	8.17	9.37	9.57	8.78	11.44	9.85	9.39
pH	3.64	3.26	3.24	3.11	3.55	3.25	3.04	3.02	3.35	3.23	3.11	2.94	3.22	3.09	3.14	3.03
Tartaric acid (g/L)	5.44	7.81	8.57	7.53	6.31	9.67	9.19	8.98	7.37	6.47	9.57	9.04	8.82	9.83	10.40	8.11
Malic acid (g/L)	2.60	3.33	1.65	2.37	1.48	1.70	1.77	1.24	2.17	2.05	2.19	2.82	3.27	4.49	3.05	2.06
Potassium (mg/L)	2151	2502	1841	1606	2122	2536	1517	1489	2060	2096	1562	1649	2073	2056	1964	1427
IFC	20.06	17.64	19.07	19.00	10.96	11.42	10.60	10.23	15.02	16.58	12.76	17.90	9.63	12.20	13.53	11.73
TPI	21.75	18.73	19.11	16.81	19.64	11.51	21.19	18.67	15.36	15.93	13.65	15.28	12.46	21.99	15.65	12.36
Tannins (g/L)	3.04	2.62	2.68	2.35	2.75	1.61	2.97	2.61	2.15	2.23	1.91	2.14	1.74	3.08	2.19	1.87
Total anthocyanins (mg/L)	767	684	1052	1556	742	630	978	1419	619	500	841	1078	295	1004	645	881
Exttractability	45.4	61.9	54.1	65.7	43.8	60.4	51.5	67.0	49.1	43.2	50.8	68.1	58.6	54.1	51.1	72.1
Extractable anthocyanins (mg/L)	417	274	483	532	416	251	474	468	315	284	413	344	124	460	315	248

Table 3. Variables for stilbenes established.

		C_o resv (mg/Kg	C_o pictnol (mg/Kg	C_o vinif (mg/Kg	C_o stilb (mg/Kg	C_{Dm} resv (mg/Kg	C_{Dm} pictnol	C_{Dm} vinif (mg/Kg	C_{Dm} stilb (mg/Kg	Dm (day)	IV resv (C_{Dm}-
SYRAH	**Jerez**	1.88	0.32	0.23	2.43	7.04	2.27	0.61	9.92	5	1.03
	Cabra	11.45	1.35	0.27	13.07	23.55	6.13	3.46	33.14	6	2.02
	Ronda	0.15	n.d.	n.d.	0.15	4.67	1.25	0.80	6.72	7	0.65
	Cadiar	1.61	0.41	0.34	2.36	7.80	2.61	1.90	12.31	7	0.88
MERLOT	**Jerez**	0.43	0.12	n.d.	0.55	2.30	1.17	0.26	3.73	5	0.37
	Cabra	0.64	0.10	n.d.	0.74	3.11	0.91	0.61	4.63	4	0.62
	Ronda	0.16	n.d.	n.d.	0.16	5.89	1.94	0.65	8.48	7	0.82
	Cadiar	0.28	n.d.	n.d.	0.28	2.01	0.76	0.27	3.04	5	0.35
CABERNET SAUVIGNON	**Jerez**	0.64	0.11	n.d.	0.75	6.15	2.24	1.63	10.02	7	0.60
	Cabra	2.17	0.30	0.20	2.67	9.34	2.86	1.65	13.85	7	1.02
	Ronda	0.04	n.d.	n.d.	0.04	1.62	0.60	0.95	3.17	7	0.23
	Cadiar	0.37	0.11	n.d.	0.48	2.36	0.95	1.19	4.50	7	0.29
PINOT NOIR	**Jerez**	1.35	0.34	0.18	1.87	7.25	1.73	1.02	10.00	6	0.98
	Cabra	1.95	0.38	0.14	2.47	6.37	1.67	0.63	8.67	6	0.74
	Ronda	0.14	n.d.	n.d.	0.14	0.89	0.22	0.35	1.46	6	0.12
	Cadiar	1.08	0.27	0.32	1.67	2.05	0.58	0.83	3.46	7	0.14

C0 resv, basal resveratrol concentration; C0 pictnol, basal piceatanol concentration; C0 vinif, basal viniferin concentration; C0 stilb, basal stilbene concentration; CDm resv, resveratrol concentration at Dm; CDm pictnol, piceatannol concentration at Dm; CDm vinif, viniferin concentration at Dm; CDm stilb, stilbene concentration at Dm; Dm, day of maximum induction of resveratrol; IV resv, induction velocity of resveratrol; fw, fresh weight; n.d., not detected.

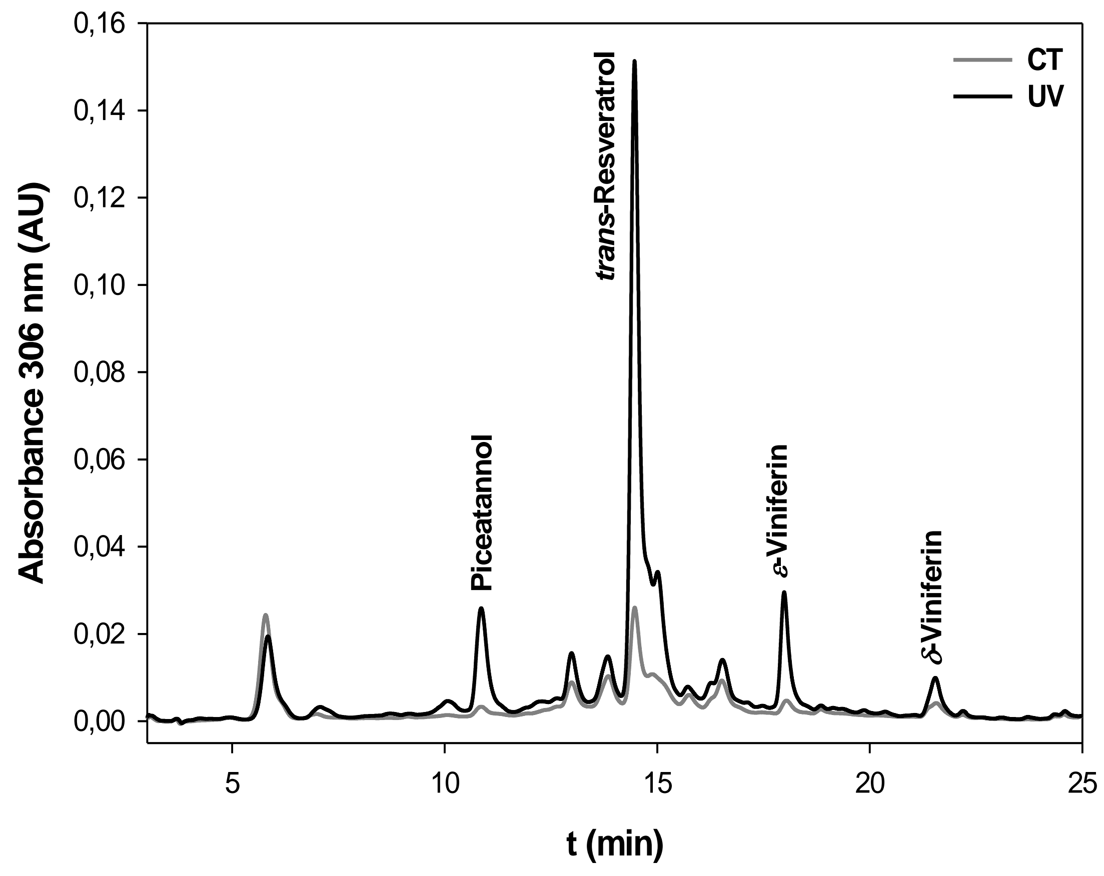

Figure 1.

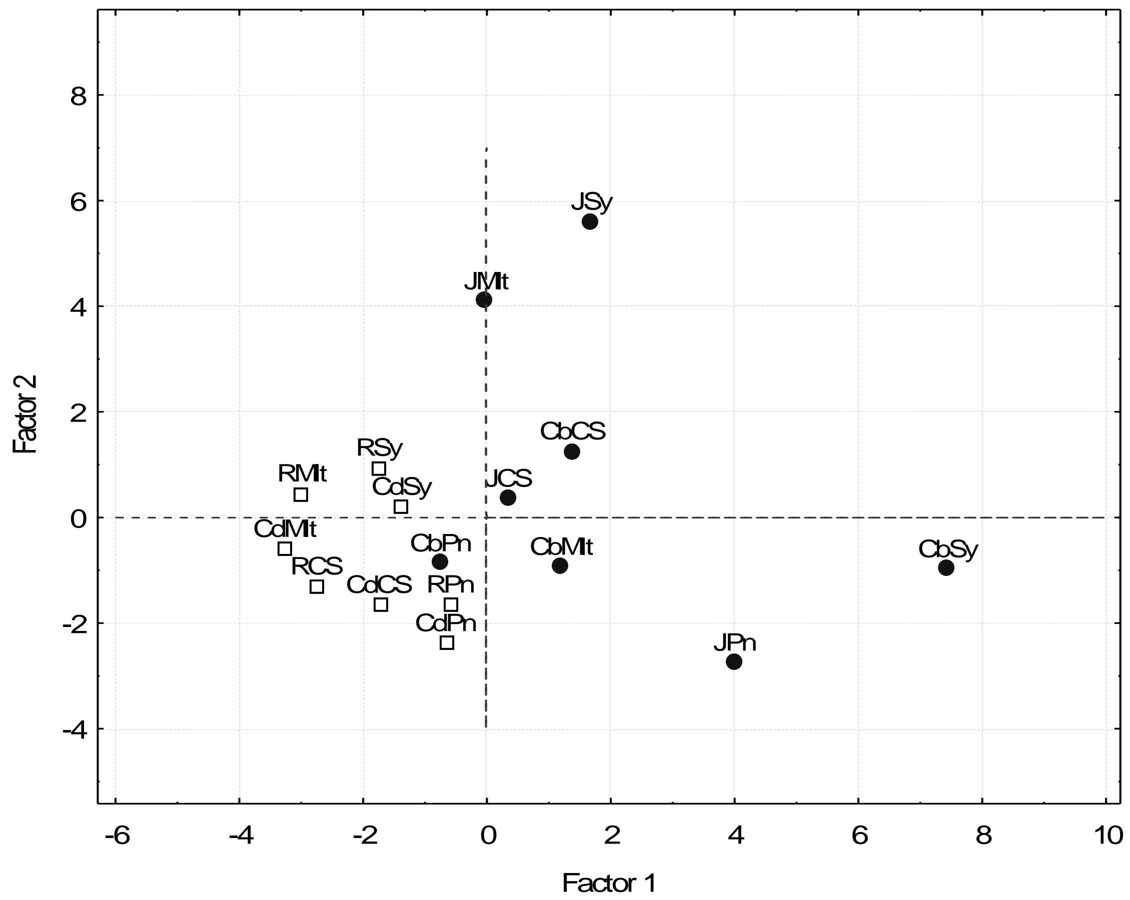

Figure 2.

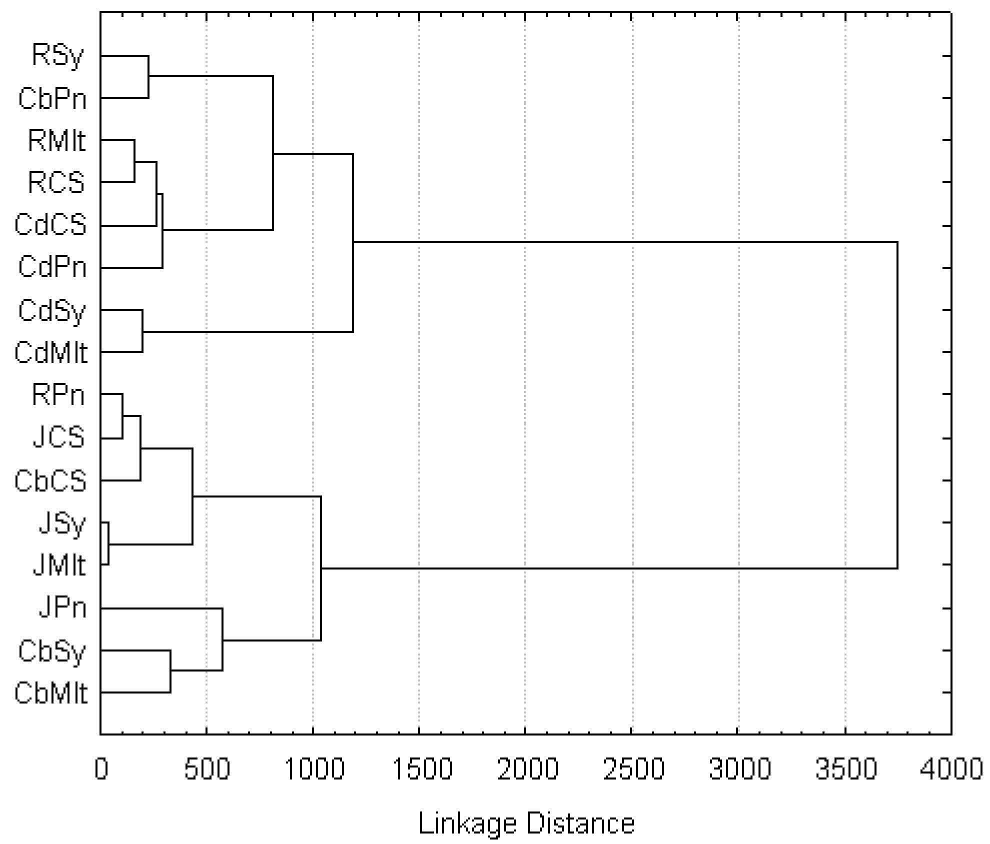

Figure 3

*Highlights

Highlights

Stilbenes are associated with health benefits

Grapes are the main source of stilbenes in the diet

Effect of both terroir and variety in stilbene concentration in grapes

UVC postharvest treatment increases stilbene content in grapes differently depending of terroir

ANEXO 4

PREHARVEST AND POSTHARVEST TREATMENTS COMBINATIONS TO INCREASE STILBENES CONTENT IN GRAPE

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Abstract

Aims: Stilbene-enriched grape is an interesting new food product with added-value due to the numerous health-promoting properties ascribed to it, mainly for its resveratrol content. The aim of this study was to evaluate the effectiveness of different elicitors on grapevine alone and combined with UVC postharvest treatment on stilbene concentration in grapes.

Methods and Results: Three preharvest treatments were tested: benzothiodiazole, (BTH), methyl jasmonate (MEJA) and chitosan (CHIT). Moreover preharvest treatments were combined with UVC postharvest treatment. The method was previously validated. As well, grape quality was evaluated.

Among preharvest treatments only BTH increased significantly *trans*-resveratrol concentration in grape before and at harvest. When combined pre- and postharvest treatments, only methyl jasmonate-ultraviolet C light (MEJA-UVC) combination was proved successful. Despite stilbenes concentration in grape after MEJA-UVC reached similar values than UVC, the time to reach maximum *trans*-resveratrol after UVC was significantly shortened.

Conclusion: MEJA-UVC combination achieves stilbene induction in grapes without altering oenological grape properties.

Significance and impact of study: The obtained results provide a treatment combination to obtained functional grapes.

Buts : Des baies de raisin enrichies en stilbènes constituent un produit alimentaire innovant avec une valeur ajoutée liée aux nombreux effets potentiellement bénéfiques sur la santé de ces composés et principalement du resveratrol. Le but de cette étude a été d'évaluer les effets de différents éliciteurs sur la concentration en stilbènes de la vigne. Ces éliciteurs ont été testés de façon isolée ou combinés avec un traitement aux UVC après la récolte.

Methodes and Resultats : Trois traitements pré-récolte ont été testés: benzothiodiazole, (BTH), methyl jasmonate (MEJA) et chitosan (CHIT). De plus, ces traitements pré-récolte ont été combiné à une exposition aux UVC après récolte. Dans un premier temps, la méthode a d'abord été validée. Dans un second temps, la qualité des baies a été évaluée.

Parmi les traitements pré-récolte seul celui avec le BHT augmente significativement la concentration en *trans*-resveratrol dans les baies avant et au moment de la récolte. Concernant la association des traitements avant récolte et l'exposition aux UVC, nos résultats indiquent que seul le traitement associant methyl jasmonate et UCV (MEJA-UVC) stimule la production des stilbènes. Même si les traitements MEJA-UVC et UVC seuls donnent des concentrations en stilbènes du même ordre de grandeur, le traitement MEJA-UVC réduit significativement la durée conduisant à une concentration maximale en *trans*-resveratrol par rapport à celui des UVC seuls.

Conclusion : La combinaison MEJA-UVC permet d'induire la production de stilbènes sans altérer les propriétés œnologiques des baies de raisin.

Impact de l'étude : Les résultats obtenus conduisent à la production de baies de raisin enrichies en stilbènes.

Keywords: benzothiadiazole; chitosan; methyl jasmonate; stilbenes; functional grape; ultraviolet C light.

INTRODUCTION

The role of grape and wine in the human diet has been widely studied in recent years. They are attributed to have beneficial health properties such as antioxidants, anti-cancer, cardioprotective, anti-inflammatory, antibacterial and antihistaminic, among others. These properties are claimed to be mainly due to polyphenols. Within this group, stilbenes (in particular, *trans*-resveratrol) can be underlined. The biological properties of resveratrol include cardioprotective, neuroprotective and anticancer actions (Guerrero *et al.*, 2009). In fact, *trans*-resveratrol seems to be one of the most promising compounds due to its bioactivity. Piceatannol and viniferins are stilbenes usually found in grape and wine at lower concentrations than resveratrol. Consequently, their bioactivity has drawn less attention although some of their health-promoting properties have been investigated (Guerrero *et al.*, 2009).

Stilbenes sources in diet are scarce. Resveratrol is found in small quantities only in peanuts, berries and grapes (skin and seeds) and their derivatives (juice and wine). Grape and wine are main sources of stilbenes in diet (Guerrero *et al.*, 2009). Resveratrol concentration in grape and wine depends on many variables: grape variety, growing conditions, climate, harvest year, and winemaking techniques. Stilbenes concentration

can be increased because they are phytoalexins and, therefore, can be induced by different stresses. Numerous stresses have been studied. For example, elicitors such as benzothiodiazole, chitosan, *Botrytis cinerea*, methyl jasmonate, jasmonic acid, salicylic acid, β-aminobutyric acid, ozone, aluminum chloride and ultraviolet C light has been used to enhance nutraceutical grape properties (Fernández-Marin *et al.*, 2012; Cisneros-Zevallos, 2003). Stilbenes-enriched products potentially offer added-value compared to traditional ones (Barreiro-Hurlé *et al.*, 2008).

Benzothiadiazole, (BTH, figure 1A) is a functional analogous to the hormone-like compound salicylic acid. It is responsible for the induction of defence genes associated with induced resistance reactions. Iriti *et al*. (2004) reduced infection by *Botrytis cinerea* under field conditions after BTH application. Treated vines showed a 40 % higher value of *trans*–resveratrol (Iriti *et al*., 2004). Methyl jasmonate (MEJA, figure 1B) is the most active derivative of jasmonic acid. Indeed, MEJA has been shown to induce the accumulation of secondary metabolites as stilbenes, especially resveratrol and ε-viniferin, in cell cultures (Belhadj *et al*., 2008), leaves and berries (Larronde *et al*., 2003). Chitosan (CHIT, figure 1C) is a natural polysaccharide with a polycationic nature, which has numerous applications in agriculture. Moreover, it is a promising material for stilbenes increase in grapes (Bautista-Baños *et al*., 2006).

Figure 1. Chemical structure of the elicitors: BTH (A), CHIT (B) and MEJA (C).

Finally, ultraviolet C light (UVC) postharvest technology has been described as one of the most effective treatment to increase stilbenes in grape (Jeandet *et al*., 1995; Cantos *et al*., 2001). In fact, this treatment is currently being used for nutraceutic production (Patent WO/2002/085137; ES 2177465; www.revidox.es). Furthermore, BTH (Iriti *et al*., 2004) and CHIT (Meng *et al*., 2008) are completely degraded in plant tissues, not

occurring in persistence and residues. MEJA is a non-toxic compound found naturally in grape berries (Kondo & Fukuda, 2001).

These stresses or their combinations can be used to target the increase of health-promoting stilbenes. Synergistic effect on phytoalexin production has been described between MEJA and ethephon (Faurie *et al*., 2009), CHIT and UVC (Romanazzi *et al*., 2006), MEJA and UVC (Larronde *et al*., 2003), MEJA and cyclodextrins (Lijavetzky *et al*., 2008), among others (Cisneros-Zevallos, 2003).

The aim of this study was to investigate the efficiency of BTH, MEJA and CHIT on vineyard and their combinations with UVC light postharvest treatment to stimulate stilbenes biosynthesis in grapes and to find possible synergies between them.

MATERIALS AND METHODS

1. Reagents

trans-Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), piceatannol (3,3′,4,5′-tetrahydroxystilbene) and methyl jasmonate (methyl (1R,2R)-3-Oxo-2-(2Z)-2-pentenyl-cyclopentaneacetate) were purchased from Sigma-Aldrich (Madrid, Spain). Benzothiadiazole (benzo-(1,2,3)-thiadiazole-7-carbothionic acid S-methyl ester, BION W.G.) was supplied by Syngenta (Madrid, Spain) and Chitosan (poly β-(1→4) *N*-acetyl-D-glucosamine, Biorend®) by Idebio S.L. (Salamanca, Spain). Analytical grades of acetic acid, methanol, acetone, sodium hydrogen carbonate and ethyl acetate dissolvent and the emulsifier Tween 80 were supplied by Panreac (Barcelona, Spain). Ultrapure water from a Mili-Q system (Millipore Corp., Bedford, MA) was used in this research.

2. Grapevine

Red grapes were grown in an experimental vineyard located in “Jerez de la Frontera” in southwestern Spain, at harvest 2010. Syrah was chosen for its high resveratrol induction capacity (Guerrero *et al*., 2010a).

3. Oenological parameters of grapes

Weight grape, Brix degree, total acidity, pH, tartaric acid, potassium, total anthocyanins, extractable anthocyanins, extractability, tannins, total polyphenols index (TPI) and maturity index (MI), were determined in grapes at harvest with the official methods (OIV, 1990).

4. Preharvest treatments

All treatments were applied in triplicates and under a complete randomized block design (10 vines for replicate). Plants were sprayed (50 mL for vine), at different time and concentration (table 1).

Table 1. Preharvest treatment applications.

BTH was performed in water suspension. A concentration 0.3 mM was sprayed on grapevine three times (Iriti *et al*., 2004): on the first, fourth and seventh day after veraison. Control plants were sprayed exactly in the same way with water. Samples were taken every two days from the last treatment to harvest date (12 day).

MEJA was performed in ethanol at 10 mM. Vines were sprayed three times (Vezzulli *et al*., 2007), on the first, third and fifth day, after veraison. Control plants were treated exactly in the same way by spraying ethanol. Samples were taken every two days from the last treatment to harvest date (12 days).

A formulation based on a 1.25 % (w/v) solution of CHIT at 10 g/L was sprayed ten days before harvesting. Control plants were sprayed exactly in the same way with deionised water at pH 5.6 (Romanazzi *et al*., 2006). Samples were taken every two days from treatment to harvest date (10 days).

After sampling, a batch of grapes from each treatment and its respective control were peeled and kept under -80°C until extraction.

5. UVC Postharvest treatment

Preharvest treatments described above were combined with ultraviolet C light postharvest treatment (UVC). Harvested grapes were divided into two batches, the first batch (CT) came from control plants and it was not treated; the second batch (UVC) came from preharvest treated grapes, and were irradiated with UVC after harvest. Grape clusters were irradiated as previously described (Guerrero *et al*., 2010a). After UVC treatment was performed, grapes were stored at 22 °C (Guerrero *et al*., 2010a). Grapes were sampling every day, peeled and kept under -80 ºC until extraction for monitoring stilbenes during one week. The parameter "maximum day" (Dm) was defined as the number of days elapsed after UVC treatment to reach the maximum *trans*-resveratrol concentration in grape (Guerrero *et al*., 2010a).

6. Stilbenes extraction

The used extraction method was described by Bavaresco *et al*. (2001) with some modifications. Skin grapes were freeze-dried (cryodos -80, TELSTAR, Spain). 0.25 g freeze-dried skin were extracted twice with 5 mL of sodium hydrogen carbonate (5%) and 5 mL ethyl acetate. The samples were ground using Ultraturrax T-25 equipment (Janke & Kunkel, Ika-Labortechnick, Deutschland, Germany) and stirred at 1200 rpm for 20 minutes. The organic phase was first dried with a vacuum centrifuge concentrator, then re-dissolved in 2 mL of methanol and, finally, filtered through a 0.22 μm filter (PVDF Teknokroma, Barcelona, Spain). Extractions were performed in triplicate at dark and low temperature to avoid oxidation and isomerisation reactions. Data was expressed in mg/Kg fresh weight.

7. Identification and quantification of stilbenes

Stilbenes were quantified as described by Guerrero *et al*. (2010a). Briefly, samples (20 μl) were analysed by a Waters HPLC system with a model 1525 pump and a Waters 996 Photodiode Array Detector. Separations were performed on a Mediterranea Sea18

column (Tecknokroma, Barcelona, Spain) (RP-18, 25×0.46 cm; 5 µm particle size) and a guard column of the same material, at 30 °C. The mobile phases consisted of a water:methanol:acetic acid mixture, solvent A (88:10:2) and solvent B (8:90:2) at a flow rate of 1 mL/min. Stilbenes (*trans*-resveratrol, piceatannol, viniferins (ε- and δ-viniferin), and total stilbenes (the sum of the previous one) were quantified at 306 nm as *trans*-resveratrol. These stilbenes had previously been identified by UPLC-MS in our lab (Guerrero *et al*., 2010a).

8. Method validation

A range of *trans*-resveratrol standards between 0.05 and 31.5 mg/L were prepared to evaluate linearity through calibration curve. The values of limit of detection (LOD) and limit of quantification (LOQ) were obtained as 3 and 10 times the signal-to-noise ratio. Intra-day and inter-day precision, and accuracy were calculated. Accuracy was evaluated by means of recovery assays, as the relative error ((concentration found – concentration spiked)/(concentration spiked) × 100%). *trans*-Resveratrol peak resolution in grape was carried out by adding standard solutions to the grape sample. Resolution was calculated as $Rs = 2\ \Delta t / (W1 + W2)$, where Δt is the difference in retention times between the two peaks; and W1 and W2 are the widths of the two peaks (in time units) (Schoenmakers, 1988).

The standard addition method (MOSA) (Harris, 1995) was applied to grape samples with 6 and 12 mg/L *trans*-resveratrol concentrations. Recovery = $(b_{MOSA} / b_{EC}) \times 100$, where b is the slope of calibration curve obtained with the spiked wine; MOSA is the Method of Standard Addition; and EC is the External Calibration. Five replicates in one working session were performed to calculate intra-day repeatability. Analyses were performed during five different working sessions in a week to calculate intermediate inter-day accuracy. Both values are expressed as RSD (%).

9. Statistical analysis

The analysis of the variance (ANOVA) and Least Significant Difference test (Tukey) were used with a significance level of α = 0.05. Statistix version 8.0 (Analitycal Software, Tallahassee, FL, USA) was used.

RESULTS AND DISCUSSION

1. Method validation

The method was validated in order to determine *trans*-resveratrol concentration (table 2). Linearity was assessed with standard solutions at concentrations ranged from 0.05 to 31.5 mg/L of *trans*-resveratrol standard, and performed in triplicate. Calibration curves were obtained by plotting the peak areas against different *trans*-resveratrol concentrations. Limits of detection and limits of quantification were 0.033 mg/L and 0.102 mg/L, respectively. These data was consistent with the obtained by other authors (Goldberg *et al*., 1996; Rodríguez-Bernaldo de Quirós *et al*., 2009).

The recovery of *trans*-resveratrol resulted 103.74% and 95.48% and accuracy showed 93.53% and 104.93%, for the two different standard concentrations respectively. Both results are acceptable according to the AOAC (Huber, 1998). Variation resulted 4.87% for intra-day and 1.15% for inter-day. The *trans*-resveratrol peak resolution in wines were 0.02 and 0.39 time units for the whole set of samples.

Therefore, the method used (a modification of the previous one (Guerrero *et al*., 2010a)) was validated as reliable for the quantitative analysis of *trans*-resveratrol in grape samples, following AOAC recommendations (Huber, 1998).

Table 2. Calibration curve, limit of detection, limit of quantification, recovery, accuracy and intra e interday variation of *trans*-resveratrol.

2. Effect of preharvest treatments on oenological parameters of grapes

The effects of preharvest treatments on quality grape were studied at harvest. The oenological quality parameters were measured on a vineyards representative sample of each treatment, and compared with its respective control (table 3).

BTH treatments affected some of the oenological parameters of grape. Treated grape showed lower Brix degree, pH and potassium, and higher total acidity when compared with its respective control, and therefore it seemed that BTH treatment delayed ripeness process (MI, table 3). These data contrasted with other authors who described slight differences when performed the same treatment on Monastrell grapes (Ruiz-García *et al*., 2012). However, our results did not show significant change in grape anthocyanin content (total and extractable). Iriti *et al*. (2004) described an increase of double anthocyanin concentration (measured by HPLC) on Merlot variety when treated with the same BTH concentration.

Table 3. Oenological parameters of grapes at harvest.

MEJA treatment did not modify any of the studied oenological parameters of grape (table 3) in agreement with authors (Ruiz-García *et al*., 2012). However, these authors have observed an increase in anthocyanin and flavonol content (measured by HPLC) in MEJA treated grapes. Additionally, an increase in anthocyanin has been described when testing MEJA in grapevine cell cultures, especially when combined with sucrose (Belhadj *et al*., 2008). Larronde *et al*. (2003) studied the effect of MEJA vapor preharvest treatment on grape stilbenes, but oenological parameters were not measured.

CHIT treatment did not affect the oenological parameters of grape (table 3). Postharvest treatment with CHIT has been proposed as an edible coating in fruit because it decreased decay incidence and reduced respiration metabolism (Olivas & Barbosa-Cánovas, 2005). However, when it was studied in preharvest condition, no effect was

observed on oenological parameters of table grape (Meng *et al*., 2008; Duxbury *et al*., 2004), which is in agreement with our data.

3. Effect of preharvest treatments on trans-resveratrol content in grapes

Some significant differences were observed on *trans*-resveratrol in grapes treated with preharvest treatments. Since piceatannol and viniferins were under quantification limit only *trans*-resveratrol was quantified (table 4). It has been described that no other stilbenes were detected in grape samples when *trans*-resveratrol concentration was low (Guerrero *et al*., 2010a).

BTH significantly increased *trans*-resveratrol content in grapes from the second sampling day (i.e. day forth after treatment application) (table 4). It is worth to mention that at harvest the *trans*-resveratrol content in treated grapes was 2.79 higher with respect to its control.

No significant differences were found between MEJA treated grape and its respective control until the fourth sampling day (i.e. day eight after treatment application) (table 4) in which treated grape contained lower resveratrol concentration than control ones. However a common tendency could be suggested. Treated grapes immediately induced a response reached 0.203 mg/Kg fw, 1.65-fold respect to its control (table 4). Subsequently, *trans*-resveratrol decreased along ripeness until harvest, being the decrease higher in MEJA treated grape that in control ones. Similar results were found in the literature. Larronde *et al*. (2003) described a *trans*-resveratrol concentration increase during 15 days after veraison when treated with MEJA vapors, but a marked decline during grape ripening was observed. Those data contrast with the obtained by other authors (Vezzulli *et al*., 2007), in which MEJA treatment on Barbera grape variety improved both resveratrol and ε-viniferin content on berries in an accumulative manner. In grapevine cell cultures, when MEJA (18 g/L) was added in the presence of sugars,

both resveratrol and piceids significantly increased (Belhadj *et al*., 2008). Other authors have described that when gaseous MEJA was used at very low concentration (0.09 mg/L) on Cabernet sauvignon grapes, resveratrol markedly increased (9-folds) before harvest. Moreover, MEJA has been proposed as a very useful resveratrol-inductor in cell cultures when combined with cyclodextrin (Lijavetzky *et al*., 2008).

CHIT treated did not show an increase in *trans*-resveratrol concentration respect to its control (table 4). When CHIT has been tested at low concentration over the canopy of Cabernet sauvignon vines, its phenolic content did not changed (Duxbury *et al*., 2004). In agreement with our data, the effect of the same doses of CHIT on table grapes did not increase resveratrol, but it had helped to control *Botrytis cinerea* infection (Romanazzi *et al*., 2006). In cell culture, a concentration of CHIT five times higher than the one used in the current experiment, led to an increase in *trans*-resveratrol content almost twice (Ferri *et al*., 2009). Furthermore, CHIT was recently assayed in the same concentration on other cell culture. In this case, it was described as less effective for stilbenes-induction (Santamaria *et al*., 2010) being the cell culture type the difference between those two studies. Ferri *et al.* (2009) obtained callus tissues from leaf petioles *V. Vinifera* L. cv. Barbera. meanwhile SantaMaria *et al*. (2010) used stem and tendril explants from *V. vinifera* cv. Italia to induce callus formation.

Table 4. *trans*-Resveratrol concentration after preharvest treatments (mg/Kg f.w.)

It can be concluded, that under this experiment conditions, MEJA and CHIT preharvest treatment did not increased *trans*-resveratrol content in grape. Only BTH preharvest treatment seemed to achieve *trans*-resverratrol content in grapes during ripeness and also at harvest.

4. Combination of preharvest treatments with UVC postharvest treatment

Synergistic effects have been described between CHIT and UVC irradiation in table grape (Romanazzi *et al*., 2006) and MEJA and UVC in cell cultures (Larronde *et al*., 2003; Zhang *et al*., 2002). In the present study, the combination of preharvest treatments in vineyard (BTH, MEJA and CHIT) with UVC postharvest treatment was tested in grapes. Once ripeness was reached, grapes from the three preharvest treatments were harvested and immediately treated with UVC. A control was used for every preharvest treatment that did not follow UVC treatment.

It was observed an increase on *trans*-resveratrol, piceatannol and ε- and δ-viniferin content (call as viniferins forehead) in UVC treated grapes. These stilbenes have previously been identified by UPLC-MS-MS in our lab (Guerrero *et al*., 2010a). The evolution of these compounds after UVC treatment was followed daily during one week to determine the day of maximum stilbenes concentration (Dm). Two requirements have been described to achieve stilbenes-enriched-wines using UVC: first, wine grape should show high induction capacity; and second, Dm should be as short as possible (minimum number of days to reach maximum stilbenes concentration) in order to preserve grape quality (Guerrero *et al*., 2010b).

We hypothesise that treatment combination (preharvest and UVC postharvest) could increase stilbenes grape content due to synergistic effect. Moreover, treatment combination might reduce the induction period and therefore Dm required by UVC posthavest treatment by previous activation of the stilbenes production metabolism through preharvest elicitors.

Figure 2. *trans*-Resveratrol concentration during the 7 days after treatment combinations.

When monitoring the stilbenes induction after different treatment combinations during seven days in grapes, it was found that there were two days in which *trans*-resveratrol

reached the maximum concentration depending on the treatment (forth and seventh days, figure 2). After four days of storage, MEJA-UVC combination showed a *trans*-resveratrol (7.23 mg/Kg fw, figure 3A, figure 2), piceatannol (1.95 mg/Kg fw, figure 3C) and total stilbenes (10.03 mg/Kg fw, figure 3G) significantly higher than the level obtained with the other treatments. This was not observed for viniferins (0.84 mg/Kg fw, figure 3E) which were significantly lower in MEJA-UVC than in the other treatments at the same day (forth day). However, UVC treatment by itself (without combination) reached the highest *trans*-resveratrol (7.97 mg/Kg fw, figure 3B, figure 2), piceatannol (2.04 mg/Kg fw, figure 3D), viniferins (3.42 mg/Kg fw, figure 3F) and total stilbenes (13.43 mg/Kg fw, figure 3H) concentrations after seven storage days. Despite the combination of preharvest treatments with UVC postharvest did not achieve the final *trans*-resveratrol content, the day of maximum induction of *trans*-resveratrol and piceatannol was reduced in three days by MEJA-UVC combination treatment respect to UVC treatment. The combination MEJA-UVC reached 7.23 and 2.04 mg/Kg fw of *trans*-resveratrol and piceatannol respectively at Dm = 4 days; in comparison with UVC treatment which reached similar concentrations 7.97 and 1.95 mg/Kg fw of *trans*-resveratrol and piceatannol respectively but at Dm = 7 days. It was expected a decrease in stilbenes concentration in MEJA-UVC at Dm= 7 in comparison with UVC, since it has been described that after treatments stilbenes reach a maximum concentration and then decrease (Guerrero *et al*., 2010a).

Figure 3. *trans*-Resveratrol, piceatannol, viniferins and total stilbenes on the fourth (d4) and seventh (d7) days after UVC postharvest treatment. Different letter showed values statistically different.

In contrast, BTH-UVC and CHIT-UVC treatments did not achieve a comparable increase in *trans*-resveratrol with UVC during the storage period (seven days) (figure 2). Regarding the other stilbenes, lower piceatannol content was observed in both BTH-

UVC and CHIT-UVC treatments (figure 3D). Similar viniferins concentration in BTH-UVC but different in CHIT-UVC were obtained after seven storage days respect to UVC (figure 3F). It has been found maximum stilbenes concentration after seven storage days for BTH-UVC and CHIT-UVC treatments. In contrast with the initial hypothesis, these preharvest treatments might attenuate *trans*-resveratrol induction and therefore the Dm could be deleted after seven days.

CONCLUSIONS

From the results discussed above, it could be concluded that, firstly, MEJA and CHIT preharvest treatment are low efficacy tools to increase *trans*-resveratrol content in grape. BTH increased *trans*-resveratrol content in grape but it seems to be linked with ripeness delay. Secondly, the combination of MEJA preharvest and UVC postharvest treatment (MEJA-UVC) is an interesting application for stilbenes-functional grapes development. Despite MEJA-UVC combination did not reached the highest stilbenes concentration in this study, the UVC storage period was reduced in three days. This fact is an important finding because it accelerated stilbenes production while it preserved grape quality. Therefore, combination MEJA-UVC treatment is suggested as an interesting application for stilbenes-enriched grape production. Functional grapes could have an application as raw material for added-value wines, since with the same ingestion of ethanol, the intake of bioactive stilbenes is significantly increased.

Acknowledgments

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FIGURE CAPTIONS

Figure 1. Chemical structure of the elicitors: BTH (A), CHIT (B) and MEJA (C).

Figure 2. *trans*-Resveratrol concentration during the 7 days after treatment combinations.

Figure 3. *trans*-Resveratrol, piceatannol, viniferins and total stilbenes on the fourth and seventh days after UVC postharvest treatment. Different letter showed values statistically different.

TABLES

Table 1. Preharvest treatment applications.

Table 2. Calibration curve, limit of detection, limit of quantification, recovery, accuracy and intra e interday variation of *trans*-resveratrol.

Table 3. Oenological parameters of grapes at harvest.

Table 4. *trans*-Resveratrol concentration after preharvest treatments (mg/Kg f.w.)

ANEXO 5

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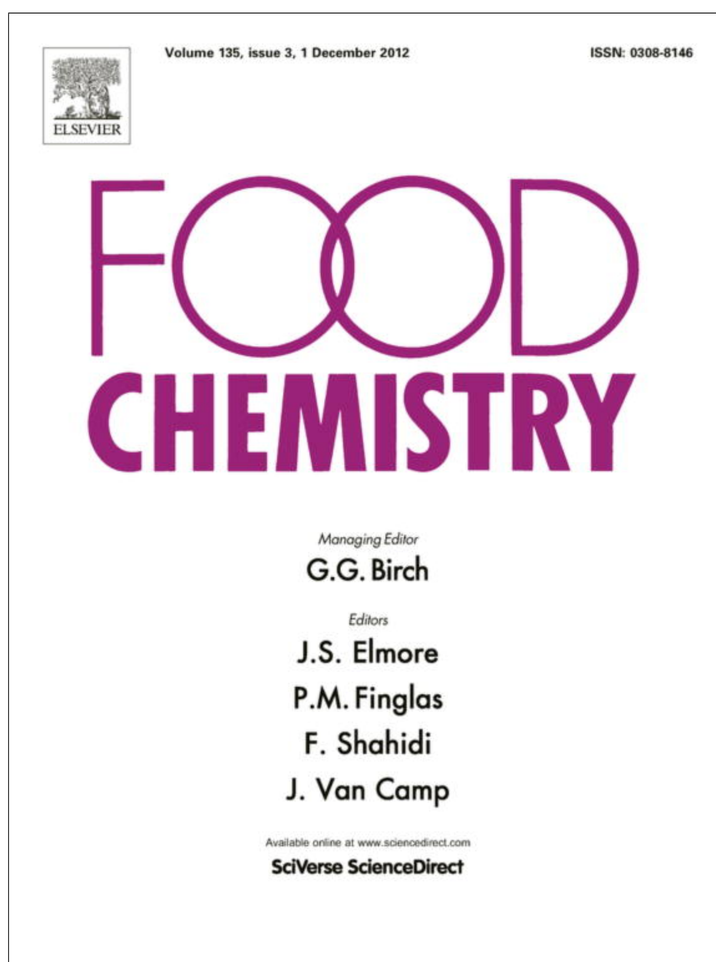

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Isorhapontigenin: A novel bioactive stilbene from wine grapes

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ABSTRACT

Stilbenes are a family of bioactive compounds found in plants. However, only a few stilbenes are present in the human diet. Grape and wine are the main dietary source of stilbenes, resveratrol and piceid being the most common ones. Ultraviolet C light (UVC) postharvest treatment was used to obtain significantly increased stilbene concentration in grapes. A new, previously undescribed-in-grapes stilbene was found after UVC treatment. The process followed to isolate and identify this unknown stilbene is described in the present work. This isolation involved several fractionation steps including counter current chromatography and semi-preparative HPLC due to its low concentration and the presence of structurally related compounds. The structure of the compound was unequivocally identified by NMR spectroscopy analyses including ^{1}H-NMR; COSY; ROESY; HSQC and HMBC. The compound was identified as isorhapontigenin (ISOR), a stilbene found in traditional Asian medicinal plants. To the best of our knowledge this is the first report of its occurrence in grapes.

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1. Introduction

Nearly 800 stilbenoids have been described in natural sources (Xiao et al., 2008). Stilbenes are non-flavonoid phenolics that occur in a number of plant families, including *Pinaceae*, *Moraceae*, *Paeoniaceae*, *Liliaceae*, *Myrtaceae*, *Fagaceae*, *Vitaceae*, *Gnetaceae*, *Iridaceae*, *Celastraceae*, *Cyperaceae*, *Dipterocarpaceae*, *Leguminosae* (Harborne, 1999). However, their dietary sources are rather limited. Stilbenes are present in peanut, pistachio, berries, dark chocolate, grapes and wine, which are its main sources in diet (Zamora-Ros et al., 2008).

Grape stilbenes include *trans*- and *cis*-resveratrol (3,5,4′-trihydroxystilbene), their glucosides (5,4′-hydroxystilbene-3-*O*-*β*-glucosides, known as piceids), picetannol (3,3′,4,5′-tetrahydroxystilbene) (Cantos, Espin, & Tomas-Barberan, 2002), astringin (5,4′,3′-trihidroxystilbene-3-*O*-β-glucoside), pterostilbene (3,4-dimethoxy-4′-hydroxystilbene) and oligomers (viniferins and hopeaphenol) (Guerrero, García-Parrilla, Puertas, & Cantos-Villar, 2009).

Stilbenes have been reported to be responsible for numerous beneficial effects: antioxidant, antibacterial, antifungal, cardioprotective, neuroprotective, antiaging and anticancer among others (Guerrero et al., 2009). Furthermore, *trans*-resveratrol increases stress resistance and lifespan in some organisms (Guerrero et al., 2009). The other stilbenes are usually found in lower concentrations than resveratrol in grape. Piceatannol shows pronounced antioxidant activity, as well as high bioactivity, showing anti-leukemic and anti-tumourigenic activities in various cell lines and animal models. Piceid and astringin show neuroprotective activity. Oligomers have been shown to have hepatoprotective and antioxidant properties, and to induce apoptosis of leukaemia B-cells (Guerrero et al., 2009).

Moreover, since stilbenes are phytoalexins, they are induced by biotic and abiotic elicitors. Pathogenic attack, pre-harvest chemical treatments, such as benzothiadiazole, methyl jasmonate or chitosan, are potent elicitors that increase resveratrol content in grapes and, consequently, in wines. In particular, ultraviolet C light postharvest treatment (UVC) has been proposed as one of the most suitable tools to obtain considerably increased stilbene content in grape and red wine (Guerrero, Puertas, Jiménez, Cacho, & Cantos-Villar, 2010b). In previous studies on grape skin, various stilbenes were induced after UVC treatment and identified by UPLC-DAD-TQD. However, in that previous work, one of the induced stilbene could not be identified (Guerrero, Puertas, Fernández, Palma, & Cantos-Villar, 2010a).

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The aim of this study was the isolation and identification of this unknown induced stilbene, as it might have interesting bioactive properties for human health. With this purpose several fractionation and isolation steps were performed to identify this unknown stilbene by NMR.

2. Material and methods

2.1. Reagents

trans-Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) was purchased from Sigma–Aldrich (Steinheim, Germany). Analytical grades of acetic acid, formic acid, methanol and diethyl ether, hexane, acetonitrile, diethyl acetate supplied by Fisher Scientific, acetone-d_6 (Eurisotop, France) and ultrapure water from a Mili-Q system (Millipore Corp., Bedford, MA) were used in this research.

2.2. Grapevine

The red grape varieties (Graciano, Merlot, Syrah and Tempranillo) used in this study were cultivated at the IFAPA Centre Rancho de La Merced, in Jerez de la Frontera (Spain) in 2009. Maturity grade was monitored weekly until optimal conditions were reached for harvest (data not shown).

2.3. UVC treatment and storage period

The UVC treatment was conducted according to the procedure described by the patent W0/2002/085137; ES 2177465 with some modification described by Guerrero et al. (2010a).

2.4. Extraction method

After UVC treatment and storage (Guerrero et al., 2010a), approximately 30 kg grapes were peeled. Grape skins were stored at −80 °C before freeze-drying (Cryodos-80, Telstar, Spain). Freeze-dried grape skins were grounded and extracted as follow: diethyl ether solvent was added (4 ml per 1 g freeze-dried skin grape) and mixed by Ultraturrax (IKA, Germany) at 21,500 rpm for 5 min until a homogeneous slurry was obtained. Subsequently, slurry was stirred for 1 hour at 1200 rpm and filtered twice consecutively with laboratory filter paper. The ether phase was evaporated until dryness by means of a rotavapor (Heidolph rotavapor Laboratora 4000 efficient, Heidolph instruments GMBH & CO. KG.), and the obtained solid extract was preserved at −80 °C before isolation. Samples were kept cold (ice bathed) and in the dark (aluminium foil) during the whole process to avoid possible isomerization.

2.5. HPLC-DAD

First of all, samples (20 μl) were analysed using a Waters HPLC system with a 1525 pump and a Waters 996 Photodiode Array Detector. Separations were performed on a Mediterranea Sea18 column (Tecknokroma, Barcelona, Spain) (RP-18, 25 × 0.46 cm; 5 μm particle size) and a guard column of the same material, at 30 °C. The mobile phases consisted of a water:methanol:acetic acid mixture, solvent A 88:10:2 and solvent B 8:90:2 at a flow rate of 1 ml min^{-1}. Stilbenes were quantified at 306 nm as *trans*-resveratrol.

2.6. UPLC-DAD-TQD

HPLC-DAD results were not enough for stilbene identification. Therefore, UPLC-DAD-TQD was used for stilbene identification. Sample in methanol (Section 2.4) was diluted 1:1 in H_2O 0.01% formic acid to improve ionisation. The Waters equipment comprised an Acquity UPLC System, an Acquity DAD (Waters, Mildford, MA, USA), and an Acquity TQD tandem quadrupole mass spectrometer (Waters, Manchester, UK). Data acquisition was performed using MassLynx 4.0 software (Waters). Reverse phase separation was conducted in an Acquity UPLC BEH C18 column (2.1 × 100 mm; 1.7 μm particle size) in gradient mode of 0.1% formic acid-water (solvent A) and 0.1% formic acid–methanol (solvent B) at flow rate of 0.5 ml/min at 40 °C. Elution conditions have been previously described (Guerrero et al., 2010a). The DAD was working at a range of 220–400 nm, and monitoring at 320 nm. Conditions of MS/MS in mode ESI- were as follows: capillary voltage, 2.50 kV; cone, 40.00 V; extractor, 3.00 V; RF, 0.1 V; source temperature, 120 °C; desolvation temperature, 350 °C; cone gas flow (N_2), 50 L/h; desolvation gas flow (N_2), 650 L/h; and collision gas flow (Ar), 0.15 ml/min.

2.7. Counter current chromatography (CCC)

Grape skin solid extracts (Section 2.4) were analysed by to two different CCC systems.

Spiral COIL-LOW SPEED Rotary CCC: separations were carried out with a Spiral Coil-LSRCCC manufactured by Pharma-Tech Research Corp. (Baltimore, MD). It was equipped with a spiral column made from a continuous piece of convoluted Teflon tubing (Inner diameter of tubing = 8.5 mm, total volume = 5600 ml). The two-phase solvent system was composed of acetronitrile/*n*-hexane (50:50; v/v). Elution mode was head-to-tail with the lighter (organic) phase acting as the stationary phase and the aqueous phase as the mobile phase. Flow rate was set at 15 ml/min and delivered by a HPLC pump (64, Knauer, Berlin).

Extract sample (5910 mg dissolved in 250 ml of solvent mixture) was injected for a single run. Fractions (F1–F13) were collected with a Pharmacia LKB super Frac fraction collector (Bromma, Sweden). Elution was monitored with a K-2501 UV/vis detector (Knauer, Berlin, Germany) at 280 nm and the resulting chromatograms were recorded by a plotter (ABB Goerz SE 120, Vienna, Austria).

High Speed CCC (HSCCC): the first four fractions from Spiral Coil-LSRCCC (F1–F4) were again fractioned with a high-speed model CCC-1000 instrument manufactured by Pharma-Tech Research Corp. (Baltimore, MD), equipped with three preparative coils, connected in series (tubing inner diameter of 2.5 mm and total volume of 800 ml). The two-phase solvent system was composed of hexane/ethyl acetate/methanol/water (3:5:3:5; v/v/v/v) (Ito, 2005). The elution mode was head-to-tail with the lighter (organic) phase acting as the stationary phase and the aqueous phase as the mobile phase. The flow rate was set at 3 ml/min and delivered by a BT 3020 HPLC pump (Jasco, Gross-Umstadt, Germany). Separation was run at 820 rpm.

Fractions F1 to F4 were grouped (442 mg) and dissolved into 25 ml of a mixture of the upper (organic) and lower (aqueous) phases (50:50; v/v). Eight fractions (FF1-FF8) of 12 ml were collected with a fraction collector (Pharmacia LKB Super Frac). Elution was monitored by a UV/vis detector at 280 nm (K-2501, Berlin, Germany).

Thin layer chromatography (TLC): HSCCC fractions were monitored by TLC on normal phase silica gel plates 60 F254 (Merck) with chloroform/ethylacetate/methanol/water (25:55:5:1; v/v/v/v) as the solvent system. Stilbenes and derivatives were revealed by means of an anisaldehyde spray reagent.

2.8. HPLC-EIS-MS/MS

CCC fractions were dried, dissolved in methanol and analysed by an HPLC-EIS-MS/MS system. An HPLC system (pump 1100 series, autosampler 1200 series) from Agilent Technologies (Böblingen,

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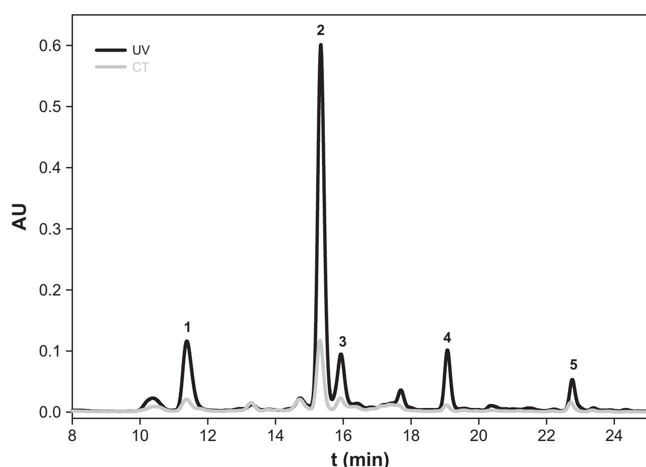

Fig. 1. HPLC-PDA Chromatogram CT and UVC grape at 306 nm. (1) Piceatannol, (2) *trans*-resveratrol, (3) unknown stilbene, (4) ε-viniferin and (5) δ-viniferin.

Germany) was connected to an Esquire LC-ESI-MS/MS from Bruker (Bremen, Germany). Mass spectra were recorded in negative mode, with capillary set at 1500 V, end plate at −500 V, capillary exit at −120.4 V, dry gas at 330 °C, gas flow at 11 L/min, nebulizer at 60 psi, target mass at m/z 500, scan range from m/z 100 to 3000, helium as the collision gas, and MS/MS fragmentation amplitude at 1.0 V. ESI-MS resolution was recorded on a Thermo Science LTQ Orbitrap mass spectrometer. An analytical C18 column (Luna C18, 250 4.6 mm, 5 μm, Phenomenex, Aschaffenburg, Germany) at a flow rate of 0.8 ml/min [solvent system of 1% acetic acid (A), acetonitrile (B)] was used. Gradient started over 0 min at 20% B, 5 min at 30% B, 15 min at 30% B, 18 min at 37% B, 29 min at 37% B, 35 min at 50% B, 57 min at 50% B, 58 min at 100% B, 61 min at 100% B, and resulted in 65 min at 20% B.

2.9. Semi-preparative HPLC

Further purification was required after HSCCC by semi-preparative HPLC on a Smartline system from Knauer (Smartline, pump 1000, manager 5000, detector UV K-2600, Berlin, Germany). Semi-preparative HPLC column (Luna C18, 250 × 15.0 mm, 5 μm, Phenomenex, Aschaffenburg, Germany) was operated with a binary solvent system of ultrapure water/methanol/acetic acid (88:10:2) (A) and methanol/ultra pure water/acetic acid (90:8:2) (B) at a flow rate of 4 ml/min. Two gradients were applied. The first gradient started over 0 min at 20% B, 40 min at 35% B, 80 min at 45% B and 100 min at 100% B. The second gradient was composed of 0 min at 35% B, 30 min at 45% B, 45 min at 55% and 120 min at 100% B. Chromatograms were monitored at 280 and 306 nm.

2.10. NMR experiments

NMR spectra were recorded on a Bruker Avance 600 NMR spectrometer (600 MHz for ^{1}H and 151 MHz for ^{13}C experiments), in acetone-d_6 at 300 K using a 5 mm TXI probe with 90° proton pulse length of 7.5 μs at a transmission power of 0 db and equipped with pulsed-gradient field utility. The chemical shift scale was calibrated on the residual of deuterated acetone at δ_H 2.05 ppm and δ_C 29.9 ppm. The following experiments were conducted: ^{1}H-NMR; (^{1}H-^{1}H)-COSY; (^{1}H–^{1}H)-ROESY; (^{1}H–^{13}C)-HSQC and (^{1}H–^{13}C)-HMBC.

3. Results and discussion

3.1. Induction by postharvest UVC treatment of an unknown stilbene

UVC postharvest treatment has been described as a useful tool to achieve stilbene concentration in grapes. In previous studies developed in our lab, we have already described how variety and season affect stilbene content in grapes before and after UVC treatment (Guerrero et al., 2010a). The following stilbenes were found by both HPLC-DAD and UPLC-DAD-TQD in the extracted samples after grape UVC treatment: piceatannol (1), *trans*-resveratrol (2), unknown stilbene (3), ε-viniferin (4) and δ-viniferin (5) (Fig. 1; Table 1). The mass spectra agree with those previously described in literature (Buiarelli, Coccioli, Jasionowska, Merolle, & Terracciano, 2007; Pezet et al., 2003; Püssa, Floren, Kuldkepp, & Raal, 2006). As it can be observed in the DAD chromatogram (Fig. 1), a new stilbene that was under detection limit before irradiation appeared after UVC treatment (compound 3, called unknown stilbene) and therefore a stilbene-like structure was expected. Its retention time (t_R = 16.2 min, Fig. 1) was close to that of *trans*-resveratrol (t_R = 15.6 min, Fig. 1) and therefore its structure is also supposed to be similar. Its DAD spectra showed a maximum at a wavelength of 325 nm and did not fit with any reviewed spectra of stilbenes. Moreover, m/z parent and daughters in ESI- mass mode of the unknown stilbene resulted in 257 uma, and main daughter ion: 241 and 224 (Table 1). According to these data by comparison with literature and MS database, there were two possibilities for the unknown compound: 3,3′,5′-trihydroxy-4-methoxystilbene, also called rhapontigenin (RHA), or 4,3′,5′ trihydroxy-3-methoxystilbene, also called isorhapontigenin (ISOR). Since the MS/MS spectra and fragmentation of both compounds are the same, they cannot be distinguished by mass spectrometric analysis (Jerkovic, Nguyen, Nizet, & Collin, 2007).

Rhapontigenin (RHA, 3,3′,5′-trihydroxy-4-methoxystilbene) is a stilbene found in Korean rhubarb rhizomes, and is the most abundant one in *Rhei undulatum* species (Ko, Lee, & Whang, 1999). Recently it has been also found in berries of *Vitis coignetiae* (Kim, Ha, Ahn, Kim, & Kim, 2009). Recent research has proven RHA a potent antioxidant, anti-allergic, anticarcinogenic, anticoagulant, and anti-inflammatory compound (Aburjai, 2000; Roupe, Helms, Halls, Yáñez, & Davies, 2005; Roupe, Remsberg, Yáñez, & Davies, 2006). Isorhapontigenin (ISOR, 4,3′,5′-trihydroxy-3-methoxystilbene) has been found in many traditional medicinal plants such as rhubarb (Matsuda et al., 2001; Matsuda, Tewtrakul, Morikawa, & Yoshikawa, 2004), as well as in many *Gnetum* spices (Ali et al., 2003; Kato, Tokunaga, & Sakan, 2009; Li, Lin, Wang, & Liu, 2004), among others. ISOR is also known to show numerous biological activities. It shows potent antioxidant activity *in vitro*, with activity being higher than

Table 1
Identification of stilbene compounds by HPLC-DAD (spectra) and UPLC-DAD-TQD (Mass data).

Compound	t_R(min)	Stilbene	[M-](m/z)	MS^2(m/z)	λmax(nm)
1	11.2	Piceatannol	243	225/201/159/143	323.8
2	15.6	*trans*-Resveratrol	227	185/143/159	306.0
3	16.2	Unknown stilbene	257	241/224	325.0
4	18.9	ε-Viniferin	453	435/411/369/359/347	322.6
5	22.7	δ-Viniferin	453	435/411/369/359/347	321.4

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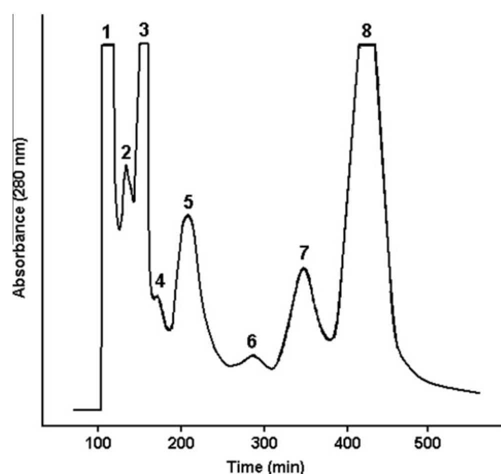

Fig. 2. HSCCC chromatogram of the spiral coil-LSRCCC from skin grape stilbene extract at 306 nm (FF1–FF8).

that shown by vitamin E (Iliya et al., 2003; Wang, Lin, & Liu, 2001), anti-allergic activity in leukemic cells (Matsuda et al., 2004), cardio-protective effect (Li et al., 2005) and antiviral activity (Liu et al., 2010). To the best of our knowledge, neither RHA nor ISOR have been described so far in grape.

An isolation process was designed to identify the unknown stilbene. First of all, raw material (grape) with high concentration of the unknown stilbene was achieved. For this goal, Graciano, Merlot, Syrah and Tempranillo varieties were selected due to their high stilbene content after UVC postharvest treatment (Guerrero et al., 2010a). A range between 0.67–1.30 mg/kg berries of unknown stilbene was obtained from approximately 30 kg of grapes, which provided sufficient quantity of crude extract to undergo the following isolation steps.

3.2. Isolation of the unknown stilbene

CCC provides a quick preparative fractionation tool for crude mixtures. Stilbene crude extracts from other sources had already been successfully separated by this technique (Winterhalter, 2009).

The grape extract was separated by two different CCC techniques: Firstly, Spiral Coil-Low Speed Rotary CCC (Spiral Coil-LSRCCC), and secondly High Speed CCC (HSCCC) were applied. Spiral Coil-LSRCC was first chosen to isolate as much sample as possible. Hexane-acetonitrile (1:1) was used as the solvent system. This hydrophobic solvent system was recommended for separation of non-polar compounds (Ito, 2005). Skin grape extract (5.9 g) was dissolved into 250 ml of a 1:1 mixture of hexane-acetonitrile and injected once the hydrodynamic equilibrium had been reached. Spiral Coil-LSRCCC separation yielded thirteen fractions. Using TLC and HPLC-EIS-MS/MS analysis, the unknown stilbene (m/z = 257) was found in the first four fractions (F1–F4), together with many other compounds mainly stilbenes (data not shown). The F1–F4 fractions containing the unknown stilbene were combined and a subsequent separation by HSCCC was carried out. HSCCC is the most versatile and powerful CCC technique (Ito, 2005). The two-phase solvent system selected was based on previous works (He et al., 2009; Ito, 2005; Wilkens, Paulsen, Wray, & Winterhalter, 2010). The solvent system selected after testing different solvents and proportions was hexane/ethyl/acetate/methanol/water (3:5:3:5; v/v/v/v). Extracts from F1–F4 (442 mg) of LSRCCC was separated in eight fractions by HSCCC (FF1–FF8). The resulting fractions were analysed by TLC and HPLC-ESI-MS/MS (Fig. 2, Table 2). Fraction 1 (FF1) was polydisperse and eluted as a hump. Fractions 2 and 3 (FF2 and FF3) were mainly composed of one compound (m/z = 453) in agreement with the mass of ε and δ-viniferins. However, the fragmentation pattern differed from previous descriptions (Pezet et al., 2003; Püssa et al., 2006). It might be tentatively identified as pallidol, which shows this mass fragmentation pattern (Mulinacci, Innocenti, Santamaria, la Marca, & Pasqua, 2010), and maximum wavelength at 280 nm, in agreement with literature (Vitrac, Monti, Vercauteren, Deffieux, & Merillon, 2002; Landrault et al., 2002). Fraction 4 (FF4) presented several unidentified compounds, whose mass spectral data could be related to oxidised dimeric stilbenes (Mulinacci et al., 2010). It must be taken into account that UVC treatment may induce both other stilbenes and oxidation reactions. A low amount of this fraction was achieved to undergo identification. Fraction 5 (FF5) mainly presented piceatannol, α-viniferin (Püssa et al., 2006) and also a resveratrol dimer, tentatively ampelopsin D, in agreement with mass data (Vergara et al., 2012). Ampelopsin D had been previously described in *Vitaceae* (Jeandet et al., 2002; Mattivi et al., 2011). Ampelopsin D had been suggested to be synthesized in the *Vitaceae* from its precursor ε-viniferin and also δ-viniferin (Püssa et al., 2006). Fractions 6 and 7 (FF6 and FF7) contained mainly δ and ε-viniferins, respectively, in agreement with available previous data (Table 1) and other authors (Pezet et al., 2003; Püssa et al., 2006). In the last fraction (FF8), the unknown stilbene co-eluted with *trans*-resveratrol (Fig. 2). Their mass spectrum ESI-MS/MS in negative mode is shown in Fig. 3. The identification of *trans*-resveratrol (Fig. 3C) was made by comparison with the pure standard and was dominated by the product ion at m/z 185 representing the loss of a ketene molecule. The unknown stilbene was characterised by its molecular ion *m/z* 257 and intense fragmentation ion at *m/z* 241. This peak was deduced from the lost of methyl radical that caused the ortho-quinone ion after re-arrangement (Fig. 3B).

For further enrichment of the unknown compound, FF8 was separated by semi-preparative HPLC. FF8 (22.31 mg) was injected in the semi-preparative HPLC system (section 2.8), and six new fractions were recovered (FFF1–FFF6). The first one (FFF1,

Table 2
Mass data and fraction quantification obtained by HSCCC.

Fraction	Amount (mg)	$[M–H]^-$ (m/z)	Main daughter ions	Main compound
F2	11.11	453	435/411/359/317/289	Pallidol
F3	12.51	453	435/411/359/ 317/289	Pallidol
F4	4.0	417	409/329/255	Unidentified compounds
		395	313/227	
F5	34.8	243	225/201/159/143	Piceatannol
		469	451/375/363	Ampelopsin A
		679	661/637/573/555/479/273/239	α-Viniferin
F6	5.27	453	435/411/369/359/347	α-Viniferin
F7	13.0	453	435/411/369/359/347	α-Viniferin
F8	22.31	227	185/143/159	Resveratrol
		257	**241/224**	**Unknown stilbene**

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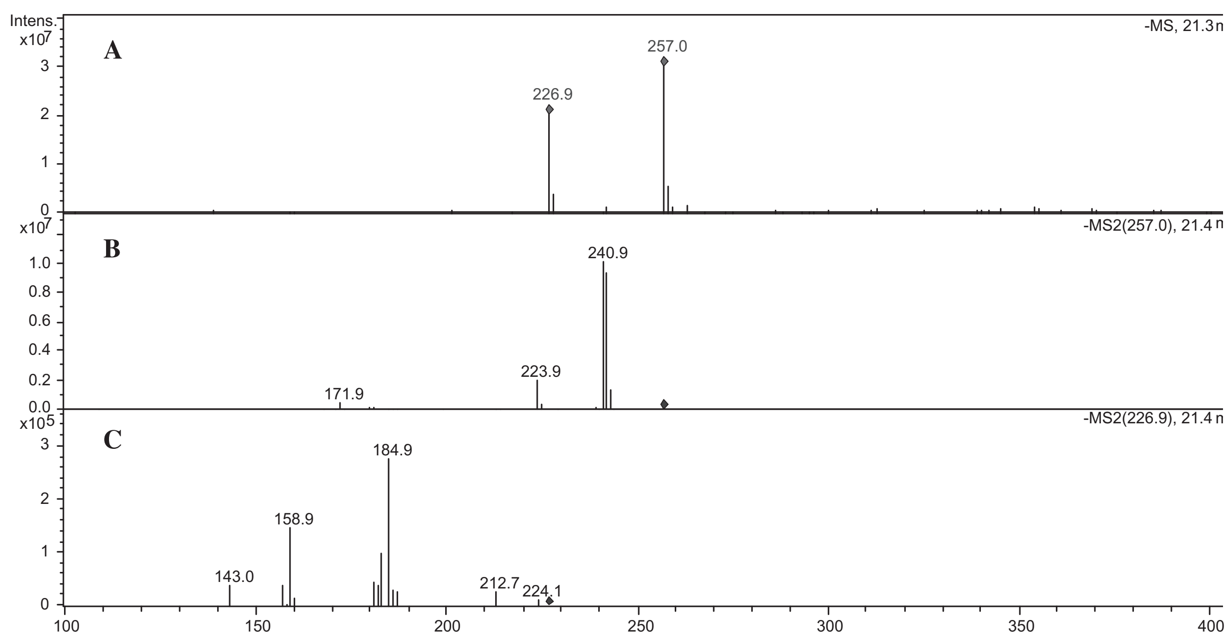

Fig. 3. MS/MS spectra and fragmentation of F8 from HSCCC. (A) peak mass detected (B) daughter ion data of 257 peak (C) MS/MS of 227 peak.

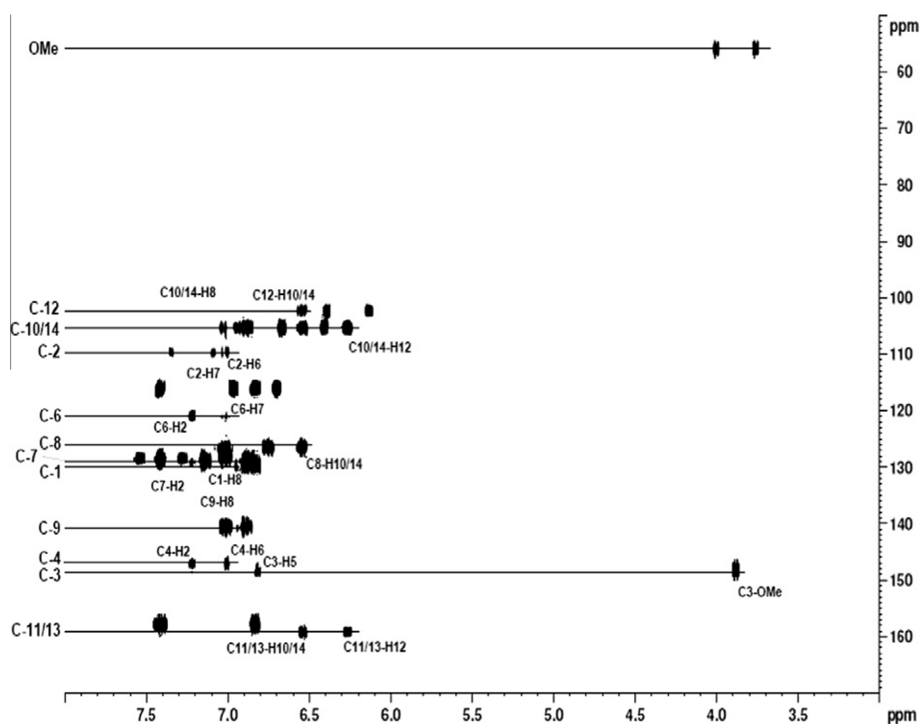

Fig. 4. HMBC spectrum of isorhapontigenin.

12.7 mg) contained the majority of the unknown stilbene and *trans*-resveratrol and, therefore, the other fractions were discarded (data not shown). This fraction was again run in the semi-preparative system. A purer fraction of 7.9 mg was achieved. It contained 97% of *trans*-resveratrol and 3% of the unknown stilbene. This extract was then used for NMR identification.

It should be noticed that a higher number of compounds were found after CCC and HPLC-EIS-MS/MS analysis, at least more than had previously been detected by direct injection of extracted samples using UPLC-DAD-TQD (Table 1 vs. Table 2). Pallidol, ampelopsin D and α-viniferin might be likely to be present in the samples. The fact that they were not detected from the beginning could be justified because of the large amount of many other compounds when extracts of diethyl ether were prepared. Further research is required to confirm these data due to the high biological activity associated with the above mentioned compounds.

3.3. NMR Elucidation of the unknown stilbene

The structures were elucidated by 1D- and 2D-NMR experimentation (1D- and 2D-(^{1}H-^{1}H)-COSY, 2D-(^{1}H-^{1}H)-ROESY, 2D-(^{1}H–^{13}C)-HSQC and 2D-(^{1}H–^{13}C)-HMBC). ^{1}H-NMR and ^{13}C-NMR

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Table 3
NMR spectral data of isorhampontigenin in acetone-*d6*.

No	δ_C	δ_H *J* (Hz)	COSY	HMBC
1	129.1	–	–	H-8
2	109.6	7.21 *d*(2.0)	H-6	H-6, H-7
3	148.5	–	–	H-5, OMe
4	147.1	–	–	H-2, H-6
5	115.9	6.82 *brd*(8.2)	H-6	–
6	120.8	6.97 *brd*(8.2)	H-2, H-5	H-2, H-7
7	128.6	7.00 *d*(16.5)	H-8	H-2, H-6, H-8
8	126.4	6.93 *d*(16.5)	H-7	H-7, H10/14
9	140.6	–	–	H-7, H-8
10/14	105.1	6.61 *brs*	H-12	H-8, H12
11/13	159.1	–	–	H12, H10/14
12	102.1	6.27 *brs*	H-10/14	H10/14
OMe	56.2	3.86 *s*	–	–

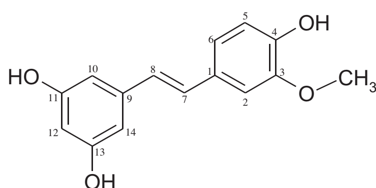

Fig. 5. Chemical structure of isorhapotigenin.

data for the unknown compound are reported in Table 3. The unknown compound resulted in isorhapontigenin. The NMR spectra of the mixture showed the presence of two compounds: *trans*-resveratrol and isorhapontigenin. The former presence was confirmed by comparison with references in literature (Mattivi, Reniero, & Korhammer, 1995). Due to overlapping signals, the latter presence was confirmed through 2D-NMR experimentation (Fig. 4). These data agree with those described for isorhapontigenin isolated from bulbs of *Scilla nervosa* (Silayo, Ngadjui, & Abegaz, 1999).

The ^{1}H-NMR data of isorhapontigenin indicated the presence of eight aryl/vinyl protons resonances. The ^{13}C-NMR spectral data showed thirteen aryl/vinyl signals, and no carbonyl resonances. ^{1}H-NMR spectral data showed a pair of olefinic proton signals at δ 6.93 and 7.00 (d, $J = 16.2$ Hz). The large coupling constant indicated a *trans* geometry (Mattivi et al., 1995), a set of three proton resonances at δ 7.21, 6.82 and 6.97 assigned to a 1,3,4-trisubstituted phenyl group, a set of two proton signals at δ 6.27, 6.61 consistent with a 1,3,5-trisubstituted second phenyl group and an aromatic methoxyl group at δ 3.86. The above data were consistent with a stilbene skeleton. The ^{13}C-NMR data of isorhapontigenin were also found in full agreement with the assigned structure (Table 3). In the 2D-(^{1}H–^{13}C)-HMBC, the correlation of *O*-methyl protons (δ 3.86, s) with C-3 (δ 148.5) was observed to suggest the location of the *O*-methyl group at C-3 (Fig. 4). The cross peak between H-2 (δ 7.21) and the *O-methyl* proton (δ 3.86) in the 2D-(^{1}H-^{1}H)-ROESY spectrum confirmed the location of this *O*-methyl group (Fig. 5).

4. Conclusion

Isorhapontigenin (ISOR), a bioactive stilbene present in many traditional medicinal plants, is firstly described in grapes, which is the only dietary source. Its concentration in grapes can be remarkably increased through elicitor treatments such as UVC postharvest. This makes grapes a relevant ISOR source in human diet.

ISOR isolated from different sources has shown potent antioxidant and protective effects. A positive concentration-effect relation is expected. More detailed studies on the biological importance of this new dietary compound are required.

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