FACULTAT DE FARMÀCIA

DEPARTAMENT NUTRICIÓ I BROMATOLOGIA

CONTRIBUCIÓN AL ESTABLECIMIENTO DE LAS BASES CIENTÍFICAS PARA EL USO DE FRACCIONES POLIFENÓLICAS Y FIBRA DIETÉTICA ANTIOXIDANTE EN LA PREVENCIÓN DEL CÁNCER

SONIA TOURIÑO EIRIN

2009

INSTITUT DE QUÍMICA AVANÇADA
DE CATALUNYA (**IQAC-CSIC**)

DEPARTAMENT QUÍMICA BIOLÒGICA I
MODELITZACIÓ MOLECULAR (QBM)

NUTRACÈUTICS I RADICALS LLIURES

UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA

**DEPARTAMENT NUTRICIÓ I
BROMATOLOGIA**

Programa de doctorat

NUTRICIÓ I METABOLISME

Bienni 2005-2007

CONTRIBUCIÓN AL ESTABLECIMIENTO DE LAS BASES CIENTÍFICAS PARA EL USO DE FRACCIONES POLIFENÓLICAS Y FIBRA DIETÉTICA ANTIOXIDANTE EN LA PREVENCIÓN DEL CÁNCER

Memòria presentada per **Sonia Touriño Eirín** per optar al títol de doctor per la Universitat de Barcelona

Director de la tesi: Tutor de la tesi:

Josep Lluís Torres Simón Rosa Mª Lamuela Raventós

SONIA TOURIÑO EIRIN
2009

Este trabajo ha sido financiado por:

Ministerio de Ciencia e Innovación

Beca de Formación de Personal Investigador (FPI): 2005-2009

AGL2004-07579-C04-02/ALI

Science is organized knowledge. Wisdom is organized life.
Immanuel Kant

Agradecimientos

Quisiera expresar mi más profundo agradecimiento a todas y cada una de las personas que han contribuido en este trabajo y que han hecho posible la consecución de un reto profesional y personal

En primer lugar, me gustaría dar las gracias el **Dr. Josep Lluís Torres** por haberme brindado la gran oportunidad de realizar esta tesis doctoral bajo su dirección y depositar en mí su confianza desde el primer día que aterricé en Barcelona. Por guiarme a lo largo de todo este período no sólo académicamente sino que también personalmente. Por hacerme participe de todos sus proyectos y por despertar en mí el entusiasmo por la ciencia.

Mi agradecimiento al Dr. Pere Clapés por tratarme de igual a igual, por conseguir que controle mi carácter pero sobretodo por su ayuda desinteresada en todo momento. Al Dr. Jesús Joglar por estar siempre dispuesto a ayudar y transmitir su buen humor. Al Dr. Lluís Julià por que una parte de esta tesis no podría ser realizada sin su colaboración.

A la Dra. Pilar Vinardell y Dra. Montse Mitjans, del departamento de Fisiologia de la Universitat de Barcelona, por su ayuda en los estudios de experimentación animal.

Al grupo de Bioquímica Integrativa i Teràpia del Càncer de Universitat de Barcelona. Especialmente, a la Dra. Marta Cascante por que sin su colaboración está tesis no podría llevarse a cabo. A la Dra. Daneida Lizárraga por que parte de esta tesis es suya. A los demás integrantes del grupo (Susana, S; Marisa, F; Silvia, M; Cecilia, M….) por acogerme como una más del equipo.

Al personal dels Serveis Científico-Tècnics de la Universitat de Barcelona, personalmente a la Dra. Olga Jáuregui, por cogerme de la mano en los primeros pasos y enseñarme a caminar en el mundo de la espectrometría de masas, ojalá pueda contar con tu ayuda en el largo camino que todavía me queda por recorrer.

Al personal del Servei d'espectrometria de masses del IADEA-CSIC, a la Dra. Roser Chaler y Maria por ayudarme con el uso del Q-TOF.

Mi más profundo agradecimiento para el Profesor Jason Morrow por ofréceme la oportunidad de realizar una estancia en su laboratorio y dar un millón de gracias al Dr. Brian Cox por su paciencia y buen humor en todo momento. Por supuesto, quisiera agradecer al Dr. Álvarez su inestimable ayuda para poder establecer los vínculos con el Profesor Morrow.

A todos mis compañeros y amigos del laboratorio, a los que están y a los que se han ido. Ellos han sido cómplices del esfuerzo, de las alegrías y también de los lloros que conllevan esta tesis. Compartir y convivir con vosotros gran parte del día a día ha sido fantástico. Mi más profundo agradecimiento a Ariadna Selga y el Dr. Carles Lozano por su amistad y por ser mis maestros durante los primeros años en el laboratorio. E incluir al Dr. Jordi Calveras y el Dr. José A. Castillo por ayudarme con mis dudas y problemas químicos. Gracias a todos por hacerme sentir como en mi propia casa, apoyándome en los momentos más difíciles.

A la Dra. Elisabet Fuguet por su ayuda incondicional y por su contribución fundamental durante estos dos últimos años. Por ser, además de consejera, amiga y por ensañarme a ver de manera analítica cada uno de mis experimentos.

A Sònia Castellanos por iluminar el laboratorio más oscuro del CSIC (la cueva) con sus radicales y sus placas solares, con su buen humor. Te echaremos de menos estos meses.

A Debi Castiella y a Sonia Lorenzo por aquellos buenos momentos que pasamos juntas en el laboratorio. Al Guillem Rocasalbas por no olvidar nunca su sonrisa, por ser realmente un amigo.

Gracias a Cristina Alonso por estar disponible cada vez que necesitaba envasar al vacío. Ya sabes que eres una más de nuestro grupo.

Gracias Anna, Xavi, Bruno y Aris por una convivencia ejemplar en el laboratorio

A Mariana por compartir su alegría con nosotros y….. su comida, su café. Por traernos los mejores alfajores del mundo. Mis mejores deseos para esta nueva etapa de la vida que comienzas.

A Livia, G. y Alda Lisa, C. por ser mis compinches dentro y fuera del laboratorio. Todavía nos quedan muchos rosarios por disfrutar.

Al Jordi P. por la paciencia de estos últimos meses y por confiar en mis indicaciones a ciegas. Estoy segura de que éste es el comienzo de una carrera científica fructificante.

Al equipo Bioglane; Susana, Sara, Dani, Ester por que el ambiente del Laboratorio no sería el mismo sin vosotros. Susana, no olvidaré nunca nuestras aventuras en la gran ciudad. Ojalá tengamos la posibilidad de poder compartir alguna más. Yo creo que somos un buen equipo.

A Marisa y a Juan por su alegría sureña, y por echarnos una mano con parte nuestro trabajo.

Gracias a los chicos del RUBAM, a los del Lab. 309 y como no, a la Patricia y el Gerard del Lab. 305 por mostrar su alegría en el día a día.

Grazas ós que me deron tanto apoio dende tan lonxe.

A todas aquellas personas y amigos que no he citado y que de alguna forma han contribuido a que esta tesis llegue a buen termino.

A todos, muchas gracias.

Índice

Índice de figuras de la introducción

TÍTULO FIGURA **PÁGINA**

TÍTULO FIGURA **PÁGINA**

Índice de tablas de la introducción

TÍTULO TABLA **PÁGINA**

Abreviaturas

$A^{\bullet}$	Radical libre no tóxico
AAPH	2,2’-azo-bis (2-amidinopropano) dihidrocloruro
AAS	R-aminoadípico semialdeído
AAT	Actividad Antioxidante Total
ABAP	2,2’-azo-bis (2-amidinopropano)
ABTS	2,2-azino-bis (3-etilbezotiazolin-6-sulfonato)
$ABTS^{\bullet+}$	Radical catión ABTS
ACE	Enzima convertidora de angiotensina I
ACN	Acetonitrilo
ADH	Alcohol deshidrogenasa
ADN	Ácido Desoxirribonucleico
AH	Antioxidante
ALDH	Aldehído deshidrogenasa
APCI	Ionización química a presión atmosférica
API	Ionización a presión atmosférica
ARP	Poder antirradicalario
ATP	Adenosín trifosfato
AUC	Área bajo la curva (del inglés, *Area under curve*)
BHA	Butilhidroxianisol
BHT	Butilhidroxitolueno
B-PE	B-Ficoeritrina
CAT	Catalasa
Cat	Catequina
CE	Electroforesis capilar
CID	Disociación inducida por colisión
Cmax	Concentración máxima
COMT	Catecol metilo transferasa
COMTs	Catecol metilo transferasas
COX	Enzima ciclooxigenasa
Cya	Cisteamina
Cya-Cat	4β-(2-aminoetiltio)catequina
Cya-Ec	4β-(2-aminoetiltio)epicatequina
Cya-EcG	4β-(2-aminoetiltio)epicatequin-3-*O*-galato
CYP	Enzima del complejo citocromo P450

DAD	Detector de diodos en línea (del inglés, *Diode array detector*)
DHA	Ácido docohexanoico
DH-IsoP	Dihomo-Isoprostanos
DMPD	radical catión N,N-dimetil-p-fenilendiamina
DMPO	5,5'-dimetil-1-pirrolín-N-oxido
DMSO	Dimetilsulfóxido
DNA	Ácido desoxirribonucleico
DPPH	Radical 2,2-difenil-1-picrilhidracilo
DSC	Calorimetría Diferencial de Barrido
DT	di-tirosina
Ec	(—)-epicatequina
EcG	(—)-epicatequina 3-*O*-galato
EGC	(—)-epigalocatequina
EGCG	(—)-epigalocatequín 3-*O*-galato
ELISA	Inmunoensayo (del inglés, *Enzyme linked inmunosorbent Assay*)
EPR	Resonancia Paramagnética Electrónica
ESI	Ionización por electrospray
EUFIC	Consejo Europeo de información sobre la alimentación (del ingles, *The European Food Information Council*)
FL	3,6-Dihidroxispiro[isobenzofuran-1(3H),9-[9H] xanten]-3-ona
FlO·	Radical fenoxil
FlOH	Flavonoide
FRAP	Reducción del Hierro/Poder antioxidante (del inglés, *Ferring Reducing/Antioxidant Power*)
G	porcentaje de galoización
GADF	Fibra antioxidante dietética (del inglés, *Grape antioxidant dietary fiber*)
GC	Cromatografía de gases (del inglés, *Gas chromatography*)
GC-MS	GC acoplada a MS
GGS	δ-glutámico semialdeído
GHz	Frecuencia de banda-X
GPx	Glutatión peroxidasa
GRed	Glutatión reductasa
GSH	Glutatión reducido
GSH	Glutatión reducido
GSSG	Glutatión oxidado
GST	Glutatión-s-transferasa
H_2O_2	Peróxido de hidrógeno

HAT	Transferencia de hidrógenos
HNE	4-Hidroxinonenal
HNTTM	Radical tris (2,4,6-tricloro-3,5-dinitrofenil)metil
HPLC	Cromatografía líquida de alta eficacia
HPLC-CE	HPLC acoplado a CE
HPLC-MS	HPLC acoplado a MS
HPLC-MS/MS	HPLC acoplado a MS/MS
HPLC-UV	HPLC acoplado a UV
HRP	Peroxidasa (del inglés, *horseradish peroxidase*)
Hx	Hidroperóxidos
IT	Trampa de iones (del inglés, *Ion Trap*)
LC	Cromatografía líquida
LDL	Lipoproteínas de baja densidad
LPH	Lactasa phlorodzin hidrolasa
MALDI	Ionización por desorción de una matriz mediante láser
MALDI-TOF	Técnica de MALDI acoplada a la técnica de TOF
MDA	Malonaldehído
mDP	Grado de polimerización medio
MeOH	Metanol
mMW	Masa molecular media
MPO	Mieloperoxidasa
MRM	Monitorización de reacciones múltiples (del inglés, *Monitoring multple reaction*)
MS	Espectrometría de masas
MS/MS ó MS^n	Espectrometría en tándem
NAC	N-acetil cisteina
NAD	Nicotín Adenín nucleótido
NADH	Nicotín Adenín nucleótido reducido
NADPH	Nicotinamida-Adenina Dinucleotido fosfato
NL	Barrido de perdidas neutras (del inglés, *Neutral loss scan*)
NOS	Nítrico oxido sintasa
NP	Fase normal (del inglés, *Normal phase*)
NP-HPLC	Cromatografía líquida de alta resolución de fase normal
O/W	Emulsión aceite en agua
OMS	Organización Mundial de la Salud
ORAC	Capacidad de absorción de radicales libres (del inglés, *Oxygen radical absorbance capacity*)

o-Tyr	*orto*-Tirosina
OWP	Extracto crudo de corteza de pino soluble en disolventes orgánicos y agua
OWH	Extracto crudo de *Hamamelis virginiana* soluble en disolventes orgánicos y agua
PAHs	Hidrocarburos policíclicos aromáticos
PAPs	fosfoadenosil-5'-fosfosulfato
PBS	Tampón fosfato salino
pH	Logaritmo negativo de base 10 de la actividad de los iones hidrógeno
PI	Barrido de ión precursor (del inglés, *precursor ión*)
PIDS	Dispersión diferencial por Intensidad de polarización (del inglés, *Polarization Intensity Diferencial Scatter*)
Q	Detector de cuadrupolo de barras
$Q^{\bullet}$	Quinonas
QqQ	Triple cuadrupolo
Q-TOF	Cuadrupolo acoplado a un detector de tiempo de vuelo
$R^{\bullet}$	Radical alquilo
RL	Radical libre
RNA	Ácido ribonucleico.
RNOS	Especies reactivas de oxígeno y nitrógeno
$RO^{\bullet}$	Radical alcoxilo
ROCl	Especies reactivas de oxígeno y cloro
$ROO^{\bullet}$	Radical peroxilo
ROONO	Alquil peroxinitrito
ROS ó ERO	Especies reactivas de oxígeno (del inglés, *Reactive oxygen species*)
RP	Fase reversa (del inglés, *Reverse phase*)
RP-HPLC	Cromatografía líquida de alta resolución en fase reversa.
SAM	*S*-adenosil metionina
SEC	Cromatografía de exclusión por tamaño
SET	Transferencia de electrones
SGLT	Proteínas con transporte sodio-glucosa
SOD	Superóxido dismutasa
ST	Sulfotransferasa
STs	Sulfotransferasas
TBARS	Sustancias reactivas al ácido tiobarbitúrico (del inglés,*Thiobarbituric acid reactive-substances*)

TEAC	Capacidad Antioxidante equivalente al Trolox (del inglés,*Trolox Equivalent Antioxidant Capacity*)
TFA	Ácido trifluoroacético
Tmax	Tiempo máximo
TNPTM	Radical tris (2,4,6-tricloro-3,5-dinitrofenil)-metilo
TOF	Detector de tiempo de vuelo (del inglés, *Time of fly*)
TOSC	Capacidad total de captación de radicales libres (del inglés,*Total oxidant scavenging capacity*)
TPTZ	Complejo 2,4,6-tripiridil-s-triazina
Trolox	Ácido 6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxílico
UDP	Uridina difosfato
UGTs	Uridina glucuroniltransferasas
UPLC	Cromatografía líquida de ultra resolución
UV	Ultravioleta (200-400nm)
UVA	Radiación ultravioleta de onda larga (320-400 nm)
UVB	Radiación ultravioleta de onda media (290-320 nm)
UV-vis	Ultravioleta-visible (200-750nm)
Vit	Vitamina
X	Xantina
XO	Xantina oxidasa
8-OH-dG	8-hidroxi-2'-deoxiguanosina
8-OHG	8-hidroxiguanina

1. INTERÉS Y JUSTIFICACIÓN

Los organismos aerobios necesitan oxígeno para vivir. La oxidación de los nutrientes para la obtención de energía fisiológica en forma de ATP implica una reducción del oxígeno escalonada en cuatro etapas monoeléctricas. Esta reducción del oxígeno a agua permite la liberación energética de manera gradual. Entre un 2 y un 5% de la reducción del oxígeno ocurre de forma incompleta en la mitocondria generando otras especies derivadas del oxígeno que se caracterizan por ser altamente oxidantes y reactivas. Las características que presentan estas especies por un lado les permite actuar como mensajeros secundarios modulando diversas funciones fisiológicas y por otro lado, sin embargo, pueden provocar daño oxidativo en elementos celulares debido a su inespecificidad y elevada capacidad oxidante. Como consecuencia, los organismos aerobios han desarrollado un sistema antioxidante capaz de mitigar el daño producido.

Factores ambientales relacionados con la dieta y la exposición a agentes pro-oxidantes (radiaciones, metales, pesticidas, contaminación, etc.) aumentan considerablemente las especies reactivas en la célula. Cuando el incremento de las especies reactivas excede la capacidad de actuación del sistema antioxidante endógeno, provoca un estado de estrés oxidativo. Daños sobre las membranas celulares y sobre el ADN son consecuencia directa de una desregulación en el estado redox de la célula. Existen evidencias que sugieren la existencia de una relación entre el estrés oxidativo y los estados patológicos de numerosas enfermedades. Es por ello que la modulación del equilibrio antioxidante fisiológico parece ser esencial para prevenir o reducir el daño al ADN y otras biomoléculas y por tanto, para disminuir la posibilidad de desarrollo de cáncer u otras enfermedades.

Estudios epidemiológicos han comprobado que una dieta rica en frutas y verduras parece ser importante para la prevención de enfermedades. Parece bastante plausible que alguno de los compuestos o la interacción de varios compuestos que constituyen frutas y verduras son responsables del efecto protector que presentan. Los flavonoides son los principales candidatos debido su abundancia en el reino vegetal y la actividad antioxidante que presentan en su estado natural.

De la ingesta total de compuestos fenólicos, se ha estimado que los ácidos fenólicos podrían representar aproximadamente 1/3 y los flavonoides los 2/3 restantes. La cantidad y la proporción de los diferentes compuestos fenólicos ingeridos varían ampliamente según el tipo de alimentos consumidos en función de los hábitos dietéticos y las preferencias. El estilo de vida al que se tiende hoy en día, ha aumentado la demanda de productos saludables que contienen compuestos biológicamente activos, conocidos por el nombre de alimentos funcionales, que además del aporte nutritivo pueden ofrecer beneficios para la salud disminuyendo el riesgo de padecer enfermedades.

En la actualidad, las investigaciones referentes a los compuestos fenólicos y a sus actividades biológicas están en auge, aunque el número de antioxidantes incluidos en las

listas positivas de ingredientes es de momento bajo. Compuestos fenólicos han sido extraídos, purificados y evaluada su actividad biológica. Los resultados prometedores en cuanto a las propiedades beneficiosas y la aceptación de los consumidores para utilizar antioxidantes naturales como complementos nutricionales han disparado el mercado de estos productos. La necesidad de disponer de grandes fuentes de compuestos fenólicos ha puesto en el punto de mira de numerosas investigaciones la obtención compuestos fenólicos a partir de subproductos de la industria agrícola y forestal. De hecho, algunos extractos obtenidos a partir de subproductos agroforestales han sido utilizados como ingredientes o suplementos nutricionales, aplicaciones dérmicas, y/o antioxidantes alimentarios. Las diferentes aplicaciones de los extractos fenólicos dependerán de factores físico-químicos (solubilidad, coeficiente de partición, estructura) y de la actividad biológica que presenten.

Una elevada proporción de los 2/3 ingeridos de flavonoides se encuentra en forma polimérica, generalmente en forma de proantocianidinas. Sin embargo, la mayoría estudios *in vitro* y *ex vivo* que parecen corroborar la capacidad de prevención de determinadas enfermedades son generalmente realizados con productos purificados que no son reflejo de una ingesta de frutas y/o verduras. Además, una gran parte de ellos utilizan enzimas que hidrolizan las conjugaciones sufridas en el organismo. Es cierto que, estos estudios facilitan el análisis y aporta información sobre la biodisponibilidad, no obstante, el hecho de no conocer las estructuras reales de los flavonoides en el organismo impide evaluar y descifrar la actividad de estos compuestos en la prevención de enfermedades.

Las proantocianidinas parecen tener una absorción limitada en el intestino, por lo que una parte de las proantocianidinas ingeridas van directamente al colon. Las bacterias que habitan el colon de los mamíferos fermentan los productos no digeridos en el estomago para obtener sustrato. Los compuestos fenólicos que no han sido absorbidos y los que se excretan vía bilis pueden ser degradados por la acción de las bacterias en compuestos más pequeños. La actividad de los metabolitos de los compuestos fenólicos y en concreto de las proantocianidinas todavía no es clara.

Un mayor conocimiento de los aspectos metabólicos relacionados con la ingesta de polifenoles ha de permitir explicar los potenciales efectos beneficiosos de los flavonoides de la dieta. Como resultado de todos los estudios con polifenoles se podría llegar a establecer recomendaciones dietéticas optimizadas en antioxidantes para distintos grupos de la población (ancianos, enfermos, deportistas, etc.) tendentes a mejorar la calidad de vida.

2. INTRODUCCIÓN

2.1 Radicales libres y especies reactivas de oxígeno

Un **radical libre** (RL) es una especie química (átomo, ión, o molécula) capaz de existir de forma independiente (de ahí, el termino de libre) y que presenta uno o más electrones desapareados en su estructura. Precisamente, debido a la existencia de electrones desapareados, son extremadamente reactivos hasta que consiguen transformarse en una molécula estable a través de una reacción de oxidación-reducción, en la cual el radical gana electrones o hidrogeniones (átomos de hidrógeno) de otra molécula. En general la mayor parte de las reacciones de oxidación son de deshidrogenación, es decir, que entrañan la perdida de átomos de hidrogeno.

Cualquier molécula es susceptible de convertirse en un radical libre tras la ganancia o pérdida de un electrón o tras la ruptura de un enlace homolítico. Un radical puede generar una cascada de reacciones en cadena clasificadas como: de **iniciación** (generación de un radical o especie reactiva), **propagación** (el número de radicales libres se mantiene constante aumentado el número de productos de la reacción y disminuyendo el de reactivos) y **terminación** (desaparición de los radicales libres por aniquilación o combinación de sus dos electrones desapareados).

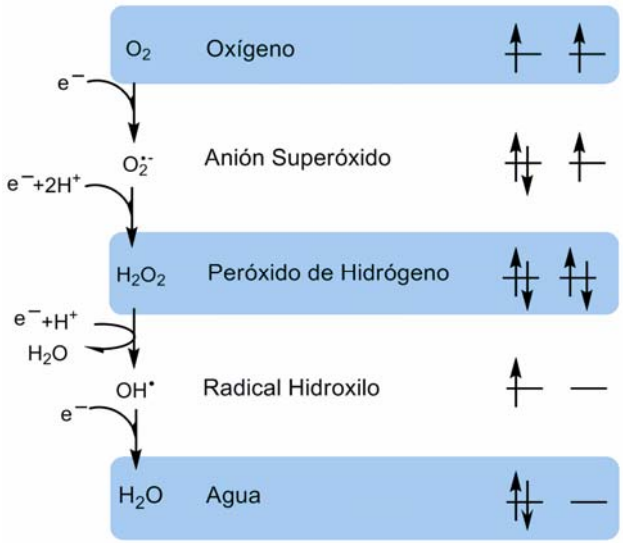


Figura 2.1: Reducción tetravalente del oxígeno molecular. Estado de los *spins* direccionales de las moléculas implicadas.

El oxígeno molecular (O_2), que necesitan todos los organismos aerobios presenta en su estructura dos electrones desapareados lo que lo convierte en un bi-radical. La disposición de estos dos electrones, en diferentes orbitales libres y ambos en el mismo sentido, permite al O_2 tener una cierta estabilidad y propiedades paramagnéticas, aunque por otro lado, impide que el oxígeno capte dos electrones simultáneamente en su reducción. En la mitocondria la reducción del oxígeno implicada en la producción de ATP tiene lugar mediante cuatro reacciones univalentes para dar lugar a dos moléculas de

agua (**Figura 2.1**). Durante este proceso se generan especies intermediarias altamente reactivas y oxidantes de naturaleza radical ($O_2^{\bullet -}$ y $OH^{\bullet}$) y no radical (H_2O_2). Ambas son incluidas en la definición de **especies reactivas de oxígeno** (ROS; del inglés *reactive oxygen species*).

Entre los más reactivos se encuentra el radical hidroxilo ($OH^{\bullet}$) que presenta un elevado potencial de reducción y una vida media de tan sólo 10^{-9} s *(Liochev et al., 1994)*. La formación del radical $OH^{\bullet}$ tiene lugar en presencia de iones metálicos y H_2O_2 por reacciones de Haber-Weiss y Fenton. Aunque es cierto que en los sistemas biológicos el Fe se encuentra generalmente en su forma férrica y en pequeñas cantidades *(Halliwell et al., 1986)*, éste puede ser reducido por el ascorbato y por el radical superóxido, con lo que se genera un ciclo de producción continua de radicales hidroxilo *(Frei, 1994)*

$$O_2^{\cdot -} + H_2O_2 \longrightarrow O_2 + OH^{\cdot} + OH^- \quad \text{(Reacción de Haber Weiss)}$$

$$Fe^{+2} + H_2O_2 \longrightarrow Fe^{+3} + OH^{\cdot} + OH^- \quad \text{(Reacción de Fenton)}$$

$$Fe^{+3} + O_2^{\cdot -} \longrightarrow Fe^{+2} + O_2$$

El peróxido de hidrógeno (H_2O_2), en ausencia de iones metálicos, es la especie menos reactiva y la más estable en condiciones de pH y temperatura fisiológicas. Se origina por dismutación del anión superóxido vía superóxido dismutasa o partir de la actividad de enzimas como la aminoácido oxidasa y la xantina oxidasa *(Lee et al., 2004)*. Es considerado un agente oxidante débil. Sin embargo tal y como se ha explicado anteriormente, el peróxido de hidrógeno puede generar radicales hidroxilo en presencia de iones metálicos y además puede producir *in vivo* oxígeno singlete al reaccionar con el anión superóxido o con cloraminas *(Stief, 2000)*. Existen otras especies reactivas que pueden generarse en los organismos aerobios. Las cuales son dependientes de otros elementos tales como nitrógeno (RNOS); cloro (ROCl), etc. Las ROS y las RNOS son las principales especies reactivas implicadas en la biología redox (Tabla 2.1) *(Halliwell, 1999)*.

Tabla 2.1: Especies reactivas de oxígeno y nitrógeno

	Radicales	**No radicalarias**
ROS	Anión superóxido ($O_2^{\bullet -}$)	Peróxido de hidrógeno (H_2O_2)
	Hidroxilo ($OH^{\bullet}$)	Ácido hipocloroso (HClO)
	Alcoxilo ($RO^{\bullet}$)	Ozono (O_3)
	Peroxilo ($ROO^{\bullet}$)	Oxígeno singlete ($^1\Delta O_2$)
RNOS	Óxido nítrico ($NO^{\bullet}$)	Anión nitrosilo (NO^-)
	Dióxido de nitrógeno ($NO_2^{\bullet}$)	Catión nitrosilo (NO^+)
		Ácido nitroso (HNO_2)
		Alquil peroxinitrito (ROONO)

2.2 Principales fuentes de ROS en la célula

La mitocondria constituye la principal fuente de ROS. La obtención de energía a partir de la reducción del O_2 tiene lugar a nivel de cadena de electrones por la acción del complejo citocromo oxidasa. En general, más de un 95% del O_2 consumido es reducido en cuatro etapas monoeléctricas, adquiriendo 4 electrones y generando 2 moléculas de agua (**Figura 2.1**). Sin embargo, el 5% restante la reducción del oxígeno no es completa formándose compuestos intermediarios: anión superóxido ($O_2^{\bullet -}$), peróxido de hidrógeno (H_2O_2) y/o hidroxilo ($OH^{\bullet}$)

Los peroxisomas son orgánulos que tienen un papel fundamental en el metabolismo lipídico y están encargados de la degradación de ácidos grasos de cadena larga para su completa oxidación en la mitocondria. Además, participan en la degradación de bases púricas de los aminoácidos (adenina y guanina) y juegan un papel importante en la detoxificación de moléculas tóxicas como el etanol *(Masters, 1998)*. Los procesos de degradación que ocurren en los peroxisomas son realizados por numerosas enzimas de tipo oxidasa que utilizan oxígeno molecular para conseguir átomos de hidrógeno de sustratos específicos generando como consecuencia grandes cantidades de H_2O_2 *(Del Río et al., 1992)*. El peróxido de hidrógeno formado en condiciones normales es eliminado por la enzima catalasa pero bajo ciertas condiciones de estrés oxidativo la actividad de la enzima catalasa disminuye liberándose grandes concentraciones de H_2O_2 en la célula *(Schrader et al., 2006)*.

Los leucocitos cuando son activados por mediadores pro-inflamatorios, productos bacterianos, víricos o parásitos, producen grandes cantidades de ROS a partir de la actividad de la NADPH oxidasa, RNOS mediante la actividad de la enzima nítrico óxido sintasa (NOS) y HClO mediante la actividad de la mieloperoxidasa (MPO) *(Babior, 2000)*. Este proceso es conocido como "estallido respiratorio".

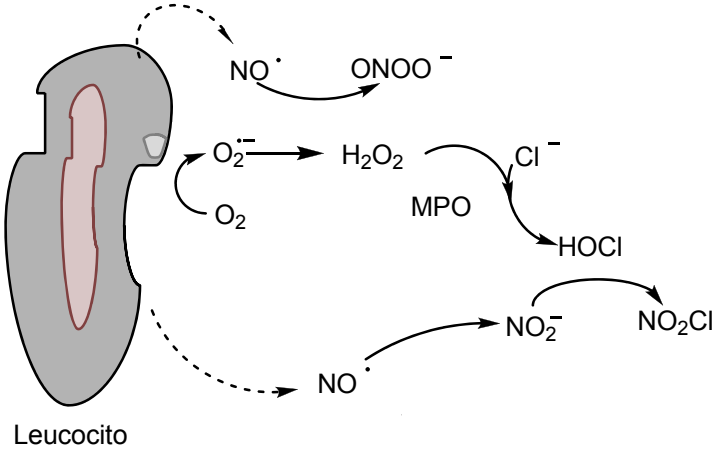


Figura 2.2: Especies reactivas generadas por leucocitos activados *(Himmelfarb et al., 2002)*

Pequeñas moléculas solubles tales como tioles, hidroquinonas, catecolaminas, flavinas y tetrahidropterinas pueden autooxidarse de forma natural generando radical superóxido que la mayor parte de las veces acaba derivando en peróxido de hidrógeno *(Martínez-Cayuela, 1995)*.

Otras enzimas, principalmente enzimas citosólicas solubles como la aldehído oxidasa involucrada en el metabolismo del etanol y la óxido nítrico sintasa responsable de transformar la L-arginina en óxido nítrico y enzimas unidas a la membrana plasmática como la lipooxigenasa y la ciclooxigenasa (COX) que participan en el metabolismo del ácido araquidónico generando radicales libres durante el ciclo de catálisis *(Balazy et al., 2008;Caro et al., 2006)*. Algunas enzimas implicadas en la hidroxilación de purinas también son responsables de contribuir a un aumento de especies reactivas en la célula. Por ejemplo, la enzima xantina oxidasa (XO) que es encargada de catalizar la reacción de hipoxantina a xantina y de xantina a ácido úrico (**Figura 2.3**). Ambas reacciones generan $O_2^{\bullet -}$ *(Vorbach et al., 2003)*.

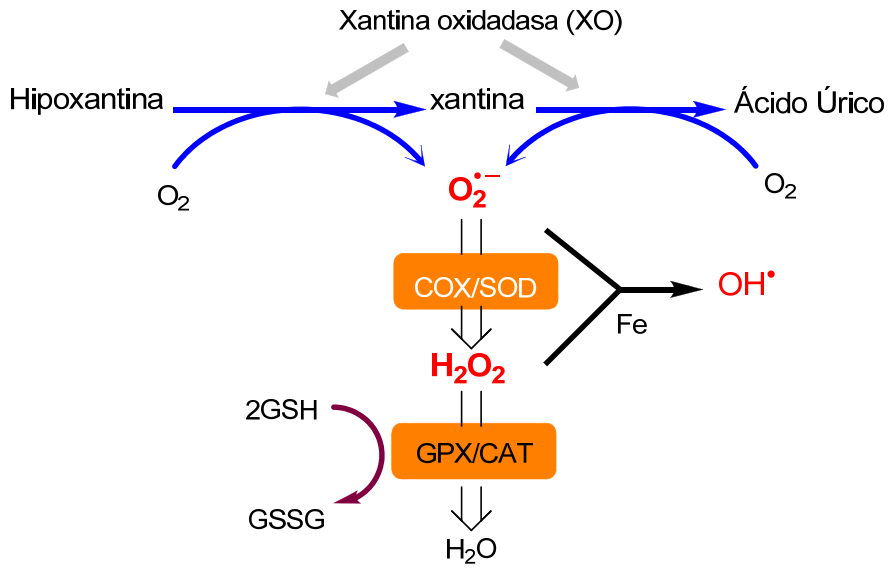


Figura 2.3: Degradación de las purinas vía xantina oxidasa (XO) y formación de ROS

2.3 Funciones biológicas de ROS

Pequeñas concentraciones de ROS parecen ser necesarias para el correcto funcionamiento de los organismo vivos *(Droge, 2002)*. Entre las funciones más destacadas de las especies reactivas de oxígeno se encuentran:

(i) Regulación de óxido nítrico generado por la enzima óxido nítrico sintasa (NOS) *(Chen et al., 2009)*;

(ii) Activación de la enzima NAD(P)H en leucocitos para la producción masiva de ROS en la defensa de microorganismos externos y en células no fagocíticas, como fibroblastos o células endoteliales vasculares *(Thannickal et al., 1995)*, que tras la producción de ROS pueden activar rutas intracelulares necesarias en la regulación de las células cardiacas y factores de crecimiento en fibroblastos *(Griendling et al., 2000;Jacobi et al., 2005)*.

(iii) Regulación del tono vascular del músculo liso *(Griendling et al., 1994)*;

(iv) Inhibición de la agregación plaquetaria *(Begonja et al., 2006)*;

(v) Detección de cambios en la concentración del oxígeno intracelular*(Droge, 2002;MacFarlane et al., 2008)*;

(vi) Programación de apoptosis *(Herdener et al., 2000)*; Pequeñas cantidades de especies reactivas juegan un papel crucial como segundos mensajeros en la transducción de la señal para la activación, diferenciación y proliferación celular. La inducción o la inhibición de la proliferación parece ser dependiente del balance ROS/antioxidantes en la célula. *(Schreck R et al., 1992)*.

(vii) Modulación de la respuesta inmunitaria de linfocitos-T y macrófagos *(Victor et al., 2004)*.

2.4 Citotoxicidad de ROS

Los radicales libres que están en exceso reaccionan rápidamente con otras moléculas cercanas para estabilizar su estructura electrónica, especialmente el radical hidroxilo que es el más reactivo ($OH^{\bullet}$) (**Figura 2.4**). De esta manera, un exceso de ROS puede oxidar pequeñas biomoléculas libres (vitaminas, aminoácidos, carbohidratos simples y lípidos), estructuras supramoleculares como membranas lipoproteicas, y material genético.

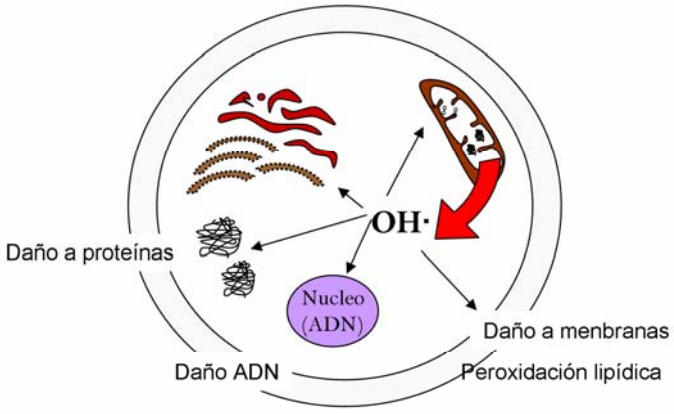


Figura 2.4: Esquema de citotoxicidad en la célula

La acción de los radicales libres viene determinada por su reactividad química, y por la disponibilidad de un sustrato susceptible en la vecindad de donde se produce el radical libre.

Daño oxidativo a lípidos: La acción de las ROS sobre los lípidos tiene lugar fundamentalmente sobre los ácidos grasos poliinsaturados generando peróxidos, los cuales son degradados hasta formar especies reactivas. La oxidación de los lípidos que forman parte de la membrana celular afecta tanto a las propiedades físicas (fluidez y permeabilidad) como a la funcionalidad de las proteínas de membrana *(Droge, 2002)*. Los radicales libres que pueden iniciar esta reacción son: el radical hidroxilo ($OH^{\bullet}$), el peroxilo ($ROO^{\bullet}$), el alcoxilo ($RO^{\bullet}$) y el radical alquilo ($R^{\bullet}$).

El mecanismo de peroxidación lipídica (**Figura 2.5**) comienza cuando un radical libre ataca a un carbono de la cadena alifática de un ácido graso, se desprende un átomo de hidrógeno, y se forma un radical alquilo. Esta reacción se produce preferentemente en los carbonos contiguos a dobles enlaces de los ácidos grasos poliinsaturados, ya que los radicales formados se estabilizan por resonancia con el enlace doble. Este radical reacciona con el O_2 y forma un radical peroxilo ($R\text{-}COO^{\bullet}$). Los radicales peroxilo pueden reaccionar con cadenas laterales de otros ácidos grasos poliinsaturados adyacentes formando un radical alquilo ($R'\text{-}CH^{\bullet}$) y un peróxido lipídico (R-COOH), con lo que se produce una reacción en cadena.

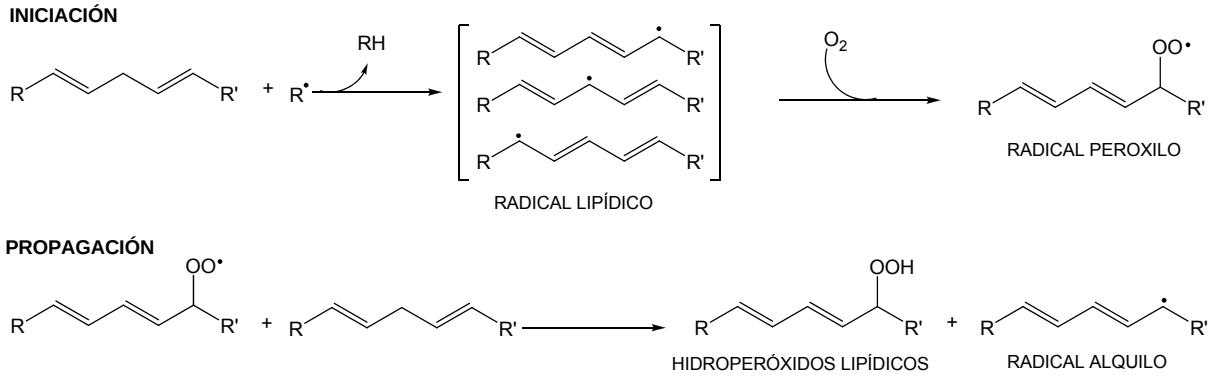


Figura 2.5: Proceso esquemático de la peroxidación lipídica.

Un único ataque de un radical libre a un ácido graso poliinsaturado puede dar lugar a la formación de un gran número de productos de oxidación, (i) productos formados a partir de la rotura de dobles enlaces carbono-carbono adyacentes a un grupo hidroperóxido, tales como son el malondialdehído (MDA) y el 4-hidroxinonenal (HNE); (ii) productos formados a partir de la reordenación y sucesivas oxidaciones (hidroperóxidos, epidióxidos, dihidroperóxidos, endoperóxidos biciclicos y otros compuestos); (iii) productos de oxidación de alto peso molecular que se generan por reacciones de polimerización.

Gran parte de los productos formados reaccionan rápidamente con los componentes celulares, por lo que causan mutaciones en el ADN, y producen daños estructurales y funcionales al reaccionar con proteínas y otras estructuras celulares *(Catalá, 2009;Spiteller, 2006)*.

La peroxidación del ácido araquidónico, componente estructural de las membranas celulares origina además de hidroperóxidos, unos peróxidos cíclicos conocidos como isoprostanoides. Estos compuestos son una familia de eicosanoides de origen no enzimático que se forman a partir de la peroxidación mediada por radicales libres *(Morrow et al., 1997;Roberts Ii et al., 1997)*

Daño oxidativo al ADN: El ADN del núcleo y de la mitocondria, son susceptibles de sufrir daños oxidativos debido, principalmente, a la cercanía donde las especies reactivas son generadas. Existen diferentes tipos de daño que sufre el ADN tales como: ruptura del esqueleto azúcar fosfato de una o de las 2 hebras; modificación de las bases nitrogenadas (saturación y fragmentación del anillo de timina) y la formación de uniones cruzadas (*cross-links*) ADN-ADN o ADN-proteína. Por ejemplo, la acción del $^{\cdot}OH^{\bullet}$ da lugar a más de 20 modificaciones y entre ellas la más frecuente es la 8-hidroxi-2'-desoxiguanosina (8-OH-dG) que tiene un potencial altamente mutagénico *(Jaruga et al., 1996)*. El daño producido en el ADN induce error frecuentemente de forma irreversible en las señales de trascripción y traducción. Los errores en la replicación y la inestabilidad genómica están asociados a carcinogénesis *(Aruoma et al., 1995;Dizdaroglu et al., 2002)*.

Daño oxidativo a proteínas: Todos los aminoácidos presentes en las proteínas tienen residuos susceptibles (sobre todo prolina, histidina, arginina y metionina) de ser atacados por los radicales libres, principalmente por el radical hidroxilo. El inicio de la oxidación proteica se ve favorecido por la presencia de iones metálicos formando complejos en el interior del la proteína. Dichos iones son capaces de catalizar la descomposición del H_2O_2 generando, un sitio especifico en la proteína para el anclaje del radical y una posterior ruptura de la proteína *(Davies, 1986)*. El radical peroxinitrito ($ONOO^{-}$) oxida esencialmente grupos –SH, y da lugar a productos estables que originan un cambio conformacional en la proteína *(Virág et al., 2003)*. La estructura proteica también puede ser atacada por productos secundarios formados en la peroxidación lipídica, tales como el MDA y HNE que generan productos de enlaces cruzados con aminoácidos específicos *(Lecomte et al., 1993)*. En cualquiera de los casos, la oxidación da lugar a un cambio conformacional en proteína que puede resultar en una pérdida o modificación de su función biológica *(Valko et al., 2007)*.

Daño oxidativo a glúcidos: Los mono y disacáridos resisten la acción de los radicales libres de oxígeno. De hecho, la glucosa es captadora de radical superóxido y la manosa y el manitol eliminan radicales hidroxilo, por lo que son considerados como agentes antioxidantes en la célula *(Albertini et al., 1996) (De Lederkremer et al., 2003)*.

El daño oxidativo a los glúcidos reviste importancia cuando se trata de polisacáridos de función estructural, ya que los polisacáridos son despolimerizados por los radicales libres dando lugar a procesos degenerativos. Un caso especial es el del ácido hialurónico cuya

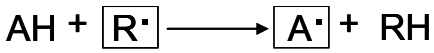
función estructural reside en mantener la viscosidad del fluido sinovial. La exposición a agentes oxidantes (sobre todo radical superóxido) provoca su fragmentación, lo que conduce a la desestabilización del tejido conectivo y a la pérdida de viscosidad del fluido sinovial, como ocurre en el caso de la artritis reumatoide *(Stern et al., 2007) (Halliwell et al., 1995b)*.

2.5 Sistemas de defensa antioxidante

Para minimizar el daño que las ROS puede producir a las biomoléculas, los organismos aeróbicos han desarrollado un sistema de defensa antioxidante ante las especies reactivas.

2.5.1 Definición de antioxidante

Un antioxidante es una molécula capaz de retardar o prevenir la oxidación de otras moléculas *(Halliwell et al., 1995a)*. Los antioxidantes (AH) actúan generalmente cediendo un electrón o hidrogenión a los radicales libres (RL) transformándose a su vez en un radical libre de naturaleza no tóxica ($A^{\bullet}$) y que en algunos casos puede ser regenerado por la acción de otros antioxidantes. De está manera, los antioxidantes puede detener reacciones de propagación e inhibir la oxidación de moléculas evitando la alteración en el funcionamiento normal de la célula.



Un compuesto es considerado antioxidante cuando cumple al menos una de las siguientes propiedades: (i) eliminar ROS y/o otras especies reactivas; (ii) disminuir la disponibilidad de especies pro-oxidantes; (iii) proteger moléculas de la oxidación.

2.5.2 Clasificación de los antioxidantes

Existen numerosas formas de clasificar los antioxidantes. Las más utilizadas son la clasificación según su naturaleza enzimática o no enzimática (**Tabla 2.2)** y según el mecanismo de acción por el cual actúan: (i) de manera preventiva (impiden la formación de RL y secuestran metales del medio); (ii) disminuyendo RL del medio y rompiendo las reacciones en cadena; (iii) reparando y reconstituyendo daños que pueden provocar los RL. En condiciones fisiológicas normales la actuación y los niveles de ambos antioxidantes está en equilibrio. Este balance es esencial para la supervivencia de los seres aerobios *(Valko et al., 2007)*

Tabla 2.2: Principales sistemas antioxidantes del organismo *(Boots et al., 2008;Halliwell et al., 1990)*

Antioxidantes enzimáticos	**Función**
Superóxido dismutasa (SOD)	Eliminación del radical superóxido
Catalasas	Eliminación de hidroperóxidos
Glutatión peroxidasas (GPx)	Eliminación de hidroperóxidos
Glutatión reductasa (GRed)	Reducción de glutatión oxidado
glutatión-S-transferasa (GST)	Eliminación de peróxidos lipídicos
Metionina sulfóxido reductasa	Reparación de residuos oxidados de metionina
Peroxidasas	Descomposición de H_2O_2 y peróxidos lipídicos
Antioxidantes no enzimáticos endógenos	
Glutatión reducido (GSH)	Sustrato de las enzimas GPx y GST y captador de RL
Ácido úrico	Captador de oxígeno singlete y radicales libres
Albúmina	Actividad peroxidasa en presencia de GSH
Bilirrubina	Captación de radicales peroxilo
Lactoferrinas, transferrinas, hemopexinas y haptoglobinas	Fijación de grupos hemo. Inhibición de la reacción de Fenton.
Glucosa, manosa y manitol	Inactiva los RL ($OH^{\bullet}$, $O_2^{\bullet -}$)
Ubiquinol (Coenzima Q)	Captador de RL
Ácido α-lipoico	Captador de $OH^{\bullet}$, $O_2^{\bullet -}$ y H_2O_2
Antioxidantes no enzimáticos exógenos (obtenidos a partir de la dieta)	
Ácido ascórbico (vit C)	Regeneración tocoferoles e inactivación de RL
α-Tocoferol (vit E)	Protección de membranas e inactivación del $OH^{\bullet}$
Compuestos fenólicos	Captación de RL y actividad quelante de metales

2.6 Estrés oxidativo

En situaciones fisiológicas, cada célula presenta un estado redox característico. En condiciones normales la cantidad de radicales libres es controlado por el sistema antioxidante endógeno. Sin embargo, existen diferentes situaciones fisiológicas en las que puede alterarse el equilibrio en favor de las especies oxidantes dando lugar a lo que se conoce como **estrés oxidativo** y éste es originado por dos motivos fundamentales *(Halliwell et al., 2004)*:

1. Disminución de los niveles de antioxidantes debido a, mutaciones que afectan a los sistemas antioxidantes o toxinas que causan depleción de las defensas antioxidantes.

2. Incremento en la producción de especies reactivas a causa de la exposición a diferentes compuestos que generan especies radicales.

Estás dos causas son mayoritariamente generadas por factores externos que desequilibran la homeostasis de los organismos aerobios.

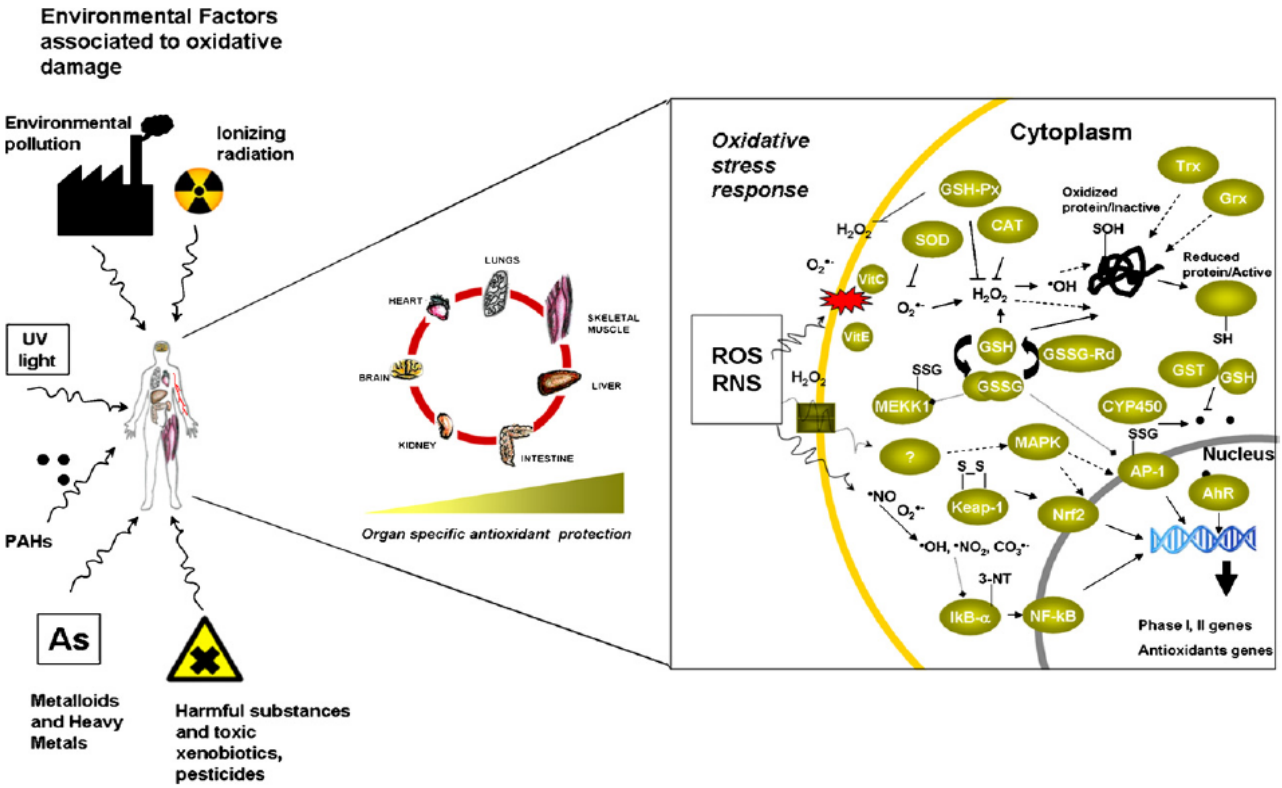
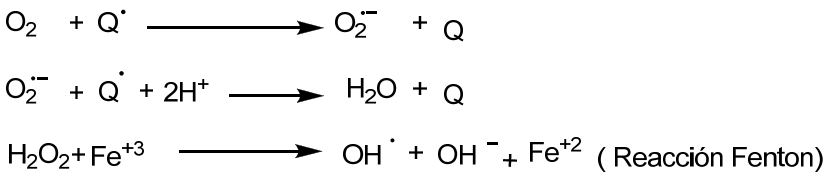


Figura 2.6: Factores exógenos que generan ROS *(Limón-Pacheco et al., 2009)*. Contaminación ambiental, radiaciones ionizantes, metales pesados, pesticidas e hidrocarburos policíclicos aromáticos (PAHs) entre otros, inducen a la formación de ROS y RNOS en células y tejidos provocando estrés oxidativo. En este estado el sistema antioxidante endógeno es sobrepasado por las elevadas proporciones de ROS, las cuales son capaces de dañar proteínas, lípidos y ADN.

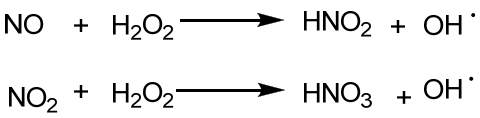
2.6.1. Factores externos implicados en el incremento de ROS

El consumo de tabaco

A pesar de que son ampliamente conocidos los riesgos de salud que conllevan el hábito de fumar, todavía existen más de 1 billón de fumadores en el mundo. Miles de compuestos promotores de radicales libres por gramo de humo inhalado son introducidos en el organismo al fumar un cigarrillo. Más de 10^{17} radicales/gramo provienen del alquitrán y aproximadamente 10^{15} radicales son introducidos en una bocanada de humo de un cigarrillo. Los radicales introducidos pueden dividirse en dos grupos: El primero consiste fundamentalmente en radicales de tipo quinona y semiquinona (representadas como $Q^{\bullet}$) generados durante el proceso de combustión a partir de la oxidación de hidrocarburos aromáticos policíclicos *(Church et al., 1985)*. Estos compuestos reducen el oxígeno molecular a radical superóxido y a peróxido de hidrógeno. La presencia de trazas de Cu y Fe en el humo del tabaco cataliza la conversión de peróxido de hidrógeno a radical hidroxilo mediante la reacción de Fenton *(Durak et al., 2002)*.



El segundo grupo son radicales de corta vida de tipo peroxilo que pueden ser detectados por técnicas de "*spin trap*". Estos radicales resultan de la oxidación del óxido nítrico al dióxido de nitrógeno, los cuales reaccionan posteriormente con otros compuestos del tabaco tales como aldehídos y olefinas para generar radicales tipo peróxido. Además, NO y NO_2 pueden reaccionar con H_2O_2 y dar lugar al radical $OH^{\bullet}$ *(Duthie et al., 2000)*



El radical libre hidroxilo tiene un gran efecto nocivo en el pulmón porque inactiva la α-1-proteasa que es responsable de la actividad antielastasa. Las infecciones en el pulmón producen inflamación y en este estado se liberan proteasas de macrófagos y células lisadas por inmunología celular en los alvéolos. Normalmente las proteasas liberadas son neutralizadas por antiproteasas como la α-1-antitripsina. Si la actividad elastasa no es bloqueada, la elastina del pulmón es degradada. Además, el radical hidroxilo y otros radicales libres favorecen la acumulación de neutrófilos en el pulmón. Los neutrófilos activados generan nuevos radicales libres provocando, por tanto, un daño tisular adicional. El resultado final puede llegar a ser la aparición de efisema pulmonar*(Duthie et al., 2000;Nakayama et al., 1985;Pryor et al., 1985)*.

Los efectos inflamatorios del humo de tabaco se relacionan con condiciones que potencian los procesos de carcinogénesis tales como la oxidación del glutatión (GSH) que incrementa los niveles de disulfuro de glutatión en el tejido pulmonar; el incremento de los niveles de 8-OH-dG; la disminución de niveles de antioxidantes en el corriente sanguíneo y el incremento de marcadores de peroxidación lipídica.

El consumo de alcohol

Un consumo excesivo y habitual de alcohol conlleva a un aumento en la producción de ROS. La hipótesis más plausible sobre el consumo de alcohol y el incremento de ROS tiene su explicación en el metabolismo del etanol a acetaldehído vía alcohol deshidrogenada (**Figura 2.7**).

Las reacciones metabólicas del etanol están localizas en diferentes compartimentos celulares: (i) en el citosol, vía alcohol deshidrogenasa (ADH); (ii) en los microsomas mediado por enzimas de complejo citocromo P450.; (iii) en los peroxisomas, vía catalasa *(Lieber et al., 2000)*.

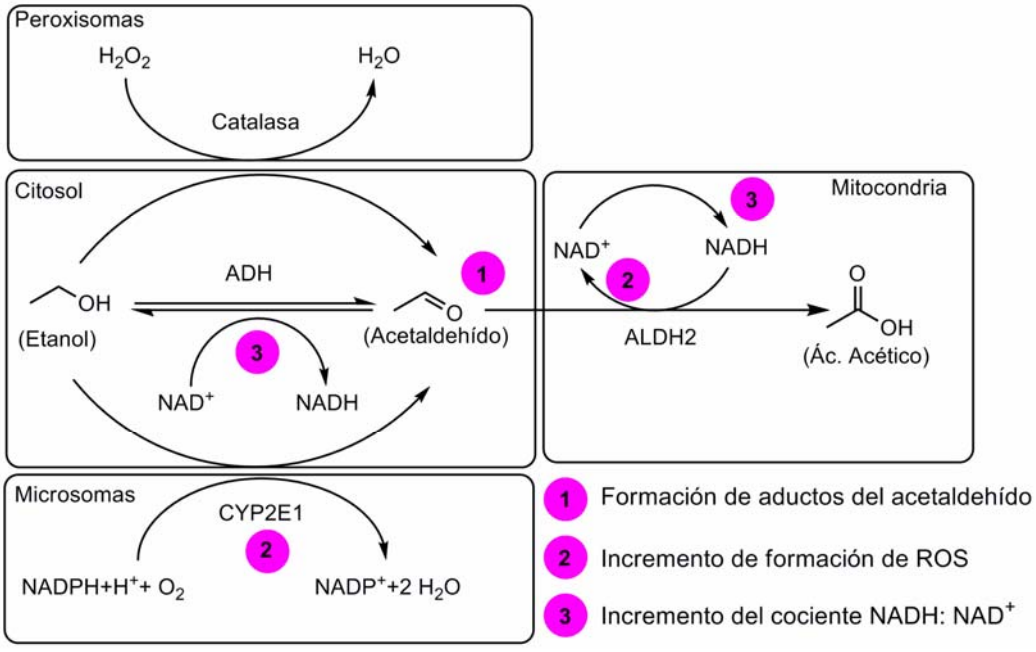


Figura 2.7: Metabolismo del etanol en los hepatocitos. ADH, alcohol deshidrogenasa; CYP2E1, citocromo P450 2E1; ALDH2, aldehído deshidrogenasa 2. *(Zakhari, 2006)*

El acetaldehído (1), producto formado en la reacción mediada por la enzima alcohol deshidrogenasa, es rápidamente metabolizado por la enzima aldehído deshidrogenasa a acetato. El Acetaldehído es capaz de formar aductos con proteínas lo que resulta en la formación de anticuerpos e inactivación de enzimas *(Dicker et al., 1988)* además de una disminución en la capacidad de reparación de errores en la replicación *(Lieber et al.,*

1989). La actividad de la isoenzima CYP2E1 (2), del complejo citocromo P-450 (localizada en los microsomas), asume un papel importante en la metabolización del etanol a acetaldehído especialmente cuando la concentración de etanol es elevada (K_m=8-10mM, comparada con 0.2- 2.0mM de la ADH hepática), pero a su vez, este proceso genera especies reactivas como hidroxietil, anión superóxido y radicales hidroxilo. El proceso de oxidación mediado por la alcohol deshidrogenasa (ADH) implica la transferencia de dos electrones del dinucleótido de nicotinamida adenina (NAD^+) el cual es reducido a NADH (3). NADH inhibe la enzima xantina deshidrogenasa promoviendo la oxidación de purinas vía xantina oxidasa y provoca un estado reductor en la célula que hace más vulnerable la defensa de las biomoléculas al ataque de ROS y a los productos formados en el metabolismo del etanol.

El consumo habitual de alcohol ha sido relacionado con un incremento de H_2O_2 en la célula debido al daño, directo o indirecto, del acetaldehído sobre la actividad de enzimas antioxidantes como la glutatión peroxidasa *(Lieber et al., 2000;Misra et al., 1992)*. Además el alcohol disminuye los niveles de GSH e induce la actividad de ciertas enzimas del complejo citrocromo P-450 (CYP2E1) que impulsan la activación de pro-carcinógenos en carcinógenos

Radiaciones ultravioletas (UVA) y ultravioletas-visible (UVB)

La exposición de la piel a radiaciones UVA (320-400nm) o UVB (290-320nm) induce a la formación de ROS (**Figura 2.8**), incluyendo el radical anión superóxido ($O_2^{\bullet -}$), peróxido de hidrógeno (H_2O_2), radical hidroxilo ($OH^{\bullet}$), oxígeno singlete ($^1\Delta O_2$) entre los más abundantes *(Zhao et al., 2001) (Nguyen et al., 2005)*.

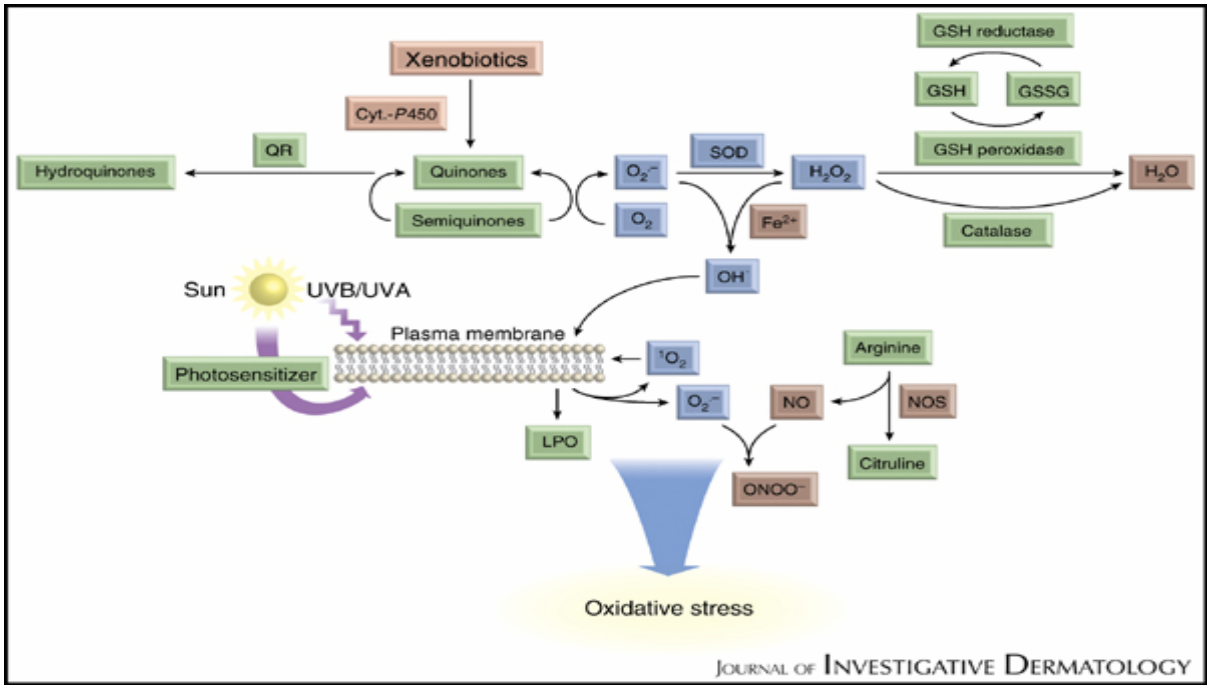


Figura 2.8: generación de ROS en la piel y defensas antioxidantes *(Bickers et al.)*

Las radiaciones solares que corresponden en más de un 90% a radiaciones UVA, penetran en la dermis causando degeneración de colágeno e inflamación por formación de ROS. La exposición continua de UVA deriva en un envejecimiento prematuro de la piel.

Las radiaciones UVB (causantes de las quemaduras solares) incrementan los niveles de ROS en células de la epidermis. Activan rutas involucradas en el crecimiento celular, diferenciación y proliferación, facilitando la expansión de tumores celulares *(Bickers et al.)*. La exposición continuada a UVB induce a la oxidación de lípidos y proteínas de las membranas epiteliales ocasionando perdida de fluidez, inactivación de enzimas y alteración de la permeabilidad de iones, que finalmente derivan en choque osmótico y ruptura celular *(Bommareddy et al., 2007)*.

Biocidas y pesticidas

Trazas de herbicidas y pesticidas son detectados en la mayoría de alimentos vegetales y aguas no comerciales que ingieren los humanos. La naturaleza de estos compuestos es variada y en ello radica su grado de toxicidad. Los pesticidas pueden inducir al estrés oxidativo por diferentes vías *(Bagchi et al., 1995;Banerjee et al., 2001)*. La alteración de enzimas del sistema antioxidante endógeno (catalasa y superóxido dismutasa) es una de las vías más frecuentes *(Braconi et al., 2008)*.

Metales

El desarrollo tecnológico, el consumo masivo e indiscriminado y la producción de desechos principalmente urbanos (pilas, pinturas, baterías de automóviles), han provocado la presencia de numerosos metales en cantidades importantes en el ambiente. Estos pueden incorporarse con los alimentos o como partículas que se respiran y se van acumulando en el organismo. La formación e incremento de ROS ha sido observada en diferentes órganos después de estar expuestos a arsénico (As) *(Bardullas et al.)*, cadmio (Cd)*(Nemmiche et al., 2007)*, cromo (Cr)*(Bagchi et al., 2002)* o mercurio (Hg)*(Göksel Şener et al., 2003)*.

Otros factores

Situaciones de estrés psicológico *(Chalmers et al., 2003)*, ejercicio físico intenso *(Bloomer et al., 2008;Ji, 2000)*, una dieta desequilibrada, rica en grasas *(Brewster et al., 2006)*, o incluso escribir una tesis *(Touriño, 2009)* pueden dar lugar a la formación y/o incremento de ROS en el organismo y derivar en un estado de estrés oxidativo.

2.6.2 Patologías relacionadas con el estrés oxidativo

Debido al daño que generan las ROS sobre las biomoléculas, las especies reactivas han sido asociadas a numerosas enfermedades crónicas (**Tabla 2.3**) y a la formación de cáncer, el cual es una de las mayores causas de mortalidad.

Tabla 2.3: Enfermedades asociadas a estrés oxidativo

Enfermedades gastrointestinales	Inflamación intestinal *(van der Vliet et al., 1992)* Hepatitis *(Cheeseman et al., 1995;Simmonds et al., 1995)* Periodentitis *(Mashayekhi F, 2005)*
Enfermedades vasculares	Aterosclerosis *(Singh et al., 2006)* Infarto miocardio *(Kaul et al., 1993)*
Enfermedades respiratorias	Asma bronquial *(Wood et al., 2003)* Neumonía *(Duflo et al., 2002;Virág, 2005)* Fibrosis pulmonar *(Pastore et al., 2003)* Bronquitis crónica *(Drost et al., 2005)* Síndrome de distrés respiratorio *(Pastore et al., 2003)*
Enfermedades neurológicas	Alzheimer *(Behl, 1999)* Parkinson *(Ebadi et al., 1996)* Esquizofrenia *(Brenner-Lavie et al., 2008)* Demencia senil *(Bagchi et al., 2007)*
Enfermedades oculares	Cataratas *(Spector, 1995)* Retinopatías *(Jarrett et al., 2008)*
Enfermedades dérmicas	Dermatitis atópica *(Omata et al., 2001)* Psoriasis *(Shilov et al., 2000)* Acné *(Ozer Arican, 2005)*
Enfermedades óseas	Sarcopenia *(Lopez et al., 2000)* Osteoporosis *(Pasco et al., 2008)*
Enfermedades endocrinas	Diabetes *(Maritim et al., 2003;Mehta et al., 2006)* Pancreatitis *(Schoenberg et al., 1995)*

2.6.3 Cáncer y estrés oxidativo

2.6.3.1. Concepto de cáncer

El concepto de cáncer es aplicado al conjunto de enfermedades en las cuales las células presentan proliferación excesiva y descontrolada con capacidad de invadir y dañar tejidos y órganos, provocando finalmente la muerte del individuo. En los países desarrollados, el cáncer es una de las principales causas de mortalidad. La OMS prevé que, a nivel mundial, la mortalidad por cáncer aumentará un 45% entre 2007 y 2030 (pasará de 7,9 millones a 11,5 millones de defunciones). El crecimiento demográfico, el envejecimiento de la población, un estilo de vida sedentario y el consumo de tabaco parecen ser las causas más obvias de esta predicción. Se estima que durante el mismo periodo el número de casos nuevos de cáncer aumentará de 11,3 millones en 2007 a 15,5 millones en 2030 *(Coleman et al., 2008)*. En el caso de Catalunya la previsión es aún más estremecedora si se tiene en cuenta que el Departamento de Salud alarmó sobre un aumento del 61,5% en los hombres y del 57,1% en las mujeres *(Ministerio de Sanidad y Consumo, 2006)*.

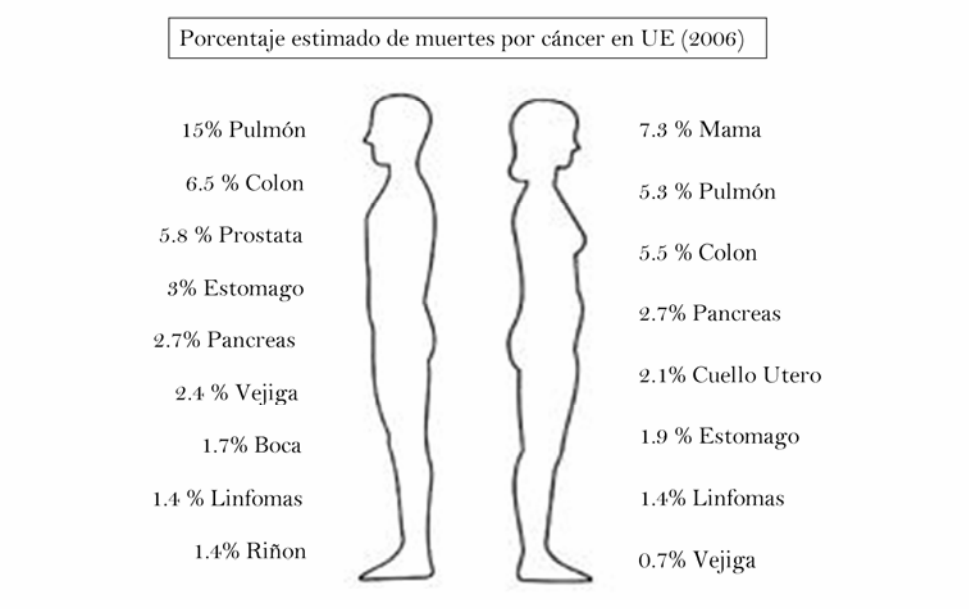


Figura 2.9: Estimación del porcentaje de muertes correspondiente a los cánceres más comunes en la Unión Europea en el año 2006 *(Coleman et al., 2008)*.

2.6.3.2. Cáncer y Especies reactivas de oxígeno

El cáncer es originado a partir de un agente agresor, al que se le denomina carcinógeno, capaz de alterar el material genético de la célula provocando una proliferación celular sin una adecuada regulación. Las células afectadas por la mutación tienden a presentar una proliferación infinita y son capaces de crecer independientemente de un soporte

estructural. Cuando un grupo de células se divide de forma anormal es llamada tumor. Si el tumor es capaz de separarse de su origen y llegar a otros órganos por el corriente sanguíneo o linfa invadiendo otros tejidos, es denominado metástasis.

Los mecanismos propuestos por numerosos autores para explicar la aparición de cáncer son: (i) incremento de síntesis y mitosis de ADN originado a partir de un carcinógeno capaz de inducir mutaciones en los genes encargados de regular la proliferación y el crecimiento celular (conocidos como pro-oncogenes) y que dan lugar a la formación de oncogenes (pro-oncogenes mutados); (ii) desequilibrio entre la proliferación de las células y la muerte celular programada (apoptosis). Estudios epidemiológicos y biológicos han demostrado que el proceso de oncogénesis ocurre en varias etapas, donde las especies radicales juegan un papel decisivo para el desarrollo del cáncer *(Frenkel, 1992;Valko et al., 2004)*.

En la **fase de iniciación**, el ADN de la célula es alterado por la acción de un carcinógeno generando a una mutación. La mutación producida no implica necesariamente que la célula se convierta en tumor, con frecuencia mutaciones generadas pueden ser reparadas o pueden ser conducidas a experimentar apoptosis celular. Pero, cuando las mutaciones producidas por los carcinógenos son irreversibles la célula comienza un proceso más o menos rápido de proliferación que recibe el nombre de fase de iniciación tumoral. Las células involucradas en esta fase se llaman células iniciadas. El daño oxidativo generado por ROS en las bases del nitrogenadas del ADN podría estar relacionado con la formación de la fase de iniciación *(Dreher et al., 1996)*.

Cuando los carcinógenos actúan de forma repetida sobre las células iniciadas, la proliferación celular comienza a ser más rápida y descontrolada aumentando la probabilidad de que se produzcan nuevas mutaciones. Este proceso es conocido como **fase de promoción** y las células involucradas se denominan células promocionadas.

Un elevado número de carcinógenos de naturaleza oxidante y no oxidante actúan inhibiendo los sistemas antioxidantes endógenos de la célula tales como SOD, la catalasa o el glutatión provocando un incremento de ROS que acaba dañando el ADN *(Nishikawa et al., 2009)*. Factores externos como los nombrados anteriormente (apartado 2.6.1) inducen la formación de especies reactivas y favorecen la promoción tumoral debido a una repetida y continua exposición en el organismo.

Curiosamente, si el nivel de estrés oxidativo es realmente elevado las células detienen la proliferación induciendo apoptosis o incluso necrosis, sin embargo si el estrés oxidativo no es demasiado elevado puede estimular la fase de promoción induciendo crecimiento tumoral (**Figura 2.10**) *(Dreher et al., 1996;Shi et al., 2004)*. De ahí que muchos autores remarquen que una regulación de la producción de ROS en esta fase es fundamental en la prevención/desarrollo de cáncer *(Valko et al., 2007)*.

Las células promocionadas siguen sufriendo mutaciones debido a la desregulación del ciclo celular tornándose más anómalas en su crecimiento y comportamiento hasta originar un tumor. La suma de estos procesos es conocida como **fase de progresión**.
Los tumores, en los que la presencia de ROS parece ser evidente *(Hsu et al., 1991)*, pueden adquirir la capacidad de invasión tanto a nivel local, infiltrando los tejidos de alrededor, como a distancia, originando metástasis. Niveles elevados de stress oxidativo han sido observados en esta fase *(Gupte et al., 2009;Nishikawa, 2008)*.

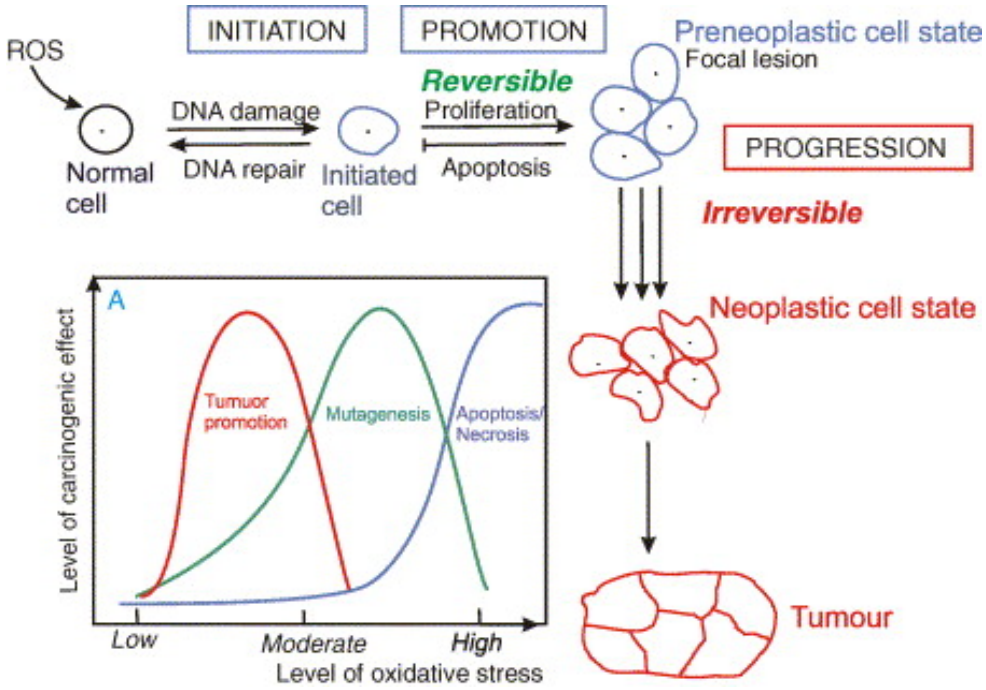


Figura 2.10: Etapas de formación del cáncer en función del estrés oxidativo *(Valko et al., 2006)*

Enfermedades crónicas y cáncer presentan en común un elevado estrés oxidativo. Todavía queda por aclarar sí el stress oxidativo es generado por los mecanismos patológicos de cada enfermedad, o si las enfermedades son causa directa o indirecta del daño celular que causan los radicales libres. Sin embargo, es clara la idea de que la presencia de estrés oxidativo en la célula convierte el sistema antioxidante endógeno insuficiente para la homeostasis. La ingesta de antioxidantes podría ser necesaria para compensar un déficit del sistema antioxidante endógeno. El ácido ascórbico (vit C) o la vitamina E son necesarias para el correcto funcionamiento celular auque no son sintetizadas en los humanos y requieren ser incorporadas por la dieta. Es por ello, que una dieta rica en antioxidantes podría prevenir enfermedades.

2.7 Los flavonoides como ingredientes de alimentos funcionales

2.7.1 La dieta en la prevención de enfermedades

Estudios epidemiológicos han demostrado, que la Dieta Mediterránea contribuye a disminuir el riesgo de padecer patologías crónicas como son las enfermedades cardiovasculares *(Fung et al., 2009)*, la obesidad *(Mendez et al., 2006)*, la diabetes *(Martínez-González et al., 2008)* y cáncer *(Benetou et al.)*. La conocida paradoja francesa esta relacionada con la prevención de enfermedades cardiovasculares *(Cheng, 2001;Vidavalur et al., 2006)*. Ambas tienen en común una elevada ingesta de frutas y verduras ricas en productos antioxidantes. Muchos de los beneficios asignados a estás dietas puede ser debido a la actividad sinérgica entre los compuestos antioxidantes ingeridos *(Rahman, 2009)*. Es por ello, que una dieta equilibrada suplementada con antioxidantes podría prevenir o/y curar enfermedades relacionadas con el estrés oxidativo.

En la actualidad, se han realizado estudios clínicos suplementando la dieta con (i) compuestos antioxidantes esenciales de la dieta tales como vitamina E y C, (ii) compuestos precursores de antioxidantes, N-acetil cisteina (NAC) y un mayor número de estudios con, (iii) antioxidantes exógenos, los cuales no son esenciales para el organismo pero que juegan un papel importante en el buen funcionamiento del organismo, tales como ácido α-lipoico y los polifenoles. Algunos de éstos estudios dieron como resultado, efectos beneficiosos en los enfermos *(Autier et al., 2007) (Christen et al., 2009;Hercberg et al., 2004)*. Sin embargo, algunos no presentaron diferencias significativas en los resultados obtenidos *(Beazley et al., 2005;Cook et al., 2007;Farouque et al., 2006;Keith et al., 2001;Kucuk et al., 2001)*, e incluso, algún estudio presentó resultados negativos *(Bairati et al., 2005)*.

La utilización de antioxidantes exógenos para la prevención y/o tratamiento de enfermedades asociadas al stress oxidativo es un tema donde todavía quedan muchos puntos que aclarar. El trabajo realizado durante la presente tesis tiene como objetivo general contribuir en un mayor conocimiento sobre el uso de antioxidantes exógenos como suplementos nutricionales e ingredientes de alimentos funcionales.

2.7.2 Alimentos funcionales. Concepto.

Los alimentos funcionales no han sido definidos hasta el momento por la legislación europea. Generalmente, se considera que son aquellos alimentos, que se consumen como parte de una dieta normal y contienen componentes biológicamente activos, que ofrecen beneficios para la salud y reducen el riesgo de sufrir enfermedades. Entre algunos ejemplos de alimentos funcionales, destacan: los alimentos que contienen determinados minerales, vitaminas, ácidos grasos o fibra dietética; los alimentos a los

que se han añadido sustancias biológicamente activas (fitoquímicos u otros antioxidantes); y los probióticos (alimentos con cultivos vivos beneficiosos) *(EUFIC, 2009)*.

2.7.3 Los flavonoides

Los flavonoides están incluidos en el grupo de los compuestos fenólicos, que se encuentran ampliamente distribuidos en la naturaleza con más de 8000 estructuras conocidas *(Harborne, 1993)*. Los compuestos fenólicos son metabolitos secundarios de las plantas y ejercen diversas funciones; desde la coloración de flores hasta la impregnación de lignina de las paredes pecto-celulósicas y son encargados de los mecanismos de defensa frente agresiones externas (radiación UVA, predadores, ataques fúngicos y víricos)*(Dixon et al., 1996)*. Los animales no son capaces de sintetizar este tipo de compuestos es por ello que tienen que incorporarlos por la dieta.

El esqueleto de los compuestos fenólicos consta de un anillo bencénico que contiene uno o diversos grupos hidroxilo. Las distintas familias de compuestos fenólicos son clasificadas principalmente por el número de átomos de carbono que presentan en su estructura (Tabla 2.4)

Tabla 2.4: Principales familias de los compuestos fenólicos.

Estructura	Familia
C_6	Fenoles simples
C_6-C_1	Ácidos fenólicos
C_6-C_2	Ácidos fenólicos y acetofenonas
C_6-C_3	Cumarinas y ácidos hidroxicinámicos
C_6-C_1-C_6	Xantonas y benzofenonas
C_6-C_2-C_6	Estilbenos
C_6-C_3-C_6	Chalconas
C_6-C_3-C_6	**Flavonoides**
$(C_6$-C_3-$C_6)_n$	**Taninos condensados**
$(C_6$-$C_1)_n$	**Taninos hidrolizables**

Los flavonoides es una de las familias de compuestos fenólicos más importante. Presentan una estructura básica de fenil-benzo-γ-pirona C_6-C_3-C_6, (**Figura 2.11**) caracterizada por un esqueleto de dos anillos bencénicos unidos por una cadena de tres átomos de carbono ciclada en un heterociclo oxigenado.

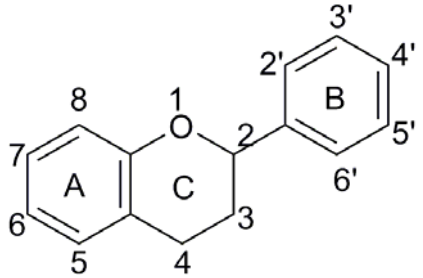
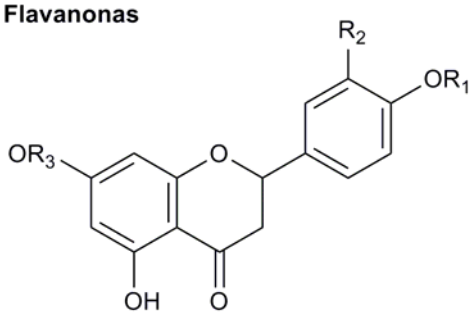


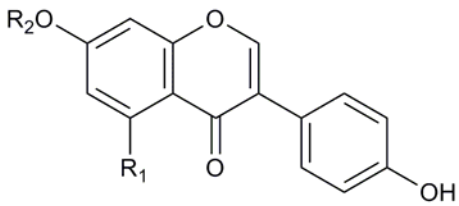
Figura 2.11: Estructura básica de los flavonoides

Flavanonas



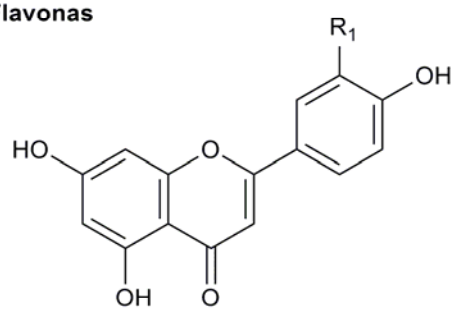
Naringina: R_1,H; R_2,H; R_3,H
Hesperidina: R_1,CH_3; R_2,OH; R_3,H

Isoflavonas



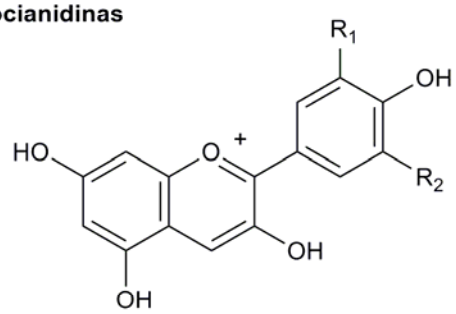
Daidzeína: R_1,H; R_2,
Genisteína: R_1,OH; R_2,H

Flavonas



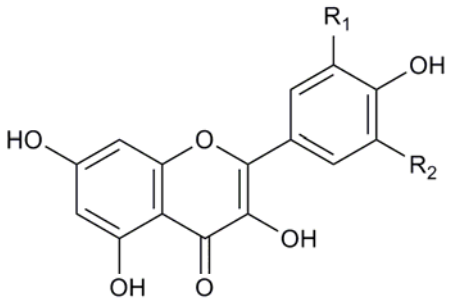
Apigenina: R_1,H
Luteolina: R_1,OH

Antocianidinas



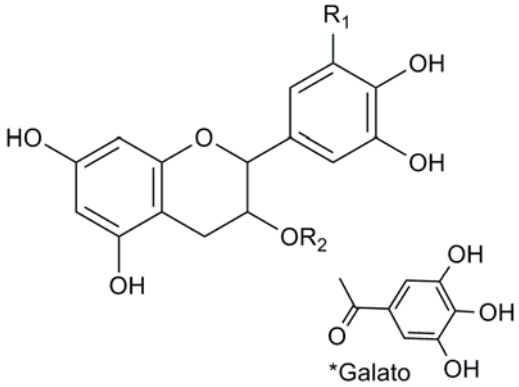
Cianidina: R_1,OH; R_2,H
Pelargonidina: R_1,H; R_2,H
Delfinidina: R_1,OH; R_2,OH

Flavonoles



kampferol: R_1,H; R_2,H
Quercetina: R_1,OH; R_2,H

Flavan-3-ol



Catequina: R_1,H; R_2,H
Epicatequina: R_1,H; R_2,H
Epigalocatequina: R_1,OH; R_2,H
Epigalocatequin-galato: R_1,H; R_2,Galato*

Figura 2. 12: Estructura química de las subclases de flavonoides más relevantes.

Las variaciones estructurales en el anillo C, subdividen a los flavonoides en seis importantes subclases; entre los que se encuentran las flavanonas, flavonas, flavonoles, isoflavonas, antocianidinas, flavan-3-ol (también llamados flavanoles). Los compuestos individuales dentro de cada grupo se distinguen por la sustitución en los anillos A y B. De esta forma, se han identificado hasta 5000 estructuras diferentes. En la **Figura 2.12**, se muestran las seis subclases nombradas anteriormente y algunos de los compuestos más representativos de cada de ellas.

Los flavonoides se pueden encontrar en estado libre (agliconas), tal y como se muestran en la **Figura 2.12**, o en forma de heterósidos (conjugados con un azúcar, y también denominados glucósidos). La presencia de un azúcar en su estructura les confiere mayor solubilidad en agua, permitiendo su acumulación en las vacuolas, por lo que, generalmente se encuentran en forma de heterósido. Hesoxas (glucosa, galactosa, ramnosa) y pentosas (xilosa y arabinosa) son los azucares preferentemente incorporados a su estructura. También se han encontrado flavonoides unidos a lípidos, aminas o ácidos carboxílicos *(Duthie et al., 2003)*. Los azucares son generalmente unidos mediante enlace β-glucosídico en la posición C-3 y C-7 (ver figura 2.11), aunque también pueden producirse en otras posiciones que contengan un grupo hidroxilo. Un caso excepcional es el de los flavan-3-ol que generalmente se encuentran en la naturaleza en forma libre o esterificados con un ácido gálico *(Manach et al., 2004)*.

Frecuentemente, los flavanoles forman polímeros $(C_6\text{-}C_3\text{-}C_6)_n$, que reciben el nombre de taninos condensados. Las proantocianidinas (**Figura 2.13**) son un ejemplo de taninos condensados formados por unidades de catequinas.

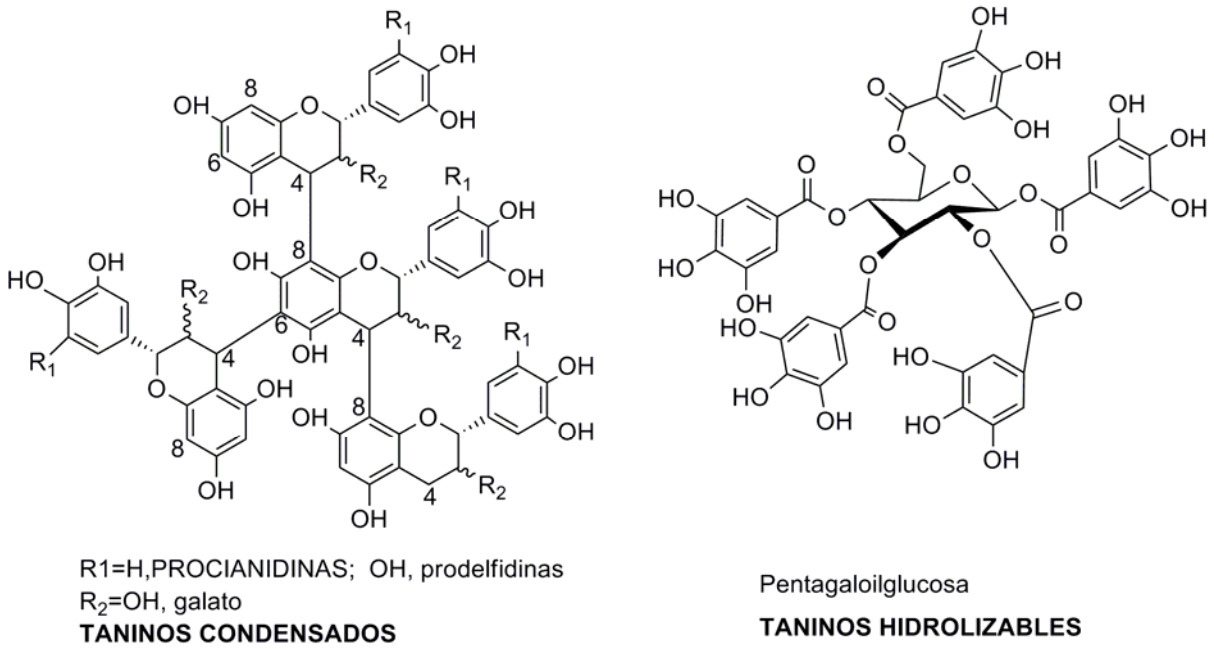


Figura 2.13: Ejemplo de taninos condensados (polímeros de flavonoides) y taninos hidrolizables (derivados de ácido gálico).

La condensación más frecuente tiene lugar entre el C-4 del heterociclo C y los C-6 ó C-8 del anillo A. Sus formas oligoméricas y poliméricas pueden llegar a presentar un número elevado de unidades.

Otro tipo de polímeros de gran importancia en la naturaleza, que no forman parte del grupo de los flavonoides, son los taninos hidrolizables (**Figura 2.13**) que están constituidos por unidades de ácido gálico. Fracciones con taninos hidrolizables y fundamentalmente, fracciones con proantocianidinas han sido objeto de estudio a lo largo de la presente tesis

2.7.4 Los subproductos agroforestales. Fuente de obtención de flavonoides y otros compuestos fenólicos

Los alimentos básicos de origen vegetal de la dieta presentan importantes cantidades de flavonoides (**Tabla 2.5**) y otros compuestos fenólicos, aunque durante el procesado, una proporción considerable de estos compuestos es eliminada o degradada.

Tabla 2.5: Ejemplos de fuentes dietéticas de flavonoides *(Beecher, 2003)*.

Fuente dietética	mg/servicio	Fuente dietética	mg/servicio
Manzanas con piel		**Zumo de naranja**	
Flavan-3-ols	13	Flavanonas	28
Flavonoles	6	Flavonoles	>1
Proantocianidinas	147	Proantocianidinas	ND
Arándanos		**Té, negro infusionado**	
Antocianidinas	82	Flavan-3-ols	6
Flavan-3-ols	1	Flavonoles	10
Flavonoles	3	Terubiginas	116
Proantocianidinas	131	**Té verde, infusionado**	
Proantocianidinas	ND	Flavan-3-ols	304
Chocolate, negro		Flavonoles	12
Flavan-3-ols	24	Terubiginas	3
Proantocianidinas	165	**Vino, tinto**	
Chocolate con leche		Antocianidinas	9–40
Flavan-3-ols	6	Flavan-3-ols	10–20
Proantocianidinas	88	Flavonoles	10
Zumo de arándanos		Proantocianidinas	77–103
Flavan-3-ols	>1	**Vino, blanco**	
Flavonoles	3	Flavan-3-ols	2–3
Proantocianidinas	42	Proantocianidinas	2–3
Tofu			
Isoflavonas	334		

N.D, no determinado.

El afán por poder descifrar los mecanismos en los que están implicados los flavonoides y con el fin de obtener concentrados con la máxima actividad biológica, libres de otros compuestos que puedan interferir o/y aportar propiedades físicas no deseables se han desarrollado metodologías especificas para la extracción y obtención de compuestos fenólicos de diferentes fuentes. Los subproductos agrícolas y forestales debido a su bajo coste y a las cuantiosas concentraciones que presentan de flavonoides han sido una de las fuentes más utilizadas *(Moure et al., 2001)*.

La industria cítrica, por ejemplo, produce grandes cantidades de pieles y pepitas como residuos. Más de un 50% del peso total corresponde a subproductos *(Bocco et al., 1998)*. El contenido total de compuestos fenólicos en los subproductos cítricos es un 15% mayor que el de la porción comestible correspondiente *(Gorinstein et al., 2001)*. Otras frutas como manzanas, melocotones y peras presentan aproximadamente el mismo porcentaje de compuestos fenólicos en la parte la parte comestibles que en la desechable.

Tabla 2 6: Ejemplos de subproductos utilizados como fuente de compuestos fenólicos

Subproductos	Compuestos fenólicos	Estudio realizado
Cáscara de almendras	Ác. Clorogénico Ác. 3-O-caffeoilquínico Ác. 4-O-caffeoilquínico Flavonoles glicosilados	*(Takeoka et al., 2003)* *(Frison-Norrie et al., 2002)*
Pieles de manzana	Flavanoles Antocianinas	*(Wolfe et al., 2003)*
Procesado alcachofas	Ác. clorogénico y neoclorogénico Derivados del ácido cafeico	*(Llorach et al., 2002)*
Procesados manzana	Flavonoles Flavanoles Dihidrochalconas Ác hidroxicinámicos	*(Schieber et al., 2003)*
Pieles de coco	Ác 4-Hidroxibenzoico Ác ferúlico	*(Dey et al., 2003)*
Procesado Remolacha	Ácido ferúlico Ác di-ferúlico	*(Saulnier et al., 1999)*
Procesado de té	Flavan-3-ol	*(Yen et al., 1997)* *(Zandi et al., 1999)*
Industrias forestales	Taninos (hidrolizables y Condensados)	*(Conde et al., 1998)*

Otro ejemplo es la industria vitivinícola que procesa anualmente más de 60 millones de toneladas de uva. Más de un 13% corresponden a subproductos del prensado que básicamente consisten en hollejo, y semillas ricas proantocianidinas y otros compuestos fenólicos *(Schieber et al., 2001;Torres et al., 2001)*

El grupo del Dr. Saura-Calixto y colaboradores ha desarrollado un nuevo concepto de fibra dietética a partir del prensando de hollejo de uva que proviene de subproductos de la industria vitivinícola *(Saura-Calixto et al., 1999)*. La fibra dietética de uva (GADF, *grape antioxidant dietary fiber*) combina los efectos beneficiosos de la fibra con los de los compuestos polifenólicos antioxidantes *(Martín-Carron et al., 1997)*. Recientemente, se han obtenido fibras dietéticas de otros subproductos generados del procesado de: los espárragos *(Fuentes-Alventosa et al., 2009)*; la coliflor *(Stojceska et al., 2008)*; el trigo *(Esposito et al., 2005)*; los cítricos *(Lario et al., 2004;Marín et al., 2007)* o subproductos derivados del procesado de la soja *(Redondo-Cuenca et al., 2008)*.

2.7.5 Actividad biológica de los flavonoides

Los compuestos fenólicos, entre ellos los flavonoides, son considerados como antioxidantes gracias a la capacidad de captar radicales libres. Los grupos hidroxilo que forman parte de su estructura donan electrones o hidrogeniones inactivando a los radicales. El radical fenoxilo generado es muy poco reactivo, debido a que se estabiliza por resonancia con los electrones π del anillo aromático (**Figura 2.14**).

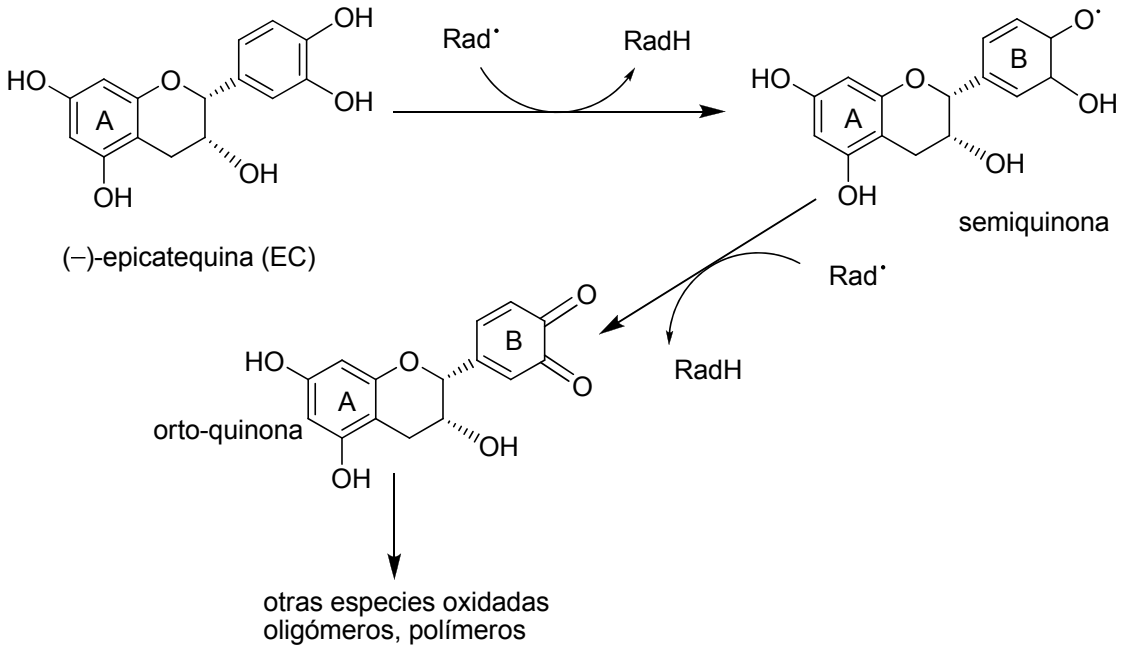


Figura 2.14: mecanismo de oxidación de la (—)-epicatequina.

Bors postuló tres criterios estructurales que definen una mayor capacidad antioxidante en los flavonoides *(Bors et al., 1990)*. Éstos son (**Figura 2.15**): (i) presencia de dos grupos hidroxilo en posición *orto* (catecol) en el anillo B, que le confiere mayor estabilidad a la forma radical y participa en la deslocalización de los electrones; (ii) un doble enlace entre el C_2-C_3 conjugado con la función 4-oxo del anillo C que es responsable de la deslocalización de los electrones en el anillo B; (iii) presencia de dos grupos hidroxilo en posición *meta* posiciones C_5 y C_7.

El flavonol quercetina, cumple estas tres características estructurales por lo que presumiblemente será un antioxidante más potente que otros flavonoides. Sin embargo, los estudios que han comparado la actividad antioxidante de la quercetina y de la cianidina, la cual carece de la segunda característica estructural, dieron resultados similares *(Rice-Evans et al., 1996)*. Otros muchos estudios han demostrado la existencia de otros muchos factores que influyen en la capacidad antioxidante de los flavonoides *(Heim et al., 2002)*. La naturaleza química de posibles sustituyentes (galato, azúcar) y la posición del hidroxilo al que se unen determinan propiedades, tales como solubilidad y facilidad de donación de electrones, que son importantes en la capacidad antioxidante. Por ejemplo, las formas heterósidas son solubles en agua pero incapaces de actuar en medios lipídicos. El grado de polimerización es otro de los factores que parece jugar un papel importante en la actividad antioxidante *(Heim et al., 2002)*. Aunque la relación estructura-actividad todavía no es clara.

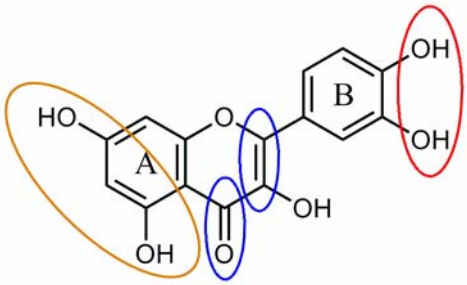


Figura 2.15: Características estructurales de los flavonoides relacionadas con la capacidad antioxidante

Los flavonoides y los taninos (tanto los condesados como los hidrolizables) presentan capacidades quelantes que contribuyen a la actividad antioxidante. Metales, como hierro y cobre, forman parte de las ferroproteínas y otros complejos necesarios en el organismo. Generalmente, estos metales se encuentran en su forma inactiva (Fe^{+3}), (Cu^{+2}). Sin embargo, cambios de pH en el medio pueden generar la forma reducida (Fe^{+2}),(Cu^{+1}) que interviene en la reacción de Fenton transformando peróxido de hidrógeno (especie poco reactiva) en el radical hidroxilo (altamente reactivo) *(Winterbourn, 1995)*.

$$H_2O_2 + Fe^{2+}(Cu^{+}) \longrightarrow OH^{\bullet} + OH^{-} + Fe^{3+}(Cu^{2+})$$ (Reacción de Fenton)

Los flavonoides actúan impidiendo la reacción de Fenton, es decir secuestran iones de hierro u otros metales. La presencia del grupo catecol del anillo B (**Figura 2.16**) parece

ser determinante en la quelación de metales *(Cheng et al., 2000)*. La actividad quelante no conlleva necesariamente a la inactivad de los flavonoides como captadores de radicales libres *(Kostyuk et al., 2001)*.

Tal y como se explicó anteriormente, las especies reactivas de oxígeno parecen estar implicadas en las distintas etapas del proceso cancerígeno, ya que, pueden oxidar directamente al ADN o activar mutágenos. Además, promotores tumorales parecen incrementar las concentraciones de ROS y la inflamación, asociada a elevadas concentraciones de ROS, esta directamente relacionada con la carcinogénesis. Por ello, las propiedades antioxidantes que presentan los flavonoides son *a priori* favorables para la prevención de la oncogénesis.

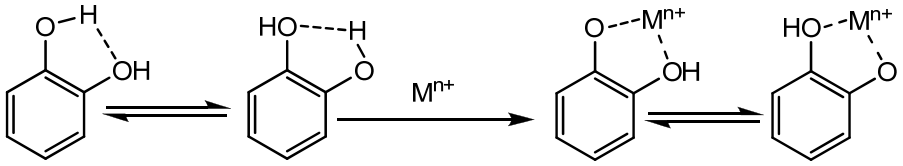


Figura 2.16: Proceso esquemático del mecanismo de quelación de metales de los flavonoides. Representación de los grupos hidroxilos en posición 3' y 4' del anillo C.

Paradójicamente, algunos flavonoides han sido directamente relacionados con una actividad pro-oxidante y mutagénica *(Galati et al., 2002;Yoshino et al., 1999)*. La actividad pro-oxidante fue directamente relacionada con un incremento en el número de grupos hidroxilos *(Cao et al., 1997)*, sin embargo, otros estudios sugieren que la presencia de un grupo pirogalol, en el anillo A o en el anillo C de los flavonoides, es el factor clave capaz de inducir la formación de H_2O_2 *(Hodnick et al., 1986)* y en algunos casos, en presencia de RNOS, provocar incluso lesiones en el ADN *(Ohshima et al., 1998)*. Existen evidencias de que el enlace insaturado entre el carbono 2 y 3 junto con la función 4-oxo del anillo C de las flavonas también puede promover la formación de ROS en medios en presencia de Cu^{+2} *(Cao et al., 1997)*.

Por otro lado, la conjugación que sufren los grupos hidroxilos *in vivo* atenúa el comportamiento pro-oxidante de los flavonoides *(Zhu et al., 1994)* y se ha observado *in vitro* que la vitamina C atenúa la formación de ROS debida a los flavonoides *(Ratty et al., 1988)*, por lo que se podría pensar que la actividad pro-oxidante de algunos flavonoides observada *in vitro* quizás *in vivo* pueda ser modulada.

Los beneficios de consumir una dieta rica en compuestos antioxidantes como frutas y verduras parecen ser evidentes. En el caso de una suplementación de la dieta con compuestos fenólicos como flavonoides y proantocianidinas es necesario un mayor conocimiento de la biodisponibilidad y de los mecanismos en los cuales estas moléculas

están implicadas. Una correcta administración de suplementos antioxidantes, especialmente a largo plazo, podría ayudar en la prevención de enfermedades crónicas.

2.7.6 Biodisponibilidad de los flavonoides.

Aunque no existe una definición única de biodisponibilidad, generalmente bajo este término se intenta incluir la suma de numerosos eventos metabólicos, tales como digestibilidad, absorción, acumulación, excreción. La FDA (*Food and Drug Administration)* define la biodisponibilidad como: "la velocidad y extensión con la cual una sustancia es absorbida y se hace disponible en el sitio de acción".

El concepto de biodisponibilidad desde un punto de vista farmacológico, biodisponibilidad absoluta, indica la velocidad y concentración de la forma inalterada de un principio activo que llega a la circulación sistémica y por lo tanto está disponible para llegar a los tejidos y producir un efecto. La cantidad absorbida suele valorarse como el área bajo la curva (AUC, *Area Under Curve*) en una representación gráfica de concentraciones plasmáticas *versus* tiempo (**Figura 2.17**). La velocidad de absorción es representada por la concentración plasmática máxima alcanzada (C max), y el tiempo requerido (Tmax) para alcanzar dicha concentración máxima. Sin embargo, desde un punto de vista nutricional, generalmente se hace referencia a términos de biodisponibilidad relativa (o comparativa) que comúnmente es usada para comparar la disponibilidad de un compuesto con otro de distinta naturaleza *(Holst et al., 2008)*.

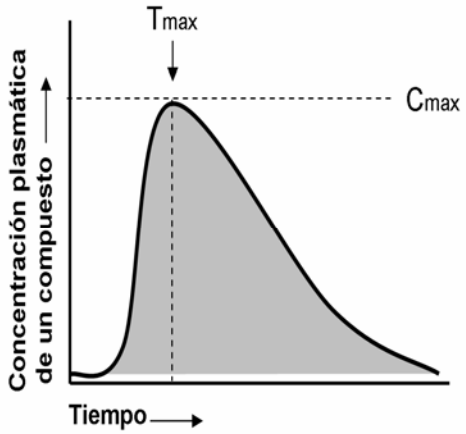


Figura 2.17: Representación de la biodisponibilidad de un compuesto

La biodisponibilidad en todos los casos integra los procesos de liberación, absorción distribución, metabolismo y excreción (generalmente acuñados bajo el acrónimo de LADME, **Figura 2.18**)

Existen numerosos factores que afectan la biodisponibilidad de un compuesto. Dichos factores pueden ser clasificados como exógenos (estructura química del compuesto, matriz alimentaría utilizada para la administración, procesado e incluso la composición y la cantidad de los compuestos co-ingeridos), o factores endógenos (el transito intestinal, la velocidad de vaciamiento gástrico y el metabolismo). La interacción de todos estos factores puede dar lugar grandes variaciones *inter-* e *intra*-individuales *(Scholz et al., 2007)*.

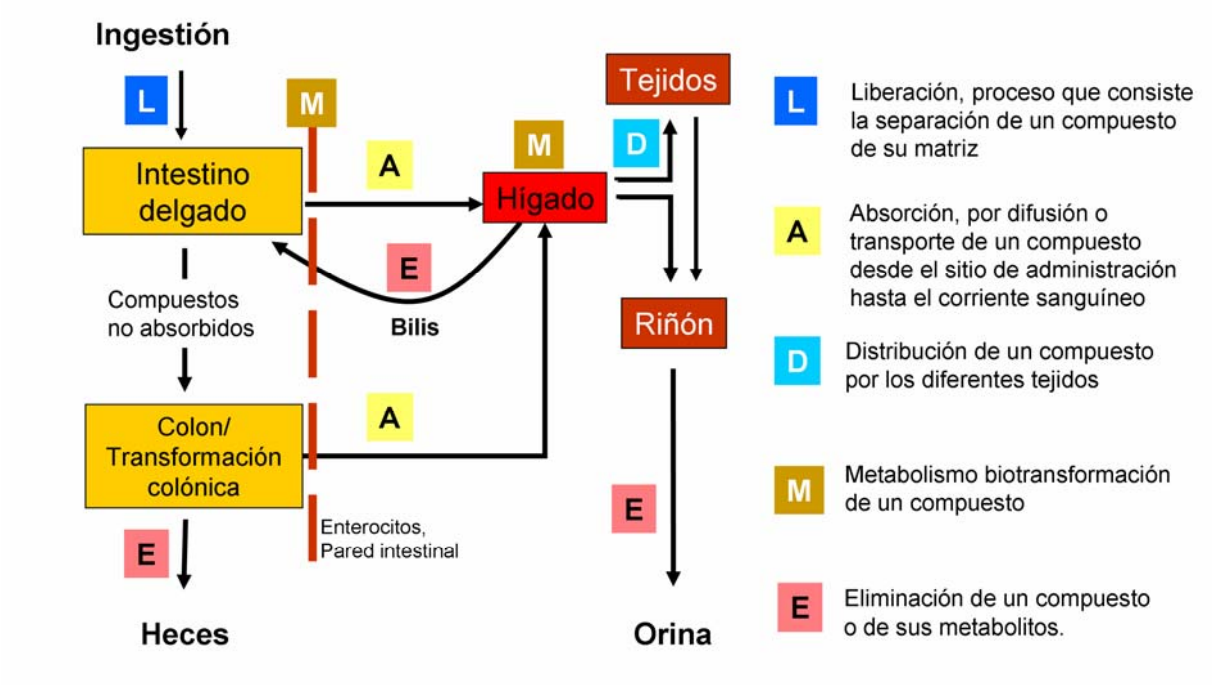


Figura 2.18: Esquema básico de las posibles rutas de los nutrientes y xenobióticos

La biodisponibilidad de los flavonoides es considerada baja en comparación con algunos macronutrientes (ácidos grasos, amino ácidos o azucares) ingeridos por vía oral. Una de las razones fundamentales que explican su baja disponibilidad es que son reconocidos como xenobióticos (término utilizado generalmente para designar una sustancia exógena al organismo) por lo que tienden a ser excretados por diferentes vías en el menor tiempo posible.

2.7.6.1 Absorción y transporte celular.

La absorción se define como el paso de un compuesto desde el sitio de administración, generalmente el tracto gastrointestinal, hasta el corriente sanguíneo. Muchas veces éste termino es utilizado de forma equivocada como sinónimo de disponibilidad.

De nuevo, una multitud de factores tanto exógenos del compuesto (solubilidad, ionización, tamaño, concentración), como endógenos (interacciones con proteínas o competencia por los transportadores de membrana) están involucrados en los procesos de absorción.

Los flavonoides glicosilados necesitan ser hidrolizados para su absorción. Así, la quercetina 4'-glucosa y genistina-7-glucosa (u otros flavonoides conjugados con una glucosa) son rápidamente hidrolizados en la saliva y absorbidos por las células epiteliales bucales. Este mecanismo de hidrólisis es gracias a la acción de las β-glucosidasa,

aunque también se ha comprobado que las bacterias de la cavidad bucal juegan un papel importante *(Walle et al., 2005)*. Otros grupo de flavonoides como las antocianinas, son hidrolizados en el intestino a la forma de aglicona mediante la enzima lactasa phlorodzin hidrolasa (LPH) y posteriormente absorbidos *(Sesink et al., 2003)*. Aunque algunos autores *(Walgren et al., 2000)*, tras haber realizado experimentos con cultivos celulares, sugieren que los glucósidos son absorbidos intestinalmente vía transporte sodio dependiente (SGLT-1)

Los flavan-3-ol, grupo de flavonoides que se encuentra de forma libre monomérica, son fácilmente absorbibles por procesos de transporte de difusión pasiva. En el caso de los esteres de galato, existen evidencias de que el ácido gálico es liberado mediante enzimas esterasas *(Yang et al., 1999)*. La absorción de oligómeros (procianidinas) y polímeros (proantocianidas) es ampliamente discutida. Algunos autores sugieren que tanto los oligómeros como los polímeros no pueden ser absorbidos por los enterocitos (células que forman la pared intestinal) de manera que llegan intactos al colon donde posteriormente son degradados por las bacterias colónicas *(Gonthier et al., 2003)*. Sin embargo, otros autores se decantan por postular la absorción de oligómeros en el intestino tras una previa hidrólisis a sus unidades monoméricas *(Spencer et al., 2001)*. Por otro lado, recientes estudios han demostrado la existencia de oligómeros, en concreto dímeros, en el corriente sanguíneo indicando que los oligómeros, pese a su gran tamaño, también pueden ser absorbidos *(Shoji et al., 2006)*.

2.7.6.2 Distribución

Pocos estudios se han realizado sobre la distribución de flavonoides, debido a la complejidad de los mismos. Experimentos con isótopos radiactivos han permitido una aproximación de la distribución de los flavonoides y sus metabolitos acumulados en los distintos tejidos. Estudios recientemente realizados de experimentación animal, en los cuales se administraba quercetina glucosa marcada radiactivamente con ^{14}C o 3-[^{3}H]epicatequina respectivamente, mostraba que estos dos flavonoides son ampliamente distribuidos y de forma similar en solo media hora por gran parte del aparato digestivo y otros órganos tales como cerebro, testículos, tejido muscular y sangre *(Abrahamse et al., 2005;Graf et al., 2005)*.

2.7.6.3 Metabolismo o biotransformación.

El término de metabolismo es de origen griego, significa cambio, y se refiere al conjunto de biotransformaciones químicas, físicas y biológicas que realizan los seres vivos, tanto en moléculas propias como en xenobióticos, con el fin de poder ser utilizadas para el desarrollo de las funciones vitales o para ser eliminadas evitando posibles daños en el organismo.

Como resultado de las reacciones de biotransformación los metabolitos (compuestos transformados) son químicamente distintos del compuesto del que provienen. En general, los metabolitos formados de aquellos compuestos considerados como xenobióticos suelen ser más hidrofílicos y de mayor tamaño que el compuesto padre. Está característica restringe la distribución de los metabolitos en los diferentes tejidos, disminuye la reabsorción y promueve una eliminación vía urinaria, biliar o fecal.

La biotransformación es generalmente mediada por procesos enzimáticos endógenos, aunque también puede ser producida por la microbiota intestinal. Procesos de hidrólisis, reducción, decarboxilación, deaminación, abertura de anillos heterocíclicos (como el anillo C de los flavonoides) entre otros, suelen producirse en contacto con los microorganismos intestinales.

2.7.6.3.1 Transformaciones enzimáticas

Los procesos enzimáticos de biotransformación son clasificados como procesos de Fase I y de Fase II. Las biotransformaciones dependen de la polaridad y la lipoficidad de los compuestos (**Figura 2.19**). No todos los xenobióticos sufren las dos biotransformaciones, algunos únicamente son sometidos a biotransformación en fase I y otros sufren biotransformación en Fase II sin haber sufrido previamente Fase I *(Liska et al., 2006)*.

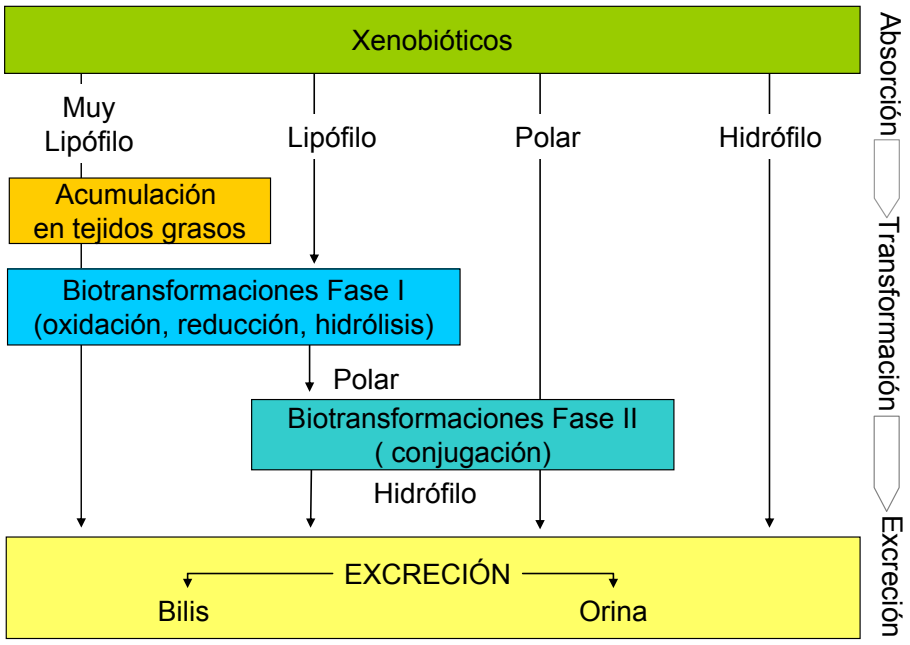


Figura 2.19: Esquema de absorción, transformación y excreción de xenobióticos.

Biotransformaciones de Fase I

Los cambios que se producen durante la Fase I del metabolismo son cambios relativamente pequeños, mientras que los cambios de biotransformaciones de Fase II suele generar metabolitos muy diferentes del compuesto padre del que proviene. Oxidación, reducción e hidrólisis son las biotransformaciones de Fase I más comunes. La finalidad de estás reacciones es exponer o adicionar grupos funcionales, como hidroxilos (—OH), carboxilos (—COOH) o amino ($—NH_2$), para aumentar la polaridad de los compuestos y permitir posteriores reacciones de conjugación en la molécula. Las moléculas que presentan grupos *N*-metilo, anillos aromáticos y las posiciones terminales de las cadenas alquílicas suelen verse afectadas por reacciones de oxidación. Los grupos nitro- azo- y carbonilo tienden a ser reducidas por la acción de las reductasas, mientras que las amidas y los esteres suelen ser hidrolizados por la acción de esterasas *(Graham, 2005)*.

Las reacciones de biotransformación de Fase I son mediadas fundamentalmente por enzimas del complejo citocromo P-450 (CYP), localizadas en el retículo endoplasmático y mitocondrias de las células. La mayor concentración de este tipo de enzimas se encuentra en las células del hígado, aunque también se ha detectado una actividad considerable en la pared intestinal, riñones, pulmones e incluso cerebro *(Lavandera et al., 2007)*. Otras enzimas como las flavin-monooxigenasas, las enzimas alcohol deshidrogenasas o las esteresas también participan en las biotransformaciones de Fase I (**Tabla 2.7**). Los flavonoides debido a su polaridad no suelen verse afectados por este tipo de biotransformaciones.

Tabla 2.7: Enzimas implicadas en las biotransformaciones de Fase I

Enzimas Fase I	Reacción
Enzimas del complejo citocromo P-450 (CYP)	Oxidación de grupos funcionales fácilmente accesibles
Flavín-monooxigenasas	Oxidación de nucleófilos de nitrógeno, azufre y fósforo.
Alcohol deshidrogenasas	Catalizan la conversión del alcohol a aldehído.
Esterasas	Hidroliza enlaces ester, liberando grupos carbonilo y grupo alcohol.

Biotransformaciones de Fase II

Las biotransformaciones de Fase II son reacciones de conjugación catalizadas por enzimas tipo transferasa. Las reacciones de conjugación permiten eliminar fácilmente a los xenobióticos por la orina o la bilis.

La glucuronidación es la reacción de conjugación más frecuente. La enzimas encargadas de está reacción son las uridina glucuroniltransferasas (UGTs) que utiliza el UDP-glucuronido como cofactor. En concreto, los flavonoides sufren transformaciones de tipo *O*-glucuronido, y son considerados como buenos sustratos para estas enzimas.*(Zhang et al., 2007)* Las UGTs están localizadas en el retículo endoplasmático, principalmente, de las células hepáticas, aunque también están presentes en otros tejidos como, intestino, bazo, riñón, piel y testículos *(Nakamura et al., 2008)*.

La reacción de sulfatación consiste en la transferencia de un grupo sulfato desde el cofactor 3´-fosfoadenosil-5´-fosfosulfato (PAPs) a un grupo hidroxilo de los flavonoides. La reacción es catalizada por sulfotransferasas (STs), enzimas solubles que se encuentran en el citosol de células que forman parte de hígado, riñón, intestino y pulmones. Las enzimas pueden conjugar más de un grupo hidroxilo en una misma molécula, siempre y cuando dispongan de PAPs. El sulfato inorgánico libre necesario para la síntesis de PAPs puede ser un factor limitante de esta reacción y genera variaciones inter/intra-individuales *(Pai et al., 2001)*. Cuando un flavonoide se conjuga con un sulfato se convierte en un compuesto ionizado, soluble en agua que se excreta por la orina.

La enzima catecol metilo transferasa (COMT) es la encargada de transferir un grupo metilo a uno de los hidroxilos que forman parte del grupo catecol en presencia de Mn^{+2} utilizando compuesto donador de grupos metilo la *S*-adenosil metionina (SAM) *(Guldberg et al., 1975)*. Las COMTs se encuentran en el citosol de casi todas las células, y al igual que las otras enzimas implicadas en los procesos de biotransformación de Fase II, el hígado es el tejido donde se concentra un mayor número *(Mannisto et al., 1999)*.

La glutationización, que consiste en la adición de glutatión (GSH), a través de su grupo sulfidrilo, y la aminoacidación, que consiste en la formación de una unión peptídica entre el grupo amino de la glicina, y un carboxilo en el xenobiótico, son otras reacciones de biotransformación de Fase II. Los flavonoides debido a su estructura no son buenos sustratos para estas reacciones.

2.7.6.3.2 Transformaciones producidas por la microbiota intestinal.

El intestino de los humanos está colonizado por un elevado número de microorganismos (en el colón, más de 10^{12} microorganismos/cm^3), que viven en simbiosis con los organismos a los que habitan. Las bacterias del tracto gastrointestinal contribuyen a aumentar el rendimiento de la digestión, fermentando proteínas y carbohidratos que no fueron digeridos en el intestino delgado, y produciendo ácidos grasos de cadena corta (butirato, acetato, propionato) que son utilizados como fuente de energía para el organismo. A diferencia de las transformaciones enzimáticas, que producen compuestos polares de mayor tamaño molecular, las transformaciones de la microbiota intestinal consisten fundamentalmente en reacciones de reducción, hidrólisis y rotura de anillos

heterocíclicos que generan compuestos menos polares de bajo peso molecular *(Shamat, 1993)*.

Los flavonoides y otros xenobióticos que no son absorbidos en el intestino delgado, llegan intactos al intestino grueso donde se exponen a la actividad de los microorganismos. A estos se le suman los flavonoides que han sido absorbidos y excretados al interior del intestino por la bilis; o aquellos que son absorbidos en los enterocitos y devueltos al tracto gastrointestinal *(Scalbert et al., 2000)*.

Estudios *in vitro (Rechner et al., 2004)*, *(Aura et al., 2002)*; *(Déprez et al., 2000)*; *(Appeldoorn et al., 2009)* e *in vivo (Ward et al., 2004)* han demostrado que los flavonoides tanto monoméricos como oligoméricos son degradados a compuestos fenólicos más pequeños tras abertura del anillo C y pueden ir sufriendo posteriores hidrólisis (**Figura 2.20**).

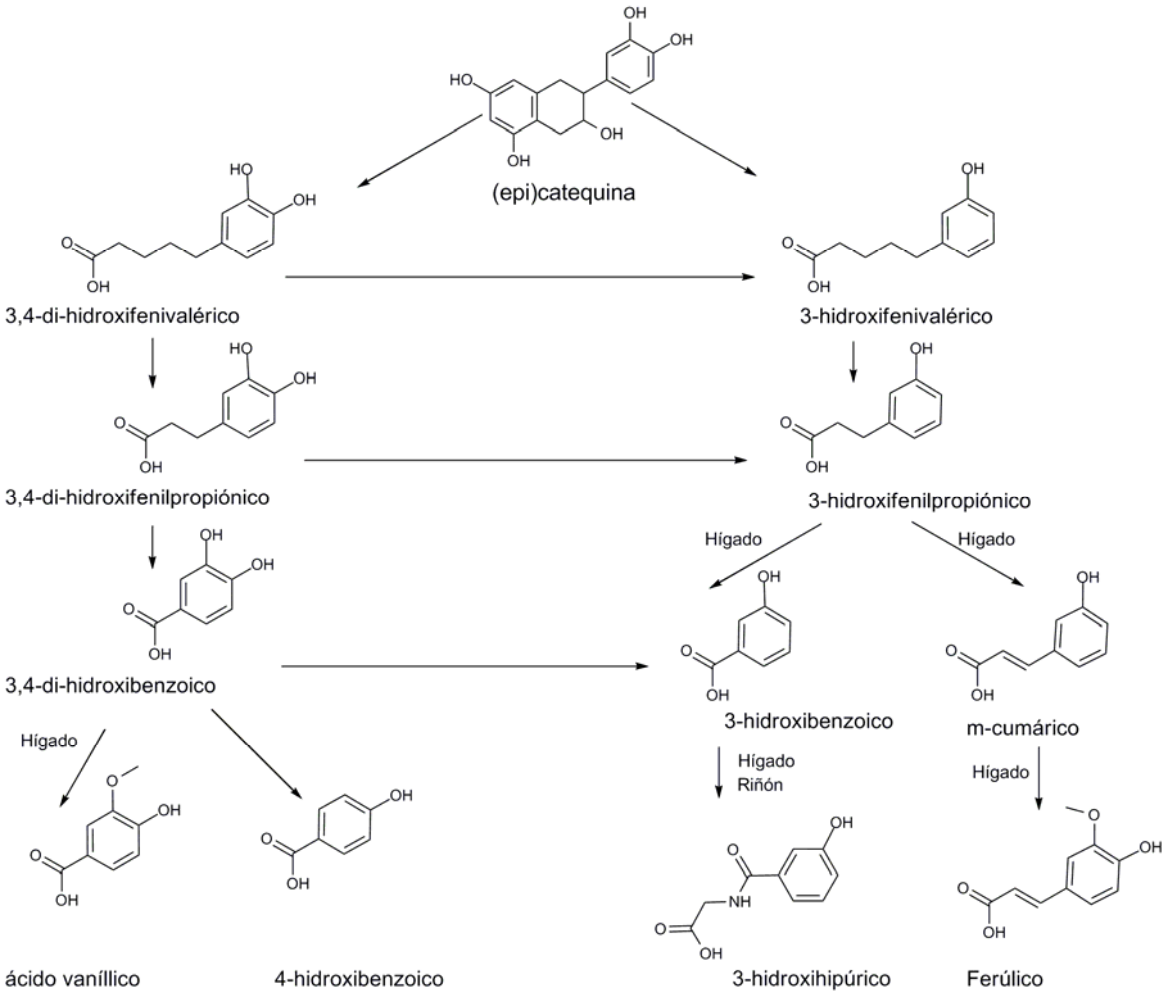


Figura 2.20: Posibles biotransformaciones colónicas de las (epi)catequinas*(Scalbert et al., 2000)*

2.8 Metodología de identificación y análisis de compuestos fenólicos.

Los extractos vegetales contienen mezclas complejas de compuestos fenólicos que varían en composición desde ácidos fenólicos simples hasta taninos condensados o hidrolizables. Para establecer una relación estructura-actividad es necesario definir los compuestos mayoritarios que componen la mezcla y, así posteriormente, poder explicar su actividad biológica.

2.8.1 Extracción de los compuestos fenólicos

La extracción de los compuestos fenólicos de una muestra vegetal se realiza mediante el uso de disolventes de diferente polaridad. La elección de los disolventes está en función de los compuestos que se quieran separar. Los compuestos menos polares son extraídos con disolventes orgánicos, mientras que los compuestos más polares (glucósidos) son extraídos con alcoholes o mezclas de alcohol-agua. El rendimiento de los métodos de extracción no sólo está en función del disolvente, la temperatura y el tiempo de extracción son otros de los factores importantes *(Spigno et al., 2007)*. Los taninos tanto hidrolizables como condensados pueden ser difíciles de extraer de la matriz vegetal que los envuelve. La cantidad de compuestos fenólicos no extraíbles puede llegar a un 90% en algunos casos y está en función de la matriz vegetal y del grado de polimerización de los taninos *(Matthews et al., 1997)*.

2.8.2 Métodos cromatográficos de separación

El extracto crudo obtenido de la fuente vegetal, como se comentó anteriormente, es una mezcla compleja que puede variar desde fenoles simples hasta compuestos fenólicos de naturaleza polimérica. Los métodos cromatográficos permiten separar los diferentes compuestos fenólicos según su tamaño, volatilidad o su grado de hidrofobicidad.

2.8.2.1 Cromatografía de gases. (GC)

Para la separación de mezclas de compuestos polifenólicos, la cromatografía de gases es generalmente descartable, debido a la baja volatilidad de los compuestos (especialmente polímeros) y al tedioso proceso de derivatización de los compuestos para aumentar su volatilidad. Sin embargo, el acoplamiento de detectores de espectrómetros de masas a la cromatografía de gases ha empujado a algunos investigadores a optimizar y aplicar métodos de separación y determinación de mezclas de compuestos fenólicos utilizando estas técnicas. *(Deng et al., 2003;Fiamegos et al., 2004;Proestos et al., 2006)*

2.8.2.2 Cromatografía de exclusión por tamaño (SEC)

Está técnica se usa para separar de manera preparativa los compuestos de un extracto polifenólico en fracciones según el tamaño molecular, aunque también se ha utilizado para determinar el peso molecular de proantocianidinas mediante el uso de patrones *(Williams et al., 1983)*. La separación de las procianidinas según el grado de polimerización se basa en el tamaño y la polaridad de los oligómeros. A medida que aumenta el grado de polimerización los polímeros son más polares.

Sephadex LH-20 y Toyopearl HW-40 son las resinas frecuentemente utilizados para separar proantocianidinas según el grado de polimerización *(Sun et al., 1998;Yanagida et al., 1999) (Jerez et al., 2007;Torres et al., 2002)*.

2.8.2.3 Cromatografía líquida de alta resolución (HPLC)

La cromatografía líquida de alta resolución (HPLC) es la técnica más utilizada en la separación y análisis de los compuestos fenólicos.

El uso de columnas de fase normal (NP, *normal phase*) ha sido preferentemente escogido para el análisis de proantocianidinas *(Lazarus et al., 1999;Rigaud et al., 1993)*. NP-HPLC permite separar y cuantificar proantocianidinas con grados de polimerización entre cinco y doce *(Adamson et al., 1999;Sudjaroen et al., 2005)*. Sin embargo, esta técnica parece presentar varias limitaciones que complican el uso rutinario tales como: la falta de resolución, el uso de disolventes clorados para la elución o una incompleta elución de las proantocianidinas de elevado grado de polimerización *(Wolfgang Hümmer, 2008)*. Recientemente, Kelm y colaboradores *(Kelm et al., 2006)* han desarrollado un método, tanto a escala preparativa como analítica, aparentemente mucho más eficaz utilizando una fase estacionaria de tipo diol.

La cromatografía líquida de alta resolución en fase reversa (RP-HPLC) es la técnica generalmente usada para separar compuestos fenólicos tanto a nivel de escala analítica como preparativa. Existen numerosos artículos de revisión de los diferentes métodos utilizados para la separación de los compuestos fenólicos *(*"Bibliography of liquid column chromatography 1971-1973 and survey of applications*," 1976;Daigle et al., 1983;Robards et al., 1997) (Merken et al., 2000)*. La separación se basa en el uso de sistemas de gradiente binarios, que consisten en una solución acuosa con un modificador de pH y un solvente orgánico soluble en agua como metanol (MeOH) o Acetonitrilo (ACN). Los modificadores de pH dependen de la técnica de detección que se utilice. Para UV-DAD el ácido trifluroroacético (TFA) es frecuentemente utilizado, sin embargo, esté ácido presenta numerosos problemas en la detección por espectrometría de masas *(Shou et al., 2005)*. El ácido fórmico y el ácido acético son otros modificadores de pH ampliamente utilizados.

Un gran porcentaje de las separaciones de los compuestos fenólicos utilizan columnas C-18 entre 100 y 300mm de longitud, con tamaños de partícula menores de 10µm *(Robards et al., 1997)*. Los factores que influyen en la separación son la estereoquímica, las sustituciones, peso molecular y la polaridad de los compuestos.

La cromatografía líquida de ultra resolución (UPLC) presenta grandes ventajas sobre el HPLC. El UPLC ha permitido mejorar la resolución, disminuir el tiempo de análisis y aumentar la sensibilidad de la detección. El uso de columnas con un diámetro de partícula muy pequeño (› 2µm) y sistemas de gradiente que operan a alta presión son las claves de está técnica *(De Villiers et al., 2006;Spácil et al., 2008)*, que posiblemente en un futuro no muy lejano dejará de lado al HPLC gracias a sus ventajas.

2.8.3 Técnicas de detección.

Las técnicas de detección acopladas al HPLC más utilizadas en el análisis de los compuestos fenólicos se describen brevemente a continuación.

2.8.3.1 Espectroscopía UV-VIS

Los espectros de absorbancia de los compuestos fenólicos consisten en dos bandas de típicas de absorción (**Tabla 2.8**) con un máximo entre 240-285nm (banda II) y otro entre 300-550nm (banda I) *(Richardson, 1985)*. Cada familia absorbe a una longitud de onda característica que depende del número, posición y tipo de sustituyentes adicionales en el anillo. Por ejemplo, los flavonoles se detectan fácilmente a 360nm mientras que las antocianidinas presentan un máximo de absorbancia a 550nm. Las longitudes de onda más empleadas para los análisis rutinarios son 214 nm y 280nm.

Tabla 2.8: Bandas de absorción UV-Vis de algunos compuestos fenólicos *(Robards et al., 1997)*.

Compuestos	Banda II (nm)	Banda I (nm)
Fenoles simples	266–295	
Ácidos fenólicos	235-305	
Ácidos hidroxicinámicos	227-245	310-332
Flavonas	250-280	310-350
Flavonoles	250-280	350-385
Isoflavonas	245-275	310-330
Flavanonas	275-295	310-330
Antocinidinas	265-275	465-560

La utilización de un detector de diodos en línea (DAD, *Diode Array Detector*) supone enormes ventajas sobre el tradicional detector de UV, ya que permite analizar una

muestra a diferentes longitudes de onda, ofreciendo un espectro en tres dimensiones y mejorando los métodos de cuantificación. La técnica DAD permite en una sola inyección analizar diferentes familias de compuestos fenólicos *(Merken et al., 2000)*.

2.8.3.2 Espectroscopía de fluorescencia

La técnica de detección por fluorescencia se usa relativamente poco para los compuestos fenólicos debido a que el número de flavonoides que exhiben de manera natural fluorescencia es limitado *(Bader et al., 2004)*. La ventaja que ofrece esta técnica es que los compuestos que pueden ser analizados son fácilmente detectados en matrices o mezclas complejas *(De Rijke et al., 2001)*. Las isoflavonas *(De Rijke et al., 2002)* y los flavonoides con un grupo hidroxilo libre en el C-3, como es el caso de las catequinas pueden ser detectadas por esta técnica *(Sengupta et al., 1979)*.

2.8.3.3 Espectrometría de masas (MS)

La espectrometría de masas es una técnica de detección basada en la ionización de los compuestos, la separación y registro de los iones producidos, según su relación masa/carga (*m/z*). Esta técnica es idónea para compuestos no volátiles, polares y termolábiles. La técnica de HPLC-MS proporciona una alta sensibilidad, ofreciendo muy buenos limites de detección.

El acoplamiento de la técnica de espectrometría de masas a la cromatografía de líquidos de alta resolución supuso bastantes problemas debido a que la MS opera a presión atmosférica ionizando los compuestos mientras que HPLC trabaja en fase líquida. Los caudales relativamente elevados que se manejan en HPLC obligan al empleo de una interfase cuya misión es compatibilizar la cromatografía líquida con el espectrómetro de masas. Las principales interfases generalmente usadas para ionizar a presión atmosférica (API) son la ionización química a presión atmosférica (APCI) y la ionización por electrospray (ESI) *(Vékey, 2001)*. En la **Figura 2.21** se muestra un esquema simple de cada una de ellas.

ESI ioniza las muestras directamente desde soluciones acuosas, orgánicas o mezclas de ambos, mediante la formación de un spray de gotas. Estas gotas van reduciendo su tamaño debido a fenómenos de evaporación del solvente (por efecto del gas y de la temperatura) y a la desintegración de las mismas gotas en otras de menor tamaño (3-10 nm). Al disminuir las gotas de tamaño, la densidad de carga eléctrica en su superficie aumenta. La mutua repulsión entre cargas de igual signo en la superficie llega a ser tan grande que se superan las fuerzas de cohesión superficial y los iones dejan las gotas en la forma que se conoce como "cono de Taylor", siendo dirigidas hacia el analizador mediante la aplicación de vacíos crecientes, que van desde presión atmosférica en la introducción de la muestra, hasta valores de vacío de 10^{-9} en la zona del analizador-detector. La vaporización de estas gotas cargadas da lugar a la producción de iones con

una o varias cargas en fase gaseosa. Los iones formados se "extraen" del spray gracias a la diferencia de potencial existente entre el capilar y el cono de entrada.

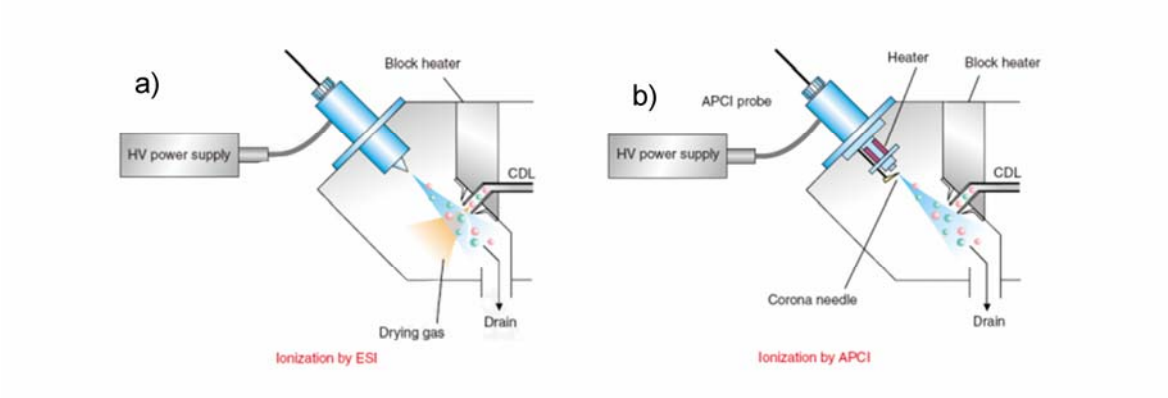


Figura 2.21: Esquema de ionización química a presión atmosférica (API)
a) Ionización por electrospray (ESI), b) Ionización química a presión atmosférica (APCI)

La sonda de APCI se utiliza para el análisis de moléculas de menos de 1000 Da, con baja o moderada polaridad. Estos analitos deben poseer además cierta volatilidad y no ser excesivamente termolábiles. Utilizando la sonda de APCI el líquido que procede del LC es nebulizado y rápidamente evaporado por la acción de una temperatura elevada (300-500 $^{\circ}$C). Para incrementar el proceso de ionización se suele aplicar una descarga en corona, del orden de 2-6 kV, justo a la salida de la sonda de APCI. Esta descarga eléctrica no sólo ioniza las moléculas de analito, sino también las del disolvente de la fase móvil, por lo que se facilitan las reacciones ión-molécula, de lo que resulta la ionización química de los mismos. Una importante ventaja que presenta la sonda de APCI es que el flujo óptimo de trabajo es muy similar al alcanzado en la cromatografía de líquidos (0,7-1,2 mL/min) *(Quintela et al., 2005;Somogyi et al., 2008)*.

La ionización por desorción de una matriz mediante láser (MALDI) es otra técnica de ionización frecuentemente utilizada para el análisis de oligómeros y polímeros (taninos condensados o hidrolizables). La desorción y la ionización del analito se inducen mediante un pulso de láser, la energía del láser es absorbida por la matriz, desprendiéndose iones del analito desde la superficie *(Stults, 1995)*. El uso de una superficie sólida para el secado de la muestra y posterior ionización hace bastante difícil el acoplamiento al HPLC. Sin embargo, dado que MALDI ofrece algunas ventajas sobre ESI para el análisis de polímeros se ha visto algunos estudios en los que el MALDI se acopla al HPLC como detector off-line *(Maslen et al., 2007)*.

Los iones formados son analizados por diferentes técnicas tales como trampa de iones, tiempo de vuelo (TOF) o cuadrupolos de barras (Q). El tipo de analizador influye en la resolución, el intervalo de masas a registrar y también en la posibilidad de realizar experimentos en tándem.

Espectrometría de masas en tándem (MS/MS ó MS^n) es una técnica que se basa en la detección de los compuestos mediante la fragmentación de los iones. La trampa de iones (IT), el triple cuadrupolo (QqQ) y el cuadrupolo-TOF (Q-TOF) permiten realizar experimentos en tándem *(Schermann, 2008)*. Todos estos diseños presentan el mismo esquema general. Primero, los iones de la muestra son formados en la fuente de ionización. Uno de los iones es seleccionado para ser analizado por tándem. El ión seleccionado es excitado y fragmentado con la ayuda de un gas inerte en una celda de colisión formando iones producto. Este proceso recibe el nombre de disociación inducida por colisión (CID). Los iones productos son nuevamente analizados de acuerdo con su relación m/z *(Vékey, 2001)*.

Los cuatro modos principales de operación MS/MS se describen a continuación para el triple cuadrupolo (QqQ) que se usa frecuentemente debido a su relativa sencillez y versatilidad *(Watson et al., 2003)*.

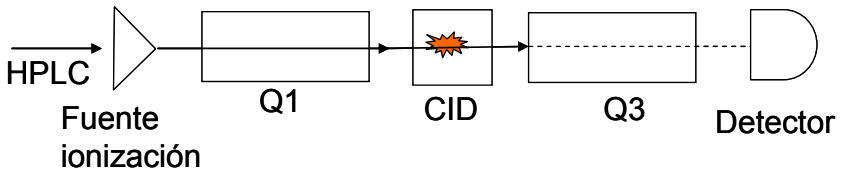


Figura 2.22: Representación esquemática de un triple cuadrupolo (QqQ).

(i) Barrido de producto de iones (*product ion scan*), este modo filtra en Q1 (**Figura 2.22**) el ión seleccionado y Q3 registra todos los iones productos formados del ión seleccionado; (ii) Barrido del ión precursor (*precursor ion scan*), este modo permite fragmentar el ión seleccionado en Q3 y el barrido en Q1 muestra todos los precursores que han sido fragmentados y que presentaban el ión precursor;.(iii) Barrido de perdidas neutras (*neutral loss scan*), este modo permite escanear en Q1 y Q3 de manera sincronizada, registrando los iones que presentan perdidas de una masa neutra especifica; (iv) Monitorización de reacción múltiple (*multiple reacction monitoring*), permite seleccionar un ión en Q1 y un ión producto en Q3.

En general, ESI y APCI han sido ampliamente utilizadas para la ionización de los compuestos fenólicos tanto en modo positivo como en modo negativo *(Cuyckens et al., 2004)*. El modo positivo es más adecuado para la detección de antocinidinas, que se encuentran en forma catiónica en medio ácido, sin embargo, cuando están derivadas con ácidos carboxílicos pueden ser analizadas en modo negativo *(Fulcrand et al., 1998)*. Entre las fuentes API más utilizadas, ESI parece presentar la mayor eficiencia de ionización sobre los compuestos fenólicos *(Jussi-Pekka Rauha, 2001)*. MALDI es fundamentalmente usada para el análisis de oligómeros y polímeros de compuestos fenólicos. Taninos condensados, polímeros de antocianinas y taninos hidrolizables han

sido analizados utilizando MALDI-TOF *(Babior, 2000) (Vivas et al., 2004)*, *(Ishida et al., 2005)*, *(Monagas et al.)*.

Las técnicas de espectrometría en tándem, gracias a su alta sensibilidad y robustez permiten el análisis de compuestos fenólicos de matrices complejas *(Liu et al., 2005a)*, *(Sánchez-Rabaneda et al., 2004)*, *(Määttä-Riihinen et al., 2004)*, el análisis de metabolitos generados tras la ingesta de compuestos fenólicos *(Dvorakova et al., 2008;Felgines et al., 2003;Meng et al., 2001;Sang et al., 2008;Xu et al., 2007)* y también, el análisis de biomarcadores de estrés oxidativo *(Watson et al., 2003)*.

2.8.4 Evaluación de la actividad antioxidante

La evaluación de la actividad antioxidante de un compuesto o en particular, la actividad antioxidante de un alimento funcional puede realizarse respondiendo a las siguientes pautas.

I. Evaluación de la actividad antioxidante *in vitro* mediante pruebas químicas.

II. Evaluación de la actividad antioxidante y de la actividad frente al daño oxidativo en cultivos celulares.

III. Evaluación de la actividad antioxidante *in vivo* tras la ingesta en animales y/o humanos.

Los métodos utilizados en cada uno de estos niveles son numerosos y la gran mayoría han sido adaptados según los requerimientos, por ejemplo, métodos químicos en solución son utilizados, tras algunas modificaciones, en cultivos celulares. Por ello es difícil etiquetar los métodos aplicados a los diferentes niveles. A continuación se describen de manera general los métodos más utilizados para evaluar la actividad antioxidante *in vitro e in vivo.*

2.8.4.1 Métodos químicos en solución

Evaluar la capacidad antioxidante *in vitro*, de compuestos puros, extractos o alimentos, aporta de manera, simple, rápida y no invasiva, información relevante sobre las posibles sinergias u otros procesos que puedan generarse como resultado de la interacción de los compuestos con el medio o la matriz de la muestra analizada. Generalmente los resultados difieren de los obtenidos *in vivo*, pero los resultados *in vitro* pueden ayudarnos a resolver problemas iniciales. Así, la capacidad antioxidante puede variar según el pH, la solubilidad, la temperatura del tratamiento al que pueda someterse la muestra o el almacenamiento de los compuestos escogidos como antioxidantes. Por lo tanto, el estudio y conocimiento de la relación estructura-actividad antioxidante de los

compuestos seleccionados nos dará una aproximación a las propiedades que pueda ejercer *in vivo*.

La transferencia de átomos de hidrógeno o/y la transferencia electrónica son los dos mecanismos fundamentales de actuación de un antioxidante. Estas reacciones difieren en la cinética y el potencial de ionización pero el resultado final de ambas es el mismo *(Prior et al., 2005)*. Ambos procesos pueden ocurrir simultáneamente y dominará uno u otro de los mecanismos en función de la estructura del antioxidante y/o del radical, de la solubilidad, del coeficiente de partición y del medio donde tenga lugar la reacción.

La necesidad de estandarizar los métodos antioxidantes usados en alimentos, extractos vegetales, nutracéuticos u otros suplementos dietéticos ha derivado en una clasificación de los métodos antioxidantes en función del mecanismo de actuación.

Los métodos de transferencia de átomos de hidrógeno (**HAT**) son los más comunes y miden la habilidad de un antioxidante para neutralizar un radical por donación de un átomo de hidrógeno. Las reacciones HAT son independientes del pH y del disolvente. Generalmente, los métodos que miden la transferencia de átomos de hidrógeno son rápidos debido a que las reacciones se completan en cuestión de minutos.

Por el contrario, los métodos basados en la transferencia electrónica (**SET**) miden la eficacia de un antioxidante para transferir un electrón y reducir un compuesto *(Wright et al., 2001)*. Las reacciones SET dependen del pH y son lentas ,por ello, los resultados de estos métodos se basan en el porcentaje de disminución del radical *(Prior et al., 2005)*.

Por lo general, la actividad antioxidante ocurre simultáneamente por ambos mecanismos, y tal como hemos comentado anteriormente, la manifestación del mecanismo dominante estará en función de la estructura del antioxidante y del pH. En ocasiones, diferenciar entre uno u otro mecanismo es bastante complicado por lo que se ha creado un grupo de clasificación de métodos antioxidantes basado en ambos mecanismos de actuación *(Prior et al., 2005)*.

2.8.4.1.1 Métodos de evaluación de la actividad antioxidante basados en ambos mecanismos de actuación: HAT y SET

Ensayo del DPPH (2,2-Difenil-1-picrilhidracilo)

Este ensayo se basa en la reducción del radical DPPH por los antioxidantes de la muestra. La acción de los antioxidantes puede ser medida por disminución de la señal del radical por EPR o por lo disminución de absorbancia que se observa a medida que el radical es reducido por el antioxidante *(Prior et al., 2005)*. Se considera un método sencillo y fácil de aplicar, los resultados que se obtienen son altamente reproducibles y

comparables con otros métodos que miden de la misma forma la capacidad secuestradora de radicales libres como es el caso del ABTS *(Gil et al., 2000)*. El DPPH es un buen método para medir la capacidad antioxidante en fracciones o alimentos. No obstante algunos antioxidantes que presentan un espectro de absorción similar al del radical, como es el caso de los carotenoides, pueden causar interferencias si son cuantificados por UV-VIS *(Nomura et al., 1997)*.

Ensayo TEAC (Trolox Equivalent Antioxidant Capacity)

El ensayo TEAC o ensayo del radical ABTS (2,2-azinobis–(3-etilbenzotiazolin-6-sulfonato)) se basa en medir la capacidad de captación del radical cation $ABTS^{\bullet+}$. En este ensayo, el ABTS es oxidado *in situ,* generalmente por peróxido de hidrógeno, hasta su forma de radical catión. El $ABTS^{\bullet+}$ presenta una coloración verde-azulada muy intensa a una longitud de onda de 734nm que disminuye a medida que los antioxidantes reducen el radical *(Sánchez-Moreno, 2002)*. El ensayo TEAC presenta otras variaciones de protocolo que consisten en hacer reaccionar el ABTS con dióxido de manganeso, persulfato potásico o ABAP (2,2'-azo-bis-(2-amidinopropano), pero estas modificaciones presentan el inconveniente de que requieren largos tiempos de incubación o altas temperaturas *(Prior et al., 2005)*.

A diferencia del DPPH, el radical catión ABTS presenta la ventaja de que es soluble tanto en medios acuosos como orgánicos, lo cual convierte al ensayo TEAC en un método apto para determinar la capacidad antioxidante en soluciones químicas y también en fluidos biológicos *(Awika et al., 2003)*.

2.8.4.1.2 Métodos de evaluación de actividad antioxidante basados en mecanismos HAT

Ensayo ORAC (oxygen radical absorbance capacity)

Este método permite medir la inhibición del radicales peróxido ($ROO^{\bullet}$) generados mediante la descomposición térmica de un compuesto azo, generalmente AAPH (2,2'-azo-bis (2-amidinopropano) dihidrocloruro). En las primeras versiones de este método se hacia reaccionar al radical peroxilo con un producto fluorescente (B-ficoeritrina, B-PE) para formar uno no fluorescente. La disminución de fluorescencia indicaba el daño producido por la reacción del radical *(Cao et al., 1994)*. Debido a la inespecificidad del B-PE, el método fue posteriormente mejorado. La utilización de FL (3,6-Dihidroxispiro[isobenzofuran-1(3H),9-[9H] xanten]-3-ona) un producto fluorescente mucho más estable y más selectivo, ha permitido medir con exactitud la capacidad antioxidante frente a radicales peróxido *(Huang et al., 2002)*.

Ensayo TRAP (total peroxyl radical-trapping antioxidant parameter)

Es un ensayo muy similar al anteriormente explicado, el ORAC. Este ensayo permite medir la eficacia con la que los compuestos antioxidantes actúan frente a los radicales peroxilo generados por compuestos azo tales como AAPH o ABAP ([2-2'-azobis(2-amidinopropano)dihidrocloridro]). Dicha habilidad se puede medir por fluorescencia mediante el uso de R-ficoeritrina *(DeLange et al., 1989)* o por absorbancia mediante el uso de ABTS (2,2-azino-bis (3-etilbezotiazolin-6-sulfonato)) *(Bartosz et al., 1998)*.
El ensayo del TRAP es generalmente escogido para medir la capacidad antioxidante de compuestos como glutatión, vitamina C y α-tocoferol en muestras de suero o plasma *(Wayner et al., 1987)*.

Ensayo TOSC (total oxidant scavenging capacity)

Este método permite medir la capacidad de transferir un átomo de hidrógeno a radicales hidroxilo, peroxilo y peroxinitrito. El método utiliza el área bajo la curva para determinar la respuesta de los antioxidantes frente a los radicales *(Regoli et al., 1999)*.

2.8.4.1.3 Evaluación de actividad antioxidante basados en mecanismo SET

Ensayo FRAP (Ferric reducing antioxidant power)

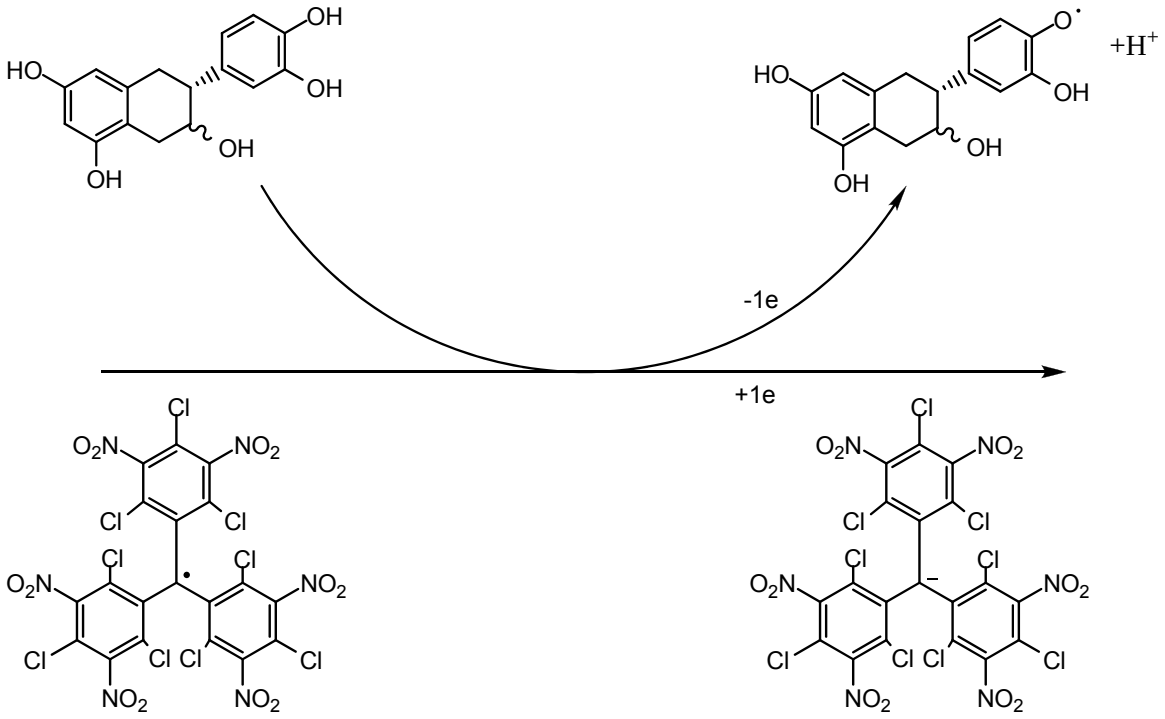
Este ensayo estima la actividad antioxidante como una medida de la capacidad de los antioxidantes para reducir el complejo 2,4,6-tripiridil-s-triazina (TPTZ) a su forma ferrosa. La forma ferrosa es un producto coloreado que permite ser cuantificado por espectrofotometría a 595nm.

Este ensayo ha sido ampliamente utilizado para medir la capacidad antioxidante en muestras de suero o plasma, sin embargo no es recomendable debido a que no detecta la capacidad de los antioxidantes que actúan mediante transferencia de hidrógenos, por lo que genera resultados erróneos *(Ou et al., 2002)*.

Ensayo del radical HNTTM (tris (2,4,6-tricloro-3,5-dinitrofenil)-metilo)

El radical HNTTM, (tris (2,4,6-tricloro-3,5-dinitrofenil)-metilo), (**Figura 2.23**) fue sintetizado en nuestro laboratorio del actual Instituto de Química Avanzada de Catalunya (IQAC). La estructura química del radical estable HNTTM, no permite la transferencia de átomos de hidrógeno de los antioxidantes debido al impedimento estérico de los seis átomos de cloro en posición -*orto*. El elevado potencial de reducción (0.58V) convierte al radical HNTTM en un buen captador de electrones.

La capacidad donadora de electrones del antioxidante al radical es cuantificada mediante la disminución de la señal del radical por EPR o UV *(Jiménez et al., 2004;Torres et al., 2003)*.



HNTTM HNTTM⁻

Figura 2.23: Transferencia electrónica entre la (—)-Ec y el radical estable HNTTM.

Ensayo del radical TNPTM tris(2,3,5,6-tetracloro-4-nitrofenil)-metilo

El radical TNPTM, al igual que el HNTTM también fue sintetizado en nuestro laboratorio, es un radical estable que permite medir únicamente la transferencia electrónica. La diferencia entre los ensayos del radical HNTTM y el radical TNPTM radica en la selectividad del TNPTM con los grupos pirogalol, es decir, sólo aquellas moléculas que presentan un grupo pirogalol en su estructura reaccionan con el TNPTM. Los ensayos con los radicales HNTTM y TNPTM permiten clasificar los polifenoles en función de su capacidad para transferir electrones *(Torres et al., 2007)*.

2.8.4.2 Ensayos en medios lipídicos

Ensayo en emulsiones O/W de aceite de maíz

Las emulsiones representan a un gran grupo de sistemas coloidales relevantes para la industria alimentaria. Uno de los problemas que afectan a este tipo de productos es la

oxidación lipídica, responsable del desarrollo del enranciamiento, con la consiguiente aparición de sabores y aromas desagradables y la disminución de su calidad nutricional.

Además, los sistemas biológicos son más semejantes a sistemas coloidales que a soluciones. Los lípidos, diana de los radicales libres, se deterioran rápidamente es por ello que la evaluación de la actividad antioxidante en emulsiones es un dato importante que puede ayudar a definir las diferentes aplicaciones de los compuestos antioxidantes.

Este ensayo determina la inhibición de la oxidación lipídica en emulsiones por cuantificación de la concentración de hidroperóxidos formados (dienos conjugados) que pueden son determinados espectrofotométricamente a 234nm o por cuantificación de hexanal mediante el uso de técnicas de cromatografía de gases *(Schwarz et al., 2000)*.

Ensayos en aceites. Evaluación de la autooxidación lipídica

La autooxidación lipídica es un proceso exotérmico, lo cual permite ser evaluada mediante calorimetría diferencial de barrido (DSC). La técnica de DSC es ideal para calcular los parámetros cinéticos de inhibición de autooxidación de ácidos grasos insaturados y para medir la actividad antioxidante frente a lípidos *(Litwinienko et al., 1999)*. Además permite determinar si los ingredientes de alimentos funcionales o fracciones polifenólicas tienden a actuar como antioxidantes o por el contrario inducen peroxidación *(Litwinienko et al., 1998)*

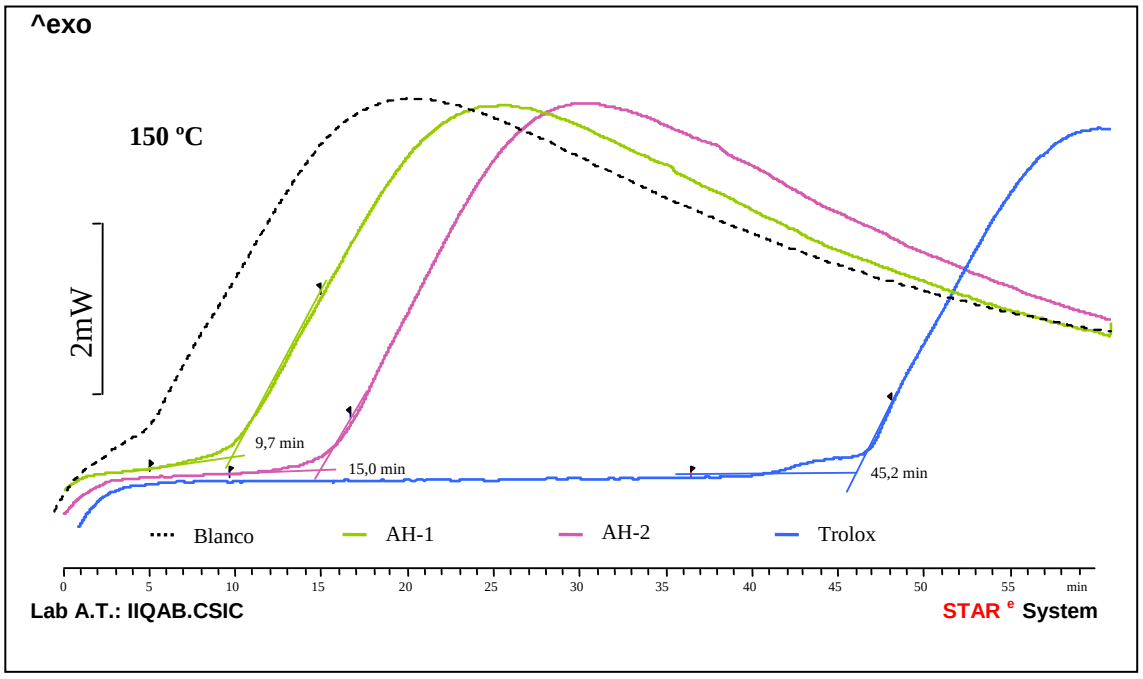


Figura 2.24: Ejemplo de un ensayo de inhibición de autooxidación lipídica medida por calorimetría diferencial de barrido (DSC). Las curvas muestran que los antioxidantes (AH-1 y 2) son menos eficaces frente a la inhibición de la autooxidación lipídica que el antioxidante de referencia (Trolox).

2.8.4.3 Ensayos con modelos celulares

Los modelos celulares son considerados una buena herramienta para evaluar la actividad antioxidante y otras actividades biológicas relacionadas en la prevención de enfermedades como el cáncer *(Liu et al., 2005b)*. Las pautas ha seguir en los ensayos celulares presentan los siguientes pasos: (i) caracterización de las dosis citotóxicas de la muestra; (ii) ensayos en cultivos celulares con dosis no citotóxicas; (iii) inducción al estrés oxidativo o ensayos en líneas celulares especificas (Caco-2, carcinoma de colon; HepG2 carcinoma hepático; U937, leucemia mieloide; Jurkat-T, leucemia) y (iv) valoración del efecto protector proporcionado *(García, 2005)*.

En la **Tabla 2.9** se incluyen algunos métodos que pueden ayudarnos entender los mecanismos de acción de los compuestos fenólicos frente al cáncer u otras enfermedades.

Tabla 2.9: modelos celulares para evaluar los efectos protectores de un compuesto antioxidante en el desarrollo de cáncer *(Liu et al., 2005b)*

Modelos celulares	Valoración del efecto protector
Evaluación de proliferación celular	Inhibición de la proliferación
Estudio del ciclo celular	Control de la etapa G1, evaluación del cociente G1/S
Estudio de apoptosis	Inhibición /Inducción de apoptosis
Angiogénesis	Inhibición de la angiogénesis
Estudio de inhibición de enzimas	Inhibición de COX-2, formación prostaglandinas
Daño oxidativo al ADN	8-OH-dG, ensayo cometa

2.8.4.4 Marcadores de estrés oxidativo.

El gran numero de fuentes por las que se pueden generar especies reactivas en un individuo y la variedad de biomoléculas con las que puede interaccionar, dificulta en gran medida realizar una predicción del daño oxidativo o de la acción de los antioxidantes introducidos por la dieta. En ciertos casos, un incremento de especies reactivas puede ocurrir sin generarse una disminución de los antioxidantes; o una oxidación lipídica puede tener lugar sin que se produzca daño oxidativo al ADN *(Han et al., 2000)*. Una alternativa a los problemas analíticos generados a la hora de medir el estrés oxidativo es

la utilización de biomarcadores. Los marcadores de estrés oxidativo reflejan cambios en los sistemas biológicos relacionados con el daño de los radicales sobre las biomoléculas. Las productos estables originados como consecuencia del daño oxidativo a las biomoléculas (ver apartado 2.4.) son utilizados como marcadores de estrés oxidativo en individuos (**Figura 2.25**). El objetivo de usar marcadores de estrés oxidativo es la detección de un cambio fisiológico en el organismo, el cual esta íntimamente relacionado con un efecto adverso de salud.

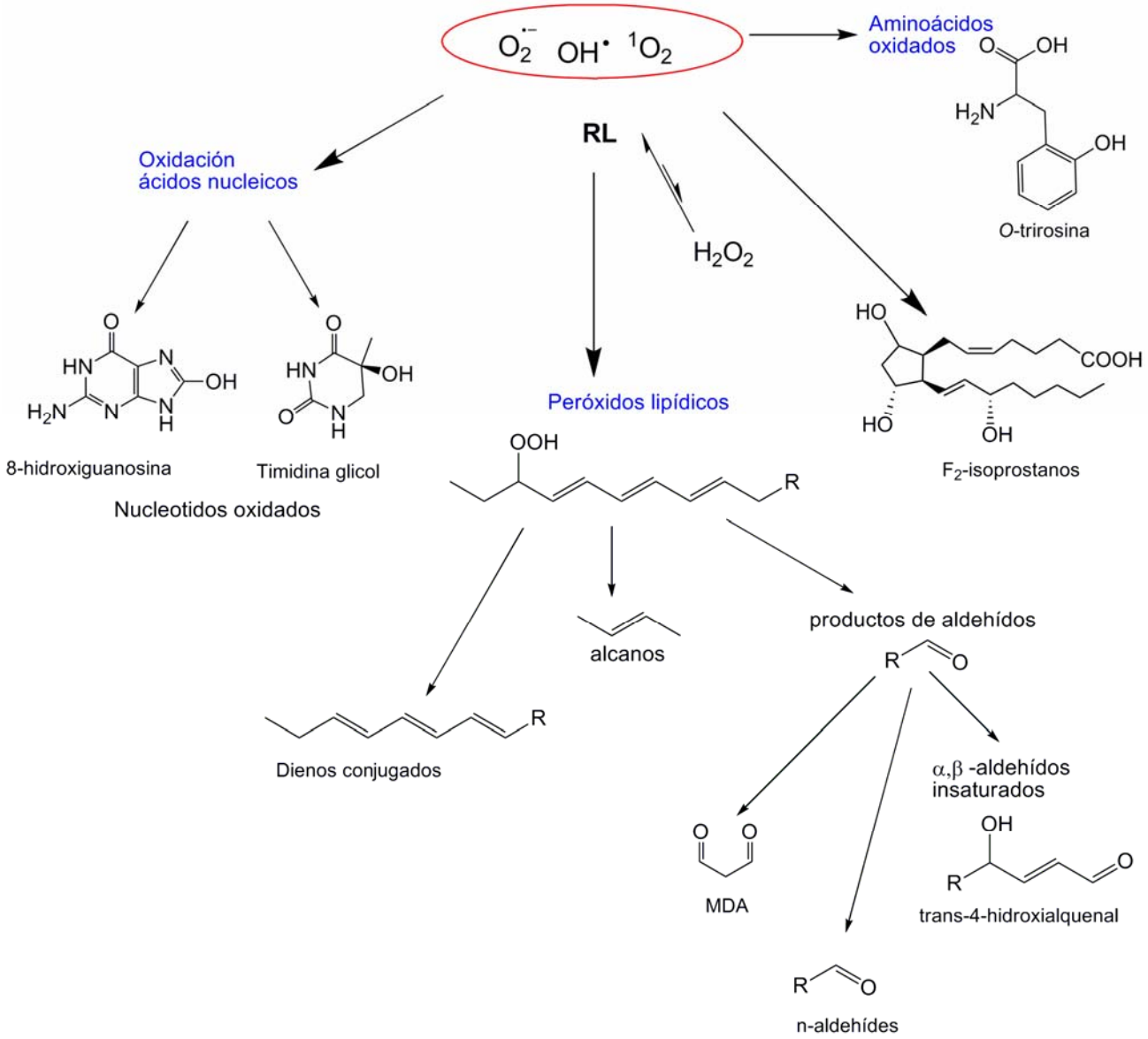


Figura 2.25 : Algunos de los productos generados tras la oxidación de biomoléculas*(De Zwart et al., 1999)*

2.8.4.4.1 Indicadores de daño oxidativo en el citosol

Cociente GSSG/GSH.

El glutatión es un tripéptido (γ-glutamil, cisteinil, glicina). Gracias al grupo SH de la cisteina, es capaz de reducir sustancias oxidantes, pasando de su forma reducida (GSH) a su forma oxidada (GSSG). GSH desempeña diversas funciones metabólicas de gran importancia, sobre todo relacionadas con la protección antioxidante de las células, entre ellas la conversión de peróxido de hidrógeno a oxígeno molecular mediada por la enzima glutatión peroxidasa, evitando la formación del radical hidroxilo. La glutatión reductasa regenera el GSSS a su forma reducida (GSH) que queda nuevamente disponible. En situaciones de estrés oxidativo, la forma oxidada (GSSG) aumenta, es por ello, que el cociente GSSH/GSH es un marcador de estrés oxidativo.

2.8.4.4.2 Indicadores de daño oxidativo en ADN

La oxidación del ADN puede derivar en gran cantidad de productos, sin embargo la oxidación del C-8 de la guanina es uno de los productos generados más comunes y resultado de una mutación en el ADN. Es por ello que la medida de 8-hidroxi-2'-deoxiguanosina (8-OHdG) y su base libre 8-hidroxiguanina (8-OHG) es el marcador más utilizado para medir el daño oxidativo en el ADN y se considera un buen marcador de mutagénesis y carcinogénesis *(Loft et al., 1992)*. Ambos aductos, 8-OHdG y 8-OHG, pueden medirse en ADN de humanos en muestras de linfocitos, placenta y orina. Las determinaciones pueden realizarse mediante pruebas cromatográficas (CG-MS, HPLC-CE y HPLC-MS/MS) después de una extracción en fase sólida *(De Zwart et al., 1999)*.

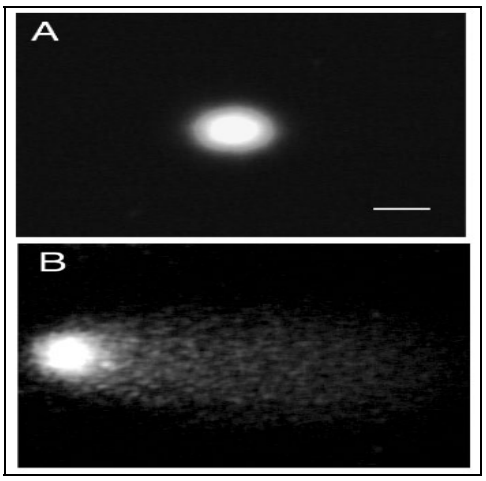


Figura 2.26: Resultados del ensayo cometa a) núcleo sin daño oxidativo y b) núcleo oxidado (fotografía obtenida de *(Lenzi et al., 2003)*)

La detección por electroforesis en gel de la rotura de hebras que componen la doble hélice (ADN) es otro de los ensayos utilizado para medir el daño producido por las especies radicales. Es conocido como ensayo cometa (*comet*), debido a que la rotura de las hebras permite que los bucles de ADN superenrrollado se desplacen en el gel durante la electroforesis dando lugar a una serie de colores que vistos al microscopio parecen un cometa. Para este ensayo se utilizan cultivos celulares obtenidos de frotis bucales, de útero o fecales *(Ferguson et al., 2006)*. El ensayo cometa permite evaluar el daño del ADN de forma relativamente simple, barata, y rápida y por lo tanto, es considerado un buen ensayo para la detección de estrés oxidativo y para determinar el efecto de los compuestos fenólicos con respecto al daño oxidativo *(Wasson et al., 2008)*.

La quercetina ha sido ampliamente evaluada con el ensayo cometa y parece proteger del daño oxidativo en distintos tipos de células, linfocitos *(Duthie et al., 1997a;Johnson et al., 2000)* colon, hepatocitos y células epiteliales *(Duthie et al., 1997b)*, melanoma *(Horváthová et al., 2005)* y macrófagos. La luteolina parece proteger frente al daño del hidrógeno de peróxido en melanoma *(Horváthová et al., 2005)* y las catequinas reducen el daño del ter-butilhidroperóxido en macrófagos *(Kanupriya et al., 2006)*.

2.8.4.4.3 Indicadores de daño oxidativo en lípidos

Medir la oxidación lipídica es realmente difícil debido a la variedad de productos que se generan. Éstos dependen de la composición lipídica de la cual son generada y de la fase en la que se encuentre el proceso oxidación. Es por este motivo, que existe un gran rango de biomarcadores para medir la oxidación lipídica. El método más rápido y sencillo es la determinación colorimétrica (532nm) de las sustancias reactivas con el ácido tiobarbitúrico (TBARS). Mediante está técnica se mide un cromógeno formado por el ácido tiobarbitúrico y malondialdehído (MDA) *(Han et al., 2000)*. Sin embargo, este método ha sido ampliamente criticado debido al gran número de artefactos existentes en muestras biológicas que absorben a la misma longitud de onda.

Una alternativa ha sido el ensayo de determinación de dienos conjugados, que son productos primarios de oxidación lipídica. Los dienos conjugados son detectados y cuantificados espectrofotométricamente a longitudes de onda de 230-235nm. Pero al igual que sucede en el método TBARS, gran cantidad de sustancias biológicas absorben en este rango de UV. Con el fin de mejorar el método, se cambiaron las técnicas de detección de las mediciones, de HPLC-UV a CG, aunque sin buenos resultados. Es por ello que el mejor indicador de peroxidación lipídica parece ser la determinación de isoprostanos *(Kadiiska et al., 2005)*.

Los isoprostanos son un grupo de compuestos formados vía no enzimática debido a la ataque de los radicales libres sobre el ácido grasos poliinsaturados. Los isoprostanos son generados *in situ* en los fosfolípidos de los cuales forman parte y posteriormente, liberados al medio por la acción de las enzimas fosfolipasa tipo A. Dependiendo del ácido

graso a partir del cual es generado se conocen los F2-isoprostanos, originados del ácido araquidónico *(Morrow et al., 1992)*; F4-neuroprostanos, originados del ácido docohexanoico (DHA) *(Roberts Ii et al., 1998)* y los dihomo-isoprostanos (DH-IsoP), generados tras la oxidación del ácido adrénico (abundante en membranas de neuronas) *(VanRollins et al., 2008)*. La determinación de isoprostanos se realiza generalmente mediante CG-MS o HPLC-MS. Aunque, se han desarrollado métodos inmunológicos para realizar esta determinación, se ha comprobado que no son demasiado sensibles ni precisos *(Kadiiska et al., 2005)*

2.8.4.4.4 Indicadores de daño oxidativo en proteínas

El daño oxidativo derivado de la acción de los radicales libres y/o del ataque de los productos secundarios formados tras la peroxidación lipídica, genera grupos carbonilo y otros grupos funcionales como hidroxilos que son resultado de oxidaciones producidas en los enlaces entre aminoácidos. La carbonilación de proteínas es una oxidación más severa que la generación de metionín sulfóxidos y derivados de cisteína por lo que indican un estrés oxidativo más severo y representan un marcador de oxidación proteica estable.

La cuantificación de los grupos carbonilos es el método mas usado aunque es poco selectivo, éste se realiza mediante un ensayo espectrofotométrico (generalmente a 370nm) tras el marcaje con dinitrofenilhidrazina. Otra alternativa es la inmunodetección, que hace uso de anticuerpos contra el grupo dinitrofenilo y puede llevarse a cabo tanto por la técnica de *Western Blot*, separando las proteínas en una o dos dimensiones como por *Enzyme Linked Immunosorbent Assay* (ELISA). Aunque estos métodos proporcionan valores absolutos diferentes, parecen presentar una buena correlación cuando se emplean en determinaciones plasmáticas.

Recientemente se han desarrollado métodos que permiten determinar los grupos carbonilo más abundantes tras la oxidación de proteínas, el (R-aminoadípico semialdeído (AAS)) y el δ-glutámico semialdeído (GGS), permiten conocer el mecanismo de oxidación y el grado de distribución in vivo después de una reacción de reducción con borohidruro de sodio ($NaBH_4$), que es medida por fluorescencia. *(Akagawa et al., 2006)*

Medir los productos de oxidación de los aminoácidos parece dar información más detallada sobre el daño de las especies radicales sobre las proteínas. Algunos ejemplos, son la medida de orto-tirosina (o-Tyr) que se genera tras la oxidación de la fenilalanina, y la formación di-tirosina (DT) , que se forma tras la oxidación de L-tirosina *(De Zwart et al., 1999)*. La formación de los productos de oxidación es generalmente medida por HPLC o CG en muestras de sangre y orina *(De Zwart et al., 1999)*.

3. OBJETIVOS

El objetivo general de la presente tesis es contribuir al conocimiento de la relación estructura/efecto(s) de los compuestos fenólicos naturales, en especial de las proantocianidinas, con relación a las diferentes aplicaciones a las que pueden ser destinados, por ejemplo como antioxidantes alimentarios, suplementos nutricionales o ingredientes funcionales. Para ello, y teniendo en cuenta que la presente tesis está enmarcada en el desarrollo de dos proyectos nacionales diferentes: PPQ2003-06602-C04-01 (Obtención de fracciones polifenólicas a partir de subproductos agroalimentarios y forestales) y AGL2004-07579-C04-02/ALI (Establecimiento de las bases científicas para el uso de la fibra dietética antioxidante de uva), los objetivos concretos planteados fueron los siguientes:

1. Relacionar el grado de polimerización y porcentaje de galoización de proantocianidinas procedentes de fracciones fenólicas con sus efectos químicos y biológicos.

2. Evaluar la biodisponibilidad (absorción/metabolización) de compuestos fenólicos, en particular proantocianidinas, procedentes de una matriz compleja parecida a un alimento (fibra antioxidante de uva).

3. Proponer explicaciones a la actividad antioxidante de polifenoles, en particular proantocianidinas, en modelos de oxidación y en el organismo vivo.

4. RESULTADOS

Los resultados de esta tesis se concretan en tres artículos publicados en revistas del *Science Citation Index* y un cuarto artículo en proceso de revisión.

Previo a cada una de las publicaciones, se acompaña un breve resumen y el material suplementario es incluido en los Anexos (apartado 8.2, *ver pág. 253*).

Los resultados se presentan en dos grandes bloques atendiendo a los objetivos marcados al inicio de la presente tesis:

4.1. Estudio de la relación entre el grado de polimerización y porcentaje de galoización de fracciones fenólicas con sus efectos químicos y biológicos.

- Publicación 1: Evaluación de la actividad antioxidante y biológica de fracciones fenólicas exentas de galoización (*ver pág. 73*)

- Publicación 2: Evaluación de la actividad antioxidante y biológica de fracciones fenólicas de elevada galoización (*ver pág. 83*)

4.2 Estudio de la absorción / metabolización de proantocianidinas

- Publicación 3: Identificación de los compuestos fenólicos de la fracción extraíble de fibra antioxidante de uva (*ver pág. 97*)

- Publicación 4: Identificación de metabolitos de fibra antioxidante de uva en orina de ratas (*ver pág. 111*)

4.1. Estudio de la relación entre el grado de polimerización y porcentaje de galoización de fracciones fenólicas con sus efectos químicos y biológicos.

4.1.1. PUBLICACIÓN 1: Evaluación de la actividad antioxidante y biológica de fracciones fenólicas exentas de galoización.

Título original: Procyanidin fractions from pine (*Pinus pinaster*) bark: Radical scavenging power in solution, antioxidant activity in emulsion, and antiproliferative effect in melanoma cells.

Autores: Sonia Touriño, Ariadna Selga, Aurora Jiménez, Lluís Juliá, Carles Lozano, Daneida Lizárraga, Marta Cascante and Josep Lluís Torres. ***Journal of Agricultural and Food Chemistry***, 2005, *53*, 4728-4735

Índice de impacto: 2,507

Clasificación y categoría: 7/ 103 (Ciencia y Tecnología de los alimentos); 8/62 (Química aplicada); 1/35 (Agricultura, Multidisciplinar)

RESUMEN:

La capacidad antioxidante de los compuestos fenólicos es responsable de que extractos de naturaleza fenólica se utilicen en diversas aplicaciones. Los subproductos de las industrias agroforestales son una importante fuente de polifenoles, es por ello que en los últimos años el número de extractos comerciales obtenidos a partir de subproductos de la industria agroforestal ha experimentado un considerable aumento. Por ejemplo, la corteza de pino es uno de los subproductos más utilizados desde que Masquelier en 1987 puso de manifiesto la elevada capacidad antioxidante de las procianidinas que forman parte de su composición *(Masquelier, 1987)*.

Un estudio previo realizado en nuestro laboratorio *(Torres et al., 2002)*, consistió en elaborar un abanico de fracciones, con diferente grado de polimerización y galoización, a partir de un extracto obtenido de bagazo de uva soluble tanto en agua como acetato de etilo. Los extractos de bagazo de uva se componen fundamentalmente de monómeros de flavan-3-ol (catequina, epicatequina, epigalocatequíngalato), proantocianidinas y diferentes clases de flavonoides glicosilados.

El porcentaje de gailozación de estos extractos es la proporción de ésteres de galato en posición 3 de la estructura de los flavanoles. El tamaño y composición medios de las diferentes fracciones fue estimado mediante una despolimerización ácida en presencia de cisteamina *(Torres et al., 2001)*. Se evaluó la capacidad antioxidante de las fracciones utilizando el ensayo del DPPH, ensayos de inhibición de la oxidación lipídica en emulsiones aceite/agua y se realizaron ensayos de antiproliferación de cada una de estas fracciones.

Los resultados de este estudio *(Torres et al., 2002)* mostraron una relación entre el grado de polimerización y un aumento de la capacidad antioxidante. Además, las fracciones más galoizadas presentaron un aumento de actividad antioxidante comparadas con las fracciones menos galoizadas pero de igual grado de polimerización. El aumento en el número de grupos hidroxilo es la explicación a esta observación.

Por otro lado, éste y otros estudios demostraron que la presencia de compuestos galoizados podía presentar efecto pro-oxidante *(Kondo et al., 1999;Long et al., 2000)*.

A partir de este trabajo y de otros, realizados por diferentes autores, se partió de la hipótesis de que el grado de galoización y polimerización son un punto clave para definir el tipo de posibles aplicaciones de los extractos.

Con el fin de evaluar la influencia de la galoización en la actividad de los extractos fenólicos, el primer trabajo de la presente tesis fue obtener un abanico de fracciones de grado de polimerización análogo al obtenido a partir del bagazo de uva y con grado de galoización cero.

Las procianidinas de la corteza de pino (*Pinus pinaster*) fueron escogidas para realizar este trabajo, ya que carecen de esteres de galato en su composición *(Packer et al., 1999)*. Una vez obtenido el extracto crudo, se realizó el mismo esquema de fraccionamiento que el utilizado en el estudio de la actividad biológica de fracciones de bagazo de uva, con el fin de obtener fracciones comparables en grado de polimerización y diferentes en cuanto al porcentaje de galoización.

El tamaño y composición de las diferentes fracciones fue estimado, al igual que en las fracciones obtenidas a partir de bagazo de uva, mediante una despolimerización ácida en presencia de cisteamina. Se realizaron ensayos para evaluar la capacidad antioxidante (DPPH; TEAC), antirradicalaria (HNTTM), inhibición de la oxidación lipídica en emulsiones aceite/agua y ensayos en la línea celular de melanoma (SK-MEL-28) para evaluar la capacidad antiproliferativa.

Los resultados mostraron que las fracciones de bagazo de uva son mejores antioxidantes que las de corteza de pino, posiblemente debido a un mayor número de grupos hidroxilo en la molécula. Las fracciones de corteza de pino resultaron ser buenos agentes antioxidantes y de baja toxicidad debido a la ausencia de esteres de galato. Los ensayos de inhibición lipídica en emulsiones aceite/agua demostraron que la presencia de galatos no incrementa la actividad antioxidante. Así, fracciones de bagazo de uva y corteza de pino presentaron una actividad antioxidante similar, e incluso algunas fracciones de corteza de pino (XP, XIP) fueron ligeramente mejores protectores que sus análogas de bagazo de uva.

Teniendo en cuenta que la presencia de esteres de galato parece jugar un papel importante en ciclo celular, las fracciones de corteza de pino podrían ser una buena

opción para aplicaciones donde se requieran agentes antioxidantes eficientes que no alteren las funciones normales de la célula.

A continuación se muestra la publicación derivada de este estudio cuyo material suplementario está incluido en los Anexos (apartado 8.2.1, *ver pág. 255*).

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Procyanidin Fractions from Pine (*Pinus pinaster*) Bark: Radical Scavenging Power in Solution, Antioxidant Activity in Emulsion, and Antiproliferative Effect in Melanoma Cells

SONIA TOURIÑO,[†] ARIADNA SELGA,[†] AURORA JIMÉNEZ,[†] LLUÍS JULIÁ,[†] CARLES LOZANO,[†,‡] DANEIDA LIZÁRRAGA,[‡] MARTA CASCANTE,[‡] AND JOSEP LLUÍS TORRES*,[†]

Institute for Chemical and Environmental Research (IIQAB-CSIC), Jordi Girona 18-26, 08034 Barcelona, Spain, and Department of Biochemistry and Molecular Biology, Associated Unit to CSIC, University of Barcelona, Martí i Franquès 1-11, 08028 Barcelona, Spain

Pine (*Pinus pinaster*) bark is a rich source of procyanidin oligomers. From a total polyphenolic extract, we have generated fractions of different procyanidin composition. The mixtures, devoid of gallate esters, were active as free radical scavengers against $ABTS^{\bullet+}$, DPPH, and HNTTM. Pine bark fractions were tested for antioxidant activity in solution (hydrogen donation and electron transfer) and emulsion (inhibition of lipid peroxidation) and compared with their galloylated counterparts from grape origin. While galloylation clearly influenced the free radical scavenging efficiency in solution, it did not seem to play a determinant role in protection against lipid peroxidation in emulsion. The fractions were very mild inhibitors of cell proliferation. Because gallate esters appear to interfere with crucial cell functions, gallate free pine procyanidins may be the innocuous chemopreventative agents of choice for many applications in food and skin protection.

KEYWORDS: ***Pinus pinaster*; polyphenols; procyanidins; catechins; antioxidants; free radical scavenging activity; emulsions; cell proliferation**

INTRODUCTION

Polyphenols from plant origin, particularly flavonoids, are widely appreciated for their putative health promoting properties (*1*, *2*). The antioxidant activity, taken in a broad sense, is believed to be responsible for the preventative properties of flavonoids. The main mechanisms behind this antioxidant activity are direct free radical scavenging (*3*, *4*), transition metal chelation (*5*, *6*), and maintenance of endogenous antioxidants such as the glutathione and superoxide dismutase systems (*7*−*9*). Moreover, the efficiency of any antioxidant very much depends on its distribution within the different biological and physicochemical environments where oxidation of key molecules (e.g., lipids, proteins, DNA) takes place (*10*, *11*). Because so many factors influence the oxidative degradation of biomolecules, the antioxidant activity should be evaluated by more than one experimental setup including methods in solution and emulsion (*12*). This is particularly true for the protection of colloidal systems such as food products and skin care formulations.

Apart from their direct antioxidant properties, flavonoids exert other activities that may or may not be related to their radical scavenging capacity. These activities, mostly mediated by receptor−ligand interactions, include antiproliferation, cell cycle regulation, and induction of apoptosis (*13*−*17*). It is becoming evident that the activity of plant flavonoids as preventive agents must be evaluated from different angles to cover not only the scavenging power but also the influence of the physicochemical environment on the antioxidant effectiveness and the occurrence of other biological activities.

Proanthocyanidins, which include both procyanidins and prodelphinidins (**Figure 1B**), are a particularly interesting type of flavonoids consisting of oligomers of flavan-3-ols (catechins). They are potent free radical scavengers, efficient antioxidants, and antiproliferative and antimflammatory agents (*18*, *19*). The size and composition of oligomeric proanthocyanidins appear to be related to their antioxidant activity, through differences in both the intrinsic scavenging capacity and the physicochemical properties governing their partition behavior within biological environments (*20*−*22*).

One of the main interests in our laboratories is to assess the suitability of proanthocyanidins (**Figure 1B**) obtained from plant byproducts as food antioxidants and preventative agents against skin aging and cancer. Oligomeric proanthocyanidins of the prodelphinidin type are present in grape skin and stems (*23*−*26*), whereas grape seeds contain only procyanidins (*27*, *28*). The three components (skin, seeds, and stems) of the grape pomace are galloylated (presence of gallate esters in C-3, **Figure 1B**) to some extent (*23*−*28*). Pine bark appears to contain only procyanidins with no measurable galloylation (*29*−*31*). The fact

* Author to whom correspondence should be addressed [telephone 34 93 400 61 12; fax 34 93 204 59 04; e-mail jltqbp@iiqab.csic.es].
[†] Institute for Chemical and Environmental Research-CSIC.
[‡] University of Barcelona.

10.1021/jf050262q CCC: $30.25
Published on Web 04/30/2005

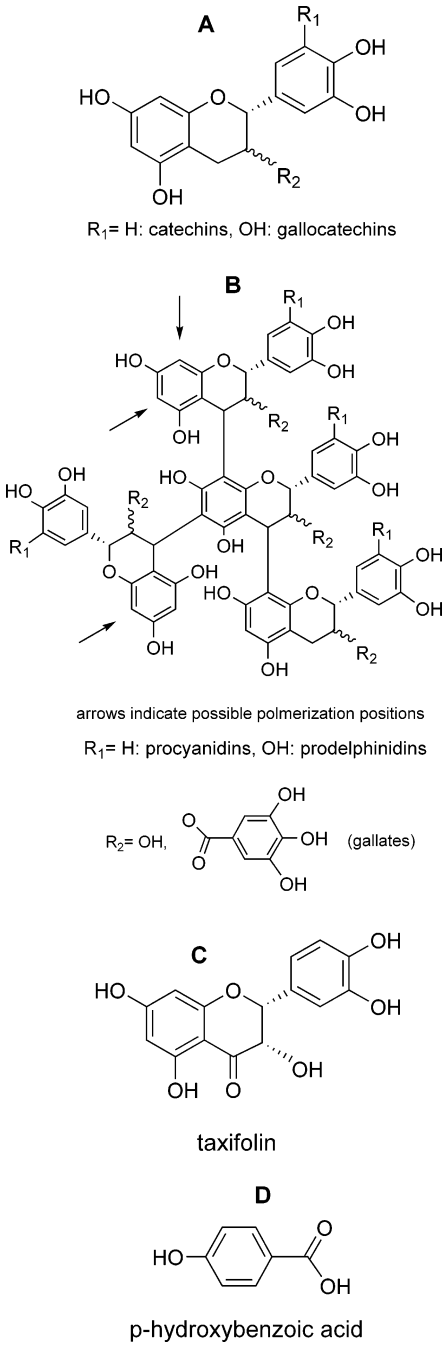


Figure 1.

that pine bark extracts are devoid of gallocatechins and gallates makes them particularly interesting because there have been reports of possible adverse effects of the pyrogallol group due to a putative prooxidant action (*32*, *33*). We have obtained a mixture of pine (*Pinus pinaster*) bark polyphenols and generated fractions with different content in monomeric catechins and oligomeric procyanidins. To evaluate the potential application of the fractions mainly in skin care formulations, and also as functional ingredients of food products, we have measured their efficiency as free radical scavengers, inhibitors of lipid peroxidation in oil-in-water emulsion, and antiproliferative activity. Because galloylation is an important parameter for defining the suitability of procyanidins and for exploring the influence of the galloyl moiety on the activity of the fractions, the results have been compared with those obtained with homologous fractions of grape origin.

MATERIALS AND METHODS

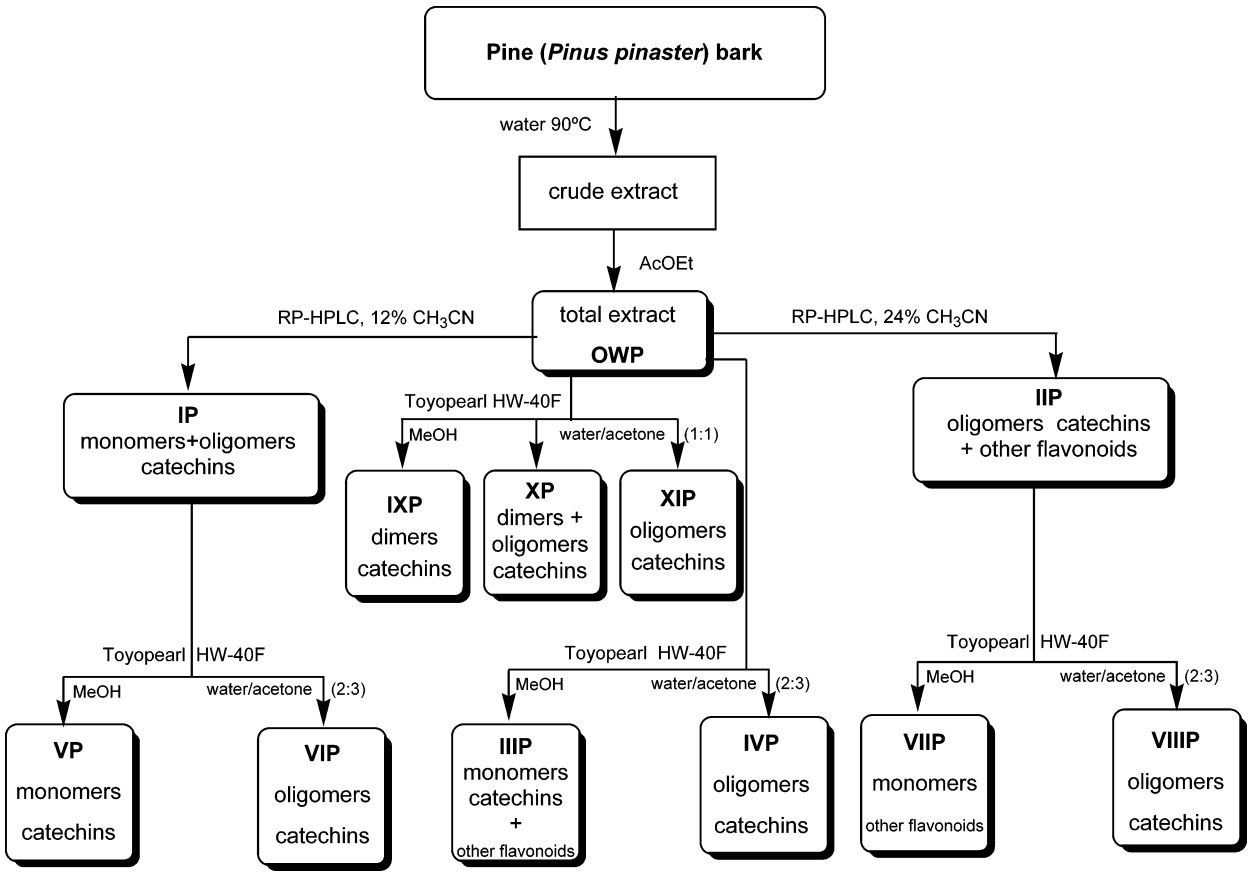
Materials and Chemicals. The starting material was pine (*Pinus pinaster*) bark (byproduct of a saw-mill) provided by Manuel Bouzas SA (Vedra, A Coruña, Spain). After air-drying, the solid was ground (GR-250 mill with 3 mm pore size from Oliver Batlle SA, Badalona, Spain) before extraction.

Water and solvents were deionized water, analytical grade MeOH (Panreac, Montcada i Reixac, Spain), and analytical grade acetone (Carlo Erba, Milano, Italy) for semipreparative chromatography; milli-Q water and HPLC grade CH_3CN (E. Merck, Darmstadt, Germany) for analytical RP-HPLC; analytical grade MeOH (Panreac) for the thioacidolysis and free radical scavenging assays; and analytical grade CH_3-Cl (Panreac) for the HNTTM assay. Trifluoroacetic acid (TFA, Fluorochem, Derbyshire, UK) biotech grade was distilled in-house. Cysteamine hydrochloride was from Sigma-Aldrich Chemical (Steinheim, Germany), and 37% HCl was from Merck. 1,1-Diphenyl-2-picrylhydrazyl free radical (DPPH) (95%) was from Aldrich (Gillingham-Dorset, UK), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) (97%) was from Aldrich (Milwaukee, WI), and (−)-epicatechin was from Sigma Chemical (Saint Louis, MO). 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) crystallized diammonium salt and horseradish peroxidase type IV (RZ $A_{403}/A_{275} < 3$) were obtained from Sigma Chemical. Hydrogen peroxide (3% v/v) was from Sigma Chemical. Tris(2,4,6-trichloro-3,5-dinitrophenyl)methyl (HNTTM) free radical was synthesized as described (*34*). Corn oil stripped of tocopherols was from Ácros Organics (New Jersey, NJ), and soybean lecithin (L-α-phosphatidylcholine) was from Sigma Chemical. Dulbecco modified Eagle's medium (DMEM) and Dulbecco's phosphate buffer saline were from Gibco-BRL (Eggenstein, Germany), fetal calf serum (FCS) was from Invitrogen (Carlsbad, CA), and Trypsin EDTA solution C (0.05% trypsin−0.02% EDTA) was from Biological Industries (Kibbutz Beit Haemet, Israel). 3-[4,5-Dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) was from Sigma Chemical.

Extraction and Solvent Fractionation. The extraction and fractionation with solvents were performed at a pilot plant (A+/LGAI, Bellaterra, Spain). The polyphenolic fraction **OWP** was obtained essentially as described before for grape pomace (*35*) except that water at 90 °C was used instead of 70% EtOH in the extraction step, which was performed in an Autoclave Engineers Reactor (Dispermax, De Dietrich Niederbronn, France). Briefly, ground pine bark (10 kg) was suspended in deionized water (35 L) and kept at 90 °C, 1 atm, under stirring (80 rpm) and continuous nitrogen flow (8 L/min) for 2 h. The solid was then filtered off (Centrifugal Filter Riera Nadeu, SA, Montcada i Reixac, Catalonia, Spain), and the filtrates were extracted with ethyl acetate (3 × 20 L) after saturating the water phase with NaCl. The organic phase was then dried under vacuum, the pellet was dissolved in deionized water, and the solution was filtered through a porous plate. The dry fraction **OWP** (8.96 g, fraction from pine bark soluble in both ethyl acetate and water) was obtained by lyophilization.

Chromatography. OWP was fractionated as summarized in **Scheme 1**. Fractions **IP** and **IIP** were obtained by preparative RP-HPLC essentially as described before (*36*). The rest of the fractions were obtained by semipreparative chromatography on Toyopearl TSK HW-40F (TosoHass, Tokyo, Japan) following a protocol previously described by us (*22*). TSK HW-40F 32−63 μm particle size stationary phase was packed into a flash chromatography type 240 × 25 mm i.d. glass column and equilibrated with MeOH. Fractions **OWP** (total of 9 mL of MeOH solution, 900 mg in three runs × 3 mL), **IP**, and **IIP** (total of 6 mL of MeOH solution each fraction, 600 mg, in two runs × 3 mL) were separately fractionated in two steps: MeOH (250 mL) and water/acetone (2:3) (250 mL) following elution protocols described in the literature (*37*, *38*). The flow rate was 10 mL/min (MeOH) and 2.5 mL/min (water/acetone). The solvent was then evaporated under vacuum, and the residue was dissolved in milli-Q water (ca. 100 mL) and lyophilized to give slightly colored fluffy solids (from fraction **OWP**, 501 mg of **IIIP** and 271 mg of **IVP**; from fraction **IP**, 341 mg of **VP** and 173 mg of **VIP**; and from fraction **IIP**, 340 mg of **VIIP** and 153 mg of **VIIIP**). Alternatively, **OWP** (four runs × 300 mg) was more extensively fractionated with MeOH and water/acetone 1:1 to generate a fraction of monomers, essentially equal to **IIIP**, 54 mg

Scheme 1



fraction **IXP**, 82 mg fraction **XP**, and 186 mg fraction **XIP**. The fractions were analyzed by RP-HPLC on a Smart System (Amersham-Pharmacia Biotech, Uppsala, Sweden) equipped with a μPeak Monitor (Amersham-Pharmacia Biotech) and fitted with a μRPC C2/C18 SC 2.1/10 (100 × 2.1 mm i.d.) column (Amersham-Pharmacia Biotech). Elution: [A] 0.10% (v/v) aqueous TFA, [B] 0.08% (v/v) TFA in water/CH_3CN 1:4, gradient 8 to 23% [B] over 38 min. Flow rate 200 μL/min. Detection by triple wavelength 214, 280, 320 nm.

Thiolysis with Cysteamine and RP-HPLC. The size and composition of the procyanidins within the fractions were estimated from the HPLC analysis of the depolymerized fractions as described (*39*). Briefly, the terminal flavan-3-ols units were released as such by acid cleavage in the presence of cysteamine, whereas the extension moieties were released as the cysteamine derivatives on the fourth position of the flavanoid system. The resulting mixtures were submitted to analytical RP-HPLC (μRPC column, gradient 8−23% [B] over 45 min), and the molar amount (nanomoles) of all of the released moieties was calculated from the peak areas and calibration curves obtained with pure samples:

mean degree of polymerization (mDP) = total nmol/nmol terminal units;

mean molecular weight (mMW) = total mass/nmol terminal units.

Free Radical Scavenging Activity. *ABTS Radical Cation Decolorization Assay*. The method is based on the capacity of a sample to scavenge the ABTS radical cation ($ABTS^{\bullet+}$) as compared to a standard antioxidant (Trolox). $ABTS^{\bullet+}$ was generated from ABTS as described (*40*, *41*) with some modifications. To prepare the initial radical solution, H_2O_2 3‰ (45 μL) was added to a reaction mixture containing ABTS (54.9 mg, 1 mM) and horseradish peroxidase (HRP, 1.1 mg, 0.25 μM) in 50 mM gly-HCl buffer pH 4.5 (100 mL). The reaction mixture was left to stand at room temperature for 15 min in the dark. Polyphenolic solutions (50 μL) at concentrations of 0.3, 0.2, 0.15, 0.10, and 0.05 mg/mL in MeOH were then added to the $ABTS^{\bullet+}$ solution (1950 μL). The total time needed to carry out each assay was 20 min, including ABTS radical generation, addition of antioxidant, and acquisition of final absorbance value. The decrease of absorbance at 734 nm with respect to the 1 mM solution of $ABTS^{\bullet+}$ was recorded. The assay was performed in triplicate. The dose−response curves obtained with the antioxidant mixtures and Trolox were plotted as the percentage of absorbance decrease against the amount of antioxidants expressed either in micromolar units or as μg/mL. The molar concentrations were calculated using the mean molecular weight values estimated by thioacidolysis. The Trolox equivalent antioxidant capacity (TEAC) was calculated as the ratio between the slopes of the dose−response curves of the samples and Trolox, and also as mass concentration of fraction equivalent to a 1 μM solution of Trolox. The total antioxidant activity (TAA) of the fractions was expressed in millimoles of Trolox equivalents.

DPPH Assay. The antiradical activity of the fractions was also evaluated by the DPPH stable radical method (*42*, *43*). The samples (0.1 mL) were added to aliquots (3.9 mL) of a solution made up with DPPH (4.8 mg) in MeOH (200 mL), and the mixture was incubated for 1 h at room temperature in the dark. The initial concentration of DPPH, approximately 60 μM, was calculated for every experiment from a calibration curve made by measuring the absorbance at 517 nm of standard samples of DPPH at different concentrations. The equation of the curve was $Abs_{517nm} = 11\,345 \times C_{DPPH}$ as determined by linear regression. The results were plotted as the percentage of absorbance disappearance at 517 nm $((1 - A/A_0) \times 100)$ against the amount of sample divided by the initial concentration of DPPH. Each point was acquired in triplicate. A dose−response curve was obtained for every fraction. ED_{50} corresponds to either micrograms or micromoles of product able to consume one-half the amount of free radical divided by micromoles of initial DPPH. The molecular amounts (micromoles) of procyanidin mixtures were calculated with the mean molecular weights (mMW) estimated by thiolysis with cysteamine. The results were expressed as antiradical power (ARP), which is the inverse of ED_{50}.

HNTTM Assay. EPR measurements were performed on a Varian (Palo Alto, CA) E-109 spectrometer equipped with a dual cavity (V-4532 mode). The fractions were dissolved in CH_3Cl/MeOH (2:1) at different concentrations. Aliquots (1 mL) were added to a solution (1 mL) of HNTTM (120 μM in CH_3Cl/MeOH (2:1)) (*34*), and the mixture

was incubated for 30 min. The exact initial concentration of radical, around 60 μM, was calculated for every experiment from calibration curves made by measuring the intensity (I_0) of the EPR signal (peak to peak line distance) of standard samples of the radical at different concentrations. The equations of the curves were $I = 1980 \times C_{radical}$ or $I = 2262 \times C_{radical}$ depending on the experiment. The results were plotted as the percentage of signal intensity disappearance [$(1 - I/I_0) \times 100$] against the amount of sample divided by the initial micromoles of the radical. Each point was acquired in triplicate. A dose−response curve was obtained for every fraction. The results were expressed as the efficient dose ED_{50} given as micromoles of fraction able to consume one-half the amount of free radical divided by micromoles of initial HNTTM. As for the DPPH assay, the mean molecular weight was estimated by thioacidolysis and RP-HPLC.

Inhibition of Lipid Peroxidation in an Oil-in-Water Emulsion. Lipid peroxidation in corn oil emulsion was monitored by measuring UV absorbance at 234 nm, corresponding to the formation of conjugated dienes upon air oxidation (*10*, *44*−*46*). The emulsion was formed essentially as described (*47*, *48*). A mixture of corn oil stripped of natural antioxidants (10% w/w) and soybean lecithin (1% w/w) in 25 mM potassium phosphate pH 5 buffer was mixed with an Ultra-turrax T25 (Ika-Labortechnik, Staufen, Germany) at 8500 rpm for 5 min until the complete emulsification. The emulsion was then homogenized with a Microfluidics Corp. (Newton, MA) 110 L high-pressure homogenizer in six cycles at 600−630 bar. The average particle size of the fresh emulsions was 0.35−0.40 μm determined with an optical microscopy Reichert Polyvar (Leica Microsystems AG, Wetzlar, Germany) equipped with a video camera and a PC with a Leica IM500 software for image capture. Polyphenolic fractions dissolved in buffer (100 μL) were added to the emulsion (9.9 mL) to obtain initial concentrations ranging from 2 to 50 μg/mL. The samples (2.5 mL) were placed into 10-mL screw-capped test tubes and air oxidized at 60 °C in a shaker bath for 4 days. Every day, aliquots (50 μL) were taken, dissolved in MeOH (10 mL), to obtain absorbance values in the linear range, and the UV absorbance at 234 nm was recorded. The lipid peroxidation was calculated as millimoles of hydroperoxydes per kilogram of oil using an absorptivity of 26 000 for linoleate hydroperoxydes (*49*) and plotted against time. The experiments were done in triplicate. The antioxidant activity was expressed as percentage inhibition of hydroperoxide formation $100 \times [(C - S)/C]$, where C is the amount of hydroperoxides in the control and S is the amount of hydroperoxides in each sample with antioxidants (*48*).

Proliferation Assay on SK-Mel-28 Human Melanoma Cell Line. SK-MEL-28 adherent cells (ATCC #HTB-72) were grown in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), in the presence of 0.1% (v/v) antibiotics (10 000 U/mL penicillin, 10 000 μg/mL streptomycin), at 37 °C in a humidified environment with 5% CO_2. The cells were split (ratio 1:2 to 1:5) by mild trypsinization every 4−5 days, and the medium was changed every 2−3 days. Cell culture used in this study was free of mycoplasm infection as shown by the EZ-PCR Mycoplasm test kit (Biological Industries) prior to the treatment with the samples.

Cell growth was determined using the Mosmann assay (*50*) with some modifications. Cells were seeded into 96-well plates at 1×10^4 cells/mL density, 200 μL/well, and incubated for 24 h in the culture medium prior to the addition of the samples dissolved in DMEM. Control wells were treated with equal volume of DMEM as the test cultures. After 72 h of culture, the supernatant was aspirated and 100 μL of sterile filtered MTT (0.5 mg/mL in DMEM) was added to each well. The plates were incubated at 37 °C−5% CO_2 during 1 h. The supernatant was removed, the blue MTT formazan precipitated was dissolved in DMSO (100 μL), and the optical density (OD) was measured at 550 nm on a multi-well reader (Merck ELISA System MIOS).

The inhibitory effect of the fractions at each concentration was expressed as a percentage [(mean OD treated cells after 72 h of incubation with the product/mean OD of control cells after 72 h of incubation with extra-medium instead of product) × 100]. The IC_{50} or sample concentration causing a 50% reduction in the mean OD value relative to the control at 72 h of incubation was estimated using GraFit 3.00 (Data Analysis and Graphics Program, Erithacus Software Ltd. Microsoft Corp., Surrey, UK) curve option: IC_{50} curve − start at 0.

RESULTS AND DISCUSSION

Characterization of the Fractions and Free Radical Scavenging Activity. The pine bark polyphenolic extract **OWP**, soluble in both ethyl acetate and water, contained catechins, both monomeric and oligomeric (**Figure 1A**,**B**), and other monomeric flavonoids, mainly taxifolin (**Figure 1C**), hydroxycinnamic acids, and *p*-hydroxybenzoic acid (**Figure 1D**). From this mixture, a set of fractions differing in composition and procyanidin structure were generated using a combination of two chromatographic techniques (**Scheme 1**). RP-HPLC discriminates among solutes by hydrophobicity, while Toyopearl HW-40 has been shown to separate monomeric flavonoids and oligomeric catechins in order of increasing size (*37*, *51*) by the combined action of gel permeation and adsorption phenomena. From the total extract **OWP**, we generated fractions homologous to those obtained from grape (*22*). The fractions of pine bark origin contained catechin monomers, mainly (+)-catechin, and other flavonoid monomers, mainly taxifolin (**VP** and **VIIP**, respectively), procyanidin dimers (**IXP**), procyanidin oligomers (**IVP**, **VIP**, **VIIIP**, **XP**, **XIP**), and different combinations of monomers and oligomers (**IP**, **IIP**, **IIIP**). Procyanidin size was estimated by thiolysis with cysteamine. The results are summarized in the second and third columns of **Table 1**. While grape procyanidins were galloylated to some extent, galloylated catechin monomers or oligomers were not detected in any of the fractions from pine bark. This was the main difference between homologous procyanidin containing fractions from grape and pine. Moreover, the pine extract was richer than the grape extract in monomeric species (flavonoids and catechins in pine bark, flavonols and catechins in grape pomace) as shown by comparing the amounts of monomeric fractions obtained: **VP** (341 mg), **IIIP** (501 mg), and particularly **VIIP** (340 mg) versus homologous grape fractions **V** (218 mg), **III** (293 mg), and **VII** (85 mg) in ref *22*. The yield in procyanidins with mDP from 2.0 to 2.9 (**IVP**, **VIP**, **IXP**, **XP** and the homologous **IV**, **VI**, **IX**, **X**) was similar for both origins, while pine bark contained less oligomers with a mean degree of polymerization of 3 and higher: **VIIIP** (153 mg) and **XIP** (186 mg) versus **VIII** (305 mg) and **XI** (243 mg) in ref *22*). The total antioxidant activity of the polyphenolic mixture **OWP** and its fractions was measured by the ABTS cation radical method, which is a widely used assay for the evaluation of natural antioxidant mixtures as well as their pure components (*52*−*55*). The results are arranged and summarized in **Figure 2**, which gives a comparative idea of the activity that can be recovered in each fraction from the total mixture **OWP**. To compare the antioxidant efficiency of the fractions, the results were also expressed in terms of relative potency as compared to Trolox, as summarized in the fourth and fifth columns of **Table 1**, using either mass or molar concentration. The former provides an idea of the weighed amount of fraction needed to exert a given antiradical effect, and the latter carries information on the mean efficiency of the procyanidin components of that fraction, using the mean molecular weight (mMW) estimated by thioacidolysis followed by RP-HPLC. All of the fractions except **VIIP** were more efficient scavengers than Trolox. **VIIP** contained part of the taxifolin from **OWP** and other material not of polyphenolic nature.

The free radical scavenging power was also measured against two stable radicals, DPPH and HNTTM. These two radicals, which can be stored indefinitely and determined accurately before every experiment, can be used to calculate the stoichiometry of the redox reaction and to discriminate between hydrogen donation and electron-transfer mechanisms of radical

Table 1. Mean Size of the Components and Antiradical Power of Polyphenolic Fractions from Pine Bark

	thioacidolysis		ABTS•+ assay		DPPH assay			
fractions	mDP[a]	mMW[a]	TEAC[b]	molar TEAC[c]	ARP[d] $(1/ED_{50}) \times 10^3$	molar ARP[e] $(1/ED_{50})$	stoichiometric value	H atoms per molecule
OWP	2.1	601	0.23	2.3	12.5	7.5	0.27	3.8
IP	1.9	546	0.23	2.2	11.6	6.3	0.31	3.2
IIP	2.9	835	0.26	3.0	9.6	8.0	0.25	4.0
VP	1.0	290[f]	0.43		6.5	1.9	1.05	0.9
IIIP	1.0		0.30		9.5			
VIIP	1.0		1.80		4.5			
IXP	1.9	559	0.18	3.2	16.8	9.4	0.21	4.7
XP	2.2	639	0.14	4.1	22.2	14.2	0.14	7.1
VIP	2.7	777	0.17	4.5	20.9	17.8	0.12	8.6
IVP	2.9	833	0.15	4.5	16.8	14.1	0.14	7.1
VIIIP	3.0	876	0.22	4.2	11.9	10.4	0.19	5.2
XIP	3.4	999	0.13	6.6	21.0	21.0	0.09	10.5
controls								
Trolox	1.0	250	0.53	1.0	15.8	3.95	0.51	2.0
EC	1.0	290			21.0	6.0	0.33	3.0

[a] Mean of three independent thioacidolysis experiments with three RP-HPLC replicate injections, ABTS•+ assay, mean of three experiments. [b] Micrograms/microliter of fraction equivalents to 1 μM (0.53 μg/mL) Trolox (the lower the more efficient). [c] Micromolar concentration of Trolox equivalent to a 1 μM solution of fraction (the higher the more efficient). [d] ED_{50} in micrograms of fraction/micromoles DPPH mean of three experiments. [e] ED_{50} in estimated micromoles of fraction/micromoles DPPH only for procyanidin fractions devoid of other flavonoids. [f] Not calculated mass corresponding to catechin monomer. Standard deviation ($n = 3$): $\leq$0.4 (molar ARP), 0.03 (stoichiometric value), 0.2 (H atoms per molecule).

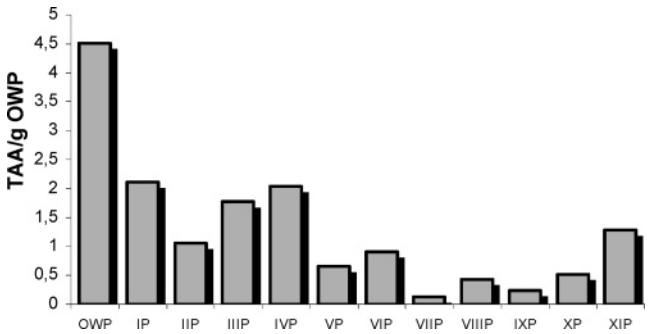


Figure 2. Total antioxidant activity (TAA) of the fractions by the ABTS cation radical method. TAA expressed as millimoles of Trolox equivalents obtained per gram of **OWP**.

scavenging. Whereas the DPPH assay measures the combined hydrogen donation and electron-transfer capacity of the polyphenols (the hydrogenated species are the end products, and no electron-transfer intermediates have been detected) (*43*, *56*, *57*), the HNTTM stable radical (*34*) exerts its action exclusively by electron transfer and the end product is the anion, as detected by UV spectrophotometry (*58*). The differential capacity of electron transfer and hydrogen donation is a relevant parameter to be measured because electron transfer is regarded sometimes as an undesired effect (*59*). Under certain conditions, flavonoids such as the pyrogallol containing (−)-epigallocatechin (EGC) and (−)-epigallocatechin-gallate (EGCG) may participate in redox cycling with the production of active superoxide radical anion ($O_2^{\bullet -}$) (*32*, *33*, *60*). Although this is less likely to occur with (−)-epicatechin or (+)-catechin or their polymers (procyanidins), redox cycling has been described also for catechols (*33*). For both DPPH and HNTTM assays, the antiradical power was expressed as the inverse of ED_{50} (micrograms or micromoles of fraction able to consume one-half the amount of free radical divided by micromoles of initial DPPH or HNTTM). By multiplying the ED_{50} by 2, the stoichiometric value (theoretical concentration of antioxidant able to reduce 100% of the radical) is obtained. The inverse of this value represents the moles of radical reduced by one mole of antioxidant and gives an estimate of the number of hydrogen atoms donated (DPPH assay) or electrons transferred (HNTTM assay) per molecule of antioxidant. **Table 1**, columns 6−9, summarizes the results of the DPPH assay. In general, the results are similar to those obtained with the ABTS cation radical. Pine bark fractions followed the general trend that the higher is the degree of polymerization, the higher is the number of hydroxyls and the higher is the free radical scavenging power per molecule. As compared to the homologous fractions from white Parellada grape pomace (*22*), pine polyphenols were less potent scavengers. This must be due to the absence of galloyl esters, which confer the extra scavenging capacity of their three hydroxyl groups. The electron-transfer capacity (HNTTM assay) of selected fractions from pine bark and grape pomace was measured and compared with the results of the DPPH assay. **Table 2** summarizes the results with the HNTTM stable radical. The fractions selected for the study contained only procyanidins of different mean degrees of polymerization (mDP). For the sake of simplicity, only the number of hydrogen atoms or electrons per molecule is shown. Homologous fractions presented essentially the same mDP. The number of hydrogen atoms per molecule increased with size (i.e., number of hydroxyls) in both cases with a plateau between 2.2 and 2.9 mDP. This was also the case for the electron-transfer capacity of grape procyanidins. Interestingly, the electron-transfer capacity of pine fractions did not completely follow this trend. Fraction **XIP** (mDP, 3.4) showed a high hydrogen donation capacity (10.5 hydrogen atoms per molecule), while keeping the electron-transfer capacity low (5.8 electrons per molecule). Because low electron transfer may imply reducing the odds for pro-oxidant effects, this result may have important implications for the use of procyanidin fractions from pine bark as safe chemopreventive agents. Alternatively, the low number of electrons transferred to HNTTM may be due to other causes such as low accessibility of the reagent in the reaction medium.

Antioxidant Activity in an Oil-in-Water Emulsion. The three methods described above provide information about the intrinsic scavenging power of the fractions in solution with solvents of different polarity going from $CHCl_3$ (HNTTM) and protic organic (MeOH, DPPH) to water ($ABTS^{\bullet +}$). The results indicate that the order of antiradical power was essentially the same for the three assays, meaning that the experimental conditions (type of radical and polarity of the solvent) did not influence the behavior of the polyphenolic fractions with the

Table 2. Hydrogen Donation versus Electron-Transfer Capacity of Pine Bark and Grape Pomace Procyanidin Fractions

	pine bark fractions				grape pomace fractions[c]		
	mDP	H/mol[a]	e/mol[b]		mDP	H/mol[a]	e/mol[b]
IXP	1.9	4.7	4.3	**IX**	2.0	7.1	4.7
XP	2.2	7.1	5.8	**X**	2.2	12.5	6.8
IVP	2.9	7.1	5.8	**IV**	2.7	12.5	8.1
XIP	3.4	10.5	5.8	**XI**	3.7	16.7	13.7

[a] Hydrogen atoms per molecule or moles of reduced DPPH per mole antioxidant. [b] Electrons per molecule or moles of reduced HNTTM per mole antioxidant. Standard deviation ($n = 3$): ≤ 0.2. [c] Grape pomace fractions and their mDP values were those obtained in ref *22*.

Table 3. Hydroxyperoxide (Hx) Formation[a] in an Oil-in-Water Emulsion after 4 days of Oxidation at 60 °C

		fraction concentration				fraction concentration		
	blank	2 μg/mL	10 μg/mL	50 μg/mL		2 μg/mL	10 μg/mL	50 μg/mL
OWP	63.5 ± 18.5	nd	25.9 ± 4.2	39.7 ± 9.1	**OW**	nd	38.5 ± 5.3	21.4 ± 4.9
IVP	66.1 ± 14.6	50.3 ± 2.8	83.6 ± 14.1	42.6 ± 1.6	**IV**	49.6 ± 2.4	43.2 ± 10.4	22.7 ± 4.1
VIIIP	78.2 ± 8.3	83.8 ± 3.4	71.3 ± 1.5	70.5 ± 12.3	**VIII**	60.2 ± 3.3	54.8 ± 3.9	28.4 ± 0.7
IXP	84.2 ± 10.3	77.4 ± 5.9	54.7 ± 14.6	58.3 ± 8.9	**IX**	76.8 ± 5.9	60.9 ± 10.4	44.6 ± 4.8
XP	64.0 ± 7.2	76.5 ± 6.9	64.9 ± 3.3	46.3 ± 0.3	**X**	61.9 ± 7.6	59.6 ± 3.0	48.3 ± 3.5
XIP	69.6 ± 28.4	70.8 ± 3.0	63.5 ± 5.2	36.4 ± 1.1	**XI**	54.5 ± 2.7	45.9 ± 2.3	31.5 ± 2.3

[a] Results expressed in mmoles Hx/kg oil ±SD, $n = 3$; nd, not determined.

possible exception of fraction **XIP** (mDP = 3.4). Because most food and living systems are colloids rather than solutions in solvents of a given polarity, and because lipids deteriorate fast at interfaces with air or water, assays in models of interfacial (e.g., lipid−water) oxidation must be part of the evaluation of antioxidants (*12*). The antioxidant activity of the fractions and Trolox against lipid peroxidation was tested in an oil-in-water emulsion made up with corn oil stripped of natural antioxidants under the conditions described in Materials and Methods. In this model, protection against lipid peroxidation is believed to depend mainly on the tendency of an antioxidant molecule to be located at water−oil interfaces (*11*, *12*, *45*, *61*). One of the goals of this paper was to establish the influence of galloylation on the antioxidant efficacy of plant procyanidins in emulsion as a model of biologically significant systems. Because the gallate moiety has been shown to be behind the influence of catechins on the cell replicating and survival machinery (cell cycle, apoptosis) (*13*, *62*), it may be preferable, in some cases, to use nongalloylated catechins for applications pertaining to food and skin protection. Our previous study with procyanidin fractions from white grape pomace recorded a relation between activity in emulsion and both size and galloylation indistinguishably (*22*). Because procyanidins from pine origin were not galloylated, they provided an excellent tool to test the influence of galloylation in lipid protection in emulsion by comparing them with grape procyanidins.

Table 3 summarizes the results obtained with pairs of significant homologous fractions of both origins. **Figure 3** depicts the percentage inhibition of lipid peroxidation exerted by the polyphenolic fractions at a concentration of 50 μg/mL after 4 days of air oxidation. The general trend was again that the galloylated fractions were the most effective. Interestingly though, nongalloylated **XP** (mDP = 2.2) and **XIP** (mDP = 3.4) were as effective as the homologous **X** (mDP = 2.2) and **XI** (mDP = 3.7), which were relatively highly galloylated (30% and 31%, respectively). This suggests that galloylation has little influence on the capacity of oligomeric procyanidins with degree of polymerization between two and four to protect lipids from peroxidation in emulsion and corroborates the hypothesis that interfacial phenomena are more important that intrinsic scavenging activity in complex multiphasic systems. This result, together with the result on low electron-transfer capacity of the same

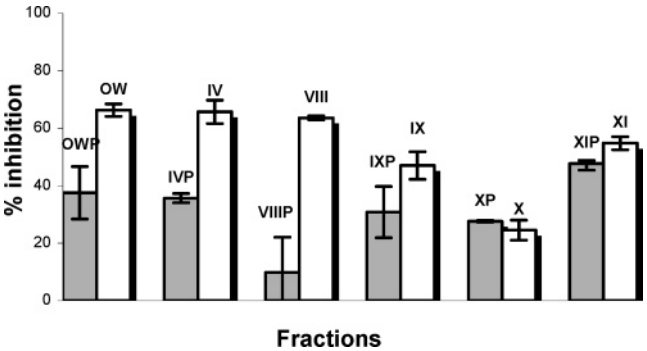


Figure 3. Inhibition of corn oil oxidation in an oil-in-water emulsion. Comparison between fractions from grape pomace and pine bark. Air oxidation for 4 days at 60 °C in the presence of procyanidin fractions at a concentration of 50 μg/mL.

procyanidins (fraction **XIP**), may have important implications in the definition of the possible applications of pine procyanidins. Pine bark procyanidins appear to protect lipids from peroxidation as effectively as other preparations, which include putatively less innocuous gallate containing components. Interestingly, fraction **XIP** contains a relatively high portion (ca. one-third) of the antioxidant activity of **OWP** (**Figure 2**) while being devoid of monomers, which, due to reports of side effects attributed to high doses of catechin (*63*, *64*), are sometimes regarded as undesirable components.

Proliferation of SK-Mel 28 Human Melanoma Cells. Selected fractions (**OWP**, **IIIP**, **IVP**, **VP**, **VIP**, **VIIIP**) showing different mean procyanidin degrees of polymerization were assayed for their influence on the proliferation of melanoma cells. Their homologous counterparts form grape origin had been assayed before on the same assay (*22*). The fractions exerted a weak antiproliferative effect on this tumoral cell line. The IC_{50} values obtained were 122 μg/mL (**OWP**), 137 μg/mL (**IVP**), 213 μg/mL (**VP**), 146 μg/mL (**VIP**), and 134 μg/mL (**VIIIP**). In overall agreement with the results obtained for grape pomace procyanidins, the monomers (**VP**) were the least efficient agents. Our results are also in agreement with those obtained with SK-MEL-1 and/or SK-MEL-28 cells after treatment with flavonoids of the flavonol and flavone type (*65*, *66*).

In conclusion, oligomeric procyanidin fractions from pine bark appear to be efficient and safe antioxidant agents. Because they do not include gallate esters, they are less potent free radical scavengers in solution than the corresponding galloylated fractions of grape origin. Interestingly, the galloyl moiety is not necessary for lipid protection in emulsion. This has important implications for the application of mixtures of this kind in food manufacturing and the formulation of skin care products. Because the galloyl moiety influences crucial biochemical pathways in cell replication and survival, efficient gallate free mixtures such as our fraction **XIP** may be the best option for products designed to offer antioxidant protection by exclusively scavenging an excess of radicals without altering the normal cell functions.

ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid); EC, (−)-epicatechin; DMEM, Dulbecco modified Eagle's medium; DPPH, 1,1-diphenyl-2-picrylhydrazyl free radical; FCS, fetal calf serum; HNTTM, tris(2,3,6-trichloro-3,5-dinitrophenyl)methyl; MTT, 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide; RP-HPLC, reversed-phase high-performance liquid chromatography; TAA, total antioxidant activity; TEAC, Trolox equivalent antioxidant capacity; TFA, trifluoroacetic acid; Trolox, 2,5,7,8-tetramethyl-chroman-2-carboxylic acid.

ACKNOWLEDGMENT

We are thankful to Drs. C. Solans and J. Esquena for their advice and help in the preparation of the emulsion.

Supporting Information Available: RP-HPLC chromatograms obtained for all of the fractions before and after thioacidolysis, plots of time course oil oxidation in emulsion, and dose−response curves from the proliferation assay. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Rusznyák, S. P.; Szent-Györgyi, A. Vitamin P: flavonols as vitamins. *Nature* **1936**, *138*, 27.
(2) Ross, J. A.; Kasum, C. M. Dietary flavonoids: Bioavailability, metabolic effects, and safety. *Annu. Rev. Nutr.* **2002**, *22*, 19−34.
(3) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933−956.
(4) Yokozawa, T.; Chen, C. P.; Dong, E.; Tanaka, T.; Nonaka, G.-I.; Nishioka, I. Study on the inhibitory effect of tannins and flavonoids against the 1,1-diphenyl-2-picrylhydrazyl radical. *Biochem. Pharmacol.* **1998**, *56*, 213−222.
(5) Brown, J. E.; Khodr, H.; Hider, R. C.; Rice-Evans, C. A. Structural dependence of flavonoid interactions with Cu^{2+} ions: implications for their antioxidant properties. *Biochem. J.* **1998**, *330*, 1173−1178.
(6) Hider, R. C.; Liu, Z. D.; Khodr, H. H. Metal chelation of polyphenols. In *Flavonoids and Other Polyphenols*; Packer, L., Ed.; Academic Press Inc.: San Diego, CA, 2001; pp 190−203.
(7) Katiyar, S. K.; Afaq, F.; Perez, A.; Mukhtar, H. Green tea polyphenol (−)-epigallocatechin-3-gallate treatment of human skin inhibits ultraviolet radiation-induced oxidative stress. *Carcinogenesis* **2001**, *22*, 287−294.
(8) Ishige, K.; Schubert, D.; Sagara, Y. Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms. *Free Radical Biol. Med.* **2001**, *30*, 433−446.
(9) Inal, M. E.; Kahraman, A. The protective effect of flavonol quercetin against ultraviolet a induced oxidative stress in rats. *Toxicology* **2000**, *154*, 21−29.
(10) Frankel, E. N.; Huang, S.-W.; Kanner, J.; German, J. B. Interfacial phenomena in the evaluation of antioxidants: Bulk oils vs emulsions. *J. Agric. Food Chem.* **1994**, *42*, 1054−1059.
(11) Frankel, E. N. Interfacial lipid oxidation and antioxidation. *J. Oleo Sci.* **2001**, *50*, 387−391.
(12) Frankel, E. N.; Meyer, A. S. The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *J. Sci. Food Agric.* **2000**, *80*, 1925−1941.
(13) Liang, Y. C.; Lin-shiau, S. Y.; Chen, C. F.; Lin, J. K. Suppression of extracellular signals and cell proliferation through EGF receptor binding by (−)-epigallocatechin gallate in human A431 epidermoid carcinoma cells. *J. Cell Biochem.* **1997**, *67*, 55−65.
(14) Ahmad, N.; Gali, H.; Javed, S.; Agarwal, R. Skin cancer chemopreventive effects of a flavonoid antioxidant silymarin are mediated via impairment of receptor tyrosine kinase signaling and perturbation in cell cycle progression. *Biochem. Biophys. Res. Commun.* **1998**, *247*, 294−301.
(15) Ahmad, N.; Cheng, P. Y.; Mukhtar, H. Cell cycle dysregulation by green tea polyphenol epigallocatechin-3-gallate. *Biochem. Biophys. Res. Commun.* **2000**, *275*, 328−334.
(16) Hayakawa, S.; Kimura, T.; Saeki, K.; Koyama, Y.; Aoyagi, Y.; Noro, T.; Nakamura, Y.; Isemura, M. Apoptosis-inducing activity of high molecular weight fractions of tea extracts. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 459−462.
(17) Williams, R. J.; Spencer, J. P. E.; Rice-Evans, C. Flavonoids: Antioxidants or signaling molecules? *Free Radical Biol. Med.* **2004**, *36*, 838−849.
(18) Santos-Buelga, C.; Scalbert, A. Proanthocyanidins and tannin-like compounds − nature, occurrence, dietary intake and effects on nutrition and health. *J. Sci. Food Agric.* **2000**, *80*, 1094−1117.
(19) Cos, P.; De Bruyne, T.; Hermans, N.; Apers, S.; Berghe, D. V.; Vlietinck, A. J. Proanthocyanidins in health care: Current and new trends. *Curr. Med. Chem.* **2004**, *11*, 1345−1359.
(20) Plumb, G. W.; De Pascual-Teresa, S.; Santos-Buelga, C.; Cheynier, V.; Williamson, G. Antioxidant properties of catechins and proanthocyanidins: effect of polymerisation, galloylation and glycosylation. *Free Radical Res.* **1998**, *29*, 351−358.
(21) Saint-Cricq de Gaulejac, N.; Provost, C.; Vivas, N. Comparative study of polyphenol scavenging activities assessed by different methods. *J. Agric. Food Chem.* **1999**, *47*, 425−431.
(22) Torres, J. L.; Varela, B.; García, M. T.; Carilla, J.; Matito, C.; Centelles, J. J.; Cascante, C.; Sort, X.; Bobet, R. Valorization of grape (*Vitis vinifera*) byproducts. Antioxidant and biological properties of polyphenolic fractions differing in procyanidin composition and flavonol content. *J. Agric. Food Chem.* **2002**, *50*, 7548−7555.
(23) Escribano Bailón, M. T.; Guerra, M. T.; Rivas Gonzalo, J. C.; Santos Buelga, C. Proanthocyanidin composition in skin and seed from grapes. *Polyphenols* **1994**, 225−226.
(24) Souquet, J.-M.; Cheynier, V.; Brossaud, F.; Moutounet, M. Polymeric proanthocyanidins from grape skins. *Phytochemistry* **1996**, *43*, 509−512.
(25) Kennedy, J. A.; Hayasaka, Y.; Vidal, S.; Waters, E. J.; Jones, G. P. Composition of grape skin proanthocyanidins at different stages of berry development. *J. Agric. Food Chem.* **2001**, *49*, 5348−5355.
(26) Souquet, J. M.; Labarbe, B.; LeGuerneve, C.; Cheynier, V.; Moutounet, M. Phenolic composition of grape stems. *J. Agric. Food Chem.* **2000**, *48*, 1076−1080.
(27) Prieur, C.; Rigaud, J.; Cheynier, V.; Moutounet, M. Oligomeric and polymeric procyanidins from grape seeds. *Phytochemistry* **1994**, *36*, 781−784.
(28) Fuleki, T.; Ricardo da Silva, J. M. Catechin and procyanidin composition of seeds from grape cultivars grown in Ontario. *J. Agric. Food Chem.* **1997**, *45*, 1156−1160.
(29) Packer, L.; Rimbach, G.; Virgili, F. Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (*Pinus maritima*) bark, pycnogenol. *Free Radical Biol. Med.* **1999**, *27*, 704−724.

(30) Rohdewald, P. A review of the French maritime pine bark extract (Pycnogenol (R)), a herbal medication with a diverse clinical pharmacology. *Int. J. Clin. Pharmacol. Ther.* **2002**, *40*, 158−168.
(31) Selga, A.; Sort, X.; Bobet, R.; Torres, J. L. Efficient one pot extraction and depolymerization of grape (*Vitis vinifera*) pomace procyanidins for the preparation of antioxidant thio-conjugates. *J. Agric. Food Chem.* **2004**, *52*, 467−473.
(32) Kondo, K.; Kurihara, M.; Miyata, N.; Suzuki, T.; Toyoda, M. Mechanistic studies of catechins as antioxidants against radical oxidation. *Arch. Biochem. Biophys.* **1999**, *362*, 79−86.
(33) Long, L. H.; Clement, M. V.; Halliwell, B. Artifacts in cell culture: Rapid generation of hydrogen peroxide on addition of (−)-epigallocatechin, (−)-epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media. *Biochem. Biophys. Res. Commun.* **2000**, *273*, 50−53.
(34) Torres, J. L.; Varela, B.; Brillas, E.; Juliá, L. Tris(2,4,6-Trichloro-3,5-dinitrophenyl)methyl radical: a new stable coloured magnetic species as a chemosensor for natural polyphenols. *Chem. Commun.* **2003**, 74−75.
(35) Torres, J. L.; Bobet, R. New flavanol-derivatives from grape (*Vitis vinifera*) byproducts. Antioxidant aminoethylthio-flavan-3-ol conjugates from a polymeric waste fraction used as a source of flavanols. *J. Agric. Food Chem.* **2001**, *49*, 4627−4634.
(36) Matito, C.; Mastorakou, F.; Centelles, J. J.; Torres, J. L.; Cascante, M. Antiproliferative effect of antioxidant polyphenols from grape in murine Hepa-1c1c7. *Eur. J. Nutr.* **2003**, *42*, 43−49.
(37) Sun, B. S.; Belchior, G. P.; Ricardo da Silva, J. M.; Spranger, M. I. Isolation and purification of dimeric and trimeric procyanidins from grape seeds. *J. Chromatogr., A* **1999**, *841*, 115−121.
(38) Labarbe, B.; Cheynier, V.; Brossaud, F.; Souquet, J. M.; Moutounet, M. Quantitative fractionation of grape proanthocyanidins according to their degree of polymerization. *J. Agric. Food Chem.* **1999**, *47*, 2719−2723.
(39) Torres, J. L.; Selga, A. Procyanidin size and composition by thiolysis with cysteamine hydrochloride and chromatography. *Chromatographia* **2003**, *57*, 441−445.
(40) Cano, A.; Hernández-Ruiz, J.; García-Cánovas, F.; Acosta, M.; Arnao, M. An end-point method for estimation of the total antioxidant capacity in plant material. *Phytochem. Anal.* **1998**, *9*, 196−202.
(41) Alcolea, J. F.; Cano, A.; Acosta, M.; Arnao, M. B. Hydrophilic and lipofilic antioxidant activities of grapes. *Nahrung* **2002**, *46*, 353−356.
(42) Blois, M. S. Antioxidant determinations by the use of stable free radical. *Nature* **1958**, *181*, 1199−1200.
(43) Brand-Williams, W.; Cuvelier, M. E.; Berset, C. Use of a free radical method to evaluate antioxidant activity. *Lebensm.-Wiss. Technol.* **1995**, *28*, 25−30.
(44) Buege, J. A.; Aust, S. D. Microsomal lipid peroxidation. *Methods Enzymol.* **1978**, *52*, 302−310.
(45) Huang, S.-W.; Hopia, A.; Schwarz, K.; Frankel, E. N.; German, J. B. Antioxidant activity of α-tocopherol and trolox in different lipid substrates: Bulk oils vs oil-in-water emulsions. *J. Agric. Food Chem.* **1996**, *44*, 444−452.
(46) Sánchez-Moreno, C.; Satué-Gracia, M. T.; Frankel, E. N. Antioxidant activity of selected spanish wines in corn oil emulsions. *J. Agric. Food Chem.* **2000**, *48*, 5581−5587.
(47) Huang, S.-W.; Satué-Gracia, M. T.; Frankel, E. N.; German, J. B. Effect of lactoferrin on oxidative stability of corn oil emulsions and liposomes. *J. Agric. Food Chem.* **1999**, *47*, 1356−1361.
(48) Schwarz, K.; Huang, S. W.; German, J. B.; Tiersch, B.; Hartmann, J. Activities of antioxidants are affected by colloidal properties of oil-in-water and water-in-oil emulsions and bulk oils. *J. Agric. Food Chem.* **2000**, *48*, 4874−4882.
(49) Chan, H. W.-S.; Lewet, G. Autoxidation of methyl linoleate. Separation and analysis of isomeric mixtures of methyl linoleate hydroperoxides and methyl hydroxylinoleates. *Lipids* **1977**, *12*, 99−104.
(50) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, 55−63.
(51) Derdelinckx, G.; Jerumanis, J. Separation of malt and hop proanthocyanidins on Fractogel TSK HW-40S. *J. Chromatogr.* **1984**, *285*, 231−234.
(52) Miller, N. J.; Rice Evans, C. The relative contributions of ascorbic acid and phenolic antioxidants to the total antioxidant activity of orange and apple fruit juices and blackcurrant drink. *Food Chem.* **1997**, *60*, 331−337.
(53) Pietta, P.; Simonetti, P.; Mauri, P. Antioxidant activity of selected medicinal plants. *J. Agric. Food Chem.* **1998**, *46*, 4487−4490.
(54) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol. Med.* **1999**, *26*, 1231−1237.
(55) Luximon-Ramma, A.; Bahorun, T.; Soobrattee, M. A.; Aruoma, O. I. Antioxidant activities of phenolic, proanthocyanidin, and flavonoid components in extracts of Cassia fistula. *J. Agric. Food Chem.* **2002**, *50*, 5042−5047.
(56) Dangles, O.; Fargeix, G.; Dufour, C. Antioxidant properties of anthocyanins and tannins: a mechanistic investigation with catechin and the 3′,4′,7-trihydroxyflavylium ion. *J. Chem. Soc., Perkin Trans. 2* **2000**, 1653−1666.
(57) Goupy, P.; Dufour, C.; Loonis, M.; Dangles, O. Quantitative kinetic analysis of hydrogen transfer reactions from dietary polyphenols to the DPPH radical. *J. Agric. Food Chem.* **2003**, *51*, 615−622.
(58) Jiménez, A.; Selga, A.; Torres, J. L.; Juliá, L. Reducing activity of polyphenols with stable radicals of the TTM series. Electron transfer versus H-abstraction reactions in flavan-3-ols. *Org. Lett.* **2004**, *6*, 4583−4586.
(59) Kovacic, P.; Sacman, A.; Wu-Weis, M. Nephrotoxins: widespread role of oxidative stress and electron transfer. *Curr. Med. Chem.* **2002**, *9*, 823−847.
(60) Kondo, K.; Kurihara, M.; Miyata, N.; Suzuki, T.; Toyoda, M. Scavenging mechanisms of (−)-epigallocatechin gallate and (−)-epicatechin gallate on peroxyl radicals and formation of superoxide during the inhibitory action. *Free Radical Biol. Med.* **1999**, *27*, 855−863.
(61) Huang, S.-W.; Frankel, E. N.; Aeschbach, R.; German, J. B. Partition of selected antioxidants in corn-water model systems. *J. Agric. Food Chem.* **1997**, *45*, 1991−1994.
(62) Liberto, M.; Cobrinik, D. Growth factor-dependent induction of p21(CIP1) by the green tea by polyphenol, epigallocatechin gallate. *Cancer Lett.* **2000**, *154*, 151−161.
(63) Jaeger, A.; Wälti, M.; Neftel, K. Side effects of flavonoids in medical practice. In *Plant Flavonoids in Biology and Medicine II: Biochemical, Cellular, and Medicinal Properties*; Cody, V., Ed.; Alan R. Liss Inc.: New York, 1988; pp 379−394.
(64) Stuhlinger, W.; Berek, K.; Grosswang, F.; Schauer, N. Several years observation of a Catergen (cyanidanol-3) induced immunohemolysis. *Schweiz. Med. Wochenschr.* **1990**, *120*, 345−348.
(65) Rodríguez, J.; Yáñez, J.; Vicente, V.; Alcaraz, M.; Benavente-García, O.; Castillo, J.; Lorente, J.; Lozano, J. A. Effects of several flavonoids on the growth of B16F10 and SK-MEL-1 melanoma cell lines: relationship between structure and activity. *Melanoma Res.* **2002**, *12*, 99−107.
(66) Lama, G.; Angelucci, C.; Bruzzese, N.; Iacopino, F.; Nori, S. L.; D'Atri, S.; Turriziani, M.; Bonmassar, E.; Sica, G. Sensitivity of human melanoma cells to oestrogens, tamoxifen and quercetin: is there any relationship with type I and II oestrogen binding site expression. *Melanoma Res.* **1998**, *8*, 313−322.

Received for review February 4, 2005. Accepted March 22, 2005. Financial support from the Spanish Ministry of Education and Science (research grants PPQ2003-06602-C04-01 and -04, and doctoral fellowships to C.L. and D.L.) is acknowledged. A private donation by Esperança and Rosa Soler Módena is also gratefully acknowledged.

JF050262Q

4.1.2. PUBLICACIÓN 2: Evaluación de la actividad antioxidante y biológica de fracciones fenólicas de elevada galoización.

Título original: Highly galloylated tannin fractions from witch hazel (*Hamamelis virginiana*) bark: Electron transfer capacity, in vitro antioxidant activity, and effects on skin-related cells.

Autores: Sonia Touriño, Daneida Lizárraga, Anna Carreras, Sonia Lorenzo, Vanessa Ugartondo, Montserrat Mitjans, Mª Pilar Vinardell, Luis Juliá, Marta Cascante and Josep Lluís Torres. ***Chemical Research in Toxicology***, 2008, *21*, 696-704.

Índice de impacto: 3,508

Clasificación y categoría: 10/73 (Toxicología); 6/41 (Química médica); 19/128 (Química, multidisciplinar)

RESUMEN:

Con el fin de poder completar el primer objetivo y continuando en la línea de los trabajos anteriormente realizados, se buscó una fuente de proantocianidinas de galoización elevada. La corteza de hamamelis (*Hamamelis virginiana*) permitió obtener un extracto rico en grupos galato gracias a la abundancia de taninos tanto condensados como hidrolizables en su composición. Los taninos condensados son polímeros formados por unidades de flavan-3-ol (en el caso de hamamelis, (epi)catequinas y (epi)galocatequinas), mientras que los taninos hidrolizables son unidades de ácido gálico unidos a una D-glucosa (galotaninos) que pueden derivar a elagitaninos cuando dos moléculas de ácido gálico sufren un acoplamiento oxidativo *(Haslam, 2007)*.

El procedimiento para generar las fracciones fue el mismo que el utilizado para los estudios de bagazo de uva y corteza de pino. Para poder comparar la actividad de las fracciones de hamamelis con las anteriormente obtenidas de corteza de pino y bagazo de uva se realizaron ensayos capacidad antioxidante/antirradicalaria, citotoxicidad (queratinocitos y fibroblastos) y actividad antiproliferativa en la línea celular de melanoma (SK-Mel-28).

Además, en este estudio se incluyó la evaluación de las fracciones de proantocianidinas frente al radical estable TNPTM *(Torres et al., 2007)*. Este radical, similar al HNTTM, únicamente capta electrones de especies altamente reactivas. Las fracciones de Hamamelis mostraron actividad frente al TNPTM, mientras que las fracciones de bagazo de uva y corteza de pino no reaccionaron con el TNPTM.

Los resultados mostraron que las fracciones de hamamelis de elevada galoización en su composición presentan mayor capacidad antioxidante (medida por los ensayos DPPH, TEAC, HNTTM) que las fracciones de bagazo de uva y corteza de pino. A la vez, el incremento del poder antioxidante resultó en un incrementó de la capacidad antiproliferativa y de la citotoxicidad de las fracciones de hamamelis.

La presencia de un elevado porcentaje de grupos pirogalol parece ser la clave de la elevada reactividad que estas fracciones presentaron. Dicha reactividad podría estar asociada a un efecto pro-oxidante. Por lo tanto, el ensayo con el radical TNPTM podría ayudar a predecir los efectos pro-oxidantes que algunos compuestos fenólicos pueden presentan.

A continuación se muestra el artículo original. El material suplementario de esta publicación se incluye en los Anexos (apartado 8.2.2, *ver pág. 273*)

Highly Galloylated Tannin Fractions from Witch Hazel (*Hamamelis virginiana*) Bark: Electron Transfer Capacity, In Vitro Antioxidant Activity, and Effects on Skin-Related Cells

Sonia Touriño,[†] Daneida Lizárraga,[‡] Anna Carreras,[†] Sonia Lorenzo,[†] Vanessa Ugartondo,[§] Montserrat Mitjans,[§] María Pilar Vinardell,[§] Luis Juliá,[†] Marta Cascante,[‡] and Josep Lluís Torres*,[†]

Institute for Chemical and Environmental Research (IIQAB-CSIC), Jordi Girona 18-26, 08034 Barcelona, Department of Biochemistry and Molecular Biology, Associated Unit to CSIC, Universitat de Barcelona, Avinguda Diagonal 645, 08028 Barcelona, and Departament de Fisiologia, Associated Unit to CSIC, Facultat de Farmàcia, Universitat de Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain

Received December 5, 2007

Witch hazel (*Hammamelis virginiana*) bark is a rich source of both condensed and hydrolizable oligomeric tannins. From a polyphenolic extract soluble in both ethyl acetate and water, we have generated fractions rich in pyrogallol-containing polyphenols (proanthocyanidins, gallotannins, and gallates). The mixtures were highly active as free radical scavengers against ABTS, DPPH (hydrogen donation and electron transfer), and HNTTM (electron transfer). They were also able to reduce the newly introduced TNPTM radical, meaning that they included some highly reactive components. Witch hazel phenolics protected red blood cells from free radical-induced hemolysis and were mildly cytotoxic to 3T3 fibroblasts and HaCat keratinocytes. They also inhibited the proliferation of tumoral SK-Mel 28 melanoma cells at lower concentrations than grape and pine procyanidins. The high content in pyrogallol moieties may be behind the effect of witch hazel phenolics on skin cells. Because the most cytotoxic and antiproliferative mixtures were also the most efficient as electron transfer agents, we hypothesize that the final putative antioxidant effect of polyphenols may be in part attributed to the stimulation of defense systems by mild prooxidant challenges provided by reactive oxygen species generated through redox cycling.

Introduction

Phenolics from plants are appreciated for their putative health-promoting properties (*1, 2*). The antioxidant activity, taken in a broad sense, is believed to be responsible for the preventative properties of flavonoids. The main mechanisms behind this antioxidant activity are direct free radical scavenging (*3, 4*), transition metal chelation (*5, 6*), and maintenance of endogenous antioxidants such as the glutathione and superoxide dismutase systems (*7*). Interestingly, polyphenols may be antioxidant and prooxidant at the same time (*8, 9*). While all phenolics are scavengers of reactive oxygen species (ROS), strongly reducing species such as pyrogallol (three adjacent phenol groups) containing (−)-epigallocatechin (EGC)[1] and (−)-epigallocatechingallate (EGCG) are able to form the superoxide radical from molecular oxygen (*10, 11*) (Figure 1). Moreover, the *ortho*-quinones formed by the loss of two electrons from pyrogallol and catechol moieties may participate in enzymatic redox cycling with the formation of superoxide and other ROS (*12, 13*). Apart from their participation in redox-related events, tannins may modify cell functions by substrate−receptor interactions (e.g., kinase inhibition), which may or may not involve redox reactions (*14*). This ensemble of activities influence cell proliferation, cell cycle regulation, and apoptosis, and the pyrogallol moieties, both on ring B and as gallate ester at C-3, appear to play a pivotal role (*15, 16*).

Whether all of these effects detected in vitro have any significance in vivo is controversial. Because polyphenols are extensively metabolized into less reactive species (*17*) and the cell redox system is too carefully regulated to be influenced by low concentrations of scavengers, it has been argued that polyphenols may not exert any significant effect on the cell redox status of complex organisms (*18, 19*). However, it can also be argued that polyphenols, particularly the less metabolized oligomeric species, may still have a significant influence on organs such as the skin and the intestinal tract (*20, 21*). In any case, flavonoid-containing nutritional supplements and over the counter drugs have become so popular and available that people risk overdosing. This is why it is important to examine the action of these plant actives from different angles and to evaluate their putative benefits and risks. Most of the information available in the literature about the antioxidant/prooxidant activities and substrate−receptor interactions of phenolics relates to monomeric EGCG and quercetin (*13, 14, 22*). Because the redox and binding properties of phenolics are affected by polymerization

* To whom correspondence should be addressed. Tel: 34 93 400 61 12. Fax: 34 93 204 59 04. E-mail: jltqbp@iiqab.csic.es.
[†] CSIC.
[‡] Department of Biochemistry and Molecular Biology, Universitat de Barcelona.
[§] Departament de Fisiologia, Universitat de Barcelona.

[1] Abbreviations: AAPH, 2,2′-azobis(amidinopropane)dihydrochloride; ARC, antiradical capacity; C, catechin; Cya, cysteamine; EC, epicatechin; ECG, epicatechin-gallate; EGC, epigallocatechin; EGCG, epigallocatechin-gallate; GC, gallocatechin; DMEM, Dulbecco's modified Eagle's medium; DPPH, 1,1-diphenyl-2-picrylhydrazyl free radical; FCS, fetal calf serum; HNTTM, Tris(2,3,6-trichloro-3,5-dinitrophenyl)methyl; HPLC-DAD, high-performance liquid chromatography with diode array detection; MTT, 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide; NRU, neutral red uptake; RP-HPLC, reversed-phase high-performance liquid chromatography; RCBs, red blood cells; TFA, trifluoroacetic acid; Trolox, 2,5,7,8-tetramethylchroman-2-carboxylic acid; TNPTM, tris(2,3,5,6-tetrachloro-4-nitrophenyl)-methyl.

10.1021/tx700425n CCC: $40.75
Published on Web 03/01/2008

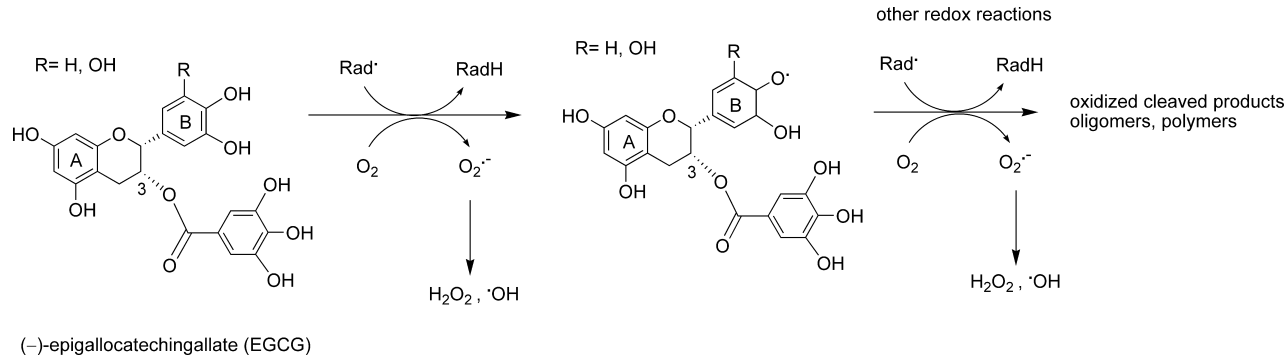


Figure 1. Scavenging of ROS and superoxide formation by catechins.

(*23, 24*), it is of great interest to evaluate the activity of the oligomers (e.g., proanthocyanidins) on cells. We have previously reported the extraction and fractionation of phenolics from grape pomace and pine bark and the evaluation of their free radical scavenging capacity, antioxidant activity in vitro, cytotoxicity on nontumoral 3T3 fibroblasts and keratinocites, and antiproliferative activity on melanoma cells (*25–27*). Polyphenols from grape and pine were essentially procyanidins (oligomeric catechins with only two hydroxyls on ring B, catechol moiety) with low gallate content or no galloylation at all, respectively. The fractions were effective free radical scavenger antiproliferative agents against skin and colon tumoral cells and weakly cytotoxic. To test the behavior of phenolics with high pyrogallol content, we have now prepared and evaluated a homologous series of fractions from witch hazel (*Hamamelis virginiana*) bark, which contains gallocatechins and prodelphinidins (monomeric and oligomeric catechins with three hydroxyls on ring B) with a high proportion of gallates. The gallates come from both condensed and hydrolyzable tannins. As compared to grape and pine, phenolics from hamamelis showed higher electron transfer capacity, cytotoxicity, and antiproliferative activity against skin-related cell lines.

Experimental Procedures

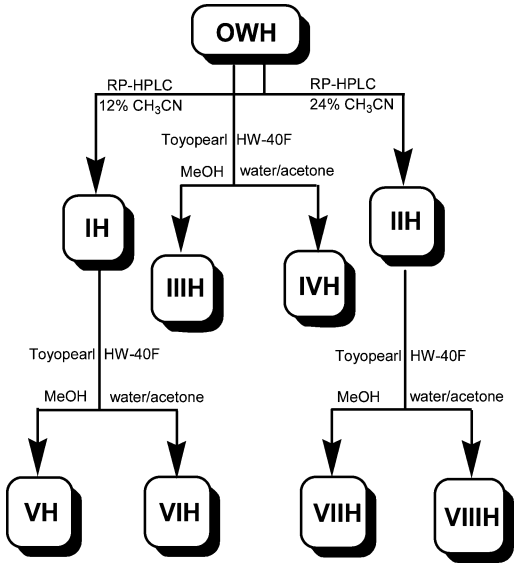
The starting material was witch hazel (*H. virginiana*) chopped stems provided by Martin Bauer GMBH (Alveslohe, Germany). The sample was stored in the dark at room temperature.

Solvents and Reagents. For extraction, deionized water, bulk EtOH (Momplet y Esteban, Barcelona, Spain), bulk acetone (Quimivita, Sant Adrià del Besòs, Spain), and bulk hexane (Quimivita) were used for polyphenol extraction. For purification, deionized water, analytical grade MeOH (Panreac, Montcada i Reixac, Spain), and analytical grade acetone (Carlo Erba, Milano, Italy) and preparative grade CH_3CN (E. Merck, Darmstadt, Germany) were used for semipreparative and preparative chromatography; milli-Q water and HPLC grade CH_3CN (E. Merck) were used for analytical reversed-phase high-performance liquid chromatography (RP-HPLC). Analytical grade MeOH (Panreac) was used for thioacidolysis and free radical scavenging assays, and analytical grade CH_3Cl (Panreac) was used for the electron transfer assays. Trifluoroacetic acid (TFA, Fluorochem, Derbyshire, United Kingdom) biotech grade was distilled in-house. Cysteamine hydrochloride was from Sigma-Aldrich Chemical (Steinheim, Germany), and 37% HCl and acetic acid were from E. Merck. Triethylamine (E. Merck) was of buffer grade. Deuterated solvents for nuclear magnetic resonance (NMR) were from SDS (Peypin, France). 1,1-Diphenyl-2-picrylhydrazyl free radical (DPPH) (95%) was from Aldrich (Gillingham-Dorset, United Kingdom), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) (97%) was from Aldrich (Milwaukee, WI), and standards of (−)-epicatechin, (+)-catechin, (+)-gallocatechin (−)-epigallocatechin, (−)-epigallocatechin 3-*O*-gallate, gallic acid, methyl gallate, and hamamelitannin were purchased from Sigma Chemical (St. Louis, MO). 4-β-(2-Aminoethylthio)catechin, 4-β-(2-aminoethylthio)epicatechin, β-(2-aminoethylthio)epicatechin-3-*O*-gallate, 4-β-(2-aminoethylthio)epigallocatechin, and β-(2-aminoethylthio)epigallocatechin-3-*O*-gallate were synthesized and purified from grape and witch hazel extracts essentially as described (*28*). 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) crystallized diammonium salt, horseradish peroxidase type IV (RZ A403/A275 <3) and 2,2′-azobis(amidinopropane)dihydrochloride (AAPH) were obtained from Sigma Chemical. Hydrogen peroxide (3% v/v) was from Sigma Chemical. Tris-(2,4,6-trichloro-3,5-dinitrophenyl)-methyl (HNTTM) and tris(2,3,5,6-tetrachloro-4-nitrophenyl)methyl (TNPTM) radicals were synthesized as described (*29, 30*). Dulbecco's modified Eagle's medium (DMEM) and Dulbecco's phosphate buffer saline were from Gibco-BRL (Eggenstein, Germany), fetal calf serum (FCS) was from Invitrogen (Carlsbad, CA), and trypsin EDTA solution C (0.05% trypsin–0.02% EDTA) was from Biological Industries (Kibbutz Beit Haemet, Israel). 3-[4,5-Dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) was from Sigma Chemical.

Extraction and Solvent Fractionation. The preparation of the crude extract was performed using already described methodology (*25, 31, 32*). Brief, witch hazel chopped stems (3 kg) were incubated with an acetone–water mixture (7:3, 10.5 L) for a period of 24 h at room temperature, with occasional shaking. The solid was filtered off, and the acetone was evaporated at reduced pressure. The remaining solution was defatted with *n*-hexane (3 × 300 mL), and the oligomeric fraction was extracted with ethyl acetate (3 × 600 mL). This organic phase was dried under vacuum, the pellet was dissolved in deionized water, and the solution was filtered through a porous plate. The dry fraction OWH (28.5 g), soluble in both ethyl acetate and water, was obtained by lyophilization.

Chromatographic Fractionation. Fractions IH (hydrophilic) and IIH (hydrophobic) were obtained by preparative RP-HPLC essentially as described before (*26, 33*). The rest of the fractions were generated from these two or directly from OWH by semipreparative chromatography on Toyopearl TSK HW-40F (TosoHass, Tokyo, Japan) following a protocol previously described by the authors (*25, 26*) (Scheme 1). The phenolics were eluted with MeOH and water/acetone 1:1, evaporated almost to dryness, redissolved in 100 mL of Milli-Q water, and freeze-dried; from OWH, 315 mg of IIIH and 573 mg of IVH; from fraction IH, 235 mg of VH and 336 mg of VIH; and from fraction IIH, 126 mg of VIIH and 468 mg of VIIIH. The fractions were analyzed by high-performance liquid chromatography with diode array detection (HPLC-DAD) using a Hitachi (San Jose, CA) Lachrom Elite HPLC system equipped with a quaternary pump, autosampler, in-line degassing unit, temperature control unit, photodiode array UV detector, and fitted with an analytical column Kromasil C18 (Teknokroma, Barcelona, Spain) (25 cm × 0.4 cm i.d., 100 Å, 5 μm particle size). Acquisitions were made using EZChrom Elite 3.1.3 from Scientific Software Inc. (Pleasanton, CA). Load, 40 μL, 10 μg; elution, (A) 0.1% (v/v) aqueous TFA and (B) 0.08% (v/v) TFA in water/CH_3CN 1:4,

Scheme 1



gradient 12−30% B over 30 min at a flow rate of 1 mL/min. DAD detection was performed from 210 to 380 nm. Data were acquired in triplicate.

Characterization by Thiolysis with Cysteamine and RP-HPLC. The size and composition of the proanthocyanidins within the fractions were estimated from the HPLC analysis of acid-catalyzed degradation of proanthocyanidins in the presence of cysteamine, followed by RP-HPLC as described (*34*). Briefly, the terminal flavan-3-ols units were released as such by acid cleavage in the presence of cysteamine whereas the extension moieties were released as the cysteamine derivatives on the fourth position of the flavanoid system. The resulting mixtures were submitted to analytical RP-HPLC using the same conditions described above for the intact samples, and the molar amount (nanomoles) of all of the released moieties was calculated from the peak areas and calibration curves obtained with pure samples. Terminal units: (+)-gallocatechin (GC), (−)-EGC, (+)-C, (−)-epicatechin (EC), (−)-EGCG, and (−)-ECG; extension units: cysteamine (Cya)-C, Cya-EC, Cya-EGC, Cya-EGCG, and Cya-ECG. Mean degree of polymerization (mDP) = total nmol/nmol terminal units.

Characterization by Chromatography Coupled to Mass Spectrometry. Liquid chromatography–mass spectrometry (LC-MS-MS) was used for the identification of gallotannins and gallates within the fractions. The analyses were carried out on an Agilent 1100 (Waldrom, Germany) coupled to an API 3000 triple quadrupole mass spectrometer (Perkin-Elmer Sciex, Concord, ON, Canada) at the Parc Cientific de la Universitat de Barcelona. Mass scan (MS) and daughter (MS/MS) spectra were measured from *m*/*z* 100 to 1500. Mass spectrometry data were acquired in the negative ionization mode.

ABTS Radical Cation Decolorization Assay. The method is based on the capacity of a sample to scavenge the ABTS radical cation ($ABTS^{\bullet+}$) as compared to a standard antioxidant (Trolox). $ABTS^{\bullet+}$was generated from ABTS as described (*35*) with some modifications (*26*). To prepare the initial $ABTS^{\bullet+}$ solution, 3‰ H_2O_2 (45μL) was added to a reaction mixture containing ABTS (54.9 mg, 1 mM) and horseradish peroxidase (HRP, 1.1 mg, 0.25 μM) in 50 mM gly HCl buffer, pH 4.5 (100 mL). The reaction mixture was left to stand at room temperature for 15 min in the dark. Then, the polyphenolic solutions (50 μL) at concentrations of 0.3, 0.2, 0.15, 0.10, and 0.05 mg/mL in MeOH were added to the $ABTS^{\bullet+}$ solution (1950 μL). The total time needed to carry out each assay was 20 min, including ABTS radical generation, addition of antioxidant, and acquisition of final absorbance value. The decrease of absorbance at 734 nm with respect to the 1 mM solution of $ABTS^{\bullet+}$ was recorded on a UV spectrophotometer Cary 300-Bio (Varian, Palo Alto, CA). The assay was performed in triplicate. The dose–response curves obtained with the antioxidant mixtures and Trolox were plotted as the percentage of absorbance decrease against the amount of antioxidants expressed as μg/mL. The total antioxidant activity (TAA) of the fractions was expressed in mmol Trolox equiv/g of OWH.

DPPH Assay. The antiradical efficiency of the fractions was evaluated by the DPPH stable radical method (*36, 37*). The samples (0.1 mL) were added to aliquots (3.9 mL) of a solution made up with DPPH (4.8 mg) in MeOH (200 mL), and the mixture was incubated for 1 h at room temperature in the dark. The initial concentration of DPPH, approximately 60 μM, was calculated for every experiment from a calibration curve made by measuring the absorbance at 517 nm of standard samples of DPPH at different concentrations. The equation of the curve was $Abs_{517nm} = 11345 \times C_{DPPH}$ as determined by linear regression. The results were plotted as the percentage of absorbance disappearance at 517 nm $[(1 - A/A_0) \times 100]$ against the amount of sample divided by the initial concentration of DPPH. Each point was acquired in triplicate. A dose–response curve was obtained for every fraction. ED_{50} corresponds to micrograms of fraction able to consume half the amount of free radical divided by micromoles of initial DPPH. The results were expressed as antiradical capacity (ARC), which is the inverse of ED_{50}. UV measurements were made on a UV spectrophotometer Cary 300-Bio (Varian).

Electron Transfer Capacity against HNTTM and TNPTM. The fractions were dissolved in CH_3Cl/MeOH (2:1) at different concentrations. Aliquots (1 mL) were added to a solution (1 mL) of HNTTM [120 μM in CH_3Cl/MeOH (2:1)] (*29*), and the mixture was incubated for 7 h. The exact initial concentration of radical, around 60 μM, was calculated for every experiment from calibration curves made by measuring the absorbance (A_0) at 385 nm of standard samples of the radical at different concentrations. The equations of the curve was $A_0 = 21170 \times C_{radical}$. The results were plotted as the percentage of absorbance disappearance $[(1 - A/A_0) \times 100]$ against the amount of sample divided by the initial micromoles of the radical as described for DPPH. Each point was acquired in triplicate. A dose–response curve was obtained for every fraction. The results were expressed as the efficient dose ED_{50} given as micromoles of fraction able to consume half the amount of free radical divided by micromoles of initial HNTTM.

The working conditions with TNPTM were essentially those described for HNTTM with some differences. The incubation time was 48 h, and the absorbance was measured at 378 nm. The equation for the calibration curve was $A_0 = 17153 \times C_{radical}$. The results were plotted as described for HNTTM. UV measurements were made on a UV spectrophotometer Cary 300-Bio (Varian). A solution of pyrogallol [60 μM in CH_3Cl/MeOH (2:1)] was stable for 48 h as ascertained by RP-HPLC under the elution conditions described before for the analysis of the fractions.

Antioxidant Activity on Red Blood Cells by the AAPH Assay. Blood samples were obtained from healthy donors by venipuncture (Blood Bank of Hospital Vall d'Ebron, Barcelona, Spain) following the ethical guidelines of the hospital and collected in citrated tubes. Red blood cells (RBCs) were separated from plasma and buffy coat by centrifugation at 1000*g* for 10 min. The erythrocyte layer was washed three times in phosphate buffer isotonic saline (PBS) containing 22.2 mM Na_2HPO_4, 5.6 mM KH_2PO_4, 123.3 mM NaCl, and 10.0 mM glucose in distilled water (pH 7.4). The cells were then suspended in isotonic saline solution at a density of 8×10^9 cells/mL. We measured the hemolysis of RBCs mediated by AAPH using a modification of the method described previously (*38*). The addition of AAPH (a peroxyl radical initiator) to the suspension of RBCs induces the oxidation of cell membrane lipids and proteins, thereby resulting in hemolysis. The erythrocyte suspension (250 μL) was incubated in the presence of AAPH at a final concentration of 100 mM for 150 min at 37 °C to achieve 100% hemolysis. Hemolysis was assessed by measuring the absorbance of the supernatant fraction, that is, the hemoglobin release, at 540 nm in a Shimadzu spectrophotometer (Shimadzu, Japan). The antihemolytic activity of the fractions was studied by adding the compounds at several concentrations (10−150 μg/mL)

to the RBCs suspension in the presence of 100 mM AAPH at 37 °C for 2.5 h. The IC_{50} (sample concentration causing 50% protection) of the hemolysis induced by AAPH was determined for each compound.

Cytotoxicity on Keratinocytes and Fibroblasts. To evaluate the cytotoxicity on nontumoral cells, we used the spontaneously immortalized human keratinocyte cell line HaCaT and the mouse fibroblast cell line 3T3. Cells were grown in DMEM (4.5 g/L glucose) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM Hepes buffer, and 1% penicillin (10000 U/mL) streptomycin (10000 μg/mL) maintained in a humidified atmosphere with 5% CO_2 at 37 °C. When 75 cm^2 culture flasks were approximately 80% confluent, the cells were seeded into the central 60 wells of 96-well plates as described previously (*39*) at a density of 5.5×10^4 cells/mL for HaCaT and 1.5×10^4 cells/mL for 3T3 (*40*). Plates were incubated at 37 °C and 5% CO_2 for 24 h. Triplicate runs were undertaken with different passage cells. After 1 day of incubation, the growth medium was removed and replaced with exposure medium (DMEM medium supplemented with 5% FBS, 2 mM L-glutamine, 10 mM Hepes buffer, and 1% antibiotic mixture), with or without the polyphenolic mixtures, which were previously sterilized by filtration. Controls, containing culture medium only, were included in each plate. Cells were then incubated at 37 °C and 5% CO_2 for 72 h.

The cell viability was assessed by the neutral red uptake (NRU) assay and performed as described (*41*) and modified to avoid the use of formaldehyde (*42*). After the treatments, medium was aspired and replaced with 100 μL/well of NR solution (50 μg/mL in RPMI medium without phenol red and serum). After 3 h of incubation at 37 °C and 5% CO_2, the medium was aspirated, the cells were washed twice with PBS, and a solution containing 50% ethanol absolute and 1% acetic acid in distilled water was added (100 μL/well) to release the dye incorporated into the viable cells into the supernatant. After 10 min on a microtiter plate shaker, the absorbance of the neutral red was measured at a wavelength of 550 nm in a Bio-Rad 550 microplate reader (Bio-Rad Laboratories, Hercules, CA).

The cytotoxicity of each fraction was expressed as a percentage of viability as compared to control wells (the mean optical density of untreated cells was set to 100% viability) in terms of its IC_{50} (concentration of product that causes 50% inhibition of growth or death of the cell population), calculated from the dose–response curves by linear regression analysis. NRU assay results were expressed as the percentage of uptake of neutral red dye by the lysosomes. Each experiment was performed at least three times using three replicates for each concentration assayed. The results were expressed as means ± SEM. Statistical significance was determined by Student's *t* test and one-way analysis of variance (ANOVA) using the SPSS software (SPSS Inc., Chicago, IL). Statistical significance was considered at $P < 0.05$.

Antiproliferative Activity on SK-Mel-28 Human Melanoma Cells. SK-MEL-28 adherent cells (ATCC #HTB-72) were grown in DMEM supplemented with 10% (v/v) heat-inactivated FCS in the presence of 0.1% (v/v) antibiotics (10000 U/mL penicillin and 10000 μg/mL streptomycin) at 37 °C in a humidified environment with 5% CO_2. The cells were split (ratio 1:2 to 1:5) by mild trypsinization every 4–5 days, and the medium was changed every 2–3 days. The cell culture used in this study was free of mycoplasm infection as shown by the EZ-PCR Mycoplasm test kit (Biological Industries) prior to the treatment with the samples. The cell viability was determined using the Mosmann assay (*43*) with some modifications. Cells were seeded into 96-well plates at 1×10^4 cells/mL density, 200 μL/well, and incubated for 24 h in the culture medium prior to addition of the samples dissolved in DMEM. Control wells were treated with equal volumes of DMEM as the test cultures. After 72 h of culture, the supernatant was aspirated and 100 μL of sterile-filtered MTT (0.5 mg/mL in DMEM) was added to each well. The plates were incubated at 37 °C and 5% CO_2 for 1 h. The supernatant was removed, the blue MTT formazan that precipitated was dissolved in DMSO (100 μL), and the optical density (OD) was measured at 550 nm on a multiwell reader (Merck ELISA System MIOS).

The inhibitory effect of the fractions at each concentration was expressed as a percentage [(mean OD treated cells after 72 h of incubation with the product/mean OD of control cells after 72 h of incubation with extra medium instead of product) × 100]. The IC_{50} or sample concentration causing a 50% reduction in the mean OD value relative to the control at 72 h of incubation was estimated using GraFit 3.00 (Data Analysis and Graphics Program, Erithacus Software Ltd. Microsoft Corp., Surrey, United Kingdom) curve option: IC_{50} curve − start at 0.

Results and Discussion

Fractionation of Witch Hazel Oligomeric Tannins. Following a combination of already described methods (*25, 28, 44*), a polyphenolic mixture of monomeric and oligomeric tannins soluble in both ethyl acetate and water (OWH) was obtained from witch hazel bark. First, a sugar free mixture was obtained by extraction with water/acetone (3:7). After the acetone was evaporated, the lipid soluble material was eliminated with hexane, and the resulting aqueous phase was extracted with ethyl acetate. The organic solvent was eliminated to yield a crude polyphenolic mixture (28.5 g from 3 kg of dry stems, ca. 1% yield). Witch hazel contained more small and medium-sized phenolics (OWH) than grape pomace (OWG, yield ca. 0.1%) (*28*) or pine bark (OWP, yield >0.1%) (*26*). This crude mixture was fractionated (Scheme 1) into eight fractions by a combination of two chromatographic techniques, namely, reversed-phase and size discrimination using the same strategy applied to grape and pine extracts (*25, 26*). RP-HPLC retains solutes by hydrophobicity while Toyopearl HW-40 has been shown to separate flavonoids in order of increasing sizes (*45*). In this way, we generated a collection of mixtures containing hydrolyzable tannins and oligomeric pronthocyanidins of different mean degrees of polymerization, galloylation, and prodelphinidin contents.

Characterization of the Fractions. The structures of significant compounds found in OWH and its fractions are depicted in Figure 2. In accordance with the literature (*44, 46*), the mixtures contained flavanol (catechin) monomers, proanthocyanidins, and hydrolyzable tannins such as hamamelitannin. Some of the mixtures also contained methyl gallate and pentagalloyl glucose. Tables 1 and 2 summarize the results obtained from the HPLC analysis after thioacidolysis (condensed tannins) and direct HPLC-DAD analysis (hamamelitannin, gallic acid, methyl gallate, and pentagalloylglucose). The mean degree of polymerization and composition in constituent monomers of the condensed tannin portion (monomers + proanthocyanidins) were estimated by thioacidolysis in the presence of cysteamine as described in the Experimental Procedures. This procedure, which uses cysteamine hydrochloride as an alternative reagent to thiol-α-toluene, was originally applied to procyanidins (catechol-containing condensed tannins). Now, we have extended the method to prodelphinidins. The appropriate pyrogallol containing new standards, namely, 4-β(2-aminoethylthio) epigallocatechin (Cya-EGC) and 4-β(2-aminoethylthio) epigallocatechin 3-*O*-gallate (Cya-EGCG), have been obtained from the polymeric fraction insoluble in ethyl acetate following essentially the procedures described before (*28*). All of the fractions contained condensed tannins, both monomers and oligomers. Interestingly, the more retained mixture on reversed-phase HPLC (IIH) contained less condensed tannins (34.7%) than IH (79.1%). Small condensed tannins from witch hazel are markedly hydrophilic as compared to phenolics from pine and grape. This is due to the presence of the pyrogallol moiety on ring B,

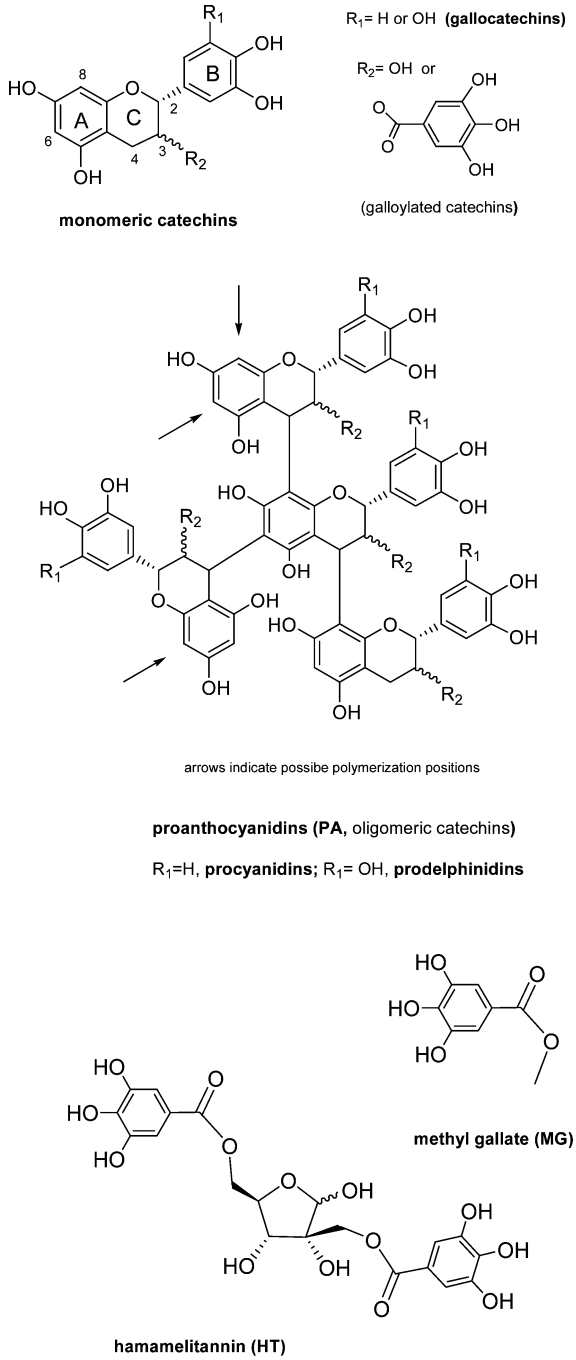


Figure 2. Structures of polyphenolics in *H. virginiana* bark extract.

which is absent in pine and very sparse in grape. Data from Table 2 show that the hydrophobic fractions IIH, VIIH, and VIIIH were low in gallocatechins, which were mainly found in IH, VIH, and IVH. The higher amounts of gallocatechins in the more retained fractions on Toyopearl indicate that they are mainly included in oligomeric structures (prodelphinidins) in contrast with tea gallocatechins, which are monomeric (*47*). This is in agreement with the composition of fraction VH, which was mainly monomeric and low in gallocatechins. The extract and fractions also contained the so-called galloylhamameloses, hydrolyzable tannins that were identified by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Di- and trigalloyl hamamelofuranoses have been described before (*46, 48*). We have identified hamamelitannin (2′,5′ di-*O*-galloyl hamamelose HT *m*/*z* [M − H]⁻483, Figure 2) and a pentagalloyl glucose (PGG, *m*/*z* [M − H]⁻ 939) as the two main galloyltannins in OWH and derived fractions. Gallic acid (GA) and methyl gallate (MG *m*/*z* [M − H]⁻ 183) were detected in some of the fractions. The pentagalloyl glucose content was particularly high in fractions IIH and VIIIH.

Table 1. Polyphenolic Composition of Fractions from Witch Hazel Bark[a]

fraction	% M + PA[b]	mDP[c] M + PA	% HT[d]	% GA[d]	% MG[d]	% PGG[e]
OWH	62.7	1.2	7.9	21.9		7.4
IH	79.1	1.1	10.9	4.1		5.8
IIH	34.7	1.7	2.5	14.7		48.2
IIIH	62.3	1.0	2.1	35.4		0.6
IVH	62.7	1.6	15.0	2.3		19.9
VH	78.8	1.1	2.0	12.1	7.0	
VIH	41.9	2.6	16.1	1.8	18.2	21.7
VIIH	7.9	1.0	0.6	43.3	30.4	17.8
VIIIH	4.3	1.1	0.5	2.4	31.8	61.0

[a] Molar percentages in the total measured phenolics. [b] M + PA, monomeric catechins and proanthocyanidins estimated from the thioacidolysis experiment. [c] mDP, mean degree of polymerization. Mean of three independent thioacidolysis experiments with three RP-HPLC replicate injections. [d] HT, hamamelitannin; GA, gallic acid; and MG, methyl gallate, estimated by HPLC and standards. [e] PGG, pentagalloylglucose, expressed as HT equivalents.

Table 2. Composition of the Condensed Tannins in Polyphenolic Fractions from Witch Hazel Bark[a]

fraction	% GC	% EGC	% C	% EC	% EGCG	% ECG
OWH	14.1	2.0	67.4	5.8	3.6	7.1
IH	11.4	1.5	73.3	7.0	1.6	5.2
IIH	2.8	0.6	70.7	6.2	2.7	16.9
IIIH	4.6	0.4	86.5	6.2	0.3	1.9
IVH	24.9	4.3	38.4	9.3	5.7	17.4
VH	2.1	0.3	89.4	6.5	0.4	1.3
VIH	29.2	4.1	32.9	10.9	4.4	18.4
VIIH	0.0	0.9	75.7	5.7	1.4	16.3
VIIIH	1.5	2.4	44.1	14.1	4.5	3.4

[a] Molar percentage. Mean of three independent thioacidolysis experiments with three RP-HPLC replicate injections.

Briefly, all of the mixtures derived from witch hazel bark presented high amounts of galloylated species pertaining to both condensed and hydrolyzable types of tannins. Some of the fractions, particularly IVH and VIH, also contained the pyrogallol moiety on ring B of their condensed tannins (gallocatechins and prodelphinidins). Because all of the fractions were rich in heavily hydroxylated phenolic molecules, we expected to obtain high free radical-scavenging activities.

Total Antioxidant Activity TAA. The total antioxidant activity of the polyphenolic mixture OWH and its fractions was measured by the ABTS cation radical method, which is a widely used assay for the evaluation of natural antioxidant mixtures such as extracts, juices, and wine (*49, 50*). OWH contained 6 mmol of Trolox equiv/g. In general agreement with the number of hydroxyls per molecule, the OWH extract showed a total antioxidant activity 70% higher than OWP (homologous extract from pine). TAA for the fractions generated from OWH are summarized in Figure 3. As compared to pine bark (*26*), hamamelis was a richer source of free radical-scavenging phenolics. The fractions retained on Toyopearl (IVH, VIH, and VIIIH), which contain bulky galloylated species, concentrated most of the activity, followed by fraction VH. To obtain information on the scavenging efficiency of the phenolics in every fraction, we then turned to the use of stable radicals.

Free Radical Scavenging and Electron Transfer Capacity. The extract and fractions were evaluated as free radical scavengers using different stable radicals, namely, DPPH and the newly introduced HNTTM and TNPTM. DPPH reacts with

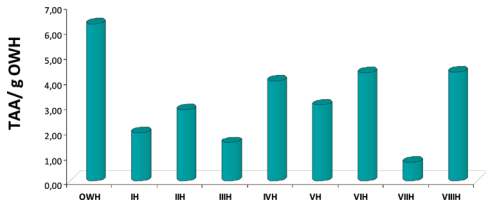


Figure 3. Total antioxidant activity (TAA) of the fractions by the ABTS cation radical method. TAA expressed as mmol Trolox equiv obtained per g of OWH.

Table 3. Hydrogen Donation and Electron Transfer Capacity of Polyphenolic Fractions from Witch Hazel Bark

	DPPH		HNTTM		TNPTM	
fractions	ED_{50}[a]	ARC[b]	ED_{50}	ARC[b]	ED_{50}[a]	ARC[b]
OWH	42.4	23.6	49.8	20.1	1225.5	0.8
IH	44.6	22.4	38.2	26.2	922.5	1.1
IIH	26.1	38.3	60.3	16.6	1592.4	0.6
IIIH	57.9	17.3	86.2	11.6	1059.3	0.9
IVH	28.6	35.0	57.2	17.5	956.0	1.0
VH	58.8	17.0	69.4	14.4	605.3	1.6
VIH	29.5	33.9	45.5	22.0	1488.1	0.7
VIIH	52.8	18.9	77.5	12.9	534.2	1.9
VIIIH	26.4	37.9	49.1	20.4	1265.8	0.8
			control			
Ec	49.3	20.3	60.9	16.4	NR	NR

[a] ED_{50} μg of fraction/μmol of radical, mean of three experiments. [b] ARC, $(1/ED_{50}) \times 10^3$; NR, no reaction.

polyphenols by mechanisms that may include both hydrogen donation and electron transfer (*37, 51*), while the new radicals are only sensitive to electron transfer (*30, 52*). Interestingly, HNTTM reacts with both catechol and pyrogallol moieties, while TNPTM will react only with the most reducing positions, namely, the pyrogallol group on ring B of condensed tannins, while being inactive against cathecols and gallates (*30*). By comparing the results generated with the three radicals, we gained information about the combined hydrogen donation and electron transfer capacity (DPPH), global electron transfer capacity (HNTTM), and the presence of highly reactive electron transfer positions (Figure 1). The results with HNTTM and particularly TNPTM may provide valuable information about the ability of some components to engage in putatively prooxidant/toxic effects involving electron transfer to oxygen. Table 3 summarizes the results obtained with the three radicals. As expected, witch hazel fractions were more potent (1.5−3-fold) scavengers than the homologous pine bark fractions. Again, fractions VIH and VIIIH, rich in bulky galloylated phenolics, were particularly efficient hydrogen donors and electron transfer agents (DPPH and HNTTM assays). Interestingly, fractions IVH (mainly condensed tannins) and VIIIH (mainly hydrolyzable tannins) were equally effective. Because the common structural feature of both fractions is the pyrogallol/gallate group, our results underscore the relevance of the trihydroxybenzene moiety for the scavenging activity of tannins. All of the fractions were active against the TNPTM radical, meaning that they contained highly reactive species. Because the ARCs were low, these reactive species are probably present as minor components. Interestingly, the most effective mixtures (VH and VIIH) were not those with the highest global electron transfer capacity. Both fractions, excluded from the Toyopearl column, were low in proanthocyanidins. Fraction VH contained monomeric catechin as the major component, and both included gallic acid and methyl gallate. None of these single molecules reacted with TNPTM when tested alone. This suggests that the mixtures might contain other reactive species. Alternatively, because all of the fractions were reactive to some extent, it may be that under the test conditions, highly reactive species are formed from otherwise inert precursors. These hypotheses are currently being tested in our laboratory.

Antioxidant Protection of Red Blood Cells. To evaluate the antioxidant protective effect of hamamelis fractions on cells submitted to oxidative stress, we used red blood cells (RBCs). Because of their susceptibility to peroxidation, RBCs have been used as a model to investigate oxidative damage in biomembranes. We investigated the oxidation of RBCs induced by AAPH, a well-known peroxyl radical initiator that causes hemolysis by means of membrane lipid and protein oxidation. Dose–response curves were analyzed, and IC_{50}(concentration triggering 50% inhibition of APPH induced hemolysis) values were obtained for some significant fractions. All of the fractions tested showed an inhibition of the in vitro AAPH-induced RBC hemolysis in a dose-dependent manner (data not shown). The IC_{50} values were 21.5 ± 1.6 (OWH), 22.6 ± 1.7 (IVH), and 24.5 ± 0.8 μg/mL (VIIIH), and all of them were more effective than the homologous fractions from grape and pine (*27*).

Cytotoxicity on Keratinocytes and Fibroblasts. To gain preliminary information about the cytotoxicity of the fractions as compared to their pine and grape homologues, the mixtures were tested on nontumoral HaCat keratinocytes and 3T3 fibroblasts. We selected the 3T3 neutral red uptake assay because this test is recommended by the U.S. National Institute of Environmental Health Science (NIEHS) Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). Nontumorigenic HaCat, a spontaneously immortalized keratinocyte cell line, provides an almost unlimited supply of identical cells, ensuring high intra- and interlaboratory reproducibility. Selected fractions showed some capacity to inhibit the proliferation of nontumoral skin cells with IC_{50} values of 41 ± 2 (OWH), 38 ± 3 (IVH), and 68 ± 10 μg/mL (VIIIH) on HaCat keratinocytes; and 51 ± 3 (OWH), 51 ± 1 (IVH), and 33 ± 3 μg/mL (VIIIH) on 3T3 fibroblasts. The cytotoxicity of the mixtures was relatively low. To visualize how safe the mixtures were for skin cells at their antioxidant active concentration, we calculated the relationship between the cytotoxicity index (IC_{50}) at 72 h in 3T3 and the antioxidant potential. We found that antioxidant concentrations were approximately 1.4−2.4-fold lower than the cytotoxic concentrations. We can conclude that an effective antioxidant activity of the fractions can be obtained at a concentration range not toxic for the nontumoral cell lines studied.

Antiproliferation of SK-Mel 28 Human Melanoma Cells. We and others have shown that plant phenolics influence the viability of eukaryotic cells by arresting the cell cycle and inducing cell death by apoptosis or necrosis (*53–55*). These effects appear to relate to the number and position of phenolic hydroxyls and, consequently, to the free radical scavenging and electron transfer capacity of the active species (*8, 55, 56*). To test the effect of the hamamelis phenolics on skin cancer cells, selected fractions (OWH, IVH, VH, VIH, and VIIIH) homologous to those from grape pomace and pine bark tested before (*25, 26*) were assayed for their influence on the proliferation of SK-Mel 28 human melanoma cells. All of the fractions showed some activity at relatively high concentrations. The IC_{50} values obtained were 26 ± 2 (OWH), 29 ± 2 (IVH), 32 ± 2 (VH), 28 ± 2 (VIH), and 39 ± 2 μg/mL (VIIIH). Interestingly again, the phenolics from witch hazel bark fractions were more efficient antiproliferative agents than those from grape and pine on this tumoral cell line. Particularly, hamamelis phenolics were between 4- and 6-fold more potent than pine bark procyanidins.

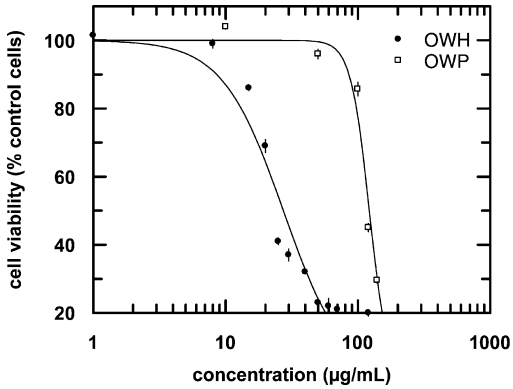


Figure 4. Percentage of proliferation of SK-Mel 28 human melanoma cells as a function of polyphenolic mixture concentration. Samples shown are the crude mixtures soluble in both ethyl acetate and water OWH (□) and OWP (●). Cells were incubated for 72 h with medium alone (control) or containing the polyphenols. $IC_{50} = 26 \pm 2$ μg/mL (OWH) and 122 ± 5 μg/mL (OWP). Data are given as the mean value ± SEM; experiments were performed in triplicate.

Figure 4 depicts the dose–response curve corresponding to the crude extract OWH as compared to the homologous mixture from pine bark (OWP). In agreement with the results on scavenging capacity, fractions IVH and VIIIH, which differed in percentage of condensed and hydrolyzable tannins but had in common a high content in trihydroxybenzene moieties (pyrogallol/gallates), were equally effective against cell proliferation.

In general agreement with the literature (*8, 27, 53, 55*), our comparative results show that the most efficient scavengers (hamamelis phenolics as compared to pine and grape components) as measured with both DPPH and HNTTM stable radicals were also the most cytotoxic/antiproliferative agents. This could be due to the so-called pro-oxidant effect of polyphenols. The generation of the superoxide radical and other ROS by EGCG, quercetin, and other phenolics in a variety of experimental setups have been reported before (*11–13, 22*) and might be behind the mild effect of phenolics on cell growth and apoptotic/necrotic death. The common structural feature mainly responsible for the high activity of witch hazel fractions appears to be the pyrogallol group both on ring B of gallocatechins/prodelphinidins and on galloyl moieties (gallates). Interestingly, fraction VH showed lower global electron transfer capacity than VIH or VIIIH as measured with HNTTM but higher electron transfer capacity as measured by TNPTM. The fact that all three fractions were equally antiproliferative against melanoma cells is in agreement with the presence in VH of the highly reactive species suggested above. The new radical TNPTM may have picked up on some relevant information by detecting the presence of putative cytotoxic species through a simple chemical test. Alternatively or complementarily, the gallate group may be interacting with relevant domains for cell replication (e.g., kinase domains of phosphorylating factors).

Concluding Remarks

Natural plant polyphenols appear to exert their action on living organisms by a combination of redox reactions and receptor–ligand interactions (*14*). They are considered antioxidants and perceived popularly as beneficial agents for the prevention of many diseases. However, what do we really mean by antioxidants? The concept is usually linked to free radical scavenging since it has been accepted that the underlying cause of cell damage is the production of ROS by mitochondrial metabolism and that ROS are essentially harmful and should be eliminated. However, ROS may not be always harmful. First, ROS as well as reactive nitrogen species (RNS) are key agents in the regulation of cell functions by acting as secondary messengers in intracellular signaling cascades (*8, 57*). Second, moderate generation of ROS may end up producing an antioxidant effect by fostering the endogenous defenses. It is becoming evident that mild prooxidant challenges such as physical exercise trigger mild transient oxidative stress with subsequent stimulation of antioxidant detoxifying defenses (*58*). Polyphenols may, at least in part, exert their activity in a similar way by providing mild prooxidant challenges through electron transfer reactions leading to moderate formation of ROS. The so-called prooxidant effect of some polyphenols may be in fact the real antioxidant activity. The results presented here on witch hazel bark phenolics, together with our previous studies with homologous fractions from pine and grape (*25–27, 55*), show that the higher the percentage of pyrogallols in the mixtures is, the higher the antiproliferative potency on epithelial cells is. Because the most cytotoxic/antiproliferative mixtures were also those with the highest electron transfer capacity, we hypothesize that tannins may provide the cell with a mild prooxidative challenge through the formation of the superoxide radical and redox cycling to oxidative species, which may stimulate the endogenous detoxifying systems. The prooxidant activity may be, at least in part, responsible for the alleged antioxidant effect of plant phenolics. The new stable radicals HNTTM and TNPTM, which are sensitive only to electron transfer and possess different redox potentials, may help to define the prooxidant and cytotoxic profile of phenolics. The abundance of pyrogallol groups appears to play a major role in the antioxidant/prooxidant effects of hamamelis phenolics.

Acknowledgment. Financial support of the Spanish Ministry of Education and Science (Research Grants PPQ2003-06602-C04-01, -04, and AGL2006-12210-C03-02/ALI and doctoral fellowships to S.T., D.L., and V.U.) is acknowledged. The assistance of Dr. Olga Jáuregui with the mass spectrometry experiments is greatly appreciated.

Supporting Information Available: RP-HPLC chromatograms obtained for all of the fractions before and after thioacidolysis and dose–response curves from the SK-Mel 28 proliferation assay. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Rusznyák, S. P., and Szent-Györgyi, A. (1936) Vitamin P: Flavonols as vitamins. *Nature 138*, 27.
(2) Ross, J. A., and Kasum, C. M. (2002) Dietary flavonoids: Bioavailability, metabolic effects, and safety. *Annu. Rev. Nutr. 22*, 19–34.
(3) Rice-Evans, C. A., Miller, N. J., and Paganga, G. (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med. 20*, 933–956.
(4) Yokozawa, T., Chen, C. P., Dong, E., Tanaka, T., Nonaka, G.-I., and Nishioka, I. (1998) Study on the inhibitory effect of tannins and flavonoids against the 1,1-diphenyl-2-picrylhydrazyl radical. *Biochem. Pharmacol. 56*, 213–222.
(5) Brown, J. E., Khodr, H., Hider, R. C., and Rice-Evans, C. A. (1998) Structural dependence of flavonoid interactions with Cu2+ ions: Implications for their antioxidant properties. *Biochem. J. 330*, 1173–1178.
(6) Hider, R. C., Liu, Z. D., and Khodr, H. H. (2001) Metal chelation of polyphenols. In *Flavonoids and Other Polyphenols* (Packer, L., Ed.) pp 190–203, Academic Press Inc., San Diego, CA.
(7) Ishige, K., Schubert, D., and Sagara, Y. (2001) Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms. *Free Radical Biol. Med. 30*, 433–446.
(8) Alanko, J., Riutta, A., Holm, P., Mucha, I., Vapaatalo, H., and Metsa-Ketela, T. (1999) Modulation of arachidonic acid metabolism by phenols: Relation to their structure and antioxidant/prooxidant properties. *Free Radical Biol. Med. 26*, 193–201.

(9) Lambert, J. D., Sang, S., and Yang, C. S. (2007) Possible controversy over dietary polyphenols: Benefits vs risks. *Chem. Res. Toxicol. 20*, 583–585.
(10) Kondo, K., Kurihara, M., Miyata, N., Suzuki, T., and Toyoda, M. (1999) Mechanistic studies of catechins as antioxidants against radical oxidation. *Archiv. Biochem. Biophys. 362*, 79–86.
(11) Kondo, K., Kurihara, M., Miyata, N., Suzuki, T., and Toyoda, M. (1999) Scavenging mechanisms of (−)-epigallocatechin gallate and (−)-epicatechin gallate on peroxyl radicals and formation of superoxide during the inhibitory action. *Free Radical Biol. Med. 27*, 855–863.
(12) Nutter, L. M., Wu, Y. Y., Ngo, E. O., Sierra, E. E., Gutierrez, P. L., and Abul-Hajj, Y. J. (1994) An o-quinone form of estrogen produces free radicals in human breast cancer cells: Correlation with DNA damage. *Chem. Res. Toxicol. 7*, 23–28.
(13) Boots, A. W., Li, H., Schins, R. P. F., Duffin, R., Heemskerk, J. W. M., Bast, A., and Haenen, G. (2007) The quercetin paradox. *Toxicol. Appl. Pharmacol. 222*, 89–96.
(14) Sang, S., Hou, Z., Lambert, J. D., and Yang, C. S. (2005) Redox properties of tea polyphenols and related biological activities. *Antioxid. Redox Signaling 7*, 1704–1714.
(15) Liang, Y. C., Lin-shiau, S. Y., Chen, C. F., and Lin, J. K. (1997) Suppression of extracellular signals and cell proliferation through EGF receptor binding by (−)-epigallocatechin gallate in human A431 epidermoid carcinoma cells. *J. Cell Biochem. 67*, 55–65.
(16) Ahmad, N., Cheng, P. Y., and Mukhtar, H. (2000) Cell cycle dysregulation by green tea polyphenol epigallocatechin-3-gallate. *Biochem. Biophys. Res. Commun. 275*, 328–334.
(17) Scalbert, A., Morand, C., Manach, C., and Remesy, C. (2002) Absorption and metabolism of polyphenols in the gut and impact on health. *Biomed. Pharmacother. 56*, 276–282.
(18) Halliwell, B. (2006) Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant Physiol. 141*, 312–322.
(19) Linnane, A. W., Kios, M., and Vitetta, L. (2007) Healthy aging: regulation of the metabolome by cellular redox modulation and prooxidant signaling systems: The essential roles of superoxide anion and hydrogen peroxide. *Biogerontology 8*, 445–467.
(20) Halliwell, B., Zhao, K., and Whiteman, M. (2000) The gastrointestinal tract: A major site of antioxidant action? *Free Radical Res. 33*, 819–830.
(21) Cross, C. E., van der Vliet, A., Louie, S., Thiele, J. J., and Halliwell, B. (1998) Oxidative stress and antioxidants at biosurfaces: Plants, skin, and respiratory tract surfaces. *Environ. Health Perspect. 106* (Suppl. 5), 1241–1251.
(22) Azam, S., Hadi, N., Khan, N. U., and Hadi, S. M. (2004) Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: Implications for anticancer properties. *Toxicol. in Vitro 18*, 555–561.
(23) Plumb, G. W., De Pascual-Teresa, S., Santos-Buelga, C., Cheynier, V., and Williamson, G. (1998) Antioxidant properties of catechins and proanthocyanidins: Effect of polymerization, galloylation and glycosylation. *Free Radical Res. 29*, 351–358.
(24) Saint-Cricq de Gaulejac, N., Vivas, N., Freitas, V., and Bourgeois, G. (1999) The influence of various phenolic compounds on scavenging activity assessed by an enzymatic method. *J. Sci. Food Agric. 79*, 1081–1090.
(25) Torres, J. L., Varela, B., García, M. T., Carilla, J., Matito, C., Centelles, J. J., Cascante, C., Sort, X., and Bobet, R. (2002) Valorization of grape (*Vitis vinifera*) byproducts. Antioxidant and biological properties of polyphenolic fractions differing in procyanidin composition and flavonol content. *J. Agric. Food Chem. 50*, 7548–7555.
(26) Touriño, S., Selga, A., Jiménez, A., Juliá, L., Lozano, C., Lizárraga, D., Cascante, C., and Torres, J. L. (2005) Procyanidin fractions from pine (*Pinus pinaster*) bark: Radical scavenging power in solution, antioxidant activity in emulsion and antiproliferative effect in melanoma cells. *J. Agric. Food Chem. 53*, 4728–4735.
(27) Ugartondo, V., Mitjans, M., Touriño, S., Torres, J. L., and Vinardell, M. P. (2007) Comparative antioxidant and cytotoxic effect of procyanidin fractions from grape and pine. *Chem. Res. Toxicol. 20*, 1543–1548.
(28) Torres, J. L., and Bobet, R. (2001) New flavanol-derivatives from grape (*Vitis vinifera*) byproducts. Antioxidant aminoethylthio-flavan-3ol conjugates from a polymeric waste fraction used as a source of flavanols. *J. Agric. Food Chem. 49*, 4627–4634.
(29) Torres, J. L., Varela, B., Brillas, E., and Juliá, L. (2003) Tris(2,4,6Trichloro-3,5-dinitrophenyl)methyl radical: A new stable coloured magnetic species as a chemosensor for natural polyphenols. *Chem. Commun.* 74–75.
(30) Torres, J. L., Carreras, A., Jiménez, A., Brillas, E., Torrelles, X., Rius, J., and Juliá, L. (2007) Reducing power of simple polyphenols by electron-transfer reactions using a new stable radical of the PTM series, tris(2,3,5,6-tetrachloro-4-nitrophenyl)methyl radical. *J. Org. Chem. 72*, 3750–3756.
(31) Dauer, A., Metzner, P., and Schimmer, O. (1998) Proanthocyanidins from the bark of *Hamamelis virginiana* exibit antimutagenic properties against nitroaromatic compounds. *Planta Med. 64*, 324–327.
(32) Deters, A., Dauer, A., Schnetz, E., Fartasch, M., and Hensel, A. (2001) High molecular compounds (polysaccharides and proanthocyanidins) from *Hamamelis virginiana* bark: Influence on human skin keratinocyte proliferation and differentiation and influence on irritated skin. *Phytochemistry 58*, 949–958.
(33) Matito, C., Mastorakou, F., Centelles, J. J., Torres, J. L., and Cascante, M. (2003) Antiproliferative effect of antioxidant polyphenols from grape in murine Hepa-1c1c7. *Eur. J. Nutr. 42*, 43–49.
(34) Torres, J. L., and Selga, A. (2003) Procyanidin size and composition by thiolysis with cysteamine hydrochloride and chromatography. *Chromatographia 57*, 441–445.
(35) Alcolea, J. F., Cano, A., Acosta, M., and Arnao, M. B. (2002) Hydrophilic and lipofilic antioxidant activities of grapes. *Nahrung 46*, 353–356.
(36) Blois, M. S. (1958) Antioxidant determinations by the use of stable free radical. *Nature 181*, 1199–1200.
(37) Brand-Williams, W., Cuvelier, M. E., and Berset, C. (1995) Use of a free radical method to evaluate antioxidant activity. *Lebensm.-Wiss. Technol. 28*, 25–30.
(38) Miki, M., Tamai, H., Mino, M., Yamamoto, Y., and Niké, E. (1987) Free radical chain oxidation of rat red blood cells by molecular oxygen and its inhibition by α-tocopherol. *Arch. Biochem. Biophys. 258*, 373–380.
(39) Ugartondo, V., Mitjans, M., Lozano, C., Torres, J. L., and Vinardell, M. P. (2006) Comparative study of the cytotoxicity induced by antioxidant epicatechin conjugates obtained from grape. *J. Agric. Food Chem. 54*, 6945–6950.
(40) Babich, H., Krupka, M. E., Nissim, H. A., and Zuckerbraun, H. L. (2005) Differential in vitro cytotoxicity of (−)-epicatechin gallate (ECG) to cancer and normal cells from the human oral cavity. *Toxicol. in Vitro 19*, 231–242.
(41) Borenfreund, E. P., and Rapad, J. (1983) A colorimetric assay to cellular growth and survival: Application to proliferation and cytotoxicity assay. *Toxicol. Lett. 24*, 119–124.
(42) Riddell, R. J., Clothier, R. H., and Ball, M. (1986) An evaluation of three in vitro cytotoxicity assays. *Food Chem. Toxicol. 24*, 469–471.
(43) Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 55–63.
(44) Dauer, A., Rimpler, H., and Hensel, A. (2003) Polymeric proanthocyanidins from the bark of *Hamamelis virginiana*. *Planta Med. 69*, 89–91.
(45) Sun, B. S., Belchior, G. P., Ricardo da Silva, J. M., and Spranger, M. I. (1999) Isolation and purification of dimeric and trimeric procyanidins from grape seeds. *J. Chromatogr. A 841*, 115–121.
(46) Hartisch, C., and Kolodziej, H. (1996) Galloylhamameloses and proanthocyanidins from *Hamamelis virginiana*. *Phytochemistry 42*, 191–198.
(47) Harbowy, M. E., and Balentine, D. A. (1997) Tea chemistry. *Crit. Rev. Plant Sci. 16*, 415–480.
(48) Haberland, C., and Kolodziej, H. (1994) Novel galloylhamameloses from *Hamamelis virginiana*. *Planta Med. 60*, 464–466.
(49) Miller, N. J., and Rice Evans, C. (1997) The relative contributions of ascorbic acid and phenolic antioxidants to the total antioxidant activity of orange and apple fruit juices and blackcurrant drink. *Food Chem. 60*, 331–337.
(50) Pietta, P., Simonetti, P., and Mauri, P. (1998) Antioxidant activity of selected medicinal plants. *J. Agric. Food Chem. 46*, 4487–4490.
(51) Dangles, O., Fargeix, G., and Dufour, C. (2000) Antioxidant properties of anthocyanins and tannins: A mechanistic investigation with catechin and the 3′,4′,7-trihydroxyflavylium ion. *J. Chem. Soc., Perkin Trans. 2* 1653–1666.
(52) Jiménez, A., Selga, A., Torres, J. L., and Juliá, L. (2004) Reducing activity of polyphenols with stable radicals of the TTM series. Electron transfer versus H-abstraction reactions in flavan-3-ols. *Org. Lett. 6*, 4583–4586.
(53) Tan, X. H., Hu, D. R., Li, S. R., Han, Y., Zhang, Y. L., and Zhou, D. Y. (2000) Differences of four catechins in cell cycle arrest and induction of apoptosis in LoVo cells. *Cancer Lett. 158*, 1–6.
(54) Kozikowski, A. P., Tuckmantel, W., Bottcher, G., and Romanczyk, L. J., Jr. (2003) Studies in polyphenol chemistry and bioactivity. 4. (1) Synthesis of trimeric, tetrameric, pentameric, and higher oligomeric epicatechin-derived procyanidins having all-4β,8-interflavan connectivity and their inhibition of cancer cell growth through cell cycle arrest. *J. Org. Chem. 68*, 1641–1658.
(55) Lizárraga, D., Lozano, C., Briede, J. J., van Delft, J. H., Touriño, S., Centelles, J. J., Torres, J. L., and Cascante, M. (2007) The importance of polymerization and galloylation for the antiproliferative properties of procyanidin-rich natural extracts. *FEBS J. 274*, 4802–4811.

(56) Lozano, C., Torres, J. L., Juliá, L., Jiménez, A., Centelles, J. J., and Cascante, M. (2005) Effect of new antioxidant cysteinyl-flavanol conjugates on skin cancer cells. *FEBS Lett. 579*, 4219–4225.
(57) Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M., and Telser, J. (2007) Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol. 39*, 44–84.
(58) Ascensao, A., Magalhaes, J. F., Soares, J. M., Ferreira, R. M., Neuparth, M. J., Appell, H. J., and Duarte, J. A. (2005) Cardiac mitochondrial respiratory function and oxidative stress: The role of exercise. *Int. J. Sports Med. 26*, 258–267.

TX700425N

4.2 Estudio de la absorción / metabolización de proantocianidinas

4.2.1 PUBLICACIÓN 3: Identificación de los compuestos fenólicos de la fracción extraíble de fibra antioxidante de uva.

Título original: High-resolution liquid chromatography/electrospray ionization time-of-flight mass spectrometry combined with liquid chromatography/electrospray ionization tandem mass spectrometry to identify polyphenols from grape antioxidant dietary fiber.

Autores: Sonia Touriño, Elisabet Fuguet, Olga Jáuregui, Fulgencio Saura-Calixto, Marta Cascante and Josep Lluís Torres. ***Rapid Communications in Mass Spectrometry***, 2008, *22*, 3489-3500.

Índice de impacto: 2,971

Clasificación y categoría: 13/ 70 (Química analítica); 9/39 (Espectrometría)

RESUMEN

La industria vitivinícola genera grandes cantidades de subproductos que son una importante fuente de compuestos de naturaleza fenólica, fibra y otras moléculas bioactivas. El término de fibra dietética es referido al conjunto de polisacáridos y lignina de los alimentos vegetales que resisten a la acción de los enzimas digestivos. Las fibras dietéticas promueven efectos beneficiosos fisiológicos como laxantes y pueden atenuar los niveles de colesterol y/o glucosa en sangre *(Guillon et al., 2000)*. Debido a que no son absorbibles en el intestino delgado, cuando estas fibras llegan al colon son susceptibles de ser degradadas por la microbiota colónica y los productos formados promueven el crecimiento y la actividad de las bacterias que habitan en el colón, es por ello que las fibras dietéticas se engloban dentro del término de alimentos prebióticos.

La fibra antioxidante dietética (GADF) patentada por el grupo del Dr. Saura-Calixto *(Saura-Calixto et al., 1999)*, es un ingrediente que además de combinar los efectos beneficiosos de una fibra dietética incluye en su composición grandes cantidades de polifenoles (20%), fundamentalmente proantocianidinas. Algunos de estos de compuestos fenólicos son absorbidos en el intestino delgado y otros, debido a que están embebidos en la matriz de lignina, son progresivamente liberados en el colon como consecuencia de la fermentación bacteriana. GADF presenta buenas condiciones de fermentabilidad y digestibilidad *(Goñi et al., 2005)*. Particularmente interesante, son los resultados obtenidos de un estudio realizado recientemente por Pérez-Jiménez y colaboradores *(Pérez-Jiménez et al., 2008)* donde se observó que GADF podía disminuir la presión arterial y el colesterol. La presencia de los compuestos fenólicos como principios activos en fibras dietéticas parece aumentar los beneficios que éstas ejercen.

La identificación de los compuestos polifenólicos mayoritarios de la fracción extraíble de la fibra fue el objetivo de este trabajo como primer paso para comprender su metabolización y la actividad que puedan ejercer en el organismo.

La fracción fenólica extraíble de GADF es una mezcla compleja con una gran cantidad de compuestos de distintas clases. Para mejorar la identificación de los compuestos fenólicos se realizó un fraccionamiento mediante técnicas cromatográficas que permitió separar monómeros, oligómeros de proantocianidinas, antocianinas y polímeros en cuatro fracciones. Para la identificación de los compuestos de cada una de las fracciones se utilizaron dos técnicas complementarias. El uso LC/ESI-TOF-MS permitió obtener un primer barrido general de cada una de las fracciones y determinar las masas exactas de cada uno de los compuestos. Los experimentos en tándem (barrido del ión precursor, barrido de perdidas neutras y barrido de iones producto) fueron realizados en un triple cuadrupolo acoplado a un HPLC (LC/ESI-MS/MS) y posibilitaron la determinación de las estructuras en base a los fragmentos característicos de cada molécula. La combinación de ambas técnicas permitió identificar un elevado número de compuestos fenólicos de diferentes clases.

A continuación se muestra la publicación original.

RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2008; **22**: 3489–3500
Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/rcm.3756

High-resolution liquid chromatography/electrospray ionization time-of-flight mass spectrometry combined with liquid chromatography/electrospray ionization tandem mass spectrometry to identify polyphenols from grape antioxidant dietary fiber

Sonia Touriño[1], Elisabet Fuguet[1], Olga Jáuregui[2], Fulgencio Saura-Calixto[3], Marta Cascante[4] and Josep Lluís Torres[1]*

[1]Institute for Chemical and Environmental Research (IIQAB-CSIC), Jordi Girona 18–26, E-08034 Barcelona, Spain
[2]Scientific and Technical Services, University of Barcelona, Josep Samitier 1–5, E-08028 Barcelona, Spain
[3]Instituto del Frío, CSIC. José Antonio Novais 10, E-28040 Madrid, Spain
[4]Department of Biochemistry and Molecular Biology, Institute of Biomedicine of the University of Barcelona (IBUB) and Centre for Research in Theoretical Chemistry, Barcelona Science Park (CeRQT-PCB), Unit Associated with CSIC, Diagonal 645, E-08028 Barcelona, Spain

Received 13 June 2008; Revised 10 September 2008; Accepted 10 September 2008

Grape antioxidant dietary fiber (GADF) is a dietary supplement that combines the benefits of both fiber and antioxidants that help prevent cancer and cardiovascular diseases. The antioxidant polyphenolic components in GADF probably help prevent cancer in the digestive tract, where they are bioavailable. Mass spectrometry coupled to liquid chromatography is a powerful tool for the analysis of complex plant derivatives such as GADF. We use a combination of MS techniques, namely liquid chromatography/electrospray ionization time-of-flight mass spectrometry (LC/ESI-TOF-MS) and liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) on a triple quadrupole, for the identification of the polyphenolic constituents of the soluble fraction of GADF. First, we separated the mixture into four fractions which were tested for phenolic constituents using the TOF system in the full scan mode. The high sensitivity and resolution of the TOF detector over the triple quadrupole facilitate the preliminary characterization of the fractions. Then we used LC/ESI-MS/MS to identify the individual phenols through MS/MS experiments (product ion scan, neutral loss scan, precursor ion scan). Finally, most of the identities were unequivocally confirmed by accurate mass measurements on the TOF spectrometer. LC/ESI-TOF-MS combined with MS/MS correctly identifies the bioactive polyphenolic components from the soluble fraction of GADF. High-resolution TOF-MS is particularly useful for identifying the structure of compounds with the same LC/ESI-MS/MS fragmentation patterns. Copyright © 2008 John Wiley & Sons, Ltd.

In recent years, there has been increasing interest in the use of plant byproducts as natural sources of compounds with useful nutritional and pharmaceutical properties.[1] Grapes (*Vitis vinifera*) are the world's largest fruit crop. Approximately 60 million tons are produced annually, most of which is used for making wine. This industrial activity generates huge amounts of byproducts that are rich in fiber, polyphenols and other bioactive molecules. Dietary fiber (non-digestible polymers such as non-starch polysacharides and lignin) is of particular interest owing to its putative benefits for human health. Fibers have laxative properties and some of them have been reported to reduce blood cholesterol and glucose.[2] Moreover, when dietary fiber reaches the colon, the polysaccharides and other non-digestible dietary compounds (e.g. resistant starches and proteins, and high molecular weight polyphenols) are fermented to some extent by the colonic microbiota. Since these compounds help to stabilize the number of colonic bacteria, they are called prebiotics and are believed to contribute to the health benefits of fiber. The polyphenolic components of some fibers may add their antioxidant potential to the putative benefits of the whole preparation. Polyphenols are powerful free-radical scavengers with antioxidant activity and antitumor effects.[3] The consumption of polyphenol-rich food and beverages has been associated with the prevention of diseases, especially different types of cancer and coronary heart conditions.[4]

Grape pomace is a particularly interesting source of dietary fiber because, apart from lignin and polysaccharides, it contains large amounts of polyphenols. These are

**Correspondence to*: J. L. Torres, Institute for Chemical and Environmental Research (IIQAB-CSIC), Jordi Girona 18–26, E-08034 Barcelona, Spain.
E-mail: jltqbp@iiqab.csic.es
Contract/grant sponsor: Spanish Ministry of Education and Science; contract/grant number: AGL2004-07579-C04-01, -02, -03; AGL2006-12210-C03-02/ALI.

associated with the fiber matrix and produce considerable antioxidant activity. Some of these phenols are absorbed in the small intestine and the rest reach the colon where, to some extent, they are hydrolyzed, metabolized and absorbed.[5,6] As a result, an array of bioactive compounds may become bioavailable at the colonic epithelial tissue and released into the bloodstream. Thus, the new concept of grape antioxidant dietary fiber (GADF) has come into being.[7] GADF incorporates the advantages of both fiber and phenols in a single dietary product.

To advance our understanding of the benefits of GADF[8] it is important to thoroughly characterize its putatively active phenolic components. Mass spectrometry (MS) alone or coupled to liquid chromatography (LC/MS) has been increasingly used in the structural characterization of complex mixtures such as bioactive plant extracts.[9–13] LC/MS with electrospray ionization (LC/ESI-MS) has been successfully used to identify anthocyanins,[14,15] flavonols,[12,16] proanthocyanidins (PAs),[17,18] pyranoanthocyanins,[16,19] and phenolic acids in wine-related products.[19–22] The identification of polymeric PAs with a high degree of polymerization is particularly complicated. The different polymers cannot be separated through conventional high-performance liquid chromatography (HPLC) since they elute together as an unresolved broad peak. ESI-MS and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) are complementary alternatives for characterizing polymeric PAs.[23,24] These soft ionization techniques provide mass spectra with little fragmentation, making it possible to define the distribution of the polymers around the mean value.[18,25–27]

The objective of this work was to identify the polyphenols in the soluble fraction of GADF with the hope of eventually establishing structure/activity relationships between the components of GADF on the one hand, and its cancer and cardiovascular chemopreventive effects on the other. Because the soluble fraction contains a huge number of polyphenolic compounds, and to facilitate their identification by LC/MS, the mixture was separated into four fractions by chromatographic methods. To identify the phenolic compounds, each fraction was analyzed using two complementary instruments: a high-resolution time-of-flight mass spectrometer (LC/ESI-TOF-MS), for the first full-scan acquisitions and to determine accurate masses; and a triple quadrupole (QqQ) mass spectrometer (LC/ESI-MS/MS) using several modalities (product ion scan, neutral loss scan and precursor ion scan) to determine structures based on fragmentation patterns. Triple quadrupole instruments have well-described MS/MS capabilities that are extensively used in the identification of phenolic compounds in natural product samples.[28,29] TOF analyzers provide greatly improved mass resolution compared to the unit resolution of traditional quadrupole instruments and, in addition, they offer significantly higher sensitivity and accuracy when acquiring full-fragment spectra. These characteristics make them suitable for both initial characterization of complex mixtures and final confirmation of component identities through the resolution of ambiguities. Thus we combined TOF and QqQ systems to qualitatively characterize the phenols in the soluble fraction of GADF.

EXPERIMENTAL

Reagents and materials

Grape antioxidant dietary fiber was obtained from red grapes (the Cencibel variety), of the vintage year 2005, from La Mancha region in Spain, as described in published patents.[30,31] For the extraction and fractionation of polyphenols, the following (analytical grade) reagents were used: methanol, ethyl acetate, and hydrochloric acid 37% from Panreac (Montcada i Reixac, Spain); acetone and diethyl ether from Carlo Erba (Milano, Italy). Acetonitrile (HPLC grade) and formic acid (analytical grade) were from Merck (Darmstadt, Germany). Water was purified by a Milli-Q plus system (Millipore, Bedford, MA, USA) to a resistivity of 18.2 MΩcm.

Standards of (−)-epicatechin (≥97%), (+)-catechin (≥98%), (−)-epicatechin gallate (≥98%), 4-hydroxybenzoic acid (≥97%), vanillic acid (≥97%), syringic acid (≥95%), dehydrocaffeic acid (>98%), protocatechuic acid (≥97%), *p*-coumaric acid (≥98%), *o*-coumaric acid (≥97%), and gallic acid (>99%) were from Sigma Chemical (St. Louis, MO, USA).

Apparatus and experimental conditions

The ESI-TOF instrument was a LCT Premier (Waters, Milford, MA, USA), equipped with a 4 GHz time-to-digital converter (TDC) combined with an Acquity ultra-performance liquid chromatography (UPLC) system. The separations were conducted using a Luna C18(2) 3 μm particle size column (50 × 2.1 mm i.d.; Phenomenex, Torrance, CA, USA) equipped with a Phenomenex Securityguard C18 column (4 × 3 mm i.d.). The mass spectrometer was equipped with a dual ESI source (LockSpray). The second sprayer provided the lock mass calibrant leucine enkephalin (*m*/*z* 556.2771). It was operated in the W-optics mode, thus providing a mass resolution of at least 10 000 full-width at half maximum (FWHM). The acquisition time per spectrum was set to 0.2 s, and the mass range was from 100 to 3000 Da. Data were acquired using a cone voltage of 50 V, capillary voltage of 2800 V, desolvation temperature of 350°C, and source temperature of 100°C. The desolvation gas flow was set at 400 L/h and the cone gas flow was set at 30 L/h. MassLynx 4.1 software from Waters was used for data acquisition and processing.

An Agilent series 1100 HPLC instrument (Agilent, Waldbronn, Germany) equipped with a quaternary pump, an ultraviolet (UV) detector, an autosampler and a column oven was used for the HPLC-UV/ESI-MS/MS experiments. The separations were conducted using the column described above. The injection volume was 5 μL, the flow rate was 400 μL/min, and the temperature 25°C. An API 3000 triple quadrupole mass spectrometer (PE Sciex, Concord, Ontario, Canada) equipped with a TurboIon spray source was used to obtain MS/MS data. The settings were: capillary voltage −3500 V (negative mode) or 5000 V (positive mode), nebulizer gas (N_2) 10 arbitrary units (a.u.), curtain gas (N_2) 12 a.u., collision gas (N_2) 10 a.u., declustering potential (DP) between −30 and −60 V, focusing potential −200 V, entrance potential 10 V, and collision energy (CE) −30 V. The drying gas (N_2) was heated to

DOI: 10.1002/rcm

400°C and introduced at a flow rate of 8000 cm^3 min^{-1}. Full-scan data acquisition was performed over values of m/z ranging from 100 to 1500 using a cycle time of 2 s with a step size of 0.1 units. Analyst 1.4.2 software from PE Sciex was used for data acquisition and processing.

Procedures

Extraction and fractionation

The soluble fraction of GADF was obtained by extraction as described in the literature.[32] In short, the sample (500 mg) was placed in a test tube and acidic methanol/water (50:50 v/v, pH 2, 20 mL) was added. The tube was shaken with an Intelli mixer RM-2L (Elmi, Riga, Latvia) for 3 h at room temperature. The tube was centrifuged at 3000 *g* for 10 min and the supernatant was recovered. Acetone/water (70:30, v/v, 20 mL) was added to the residue, and the mixture was subjected to shaking and centrifugation as before. The methanolic and acetone extracts were combined and evaporated under vacuum. The pellet was dissolved in deionized water, and the solution was filtered through a porous plate and lyophilized to obtain the dry soluble fraction of dietary fiber. To separate this complex polyphenolic mixture into different families according to their physicochemical properties, the mixture was fractionated by semi-preparative chromatography using LiChroprep RP 18 (25–40 μm; Merck, Darmstadt, Germany) and Toyopearl TSK HW-40F (32–63 μm; TosoHass, Tokyo, Japan) stationary phases, which were packed into flash-chromatography-type glass columns (12 × 1.5 mm i.d.). The equilibration and elution protocols followed those described by Sun *et al*.[33] The fractionation procedure is summarized in Fig. 1.

HPLC/MS experiments

Different HPLC procedures were used for the analysis of the polyphenols in the fractions. For the monomeric and oligomeric fractions, gradient elution was performed with a binary system consisting of [A] 0.1% aqueous formic acid and [B] 0.1% formic acid in CH_3CN (acetonitrile). An increasing linear gradient (v/v) of [B] was used, [*t*(min), %B]: 0,8; 10,23; 15,50; 20,50; 21,100; followed by a re-equilibration step. For the analysis of anthocyanins the gradient elution was carried out with [C] 5% aqueous formic acid and [D] 0.5% formic acid in CH_3CN and the gradient (v/v) was

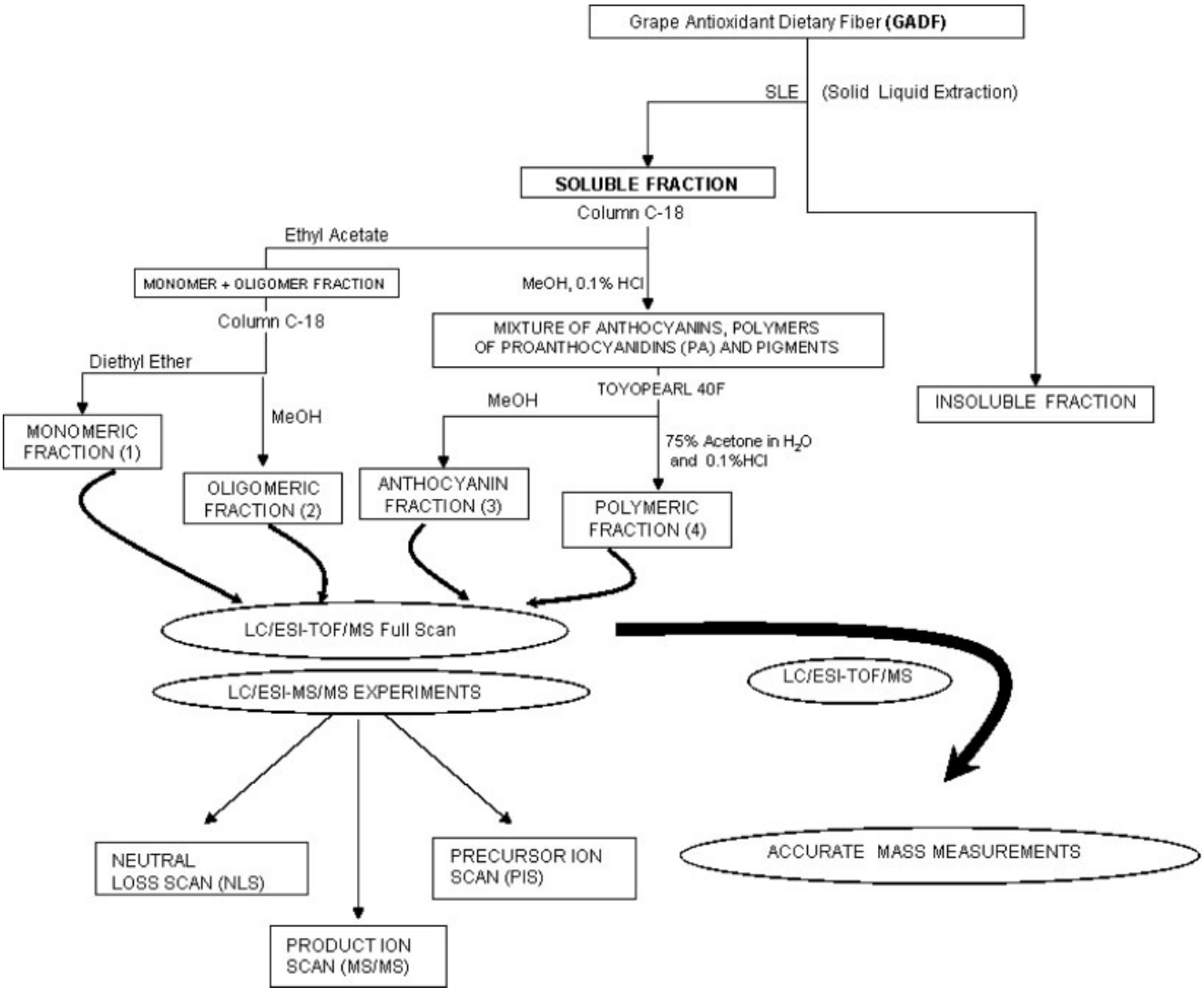


Figure 1. Scheme of GADF fractionation and MS analysis.

DOI: 10.1002/rcm

[*t*(min), %D]: 0,10; 20,40; 21,100. Detection was carried out at 280 nm (monomeric and oligomeric fractions) and 530 nm (anthocyanins). MS/MS experiments were performed in the negative mode for monomeric and oligomeric fractions (1, 2) and in the positive mode for the anthocyanin fraction (3). The polymeric fraction (4) was analyzed by direct infusion into the ESI-TOF-MS system in the negative mode. The sample was dissolved in formic acid/water/acetonitrile (5:15:80 v/v/v) at a concentration of 1 mg/mL, filtered through a 0.45 μm syringe filter (Millex-LH; Millipore, Bedford, MA, USA), and infused at a speed of 5 μL/min into the ESI source with a syringe pump (Cole-Parmer, Vernon, IL, USA).

RESULTS AND DISCUSSION

The methanol/acetone/water extract of GADF was very rich in polyphenolic species. Initial HPLC/MS analysis using the TOF system in both positive and negative modes revealed that the mixture was too complex to be sorted without further sample pre-treatment. To minimize the number of co-eluting compounds and simplify identification, the extracts were subjected to chromatographic fractionation[33] and four fractions were obtained. The monomeric fraction (1) was rich in phenolic acids and their derivatives, catechins (monomeric units of proanthocyanidins) and flavonols; the oligomeric fraction (2) was mainly composed of oligomeric PAs (up to tetramers) and flavonol derivatives; the anthocyanin fraction (3) included conjugates of anthocyanidins with sugar moieties and hydroxy acids; and finally fraction 4 contained the polymeric PAs. Figure 1 summarizes the fractionation and analysis process.

Fractions 1, 2 and 3 were analyzed using the LC/ESI-TOF system in the full-scan mode. Then the ions identified were submitted to precursor ion scan, neutral loss scan, and product ion scan experiments using the LC-UV/ESI-MS/MS system. Finally, to achieve the maximum number of identification points, the accurate masses of the target compounds were measured by high-resolution LC/ESI-TOF-MS. When available, standards were used to confirm identities. Tables 1 and 2 summarize the information generated through the MS/MS and TOF experiments for each compound: molecular ion ($[M–H]^-$ or M^+), product ions (MS/MS ions), neutral losses (NL), precursor ions (PI), accurate mass, error associated with the accurate mass (ppm) and the molecular formula. Because polymeric PAs are poorly separated in conventional reversed-phase (RP)-LC, the polymeric fraction (4) was analyzed by direct infusion into the ESI-TOF-MS system and the mass spectra revealed the degree of polymerization and galloylation of the polymeric PAs, as shown in Table 4.

Monomeric fraction 1

Small phenols are the first to be absorbed in the small intestine, and the most immediate effects of consuming GADF are probably related to them. Hydroxybenzoic acids, together with some of their derivatives, and monomeric flavonoids (catechins and flavonol aglycones) were found in this fraction (Table 1). The UV profile at 280 nm showed eight main components and some other minor peaks. Protocatechuic acid (23), *p*-hydroxybenzoic acid (1), vanillic acid (24), syringic acid (25), 3,4-dihydroxyphenylacetic acid (40), and sinapic acid (71) were all present in the fraction. The typical loss of 44 Da corresponding to the carboxylic acid moiety was observed for all of these compounds. Fertaric acid (13) was also identified, since the MS/MS experiments yielded the $[M–H]^-$ ion at 193 Da, corresponding to the loss of the tartaric acid moiety (neutral loss of 132 Da).[34] *cis*-Coutaric acid and caftaric acid were not detected in the mixture, maybe because they are eliminated during the grape fermentation process. Other phenolic derivatives identified were *p*-hydroxybenzaldehyde (9), coniferyl aldehyde (7), syringic aldehyde (56), and coumaric acid glucosides (8, 11, 15). The latter gave neutral losses of 162 Da (glucose unit), and showed typical fragments of coumaric acid in the product ion scan (*m*/*z* 163, 145, 119).[35]

Monomeric flavan-3-ols were also present in this fraction. (+)-Catechin (4) and (−)-epicatechin (19) gave the same deprotonated ion $[M–H]^-$, and originated several fragments at *m*/*z* 179, 205, 245 and 271. The fragment at *m*/*z* 205 corresponded to the loss of the flavonoid A-ring (Fig. 2(a)),[22] whereas the one at 179 Da was due to the loss of the B-ring. The two stereoisomers were identified by the use of standards. For epicatechin-3-*O*-gallate (42), also identified in this fraction, the fragment ions were the result of the cleavage of the ester bond between the epicatechin unit (fragment at *m*/*z* 289) and the gallic acid moiety (*m*/*z* 169).

Kaempferol (70) and quercetin (65) (flavonol aglycones) were also found in the monomeric fraction. Both molecular ions yielded fragments in agreement with the fragmentation pattern of flavonols, mainly consisting of the breakage of the C-ring through a *retro*-Diels-Alder (RDA) reaction (Fig. 2(b)), to give the A- and B-ring residues. For kaempferol, the loss of the A-ring residue gave fragments at *m*/*z* 151 and 107 Da, whereas for quercetin the same fragmentation pattern generated ions at *m*/*z* 179 and 151 Da.

Oligomeric fraction 2

Oligomeric PAs are phenolic components of GADF which may be partially absorbed in the small intestine and partially metabolized in the colon.[36,37] Galloylated PAs are particularly interesting as they are more resistant to metabolization than non-galloylated PAs and therefore the former are more bioavailable.[38] This is important for the structure/activity relationships of GADF, since, as we have described,[39] there is a relationship between galloylation and the activity of PAs against colon cancer cells. Most of the compounds identified in this fraction were flavonoids (oligomeric PAs, flavonols, flavones, and flavanones) and all of them are listed in Table 1. Figure 3 shows the full-scan chromatogram for this fraction obtained by LC/TOF-MS. As mentioned above, the higher sensitivity of the TOF system in full-scan mode as compared to the triple quadrupole system provided quite a sharp profile from which the main components of the fraction can be easily identified. Low intensity signals corresponding to monomeric flavanols and phenolic acids (*p*-hydroxybenzoic acid (1), catechin (4), epicatechin (19), vanillic acid (24), syringic acid (25), quercetin (65) and sinapic acid (71)) were also recorded in this analysis. Coumaric acid glucosides

DOI: 10.1002/rcm

Table 1. Identification of phenolic compounds in fractions 1 (monomers) and 2 (oligomers) of the soluble fraction of GADF

Peak No.	Identity assigned	Fraction	$[M–H]^-$	MS/MS ions	NL	PI	Acc Mass	ppm	M F
1	*p*-Hydroxybenzoic acid	1,2	137	107/93/79/53			137.0233	−4.4	$C_7H_5O_3$
2	Dimer PA1	2	577	451/425/407/289		289	577.1339	−1.2	$C_{30}H_{25}O_{12}$
3	Dimer PA2	2	577	451/425/407/289		289	577.1342	−0.7	$C_{30}H_{25}O_{12}$
4	Catechin	1,2	289	245/169			289.0701	−3.8	$C_{15}H_{13}O_6$
5	Vanillic acid glucoside 1	2	329	167	162		329.0862	−3.3	$C_{14}H_{17}O_9$
6	Trimer PA1	2	865	739/577/451/407/289		289	865.1996	1.8	$C_{45}H_{37}O_{18}$
7	Coniferyl aldehyde	1,2	177	149/133/105/89/77			177.0552	−7.9	$C_{10}H_9O_3$
8	Coumaric acid glucoside 1	1,2	325	265/205/163/145/119	162		325.0905	−5.5	$C_{15}H_{17}O_8$
9	*p*-Hydroxybenzaldehyde	1	121	92			121.028	−8.3	$C_7H_5O_2$
10	Trimer PA2	2	865	577/289		289	865.2002	2.5	$C_{45}H_{37}O_{18}$
11	Coumaric acid glucoside 2	2	325	265/205/163/145	162		325.0902	−6.5	$C_{15}H_{17}O_8$
12	Dimer PA3	2	577	451/425/407/289		289	577.1354	1.4	$C_{30}H_{25}O_{12}$
13	Fertaric acid	1	325	193/133/87			325.0912	−3.4	$C_{15}H_{17}O_8$
14	Unknown	2	311	243/227/137/122					
15	Coumaric acid glucoside 3	2	325	265/205/163/145/119	162		325.0911	−3.7	$C_{15}H_{17}O_8$
16	Dimer PA4	2	577	451/425/407/289		289	577.1332	−2.4	$C_{30}H_{25}O_{12}$
17	Trimer PA3	2	865	739/577/451/407/289		289	865.1997	2.0	$C_{45}H_{37}O_{18}$
18	Vanillic acid glucoside 2	2	329	167	162		329.0873	−3.3	$C_{14}H_{17}O_9$
19	Epicatechin	1,2	289	245/169			289.0703	−3.1	$C_{15}H_{13}O_6$
20	Trimer PA4	2	865	577/289		289	865.1993	1.5	$C_{45}H_{37}O_{18}$
21	Trimer PA monogallate 1	2	1017	865/729/591			1017.2104	1.5	$C_{52}H_{41}O_{22}$
22	Unknown	2	311	243/227/137					
23	Protocatechuic acid	1	153	125/107/109/83/69			153.0181	−4.6	$C_7H_5O_4$
24	Vanillic acid	1,2	167	123/107			167.035	3.6	$C_8H_7O_4$
25	Syringic acid	1,2	197	169/125/97/81			197.0443	−3.6	$C_9H_9O_5$
26	Dimer PA monogallate 1	2	729	577/559/441/407/289		289	729.1454	−0.3	$C_{37}H_{29}O_{16}$
27	Trimer PA5	2	865	577/289		289	865.1996	1.8	$C_{45}H_{37}O_{18}$
28	Dimer PA monogallate 2	2	729	577/559/441/407/289		289	729.1447	−1.2	$C_{37}H_{29}O_{16}$
29	Trimer PA6	2	865	577/289		289	865.1986	0.7	$C_{45}H_{37}O_{18}$
30	Dimer PA monogallate 3	2	729	577/559/441/407/289		289	729.1453	−0.4	$C_{37}H_{29}O_{16}$
31	Tetramer PA	2	1153			289	1153.2621	0.6	$C_{60}H_{50}O_{24}$
32	Trimer PA7	2	865	577/289		289	865.1982	0.2	$C_{45}H_{37}O_{18}$
33	Dimer PA5	2	577	451/425/407/289		289	577.1342	−0.7	$C_{30}H_{25}O_{12}$
34	Dimer PA monogallate 4	2	729	577/559/441/407/289		289	729.1456	0.0	$C_{37}H_{29}O_{16}$
35	Myricetin-3-*O*-glucoside	2	479	317/179	162	317	479.0824	0.4	$C_{21}H_{19}O_{13}$
36	Trimer PA monogallate 2	2	1017	865/729/591		289	1017.2111	2.2	$C_{52}H_{41}O_{22}$
37	Trimer PA8	2	865	713/577/425/289		289	865.1985	0.6	$C_{45}H_{37}O_{19}$
38	Unknown	1,2	339	192/145					
39	Naringenin-7-*O*-glucoside	2	433	271	162	271	433.1096	−3.2	$C_{21}H_{21}O_{10}$
40	3,4-Dihydroxyphenylacetic acid	1	167	125/123/107/99/89			167.0349	3.0	$C_8H_7O_4$
41	Unknown	2	591	439/301/289/150		289			
42	Epicatechin-3-*O*-gallate	1,2	441	331/289/169		289	441.0817	−1.1	$C_{22}H_{17}O_{10}$
43	Kaempferol-diglucoside	2	609	329/285			609.1468	2.0	$C_{27}H_{29}O_{16}$
44	Dimer PA digallate	2	881	441			881.1588	2.6	$C_{44}H_{33}O_{20}$
45	Quercitin-3-*O*-glucoside	2	463	301	162	301	463.0862	−3.2	$C_{21}H_{19}O_{12}$
46	Trimer PA monogallate 3	2	1017	865/729/591		289	1017.2097	0.8	$C_{52}H_{41}O_{22}$
47	Kaempferol-hexoside	2	447	285	162	285	447.0919	−1.3	$C_{21}H_{19}O_{11}$
48	Dimer PA6	2	577	451/425/407/289		289	577.1344	−2.9	$C_{30}H_{25}O_{12}$
49	Laricitrin-3-*O*-glucoside	2	493	331/330/179	162		493.0979	−0.6	$C_{22}H_{21}O_{13}$
50	Quercetin-3-*O*-rutinoside	2	609	300	308		609.1468	2.0	$C_{27}H_{29}O_{16}$
51	Quercetin-3*O*-galactoside	2	463	300/179/151	162		463.0862	−3.2	$C_{21}H_{19}O_{12}$
52	Trimer PA monogallate 4	2	1017	865/729/591		289	1017.2104	1.5	$C_{52}H_{41}O_{22}$
53	Isorhamnetin-3-*O*-rutinoside	2	623	315	308	315	623.1385	−2.6	$C_{28}H_{31}O_{16}$
54	Trimer PA 9	2	865	739/577/451/407/289		289	865.1985	0.6	$C_{45}H_{37}O_{18}$
55	Quercetin-3-*O*-glucuronide	2	477	301	176	301	477.1022	−2.3	$C_{22}H_{21}O_{12}$
56	Syringic aldehyde	1	181	153/109			181.05	−0.6	$C_9H_9O_4$
57	Kaempferol-hexoside	2	447	325/284	162		447.0918	−2.0	$C_{21}H_{19}O_{11}$
58	Unknown	1	353	191/179					
59	Unknown	2	637	329	308				
60	Isorhamnetin-3-*O*-glucoside	2	477	315	162	315	477.0671	−2.3	$C_{21}H_{17}O_{13}$
61	Kaempferol-hexoside	2	447	327/299/285/151	162	285	447.0923	−0.9	$C_{21}H_{19}O_{11}$
62	Isorhamnetin-3-*O*-galactoside	2	477	357/314/151		315	477.0656	−2.7	$C_{21}H_{17}O_{13}$
63	Trimer PA monogallate 5	2	1017	865/729/591		289	1017.2104	1.5	$C_{52}H_{41}O_{22}$
64	Unknown	2	637	491/371/329	308				

Continues

DOI: 10.1002/rcm

Table 1. (Continued)

Peak No.	Identity assigned	Fraction	$[M–H]^-$	MS/MS ions	NL	PI	Acc Mass	ppm	M F
65	Quercetin	1,2	301	273/179/151/107			301.0348	−3.3	$C_{15}H_9O_7$
66	Unknown	2	223	155/111					
67	Unknown	2	229	211/179					
68	Unknown	1,2	329	293/229/211/171/139/127					
69	Unknown	2	193	175/149/103/79					
70	Kaempferol	1	285	151/107			285.0399	−1.4	$C_{15}H_9O_6$
71	Sinapic acid	1,2	223	207/179/165			223.0606	−4.9	$C_{11}H_{11}O_5$
72	Unknown	2	193	177/123/103/79					

NL, neutral losses; PI, precursor ions; Acc Mass, accurate mass; ppm, error associated with the accurate mass; MF, molecular formula.

Table 2. Identification of phenolic compounds in fraction 3 (anthocyanins) of the soluble fraction of GADF

Peak No.	Identity assigned	M^+	MS/MS ions	NL	PI	Acc. Mass	ppm	M F
73	Delphinidin-3-*O*-glucoside	465	303	162	303	465.1024	−1.9	$C_{21}H_{21}O_{12}$
74	Cyanidin-3-*O*-glucoside	449	288/187/164	162	287	449.1073	−2.4	$C_{21}H_{21}O_{11}$
75	Petunidin-3-*O*-glucoside	479	317/302/274	162	317	479.1184	−1.3	$C_{22}H_{23}O_{12}$
76	Peonidin-3-*O*-glucoside	463	301/286/258	162	301	463.1237	−1.1	$C_{22}H_{23}O_{11}$
77	Type-B vitisin of petunidin-3-*O*-glucoside	503		162		503.1176	−2.8	$C_{24}H_{23}O_{12}$
78	Malvidin-3-*O*-glucoside	493	331/315/299	162	331	493.1342	−0.8	$C_{23}H_{25}O_{12}$
79	Type-B vitisin of peonidin-3-*O*-glucoside	487		162		487.124	−1.0	$C_{24}H_{23}O_{11}$
80	Type-A vitisin of peonidin-3-*O*-glucoside	531	369/353	162		531.1129	−1.9	$C_{25}H_{23}O_{13}$
81	Vitisin A	561	399/317	162		561.1237	−1.2	$C_{26}H_{25}O_{14}$
82	Vitisin B	517	355/339	162		517.1345	−0.2	$C_{25}H_{25}O_{12}$
83	Delphinidin-3-*O*-glucuronide	479	303/273/257/229		303	479.0836	2.1	$C_{21}H_{19}O_{13}$
84	Petunidin-3-*O*-(6-*O*-acetyl)-glucoside	521	317			521.132	4.8	$C_{24}H_{25}O_{13}$
85	Acetone derivative of peonidin-3-*O*-glucoside	501		162		501.1405	1.6	$C_{25}H_{25}O_{11}$
86	Acetone derivative of malvidin-3-*O*-glucoside	531	369/353	162		531.1509	1.1	$C_{26}H_{27}O_{12}$
87	Unknown	525		324				
88	Cyanidin-3-*O*-glucuronide	463	287		287	463.0919	9.1	$C_{21}H_{19}O_{12}$
89	Petunidin-3-(6-*O*-caffeoyl)glucoside	641	331		317	641.1506	2.3	$C_{31}H_{29}O_{15}$
90	Peonidin-3-*O*-(6-*O*-acetyl)glucoside	505	301		301	505.1346	−4.6	$C_{24}H_{25}O_{12}$
91	Delphinidin-3-*O*-(6-*O*-*p*-coumaroyl)glucoside	611			303	611.1401	4.1	$C_{30}H_{27}O_{14}$
92	Malvidin-3-*O*-(6-*O*-acetyl)glucoside	535	331/316		331	535.1476	4.5	$C_{25}H_{27}O_{13}$
93	Malvidin-3-*O*-(6-*O*-caffeoyl)glucoside	655	331		331	655.1669	0.9	$C_{32}H_{31}O_{15}$
94	Peonidin-3-*O*-(6-*O*-caffeoyl)glucoside	625	301		301	625.1582	4.0	$C_{31}H_{29}O_{14}$
95	Cyanidin-3-*O*-(6-*O*-*p*-coumaroyl)glucoside	595	287		287	595.1452	4.9	$C_{30}H_{27}O_{13}$
96	Malvidin-3-*O*-(6-*O*-*p*-coumaroyl)glucoside *cis*	639	331		331	639.1718	0.6	$C_{32}H_{31}O_{14}$
97	Petunidin-3-*O*-(6-*O*-*p*-coumaroyl)glucoside	625	317		317	625.1599	6.7	$C_{31}H_{29}O_{14}$
98	Peonidin-3-*O*-rutinoside	609	301		301	609.1812	−1.1	$C_{28}H_{33}O_{15}$
99	Malvidin-3-*O*-(6-*p*-coumaroyl)glucoside *trans*	639	331		331	639.1714	−2.2	$C_{32}H_{31}O_{14}$
100	Unknown	609	447/431		301			
101	Malvidin-3-*O*-rutinoside	639	331		331	639.1951	4.1	$C_{29}H_{35}O_{16}$
102	Malvidin-3-*O*-(6-*O*-*p*-coumaroyl)glucoside-4-vinylphenol adduct (pigment B)	755	447			755.1996	2.6	$C_{40}H_{35}O_{15}$

NL, neutral losses; PI, precursor ions; Acc Mass, accurate mass; ppm, error associated with the accurate mass; MF, molecular formula

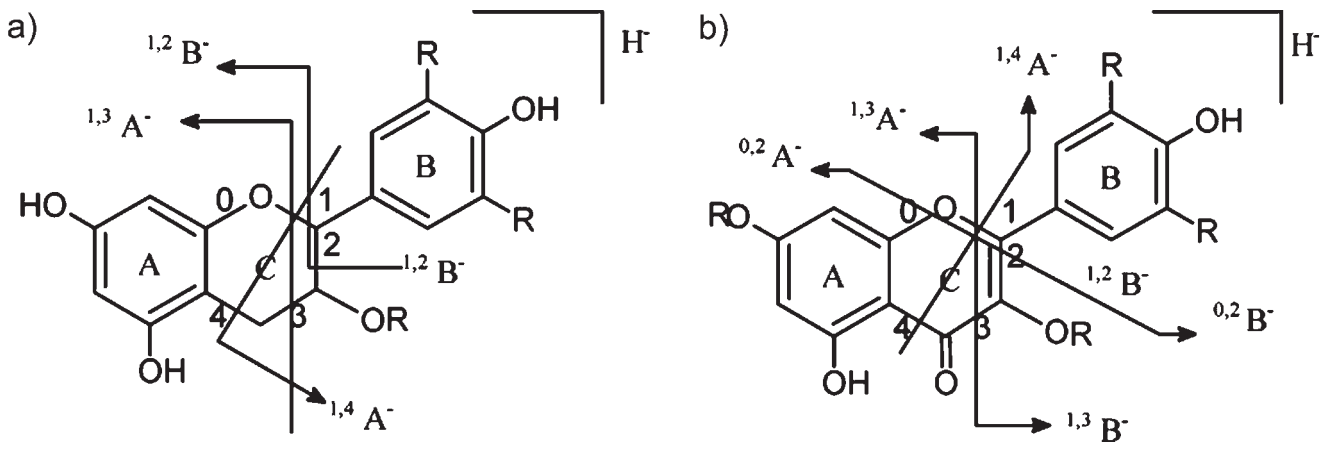


Figure 2. Structure and fragmentation pathways of flavanols (a) and flavonols (b).

DOI: 10.1002/rcm

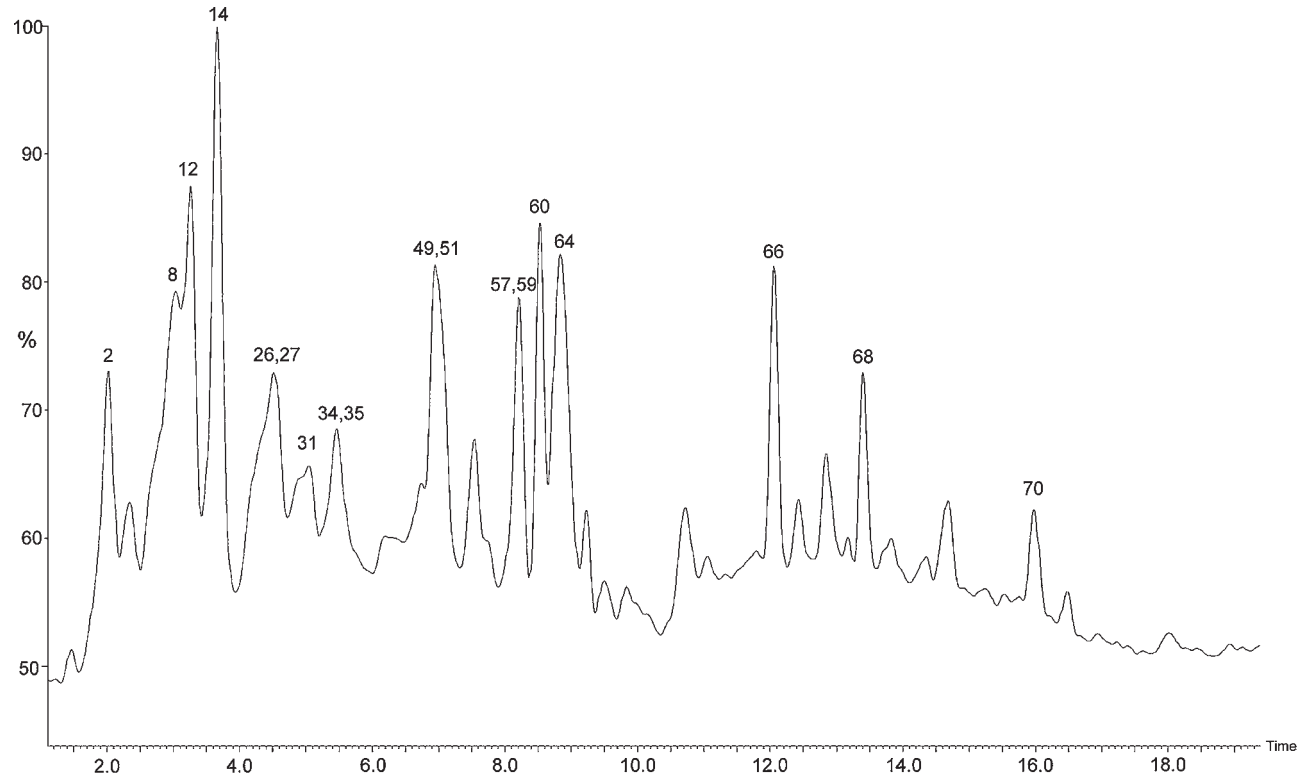


Figure 3. HPLC/TOF-MS full-scan chromatogram of the oligomeric fraction (2). The main components are labeled. For a complete list of compounds identified, see Table 1.

(8, 11, 15), as well as esters of vanillic acid (5, 18) (glucoside derivatives), were detected in this fraction.

To identify the different types of moieties present in PA oligomers, precursor ion scan experiments were performed at the characteristic *m/z* of the possible monomeric units: *m/z* 289 for catechin and epicatechin, *m/z* 305 for gallocatechin or epigallocatechin, and *m/z* 441 for epicatechin gallate. Whereas intense and fairly intense signals were obtained for the precursors at 289 and 441 Da, no significant signals were observed at 305 Da, indicating either that no prodelphinidins (PAs with gallocatechin units) were present in the extract, or that they were below the detection limit of the technique. Signals corresponding to multiple catechin/epicatechin dimers, trimers, and tetramers, resulting from different linking possibilities of the monomeric units, were observed. Dimeric structures (2, 3, 12, 16, 33, 48) ($[M–H]^-$ at 577) appeared at six different retention times, and product ion scans were performed on all of them, always giving the same fragmentation pattern. The main fragments were *m/z* 451, corresponding to the loss of a pyrogallol unit (126 Da) by heterocyclic ring fission (HRF) on the C ring; *m/z* 425 and 407, resulting from the elimination of the B-ring through a RDA reaction of the C-ring;[40] and *m/z* 289, which is the epicatechin/catechin precursor. Up to nine peaks at different retention times were observed for trimers (6, 10, 17, 20, 27, 29, 32, 37, 54) ($[M–H]^-$ at 865). PA trimers follow exactly the same fragmentation pattern as dimers. In this case, a first loss of a pyrogallol unit leads to the fragment at 739 Da, and the RDA reaction to the one at 713 Da. The loss of an epicatechin unit results in a dimeric structure. Therefore, the typical fragments already described for dimers could also be observed in the product ion scans of the trimers. In this fraction there was only one peak corresponding to a PA tetramer (31) ($[M–H]^-$ at 1153), whose product ion scan gave no fragments at all, even at high collision energies (CE up to 70 V). The accurate mass calculation matched a tetramer structure with a very low error (0.6 ppm). In agreement with the precursor ion scan experiments, PA oligomers containing epicatechingallate (ECG) moieties (galloylated PAs) were detected in this fraction. Three different structures containing this subunit were detected: dimers with one ECG unit (26, 28, 30, 34) ($[M–H]^-$ at 729 Da), trimers with one EGC unit (21, 36, 46, 52, 63) ($[M–H]^-$ at 1017 Da), and a dimer with two ECG units (44) ($[M–H]^-$ at 881 Da). Again, multiple peaks with the same molecular ion were encountered for some of these compounds: four, five and one isomers, respectively. Table 1 summarizes the main fragments obtained for the galloylated PAs in the product ion scan experiments. In general they follow the same fragmentation scheme as the non-galloylated PA dimers and trimers already mentioned, through RDA reactions and the loss of monomeric units.

The rest of the compounds identified in fraction 2 were glycosylated flavonols, flavones and flavanones. Flavonoid glycosides follow typical fragmentation patterns, which depend on the number and nature of their *C*- or *O*-glycosidic linkages.[41] Hexosides were identified through MS/MS analysis in neutral loss scan mode at 162 mass units, which corresponds to the loss of a glucose or galactose unit, and makes the aglycone easily identifiable.[9] Other precursor ion scan MS/MS experiments were performed at the *m/z* values corresponding to each of the aglycones: 317 for myricetin, 301 for quercetin, 285 for luteolin and kaempferol, 315 for isorhamnetin, 271 for naringerin, 269 for apigenin and genistein, and 283 for acacetin. Myricetin-3-*O*-glucoside (35)

DOI: 10.1002/rcm

and naringerin-7-*O*-glucoside (39) were directly identified through these experiments. Two quercetin hexosides were detected, most probably quercetin-3-glucoside (45) and quercetin-3-galactoside (51), as described for grapes.[20,21] The glucoside and galactoside were distinguished by their retention times, since it has been described[42] that, under the analytical conditions of this work, glucosides elute earlier than galactosides. Both spectra presented the aglycone fragment at 301 Da, and the galactoside also yielded the radical anion of the aglycone at *m/z* 300. The presence of radical aglycones in the product ion scan of flavonol-3-*O*-glycosides has been reported in the literature.[43] The relative abundance of the radical ion varies according to the nature and position of the sugar moiety, increasing with the number of hydroxyl substituents in the B-ring (myricetin > quercetin > kaempferol-glucoside). A precursor ion scan at *m/z* 285 revealed the presence of three compounds at *m/z* 447, all of them kaempferol or luteolin -*C*- or -*O*-hexosides. The radical anion of the aglycone at *m/z* 284 was also detected. Although product ion scan spectra were recorded for all of them, it proved hard to assign a structure on the basis of the fragment ions obtained alone. As the molecular formulas of these compounds (combinations of a kaempferol or luteolin unit with a hexose) are identical, measuring the accurate masses is of no help. Although luteolin derivatives have been found in several natural products, they have not been reported in grape products. The presence of kaempferol derivatives has, however, been widely reported.[21,44,45] The three peaks at *m/z* 447 (47, 57, 61) therefore probably belong to kaempferol -*C*- or -*O*-hexosides. Precursor ion scan MS/MS experiments at *m/z* 315 led to the identification of isorhamnetin-3-*O*-glucoside (60) and isorhamnetin-3-*O*-galactoside (62). As for quercetin, the two hexoses were differentiated by their retention times. Laricitin-3-*O*-glucoside (49) ($[M–H]^-$ 493), a compound already found in red grapes,[46] was also present in this fraction. The product ion spectra showed an intense aglycone peak (*m/z* 331) as well as one for its radical anion (*m/z* 330). Neutral loss scan MS/MS experiments at 176 mass units (glucuronide) revealed the presence of quercetin-3-*O*-glucuronide (55). Some rutinosides such as quercetin-3-*O*-rutinoside (50) and isorhamnetin-3-*O*-rutinoside (53) were identified through neutral losses of 308 mass units. The assignment of the glycoside substitutions to position 3 of the flavonoid ring was based on the literature cited. The accurate masses measured by the high-resolution system matched the structures proposed with very low errors.

Anthocyanin fraction 3

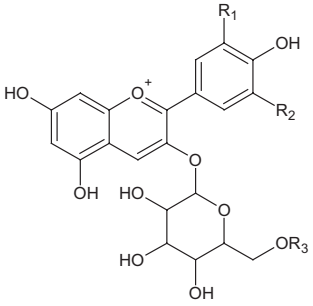
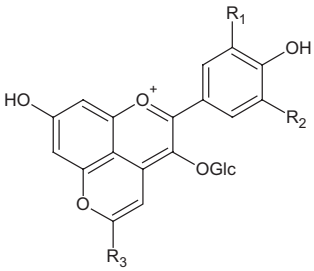
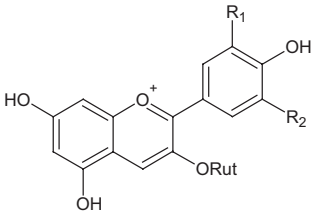
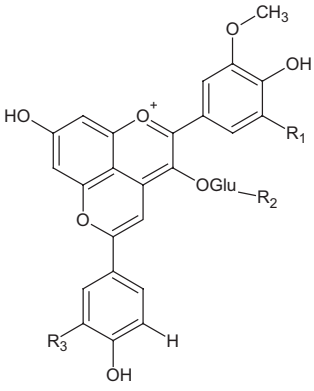
Anthocyanins constitute another biologically interesting polyphenolic fraction of GADF. They are absorbed in the digestive tract and have been found intact in a variety of tissues.[47] They even appear to be able to cross the blood brain barrier and enter the brain, where they might perform some neuroprotective activity.[47,48] LC/ESI-TOF enabled us to identify a number of putatively active small anthocyanins. Because anthocyanins are already positively charged in their natural form, this fraction was analyzed in the positive mode and M^+ molecular ions were detected. The results are summarized in Table 2. We identified six different groups of compounds by precursor ion scan experiments, corresponding to derivatives of six aglycones (anthocyanidins): delphinidin (*m/z* 303), cyanidin (*m/z* 287), petunidin (*m/z* 317), peonidin (*m/z* 301), pelargonidin (*m/z* 271), and malvidin (*m/z* 331). Neutral loss scan experiments helped us to identify the sugar moieties. Anthocyanins produced less fragmentation than flavonols and PAs did. On some occasions the aglycone fragment was the only one present in the product ion scan spectra, and this made the measurement of the accurate mass crucial to be able to assign a structure. Table 3 summarizes the chemical structures of the anthocyanins identified in this fraction.

The compounds eluting first (73, 74, 75, 76 and 78) on LC were the monoglucoside derivatives of delphinidin, cyanidin, petunidin, peonidin, and malvidin, respectively, as revealed by the characteristic fragment corresponding to the loss of glucose and confirmed by the exact mass measurements. Acetylglucoside derivatives of petunidin (84), peonidin (90) and malvidin (92) were also identified through the loss of 204 mass units (162 Da from the glucose moiety plus 42 Da from the acetyl moiety). In the same way, glucuronides of delphinidin (83) and cyanidin (88) were detected (loss of the glucuronide moiety, 176 Da). Another group of compounds found in this fraction showed a loss of 324 Da in the product ion scan spectra. This mass is compatible with the loss of two glucose units (diglucosides) or the loss of a caffeoylglucose unit. Two criteria allowed us to identify the compounds as caffeoylglucose derivatives: the absence of signals corresponding to loses of glucose in the neutral loss experiments (as we would expect if diglucosides were present) and the accurate mass measurements (which matched structures with caffeoylglucose moieties). Thus, the caffeoylglucoside derivatives of petunidin (89), malvidin (93) and peonidin (94) were unequivocally identified. A number of ions yielded a loss of 308 Da. This loss may be attributed to either rutinoside or coumaroylglucoside moieties. Again, the fragmentation was too low to decide between the two and LC/ESI-TOF was crucial to identify the compounds. Rutinosides and coumaroylglucosides have different molecular formulas, so the rutinoside derivatives of peonidin (98) and malvidin (101) were easily identified by measuring the accurate masses. In the same way compounds 91, 95, 96, 97, and 99 were identified as coumaroylglucoside derivatives of delphinidin, cyanidin, malvidin, petunidin, and malvidin, respectively. The two malvidin derivatives corresponded to the *cis*- (96) and *trans*- (99) isomers, which were distinguished by their retention times.[49] Both isomers have previously been detected in grapes[50] and wine.[49,51] Some of the structures (91–97) were identified as coumaroylglucosides. Although accurate mass measurements could not differentiate between coumaroylglucoside and glucoside-vinylguaiacol derivatives (which have identical molecular formula) the loss of a 308 Da fragment is quite unlikely to be from vinylguaiacol derivatives because this would imply the fragmentation of the main structure of the aglycone. Furthermore, according to the literature,[52,53] glucoside-vinylguaiacol derivatives lose the glucose moiety easily, and this was not observed in compounds 91–97.

DOI: 10.1002/rcm

Pyranoanthocyanins,[54] which are pigments formed during wine fermentation by the cycloaddition of pyruvic acid, acetaldehyde, acetone or 4-vinylphenol, and anthocyanins (monoglycosides and their acyl derivatives) were also detected in fraction 3. Adducts with pyruvic acid or acetaldehyde are named type-A and type-B vitisins, respectively. Compounds 80 and 81 were identified as type-A vitisins of peonidin and malvidin. They showed the characteristic aglycone (*m*/*z* 369 for peonidin, and *m*/*z* 399 for malvidin) after the loss of the glucose moiety. In the same way, compounds 77, 79 and 82 were identified as type-B vitisins of petunidin, peonidin, and malvidin. The malvidin derivatives were the ones originally named vitisin A and B.[55,56] Pyranoanthocyanins which originate in the reaction of acetone with peonidin and malvidin (85 and 86, respectively) were also identified. This kind of pigment has been detected in grape skin extracts[52] and synthetic media containing acetone and extracts of grape anthocyanins after fermentation.[57] Compounds 80 and 86 gave the same fragments in the product ion scan experiment and the same molecular ion at *m*/*z* 531. Since the proposed structures have different molecular

Table 3. Structures of the anthocyanins identified in fraction 3 of GADF

Structure	Compound	R_1	R_2	R_3
	73	OH	OH	H
	74	OH	H	H
	75	OCH_3	OH	H
	76	OCH_3	H	H
	78	OCH_3	OCH_3	H
	83	OH	OH	Glucuronide
	88	OH	H	Glucuronide
	84	OCH_3	OH	Acetyl
	90	OCH_3	H	Acetyl
	92	OCH_3	OCH_3	Acetyl
	89	OCH_3	OH	Caffeoyl
	93	OCH_3	OCH_3	Caffeoyl
	94	OCH_3	H	Caffeoyl
	91	OH	OH	Coumaroyl
	95	OH	H	Coumaroyl
	96	OCH_3	OCH_3	Coumaroyl
	97	OCH_3	OH	Coumaroyl
	99	OCH_3	OCH_3	Coumaroyl
	77	OCH_3	OH	H
	79	OCH_3	H	H
	80	OCH_3	H	COOH
	81	OCH_3	OCH_3	COOH
	82	OCH_3	OCH_3	H
	85	OCH_3	H	CH_3
	86	OCH_3	OCH_3	CH_3
	98	OCH_3	H	
	101	OCH_3	OCH_3	
	102	OCH_3	Coumaroyl	H

DOI: 10.1002/rcm

Table 4. Identification of phenolic compounds in fraction 4 (PA polymers) of the soluble fraction of GADF

	Type-B PAs								Type-A PAs			
			Monogallates		Digallates		Trigallates				Monogallates	
n	$[M–H]^-$	$[M–H]^{2-}$	$[M–H]^-$	$[M–H]^{2-}$	$[M–H]^-$	$[M–H]^{2-}$	$[M–H]^-$	$[M–H]^{2-}$	$[M–H]^-$	$[M–H]^{2-}$	$[M–H]^-$	$[M–H]^{2-}$
1	289.1		441.1									
2	577.1		729.1		881.2				575.1			
3	865.2		1017.2	508.6	1169.2		1321.3		863.2		1015.2	
4	1153.3		1305.3	652.6	1457.3		1609.3		1151.2	575.6	1303.3	
5	1441.3	720.6	1593.3	796.7	1745.4				1439.3	719.6	1591.3	795.7
6	1729.4	864.7				1016.7		1092.7	1727.4	863.7		
7		1008.7								1007.7		
8		1152.7								1151.7		
9		1295.7										
10		1440.8										
11		1584.8										
12		1728.9										

n, number of flavanol (catechin or epicatechin) units.

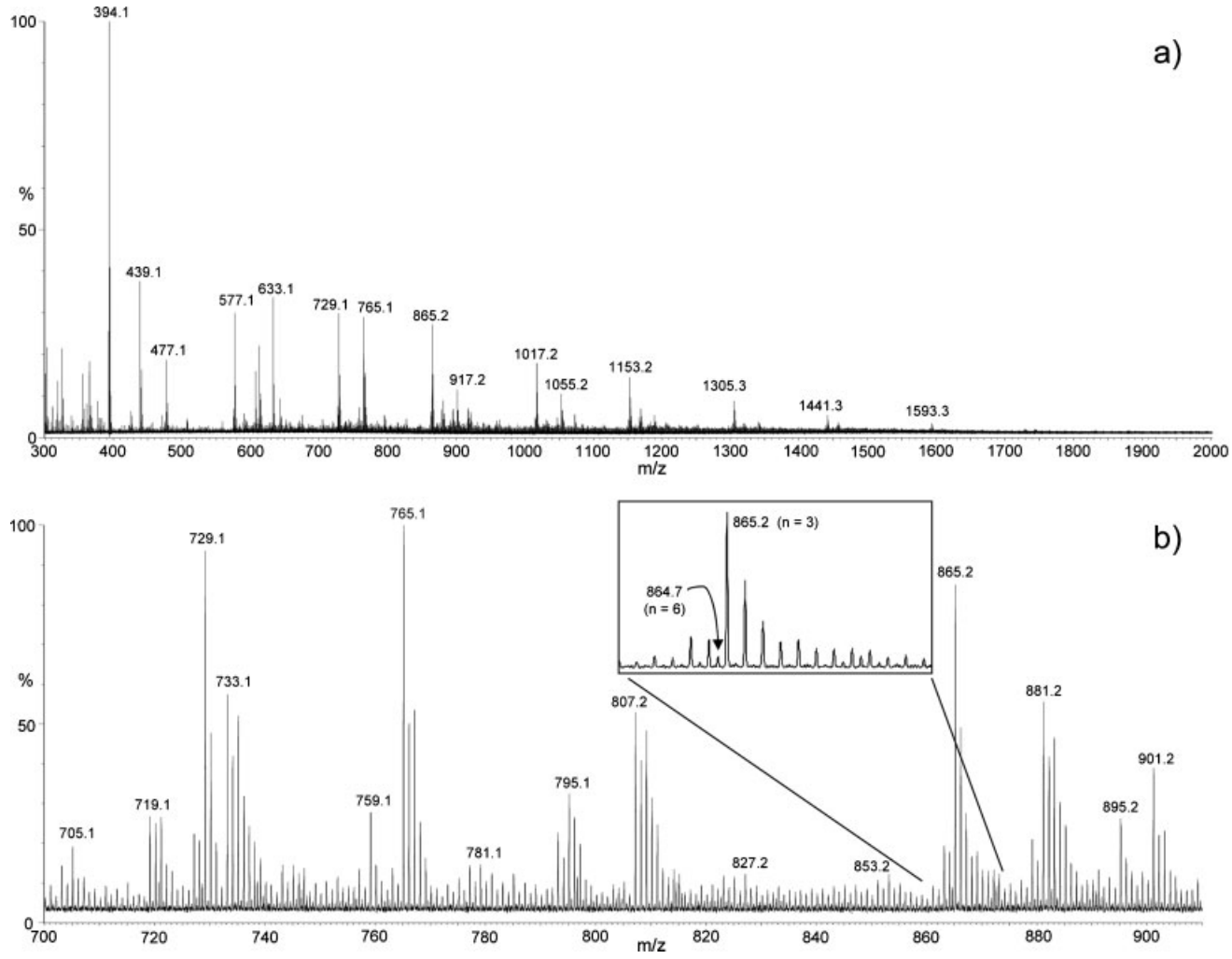


Figure 4. ESI-TOF-MS spectra in negative ion mode of the polymeric fraction (4): (a) from *m/z* 300 to 2000 and (b) from *m/z* 700 to 910.

formulas, they were easily differentiated by measuring the accurate mass with the LC/ESI-TOF system. Compounds 100 and 102 displayed the same fragment ion at *m/z* 447 in their respective product ion scans. Compound 100 gave a signal at *m/z* 301 in the precursor ion scan experiment, which identified the compound as a peonidin derivative; probably a glucose derivative as the 447 Da fragment implies the loss of glucose. The accurate mass measurement was compatible with the peonidin-3-*O*-glucoside-4-vinylguaiacol adduct. However, because the loss of 308 Da is not favored for vinylguaiacols, we do not suggest any structure. Compound 102 was identified as

DOI: 10.1002/rcm

the malvidin-3-*O*-(6″-*p*-coumaroyl)glucoside-4-vinylphenol adduct, also named pigment B.[58] In this case, *m/z* 447 corresponded to the aglycone (see Table 3) after the loss of the coumaroylglucoside moiety.

Polymeric fraction 4

Polymeric PAs are a particularly important fraction of GADF. Studies *in vitro* and *in vivo* have suggested that only the smaller oligomers (up to trimers) are adsorbed to some extent through the gut barrier, whereas larger PAs reach the colon intact.[36,37] There, these larger PAs are degraded by the colonic microbiota into smaller units, which in turn are degraded to phenolic acids, which are then absorbed.[6,59] All these molecules are bioavailable and bioactive in the colon where they may greatly contribute to the preventative effects of GADF. To characterize the polymeric fraction, we used ESI-TOF-MS in negative mode. As RP-HPLC was not able to separate the higher molecular mass polymers, the fraction was injected directly into the MS system. In a first attempt to identify the masses corresponding to PAs with high degree of polymerization, the fraction was scanned over a wide range of mass units (300–3000). Above 2000 mass units there was no significant signal from any possible ions of interest. To increase sensitivity, the fraction was analyzed over ranges of only 210 mass units (300–510, 500–710, and so on). Table 4 summarizes the PA polymers found in the fraction, indicating the degree of polymerization and galloylation. Figure 4(a) shows the whole spectrum of the fraction, and Fig. 4(b) includes expansions of the range from *m/z* 700 to 910. The most intense peaks corresponded to polymeric type-B PAs, with and without galloyl units attached. Two regular series of peaks were clearly observed with a peak occurring every 288 mass units, starting at 577 (epicatechin dimer) and 441 (monogallate monomer) mass units. A more accurate inspection of the spectrum revealed peaks corresponding to digallate and trigallate PAs, which generated series of signals at intervals of 152 (galloyl moiety) mass units. Doubly charged ions (see Fig. 4(b)) allowed us to identify PAs with a higher degree of polymerization. Polymers of up to 12 catechin units were observed for type-B PAs. The highest degree of polymerization for gallates was 5 (monogallates) and 6 (digallates and trigallates). Signals from triply charged ions were not observed.

Type-A PAs were also detected. Compared to type-B PAs, type-A PAs contain an extra interflavanic linkage (C2–C7) between catechin units. Therefore, type-A PA signals are 2 mass units lower than the corresponding type-B PA signals. Table 4 includes the type-A PAs. The highest degree of polymerization was 8 for catechin/epicatechin polymers and 4 for monogallates. MS techniques, particularly ESI-TOF, provide a wealth of information about the distribution of PA sizes and their galloylation that may eventually be related to the activity of GADF in the colon.

CONCLUSIONS

To advance the study of the structure/activity relationships of GADF, thorough identification of its bioactive polyphenols is of the utmost importance. We applied a combination of LC/MS techniques, namely high-resolution LC/ESI-TOF-MS and LC/ESI-MS/MS, to accurately identify many important polyphenolic compounds from the soluble fraction of GADF. The phenols include hydroxycinnamic acids, anthocyanins, flavonols, flavones, flavanones, catechins and PAs. Compared to LC/ESI-MS/MS on a triple quadrupole, LC/ESI-TOF-MS gave more sensitive and better-resolved full-fragment mass spectra, which facilitated the initial identification of the components from each LC run. Then LC/ESI-MS/MS fragmentation experiments suggested structures which were confirmed by the accurate masses as measured by LC/ESI-TOF-MS. High-resolution LC/ESI-TOF-MS has proved to be a particularly useful technique for discriminating between possible structures that are equally compatible with the fragmentation pattern generated by MS/MS.

Acknowledgements

Financial support from the Spanish Ministry of Education and Science (research grants AGL2004-07579-C04-01, -02, -03; AGL2006-12210-C03-02/ALI, doctoral fellowship to S.T. and post-doctoral contract to E.F.) is acknowledged.

REFERENCES

1. Schieber A, Stintzing FC, Carle R. *Trends Food Sci. Technol.* 2001; **12**: 401.
2. Guillon F, Champ M. *Food Res. Int.* 2000; **33**: 233.
3. Rice-Evans C, Miller NJ. *Biochem. Soc. Trans.* 1996; **24**: 790.
4. Scalbert A, Manach C, Morand C, Rémésy C, Jiménez L. *Crit. Rev. Food Sci. Nutr.* 2005; **45**: 287.
5. Manach C, Williamson G, Morand C, Scalbert A, Rémésy C. *Am. J. Clin. Nutr.* 2005; **81**: 230S.
6. Gonthier M-P, Donovan JL, Texier O, Felgines C, Rémésy C, Scalbert A. *Free Radical Biol. Med.* 2003; **35**: 837.
7. Saura-Calixto F. *J. Agric. Food Chem.* 1998; **46**: 4303.
8. Jiménez JP, Serrano J, Tabernero M, Arranz S, Díaz-Rubio ME, García-Diz L, Goñi I, Saura-Calixto F. *Nutrition* 2008; **24**: 646.
9. Cuyckens F, Claeys M. *J. Mass Spectrom.* 2004; **39**: 1.
10. Kammerer DR, Saleh ZS, Carle R, Stanley RA. *Eur. Food Res. Technol.* 2007; **224**: 605.
11. Liu R, Sun J, Bi K, Guo D-a. *J. Chromatogr. B* 2005; **829**: 35.
12. Flamini R. *Mass Spectrom. Rev.* 2003; **22**: 218.
13. Sanoner P, Guyot S, Marnet N, Molle D, Drilleau JF. *J. Agric. Food Chem.* 1999; **47**: 4847.
14. Mozetic B, Trebse P. *Acta Chim. Slov.* 2004; **51**: 151.
15. Vidal S, Hayasaka Y, Meudec E, Cheynier V, Skouroumounis G. *J. Agric. Food Chem.* 2004; **52**: 713.
16. Monagas M, Bartolomé B, Gómez-Cordovés C. *Crit. Rev. Food Sci. Nutr.* 2005; **45**: 85.
17. Peng Z, Hayasaka Y, Iland PG, Sefton M, Høj P, Waters EJ. *J. Agric. Food Chem.* 2001; **49**: 26.
18. Hayasaka Y, Waters EJ, Cheynier V, Herderich MJ, Vidal S. *Rapid Commun. Mass Spectrom.* 2003; **17**: 9.
19. Pozo-Bayón MA, Monagas M, Polo MC, Gómez-Cordovés C. *J. Agric. Food Chem.* 2004; **52**: 1300.
20. Kammerer D, Claus A, Carle R, Schieber A. *J. Agric. Food Chem.* 2004; **52**: 4360.
21. Monagas M, Garrido I, Bartolomé B, Gómez-Cordovés C. *Anal. Chim. Acta* 2006; **563**: 401.
22. Bravo MN, Silva S, Coelho AV, Boas LV, Bronze MR. *Anal. Chim. Acta* 2006; **563**: 84.
23. Reed JD, Krueger CG, Vestling MM. *Phytochemistry* 2005; **66**: 2248.
24. Ohnishi-Kameyama M, Yanagida A, Kanda T, Nagata T. *Rapid Commun. Mass Spectrom.* 1997; **11**: 31.
25. Vivas N, Nonier MF, Vivas De Gaulejac N, Absalon C, Bertrand A, Mirabel M. *Anal. Chim. Acta* 2004; **513**: 247.
26. Guyot S, Doco T, Souquet JM, Moutounet M, Drilleau JF. *Phytochemistry* 1997; **44**: 351.
27. Ishida Y, Kitagawa K, Goto K, Ohtani H. *Rapid Commun. Mass Spectrom.* 2005; **19**: 706.

DOI: 10.1002/rcm

28. Marchese S, Gentili A, Perret D, D'Ascenzo G, Pastori F. *Rapid Commun. Mass Spectrom* 2003; **17**: 879.
29. Fletcher AT, Mader JC. *J. Plant Growth Regul.* 2007; **26**: 351.
30. Saura-Calixto F, Goñi-Cambrodón I. In CSIC-UCM, ES 2259258 A1.
31. Saura-Calixto F, Larrauri-García JA. In CSIC, ES 2130092 A1.
32. Pérez-Jimenez J, Saura-Calixto F. *Int. J. Food Sci. Technol.* 2008; **43**: 185.
33. Sun B, Leandro MC, de Freitas V, Spranger MI. *J. Chromatgr. A* 2006; **1128**: 27.
34. Maier T, Sanzenbacher S, Kammerer DR, Berardini N, Conrad J, Beifuss U, Carle R, Schieber A. *J. Chromatgr. A* 2006; **1128**: 61.
35. Määttä-Riihinen KR, Kähkönen MP, Törrönen AR, Heinonen IM. *J. Agric. Food Chem.* 2005; **53**: 8485.
36. Jimenez-Ramsey LM, Rogler JC, Housley TL, Butler LG, Elkin RG. *J. Agric. Food Chem.* 1994; **42**: 963.
37. Terrill TH, Waghorn GC, Woolley DJ, McNabb WC, Barry TN. *Br. J. Nutr.* 1994; **72**: 467.
38. Meselhy R, Nakamura N, Hattori M. *Chem. Pharm. Bull.* 1997; **45**: 888.
39. Lizárraga D, Lozano C, Briede JJ, van Delft JH, Touriño S, Centelles JJ, Torres JL, Cascante M. *FEBS J.* 2007; **274**: 4802.
40. Gu L, Kelm MA, Hammerstone JF, Zhang Z, Beecher G, Holden J, Haytowitz D, Prior RL. *J. Mass Spectrom.* 2003; **38**: 1272.
41. Ferreres F, Llorach R, Gil-Izquierdo A. *J. Mass Spectrom.* 2004; **39**: 312.
42. Sánchez-Rabaneda F, Jáuregui O, Lamuela-Raventós RM, Viladomat F, Bastida J, Codina C. *Rapid Commun. Mass Spectrom.* 2004; **18**: 553.
43. Hvattum E, Ekeberg D. *J. Mass Spectrom.* 2003; **38**: 43.
44. Kammerer D, Carle R, Schieber A. *Rapid Commun. Mass Spectrom.* 2003; **17**: 2407.
45. Flamini R, Panighel A. *Mass Spectrom. Rev.* 2006; **25**: 741.
46. Ruberto G, Renda A, Daquino C, Amico V, Spatafora C, Tringali C, Tommasi ND. *Food Chem.* 2007; **100**: 203.
47. Kalt W, Blumberg JB, McDonald JE, Vinqvist-Tymchuk MR, Fillmore SAE, Graf BA, O'Leary JM, Milbury PE. *J. Agric. Food Chem.* 2008; **56**: 705.
48. Andrés-Lacueva C, Shukitt-Hale B, Galli RL, Jáuregui O, Lamuela-Raventós RM, Joseph JA. *Nutr. Neurosci.* 2005; **8**: 111.
49. Alcalde-Eón C, Escribano-Bailón MT, Santos-Buelga C, Rivas-Gonzalo JC. *J. Mass Spectrom.* 2007; **42**: 735.
50. Núñez V, Monagas M, López-Cordovés MC, Bartolomé B. *Postharvest Biol. Technol.* 2004; **31**: 69.
51. Monagas M, Núñez V, Bartolomé B, Gómez-Cordovés C. *Am. J. Enol. Vitic.* 2003; **54**: 163.
52. Hayasaka Y, Asenstorfer RE. *J. Agric. Food Chem.* 2002; **50**: 756.
53. de Villiers A, Vanhoenacker G, Majek P, Sandra P. *J. Chromatgr. A* 2004; **1054**: 195.
54. Rentzsch W, Schwarz M, Winterhalter P. *Trends Food Sci. Technol.* 2007; **18**: 526.
55. Bakker J, Timberlake CF. *J. Agric. Food Chem.* 1997; **45**: 35.
56. Fulcrand H, Benabdeljalil C, Rigaud J, Cheynier V, Moutounet M. *Phytochemistry* 1998; **47**: 1401.
57. Benabdeljalil C, Cheynier V, Fulcrand H, Hakiki A, Mosaddak M, Moutounet M. *Sci. Aliment.* 2000; **20**: 203.
58. Fulcrand H, dos Santos PJC, Sarni Manchado P, Cheynier V, Favre Bonvin J. *J. Chem. Soc. Perkin Trans. 1* 1996; 735.
59. Déprez S, Brezillon C, Rabot S, Philippe C, Mila I, Lapierre C, Scalbert A. *J. Nutr.* 2000; **130**: 2733.

DOI: 10.1002/rcm

4.2.2 PUBLICACIÓN 4: Identificación de metabolitos de fibra antioxidante de uva en orina de ratas.

Título original: Phenolic metabolites of grape antioxidant dietary fiber in rat urine.

Autores: Sonia Touriño, Elisabet Fuguet, Mª Pilar Vinardell, Marta Cascante, Josep Lluís Torres. ***Journal of Agricultural and Food Chemistry*** *(En proceso de revisión)*

Índice de impacto: 2,507

Clasificación y categoría: 7/ 103 (Ciencia y Tecnología de los alimentos); 8/62 (Química aplicada); 1/35 (Agricultura, multidisciplinar)

RESUMEN:

La fibra dietética de uva es particularmente interesante como suplemento nutricional gracias a sus efectos beneficiosos sobre la salud cardiovascular *(Pérez-Jiménez et al., 2008)*. Esta fibra dietética se caracteriza por presenta una elevada cantidad de flavonoides y proantocianidinas superando en porcentaje a otras fibras dietéticas similares *(Goñi et al., 2009)*. La actividad biológica de los flavonoides y proantocianidinas podría ser directamente responsable de los beneficios a la salud cardiovascular que aporta GADF. Los flavonoides son metabolizados de manera extensiva cuando son ingeridos por lo que las actividades biológicas podrían estar relacionadas con los metabolitos más que con los compuestos en su forma original en la fuente vegetal.

El objetivo de este trabajo fue identificar los metabolitos mayoritarios tras la ingesta de GADF. Para alcanzar este objetivo se realizó un estudio con animales de experimentación, ratas Sprague-Dawley (n=12), divididos en tres grupos (n=4) que correspondían a un grupo control, un grupo al que se le administró Epicatequina pura (1g /kg) y un grupo al que se le administró GADF (1,6g/kg). Posterior a la administración, se recogió muestra de orina a diferentes horas (2, 4, 6, 8, 10 y 24h). Las muestras fueron purificadas por extracción en fase sólida (SPE) previamente al análisis por LC-MS y LC-MS/MS.

Experimentos en tándem (MRM, NL, PI y barrido de iones producto) permitieron identificar 18 metabolitos de epicatequina (mono-, di-, tri- conjugada con metilo, sulfato y glucurónido) que podrían proceder de oligómeros de proantocianidinas

despolimerizados a lo largo del intestino y de ácidos fenólicos libres procedentes de las transformaciones colónicas. También fueron identificados ácidos fenólicos conjugados con sulfato y glucuronido. Éstos podrían ser productos de las fermentaciones de la microbiota colónica absorbidos y metabolizados probablemente en el hígado.

Los experimentos en tándem realizados, también aportaron información estructural de las posiciones de conjugación en los compuestos fenólicos. Esta información es particularmente interesante si se tiene en cuenta que algunos de los metabolitos identificados en este estudio presentaron el grupo catecol libre, lo cual indica que cierta capacidad antioxidante *in vivo* persiste durante los procesos de biotransformación.

El estudio de la identificación de los metabolitos de GADF aportó un mayor conocimiento sobre la biodisponibilidad de los polifenoles en mezclas complejas. Teniendo en cuenta que frutas y verduras no procesadas contienen grandes cantidades de proantocianidinas, los resultados de este estudio son una contribución al conocimiento de los posibles efectos beneficiosos de los polifenoles dietéticos en la salud.

A continuación se muestra la publicación original. El material suplementario de este artículo se incluye en los Anexos (apartado 8.2.3, *ver pág. 287*)

Phenolic metabolites of grape antioxidant dietary fiber in rat urine

SONIA TOURIÑO[†], ELISABET FUGUET[†+], MARÍA PILAR VINARDELL[‡], MARTA CASCANTE[§] and JOSEP LLUÍS[†] TORRES*[†]

[†] Institute for Advanced Chemistry of Catalonia, CSIC (ICAQ-CSIC). Jordi Girona 18-26, 08034-Barcelona, Spain.
[+]Current address: Departament de Química Analítica, Universitat de Barcelona, Martí i Franquès 1-11, 08028-Barcelona, Spain
[‡] Department of Physiology, Faculty of Pharmacy, University of Barcelona, Av. Joan XXIII s/n, 08028-Barcelona, Spain
[§] Department of Biochemistry and Molecular Biology, Institute of Biomedicine of the University of Barcelona (IBUB) and Centre for Research in Theoretical Chemistry, Barcelona Science Park (CeRQT-PCB), Unit Associated with CSIC, Diagonal 645, 08028-Barcelona, Spain

Grape antioxidant dietary fiber (GADF) combines the putative health benefits of fiber and polyphenols. Polyphenolic metabolites may play a key role in the overall biological effects of this supplement. We identified phenolic GADF metabolites in rat urine at different times after oral administration, using HPLC-ESI-MS/MS techniques. The phenolic metabolic outcome of GADF is essentially an array of mono and polyconjugated epicatechins and free or conjugated smaller phenolic acids, some of them never reported before. We have detected 18 mono-, di-, and tri-conjugates of EC with glucuronide, methyl and sulfate moieties and small phenolic acids both free and conjugated. Procyanidin oligomers are depolymerized in the small intestine and the polymers are degraded by the colonic microbiota. For several hours after ingestion of GADF, a great variety of phenolic species including some with an intact catechol group, are in contact with the digestive tract tissues before, during and after metabolization, and many of them are systemically bioavailable before being excreted.

KEYWORDS: grape antioxidant dietary fiber, polyphenols, procyanidins, epicatechin, metabolites, bioavailability, mass spectrometry, urine.

INTRODUCTION

Dietary fiber is believed to play an important role in maintaining the functional integrity of the gastrointestinal tract. A high fiber intake is associated with body weight control (*1*) and a reduced risk of diseases such as colon cancer (*2*) and atherosclerosis (*3*). However, not all fibers are equally good and the benefits of a fiber-rich diet greatly depend on the solubility and fermentability of the fiber eaten as well as on other functional constituents, particularly antioxidant polyphenols (*4, 5*). Polyphenols are scavengers of reactive oxygen species (ROS) and appear to interact with biologically significant proteins. Grape antioxidant dietary fiber (GADF) is a particularly interesting nutritional supplement obtained from grape pomace (*6*). GADF is superior to other fibers in terms of digestibility and intestinal fermentation (*7*). Most promisingly, GADF may contribute to cardiovascular health, mainly by lowering blood pressure and levels of cholesterol (*8*). GADF is 58% insoluble fiber, 16% soluble fiber and it contains a large proportion (20%) of polyphenolic compounds, including phenolic acids and flavonoids, mainly polymeric procyanidins (*8, 9*). Oligomeric procyanidins, rather than other polyphenols, are reported to be responsible for lowering blood pressure in humans (*10*).

*Autor to whom correspondence should be addressed, [Phone, +34 93 400 61 12; Fax, +34 93 204 59 04; e-mail, josepllluis.torres@iqac.csic.es]
† Institute for Advanced Chemistry of Catalonia-CSIC (IQAC-CSIC)
‡, § University of Barcelona

Therefore, the cardiovascular benefits of GADF may very well be due to the procyanidins it contains, or perhaps more precisely to their metabolites.

Procyanidins are extensively metabolized in the intestinal tract and liver, as are other polyphenols. It therefore follows that the putatively beneficial effects of polyphenols at the systemic level should be attributed to the metabolites rather than to the parent compounds. Flavonoid metabolites are formed by Phase I (cleavage/degradation) and Phase II (derivatisation) metabolism to give and array of structures. Some of them are shown in **Figure 1**. The number of possible metabolites is enormous and the amount of each individual metabolite may be small. That is why most studies in the literature use enzymes to strip off the derivatizing moieties and detect only the hydroxylic form of the metabolite (*11, 12*). However, in this way valuable information on what particular active compounds may actually be bioavailable is lost. The literature on metabolization of procyanidins is still incomplete. The most accurate accounts in terms of epicatechin (EC) derivatization (glucuronates, sulfates, methyl ethers) lack crucial information on phenolic acids (*13*), whereas the reports on colonic metabolites (phenolic acids) miss the variability of derivatized species (*11, 14*).

The aim of this study is to identify the main phenolic metabolites present in rat urine after GADF ingestion, using a combination of mass spectrometry (MS)

techniques (multiple reaction monitoring: MRM; neutral loss (NL) experiments; precursor ion (PI) experiments and product ion experiments) on a triple quadrupole apparatus. The studies of polyphenol metabolites referenced above examined the metabolic fate of pure products (e.g., EC) or simple plant extracts (e.g., purified procyanidins). The complex GADF we examined is a much more faithful model for dietary intake of whole fruit. The information provided here may help explain the biological reactions to GADF and the possible beneficial effects of a diet rich in fruit and vegetables.

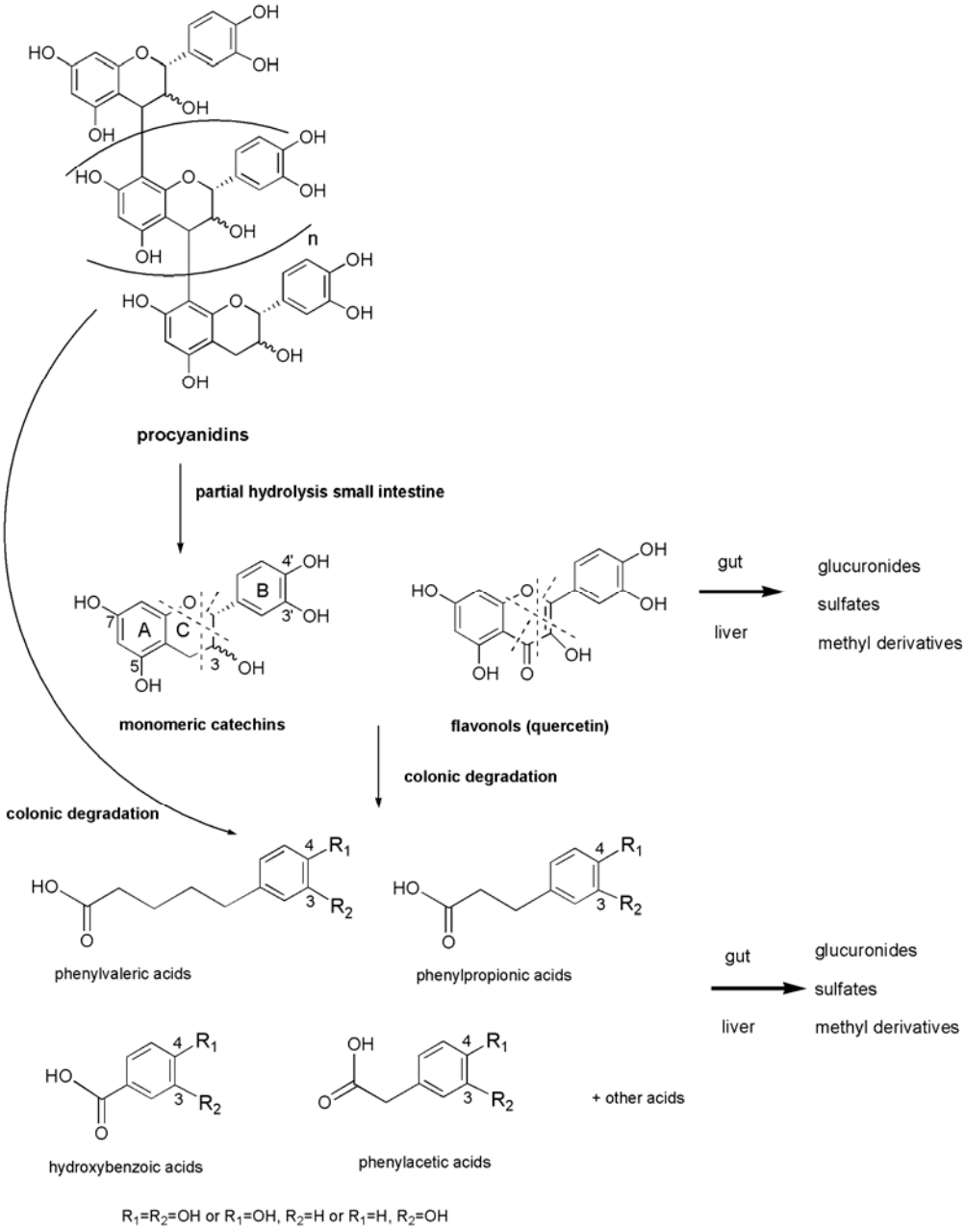


Figure 1: Metabolization of procyanidins and other flavonoids

MATERIALS AND METHODS

Chemicals and reagents. GADF was obtained from red grapes (the *Cencibel* variety) harvested in the vintage year 2005 in *La Mancha* region in Spain, as described in a published patent (*6*). Standards of (–)-epicatechin (≥ 97%), (+)-catechin (≥ 98 %), 3- and 4-hydroxybenzoic acid (≥ 97 %), vanillic acid (≥ 97 %), caffeic acid (≥ 95 %), 3,4-di-hydroxyphenylpropionic acid (DHPhPA; >98%), 4-hydroxyphenylpropionic acid (HPhPA; >98%), protocatechuic acid (≥ 97 %), *p*-coumaric (≥ 98 %), *m*-coumaric (≥ 97%) and taxifolin (≥ 85%) were obtained from Sigma Chemical (Saint Louis, MO, USA). Methanol (analytical grade) and 37% hydrochloric acid

were purchased from Panreac (Castellar del Vallés, Barcelona, Spain). Acetonitrile (HPLC grade) and formic acid (analytical grade) were obtained from Merck (Darmstadt, Germany). Water was purified by a Milli-Q plus system from Millipore (Bedford, MA, USA) to a resistivity of 18.2 MΩcm.

Animal experiments. Female Sprague-Dawley rats (SD, n = 12, body weight = 233 ± 9.3 g, 12 weeks of age) were provided by Harlan Interfauna Ibérica S.L (Barcelona, Spain). The animals were fed with a polyphenol-free diet (TD94048) purchased also from Harlan Interfauna Ibérica S.L and maintained in plastic cages at room temperature (22 ± 2 ˚C) and 55 ± 10 % relative humidity, with a 12 h light/dark cycle for one week, according to European Union Regulations. The rats were randomly divided into three groups (n =4) and then housed individually in metabolic cages. Each group was administered either a GADF suspension, or a (–)-epicatechin solution or tap water. The GADF and EC were dissolved in tap water (1 g/6 ml) and administered by oral gavage in single doses of 1 and 1.6 g/kg body weight respectively. Tap water was administered orally to the rats at a dose of 10 ml/kg body weight for blank urine collections. Prior to the administration, the rats were deprived of food for 12 h, with free access to water. Urine samples were collected and acidified with HCl (~5 µL) at 2, 4, 6, 8, 10 and 24 h after administration. Samples were stored at –80ºC until extraction and analysis. Finally, the animals were killed by an overdose of anesthetic (isofluoran gas).

These experimental protocols were approved by the Experimental Animal Ethical Research Committee of the University of Barcelona (permission number: DMA3123) in accordance with current regulations for the use and handling of experimental animals.

Sample processing. Urine samples were concentrated and then re-suspended in 2 mL of acid water. Then, 50 µL of a 50 ppm solution of taxifolin was added to each sample as an internal standard, to obtain a final concentration of 5 ppm. An Oasis HLB (60 mg) cartridge from Waters Corp. (Mildford, MA, USA) was used for the solid phase extraction (SPE). The cartridge was activated with 1 mL of methanol and 2 mL of acid water (10^{-3} M HCl) and the samples were loaded onto the cartridge. To remove interfering components, the sample was washed with 9 mL of acid water. The phenolic compounds were then eluted with 1 mL of methanol. The eluate was evaporated under nitrogen and the residue reconstituted with 500 µL of the HPLC starting mobile phase ([A], see below). The temperature of evaporation was kept under 30ºC to avoid deterioration of the phenolic compounds. The samples were filtered through a polytetrafluoroethylene (PTFE) 0.45-µm membrane from Waters Corp. into amber vials for HPLC-MS/MS analysis.

HPLC-ESI-MS/MS analysis. An Applied Biosystems (PE Sciex, Concord, Ontario, Canada) API 3000 triple quadrupole mass spectrometer with a TurboIon spray source was used in negative mode to obtain mass spectrometry (MS) and MS/MS data. Liquid chromatography separations were performed on an Agilent 1100 series system (Agilent, Waldbronn, Germany) liquid chromatograph equipped with a Phenomenex (Torrance, CA, USA) Luna C18 (50 x 2.1 mm i.d.) 3.5-µm particle size column and a Phenomenex Securityguard C18 (4 x 3 mm i.d.) column. Gradient elution was performed with a binary system consisting of [A] 0.1% aqueous formic acid and [B] 0.1% formic acid in CH_3CN. An increasing linear gradient (v/v) of [B] was used, [t(min), %B]: 0,8; 10,23; 15,50; 20,50; 21,100; followed by a re-equilibration step.

Metabolites in urine samples were identified by (i) MRM transitions of the putative metabolites using a dwell time of 100 ms, (ii) NL and PI scans and posterior comparison of the results with the retention time obtained by MRM, and (iii) product ion scan experiments to confirm the identity of each metabolite. The cycle time used was 2 s. The declustering potential (DP) and collision energy (CE) used in the product ion scan mode were –80 V and –35 V respectively.

RESULTS

Analytical strategy. In previous studies, we observed that GADF is particularly rich in (epi)catechin ((–)-epicatechin and (+)-catechin) polymers (*8, 9*). The monomeric (epi)catechin content of GADF is less than 0.01% by HPLC-UV. Consequently we expected the metabolic outcome of GADF in rat urine to be mainly that of oligomeric and polymeric (epi)catechin. In the present work we compare the metabolites generated after GADF ingestion with those generated by pure monomeric EC. With the tools provided by HPLC-MS/MS we searched for intact flavonoids (catechins and others) and phenolic acids, as well as glucuronidyl, methyl and sulfate conjugates of (epi)catechin, conjugates with other flavonoids and metabolites generated by ring fission (**Figure 1**). The metabolites were identified on the basis of published literature (*15-17*).

To gain information on the kinetics of the metabolization/absorption/metabolization and excretion, urine samples were collected at different times (2, 4, 6, 8, 10, 24 hours) after ingestion. The samples were first analyzed using an HPLC-MS system in the full-scan mode (FS). Some compounds detected in samples collected after ingestion of GADF and EC were also present in the controls and therefore were not considered GADF metabolites. We also detected metabolite conjugates with chloride coming from the HCl added to the samples before freezing. All the metabolites conjugated with chloride were also detected in the non-chlorinated form. The identity of the putative metabolite found in the FS mode was corroborated by MRM, which yields the highest selectivity and sensitivity in HPLC-MS/MS (*18*). MRM transitions in urine samples obtained after GADF and EC ingestion were compared in detail with the blank samples. To eliminate false positives and to obtain further structural information, NL experiments and PI experiments were run. NL experiments helped to identify the glucuronides (M-176) and sulfates (M-80). PI scanning helped to detect the main metabolites of the flavonoid aglycones which we had been previously found in the GADF soluble fraction (*9*).

Finally, the identity of putative GADF metabolites detected in the previous experiments was confirmed by product ion scan MS/MS experiments on the molecular ions and/or fragment ions.

Table 1: Conjugated metabolites of EC in urine from rats fed with GADF or EC (HPLC-MS/MS identification).

Metabolite	Number	MRM	NL	PI	MS^2 fragments (relative abundance %)
Gluc-EC-1	1	465→289	176	289	465(1);289(100);245(25);175(6)113(7)
Gluc-EC-2	2	465→289	176	289	465 (12)327(9);289(100);245(31);203(12);175(16);151(12);113(10)
Gluc-EC-3	3	465→289	176	289	465(1);289(100);245(25);203(9);179(20);137(9)
Sulf-EC-1*	4	369→289	80	289	369(37);289(100);245(80);217(33);203(44);137(62)
Sulf-EC-2*	5	369→289	80	289	369(16);289(100);245(45);231(23);151(15);137(30)
Me-Sulf-EC-1	6	383→303	80		383(13);303(100);259(22);245(36);220(24);204(15);165(18);151(6);137(11)
Me-Sulf-EC-2	7	383→303	80		383(15); 303(100);285(20);244(13);217(16);137(50)
Me-Sulf-EC-3	8	383→303	80		383(39);303(100);244(20);217(17);137(33)
Me-Sulf-EC-4	9	383→303	80		383(25);303(79);217(25);137(100)
Gluc-Me-EC-1	10	479→303	176		479(5);303(100);285(13);244(10);165(6);137(7);113(15)
Gluc-Me-EC-2	11	479→303	176		479(10);303(100);285(9);259(15);175(10); 137(10);113(30)
Gluc-Me-EC-3	12	479→303	176	289	479(1);313(5);303(20);289(100);245(35);203(11);137(10)
Gluc-Me-EC-4	13	479→303	176	289	313(7);303(1);289(100);245(28);203(10);179(8)
Gluc-Sulf-EC-1	14	545→369	176;80	289	545(5);465(22);369(10);289(20);271(100);151(15)
di-Sulf-EC-1	15	449→369	80		369(100);289(15);245(15);231(19);217(13)
di-Gluc-EC-1	16	641→465	176		641(90);465(50);289(20)
di-Me-Gluc-EC-1	17	493→289	176	289	493(6);475(8);317(11); 313(10)289(100);245(25);205(11)137(16)
di-Me-Gluc-EC-2	18	493→289		289	313(3); 303(6);289(100);245(25);205(10)179(6)
di-Me-Gluc-EC-3	19	493→289	176		317(6); 303(100);259(11);244(17);151(10);137(23)
Me-di-Sulf-EC-1*	20	463→383	80		383(100);303(40);245(35);217(17);165(9);137(10)
Me-di-Sulf-EC-2	21	463→383	80		383(80);303(23);273(100);245(35);231(45);217(25):189(16);137(20)
Gluc-Me-Sulf-EC-1*	22	559→383			625(2);335(100);289(4);193(12);175(52);159(91);113(35)
Gluc-Me-Sulf-EC-2*	23	559→383			559 (100);313(11);289 (70);245 (9);231(29);203(6);149 (6)
tri-Sulf-EC-1*	24	529→289			449(25);369(100)

* Only detected in the EC group

Epicatechin metabolites. EC oligomers appeared to be extensively but not completely depolymerized before absorption. Non-conjugated EC dimers were present in urine samples taken 2 hours after ingestion of GADF. Most of the EC identified in urine samples occurred as conjugates. Non-conjugated EC (m/z 289) was only detected in samples collected early after EC ingestion. No signals corresponding to either conjugated dimers or to products resulting from the opening of the C-ring ($[M–H]^-$ at 579 and 581) (*14, 19*) were recorded. We detected more EC conjugates than those previously reported (*13, 20*). The conjugates include mono-glucuronidated and mono-sulfated EC as well as di-conjugates and tri-conjugates resulting from combinations of glucuronidyl, methyl and sulfate moieties. **Table 1** lists all the EC conjugates detected by HPLC-MS and HPLC-MS/MS in urine samples coming from rats fed both GADF and EC. A Table with the times at which all these metabolites were detected is provided as supplementary material.

Mono-conjugated metabolites of EC. The MRM transition 465→289 corresponding to EC glucuronides (Gluc-EC) under negative mode revealed the presence of three compounds: 1, 2 and 3. The identity of these metabolites with molecular ions at *m/z* 465 was corroborated by the NL (M-176) and PI (*m/z* 289) experiments. These glucuronidated metabolites of epicatechin appeared in samples collected at all the different times from rats fed GADF or pure EC. In the product ion scan mode, each Gluc-EC generated the corresponding molecular ion at *m/z* 465 ($[M–H]^-$) that released a main MS/MS (MS^2) fragment at *m/z* 289 (EC after loss of the glucuronide moiety) and the characteristic fragments of the glucuronide moiety at *m/z* 175 and 113 (*21*). Other MS^2 fragments from these metabolites were useful for establishing the position of the glucuronide moiety on the EC molecule. Ions at *m/z* 245 (loss of CO_2 from the EC moiety) and an intense signal at *m/z* 137 (a fragment of the A-ring) suggested that the glucuronide moiety in compounds 1 and 3 was attached to the A-ring. Indeed, it has been reported that in rats, glucuronidation of (–)-epicatechin occurs at position 7 of the A-ring (*22*). Thus, Gluc-EC-3 (3), which gave the most intense peaks in urine samples from both groups, may be assigned to 7-Gluc-EC, and Gluc-EC-1 (1) to 5-Gluc-EC. Gluc-EC-2 (2) gave one fragment at *m/z* 327 corresponding to a B-ring fragment plus the glucuronide moiety (151+176), resulting from the retro Diels-Alder (RDA) fission of the EC C-ring (*23*). This indicates that in this case, glucuronidation occurred at the positions 3' or 4' of the B-ring. Peak 2 is nearly as intense as peak 3 in the urine of rats fed GADF fed rats, in contrast to the urine from rats fed EC (**Figure 2**). This suggests that the catechol moiety (B-ring) may be bioavailable as a combination of intact and conjugated forms.

Two peaks (compounds 4 and 5) were assigned to sulfates of EC (Sulf-EC) which were only found in urine from rats fed EC. Product ion scans of these compounds generated common fragments at *m/z* 369 ($[M–H]^-$) and 289 (EC, loss of sulfate moiety) and at *m/z* 245 (EC–CO_2). Metabolite Sulf-EC-1 (peak 4) produced fragments at m/z 217 (Sulfate + A-ring fragment by RDA fission) and m/z 137 (A-ring fragment) indicating that the sulfate occurred at position 5 or 7 of the A-ring. Sulf-EC-2 (peak 5) produced fragments at *m/z* 151 and at *m/z* 231 corresponding to B-ring and "B-ring plus sulfate" fragments at positions 3' and 4' respectively. We did not detect any mono-methylated metabolite in the urine samples.

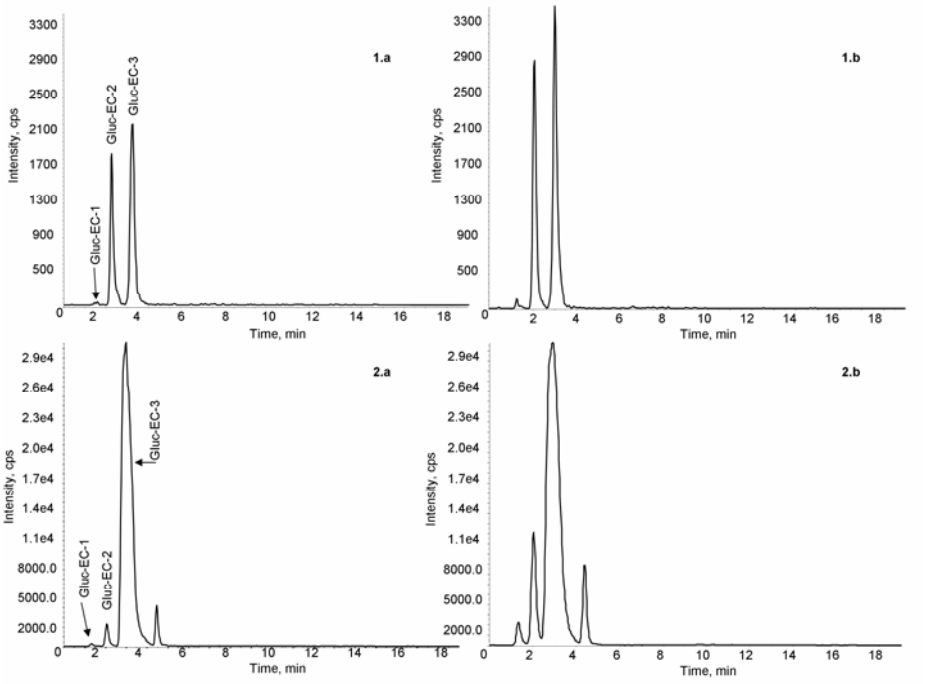


Figure 2: HPLC-ESI-MS/MS profile corresponding to the detection EC glucuronides in urine samples after feeding: 1) GADF and 2) EC. Samples collected at 2 h (a) and 6 h (b). Detection in the multiple reaction monitoring (MRM) mode, transition 465→289.

Di-conjugated metabolites. Four sulfated and methylated EC conjugates (Me-Sulf-EC; 6, 7, 8, 9) were detected in low quantities in urine samples from rats fed GADF by MRM transition 383→303. They were identified by comparison with stronger signals detected in samples from animals fed EC. The four Me-Sulf-ECs yielded a molecular ion at *m/z* 383.

However, two different fragmentation patterns were observed. Compound 6 produced specific ions at *m/z* 245 and *m/z* 165, which were not present in the fragmentation of the other isomers. The fragment at *m/z* 165 corresponds to the characteristic B-ring fragment at *m/z* 151 plus a methyl moiety, and the fragment at *m/z* 245 corresponds to the same fragment (151) plus methyl and sulfate. These two ions and the absence of the sulfated A-ring fragment (*m/z* 217, 137+80) are clear evidence that methylation and sulfation of this metabolite occurred on the B-ring. In contrast, compounds 7-9 all gave the same product ion fragments at *m/z* 303, 217 and 137, which indicates that sulfation occurred on the A-ring for all of them.

By monitoring the transition 479→303 we observed three intense peaks (10, 11, 12) corresponding to methylated and glucuronidated EC (Gluc-Me-EC). Another Gluc-Me-EC (13) was later detected through the product ion scan (**Figure 3**).

Compound 13 was not detected by MRM because the molecular ion at m/z 479 produced an intense signal at m/z 289 instead of 303. Moreover, the NL of 176 Da (Gluc) and PI of 289 Da (EC) ruled out false positive identifications. The molecular ions of Gluc-Me-EC-1 and Gluc-Me-EC-2, corresponding to peaks 10 and 11, generated MS^2 fragments at *m/z* 303 (loss of a glucuronide moiety), which suggests that the glucuronide moiety was attached at position 3' of the EC skeleton; said to be the most labile position (*24*).

Compounds 12 and 13 showed MS^2 fragment ions at *m/z* 313 and m/z 289 (intense). Fragments at *m/z* 313 correspond to an A-ring fragment from RDA fission plus one glucuronide moiety (137+176). The different fragmentation pattern suggests that the glucuronidation position is different for 10 and 11 on the one hand, and 12 and 13 on the other.

Gluc-Me-EC-1 (10) was clearly present in samples collected from the GADF group at all the different times (**Figure 3**). In samples from rats fed EC, this metabolite first appeared later (after 6 h). In contrast, peak 12, corresponding to Gluc-Me-EC-3, was clearly more intense in samples from the EC group.

Glucuronidated and sulfated EC (peak 14) was detected by monitoring the transition 545→369. The product ion scan of this metabolite generated fragments at *m/z* 465 (M–Gluc), 369 (M–Sulf) and 289 (EC), as well as an ion giving a strong signal at *m/z* 271, which could correspond to a posterior loss of water (289–18). This metabolite was clearly present in urine samples from the EC group and only traces were detected in samples from the GADF group.

Disulfated EC (compound 15) was detected in samples from the GADF group. The two sulfation sites are probably located on two different rings of the EC moiety. The ions at *m/z* 231 and *m/z* 217 correspond to a sulfated B-ring (151+80) and A-ring (137+80), respectively.

EC diglucuronide 16 produced MS^2 fragments at *m/z* 465 and *m/z* 289 from the molecular ion $[M–H]^-$ at *m/z* 641. In this case we did not have enough experimental evidence to suggest a glucuronidation position. Again, only traces of this metabolite were found in samples from animals fed GADF, while it was a main component of the samples from the EC group.

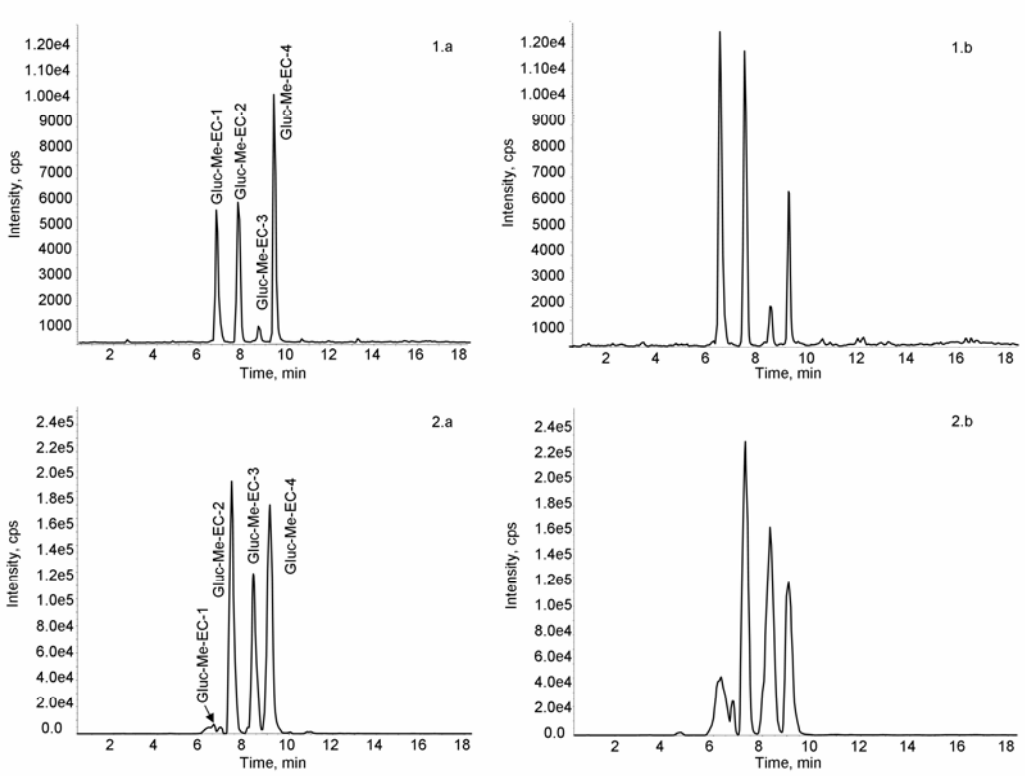


Figure 3: HPLC-ESI-MS/MS profile corresponding to the detection EC glucuronides in urine samples after feeding: 1) GADF and 2) EC. Samples collected at 2 h (a) and 6 h (b). Detection in the multiple reaction monitoring (MRM) mode, transition 465→289.

Tri-conjugated metabolites. EC conjugated with two methyl and one glucuronide moieties (17, 18 and 19) were detected by MRM transition 493→289. Compounds 17 and 18 yielded a main MS^2 fragment at *m/z* 289 (EC) and a second fragment at *m/z* 313, which indicates that the Gluc moiety was located on the A-ring (137+176). In contrast, compound 19 yielded a main MS^2 ion at *m/z* 303 and minor ions at *m/z* 259 and 244. The absence of a fragment at *m/z* 313 indicates that the Gluc moiety was not attached to the A-ring. Methylated di-sulfate conjugates of EC (20, 21) were detected as very weak signals in urine samples from rats fed GADF.

The identity of these metabolites was established by comparison with the samples from rats fed EC. These gave strong signals at all collection times. The product ion spectrum of the molecular ion $[M–H]^-$ at *m/z* 463 showed MS^2 fragments at *m/z* 383 $[M–H–Sulf]^-$ and *m/z* 303 $[M–H–2Sulf]^-$ in both cases. Other MS^2 fragments provided further structural information. Me-di-Sulf-EC-1 (20) gave characteristic peaks at *m/z* 245 (B-ring fragment + sulfate + methyl), *m/z* 217 (A-ring fragment + sulfate), and *m/z* 165 (B-ring fragment + methyl). This suggests that the two sulfate moieties were attached to different rings, and the methyl moiety to the B-ring. The presence of the fragments at *m/z* 245 (B-ring + sulfate + methyl), *m/z* 231 (B-ring + sulfate) and *m/z* 217 (A-ring + sulfate) from the molecular ion of Me-di-Sulf-EC-2 (21) suggested that this isomer also incorporated one sulfate on the A-ring, together with the methyl group and the other sulfate on the B-ring. Although compounds 20 and 21 have the same moieties on each ring, the substitution positions must be different because they elute at different retention times in HPLC-MS/MS.

Tri-substituted ECs with glucuronide, methyl and sulfate (22 and 23) were also detected in minute amounts in urine samples from rats fed GADF by the MRM transition 559→289. The identity of the metabolites was established thanks to the samples from rats fed EC. Product ion scans of metabolite 22 showed fragments at *m/z* 559 (molecular ion), 479 (559–Sulf), 461(479–H_2O), 369 (EC+Sulf), 303 (loss of a glucuronide moiety from 479, Me-EC), 289 (EC), 217 (which is the characteristic ion of RDA fragmentation with an attached sulfate moiety; 137+Sulf) and 137 (which typically results from the A-ring after RDA fission). Again, the sulfate moiety was attached to the A-ring as shown by the MS^2 fragments at *m/z* 217 and 137. The conjugation positions of the glucuronide and methyl groups could not be established for this metabolite. The glucuronidation position could be established for metabolite 23 from the information generated by the product ion scan. The MS^2 fragments were *m/z* 313 (glucuronide moiety attached to an A-ring fragmentation product; 137+176), 289 (EC), 245 (B-ring fragment + sulfate + methyl), and 231 (corresponding to a sulfate moiety attached to a B-ring fragment; 151+80).

So we concluded that the glucuronide was attached to the A-ring, and the methyl and sulfate moieties were attached to the B-ring. Finally, we report one more tri-substituted metabolite; tri-Sulf-EC (24). This EC conjugated with three sulfate moieties generated two fragments at *m/z* 449 and *m/z* 369, which correspond to the successive loss of sulfate moieties. A fragment at 289 (EC) was also detected by PI scan**.**

Table 2: Identification and occurrence of phenolic acids in urine from rats fed with GADF or EC.

Metabolite	MRM Parent	MRM Daugther	MS^2 fragments (% relative abundance)
3-hydroxyphenylvaleric acid (3-HPhVA)	193→175	—	—
3,4-di-hydroxyphenylpropionic acid (DHPhPA)	181→137	—	181(5);137(35);135(47);121(73);109(3)
4-hydroxyphenylpropionic acid (4-HPhPA)	165→121	—	165(30);121(56);93(100);77(100)
caffeic acid	179→135	—	135(100);107(9);89(8)
p-coumaric acid	163→119	—	163(1);119(100);93(4);91(2)
m-coumaric acid	163→119	—	163(3);119(100);93(5);91(10)
3,4-di-hydroxyphenylacetic acid (DHPhAcA)	167→123	—	123(100);105(7);95(17)
3-hydroxyphenylacetic acid (3-HPAcA)	151→107	—	107(100);91(23)
4-hydroxybenzoic acid (4-HBA)	137→ 93	—	137(57);119(8);108(100);93(64)
Sulf-di-hydroxyphenylpropionic acid (Sulf-DHPhPA)	261→181	181→137	261(10);199(37);181(85);137(100)
Gluc-di-hydroxyphenylpropionic acid (Gluc-DHPhPA)	357→181	181→137	181(58);166(100);121(9)
Sulf-hydroxyphenylpropionic acid (Sulf-HPhPA)	245→165	165→121	245(5);165(35);121(100);119(40);106(18)
Sulf-coumaric acid	243→163	163→119	243(5);163(15);119(100)

Phenolic acids. Both non-conjugated and conjugated phenolic acids were detected and identified in urine samples from rats fed GADF. Only a limited number of non-conjugated acids were detected in samples from the EC group. The results are summarized in **Table 2**. The species listed were confirmed by the use of standards and gave MS signals that were clearly stronger than those from the control animals. Some of phenolic acids described in the literature as metabolites of EC (e.g., 3-HBA) are not included in the list because the signals they yielded were no stronger than those from the control samples obtained from non-supplemented animals. A Table with the times at which all these metabolites were detected is provided as supplementary material.

Phenolic acids derived from GADF diet. Hydroxyphenylvaleric acid (HPhVA) detected at 24 h was one of the main metabolites in rats fed GADF. *p*-coumaric and *m*-coumaric were detected and identified in GADF samples collected all times. *p*-coumaric signals were more intense than *m*-coumaric signals in all the samples, and were clearly stronger at 8 h and 10 h. 3,4-di-hydroxyphenylacetic acid (DHPhAcA) was detected at 24 h by MRM transition 167→123. Hydroxyphenylacetic acid (HPhAcA) and 4-hydroxybenzoic acid (4-HBA) were also detected from 6 to 24h.

Conjugated phenolic acids were detected in urine samples from the GADF group by MRM and product ion scan on parent/daughter pairs of ions. 3,4-DHPhPA conjugated with either a sulfate (**Figure 4a**) or a glucuronide moiety gave transitions: 261→181 plus 181→137, and 357→181 plus 181→137, respectively, and were confirmed by the product ion scan. We also detected sulfated coumaric acid (243→163 plus 163→119) as early as 2 h after ingestion of GADF and sulfated HPhPA (245→165) some time later (from 8 to 24 h) (**Figure 4b**).

Phenolic acids derived from EC diet. The colonic metabolization of pure EC gave different phenolic acids with the three-carbon atom scaffold. Caffeic acid (179→135) gave signals of increasing intensity from 8 to 24 h. 3,4-DHPhPA, also called dihydrocaffeic acid (181→137) was only detected after 24 h. 4-HPhPA was detected from 8 to 24 h. *p*-coumaric and *m*-coumaric acids gave signals of increasing intensity from 8 to 24 h.

We also detected shorter phenolic acids after EC ingestion. 3-HPhAcA and 4-HBA appeared at 10 and 24 h. We also observed MRM signals corresponding to sulfated caffeic acid, which were confirmed by a second transition of the free acids at the same retention time. We do not include this metabolite in **Table 2** because the less sensitive product ion scan did not yield any fragment.

DISCUSSION

Food polyphenols such as monomeric (epi)catechins and phenolic acids are rapidly glucuronidated and methylated in the small intestine (*25*), absorbed into the bloodstream and further conjugated (glucuronidated, sulfated) in the liver of both rats and humans (*26-29*). Those species that are not absorbed in the small intestine (e.g., polymeric (epi)catechins) reach the colon where they are fermented by the colonic microbiota and absorbed in the form of smaller phenolic acids (*30*). These smaller species may in turn be conjugated in the liver. The metabolites found in urine are the result of all these processes and may have exerted their biological effect during the period while they crossed membranes and circulated in the bloodstream.

GADF contains a complex mixture the polyphenols including monomers, oligomers and polymers of EC, anthocyanins, flavonols and hydroxycinnamic acids associated with a fiber matrix of both soluble and insoluble polymers such as polycaccharides and lignins (*8, 9*) which may influence the absorption of the putatively bioactive GADF components. We report here that the phenolic metabolites in rat urine after the ingestion of GADF are mainly conjugates of (–)-epicatechin and phenolic acids. An important question is where the different polyphenolic components of GADF are absorbed and metabolized, as the putative protective effects of the polyphenols will be exerted where they are bioavailable..

During the metabolization/absorption/metabolization process, different phenolic mixtures are in contact with the different of the GADF's oligomeric procyanidins in the small intestine. The extent to which procyanidins are processed in the small intestine is unclear in the literature. Some studies suggest that oligomers of EC are poorly absorbed (*30, 31*) and that all the (epi)catechin found in rat tissues after the ingestion of grape seed extract must come from the monomeric fraction (*13*). However, others show that procyanidin dimers and trimers may be absorbed and excreted via urine intact (*13*) or as conjugates (*32, 33*). Still others report that EC oligomers may be hydrolyzed to EC units and metabolized (*34*). Our results suggest that most of the array of EC derivatives detected in urine after GADF ingestion come from (epi)catechin-based soluble polyphenols (i.e., procyanidins) which are depolymerized, derivatized and absorbed in the small intestine, and further processed in the liver. The amount and variety of EC derivatives (**Table 1**) cannot come exclusively from the monomeric EC present in GADF (less than 0.01%). As there seems to be agreement that depolymerization into EC monomeric units does not place in the colon (*14, 30, 35*) depolymerization must take place in the small intestine. We also detected EC dimers in urine samples collected 2 h after GADF ingestion. These dimers may come directly from GADF or they may be the result of partial depolymerization of procyanidins. The hypothesis that EC metabolites do not organs. We believe that the EC metabolites in rat urine must come from the partial depolimerization come from EC monomers in GADF is corroborated by comparing the profile of EC conjugates excreted by animals fed GADF to that of animals fed only EC. All the conjugates detected in the EC group samples also appear in the GADF group samples, but the intensity of the MS signals differs significantly for many of the metabolites. This is exemplified in **Figures 2** and **3** for the case of mono-glucuronidated ECs and glucuronidated and methylated ECs respectively. Moreover, the intensity profiles reveal differences between the GADF and EC groups in the time at which the EC metabolites first appear. Here, we would like to point out that, even after extensive metabolization at the different levels, intact dimers and conjugated species with intact catechol moieties on the B-ring were detected in urine. **Figure 2** also shows this. Note that intact catechol containing compounds such as Glu-EC-3 persist not only after an overdose of monomer (**Figure 2b**), but also after intake of a much lower amount of monomeric and oligomeric catechins (GADF, **Figure 2a**). As the catechol group is a necessary structural requirement for the free radical scavenging activity of polyphenols this observation suggests that some scavenging activity is bioavailable for several hours to different tissues after GADF ingestion.

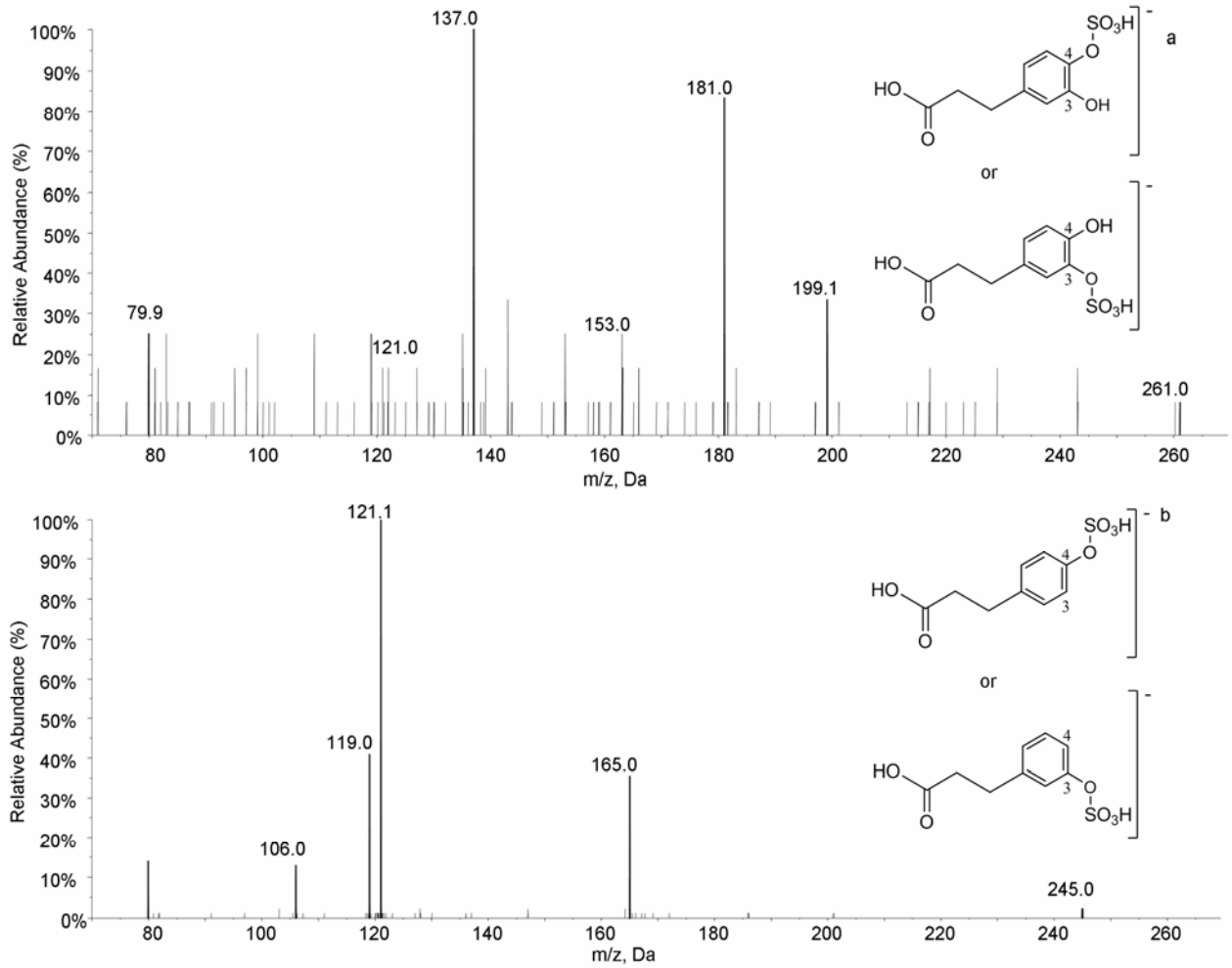


Figure 4: HPLC-ESI-MS/MS product ion scan spectra of sulfated di-hydroxyphenylpropionic acid (m/z 261), (a) and sulfated hydroxyphenylpropionic acid (m/z 245) (b)

The EC metabolites probably came from oligomers with low degree of polymerization (dimers, trimers). Bulkier oligomers and polymers are associated with proteins (*36*). The protein-associated material, as well as both soluble and insoluble procyanidins more or less associated with the fiber matrix (polysaccharides and lignin) and maybe the remaining monomers, eventually reach the colon, where the colonic microbiota break them into smaller compounds (phenolic acids) as described for both rats (*30*) and humans (*11, 37*). It is believed that procyanidins are directly degraded to phenolic acids, without previous depolymerization into (epi)catechin units (*14*). In agreement with the observations from other authors working with purified oligomers (*14, 30, 35*), we found that the urine samples from animals fed GADF contained more phenolic acids than the samples from animals given only monomeric EC. The GADF colonic metabolites (**Table 2**) most probably come from the colonic fermentation of polymeric EC. The main MS signals corresponded to 3-HPhVA, 4-HPhPA and 3-HPhAcA. All these metabolites may be generated by ring cleavage of the EC moieties within the procyanidin polymers (*14, 35*). *m*- and *p*-coumaric acids were detected in samples from both the GADF and EC groups. A small proportion of the coumaric acids may come directly from the fiber supplement, as they are minor components of GADF (*9*). Since coumaric acids have been shown to be absorbed rapidly in the small intestine (*38*), they may account for some of the phenolics detected soon after ingestion (2 h). The rest must be generated by degradation of the EC skeleton. We detected sulfated and glucuronidated conjugates of hydroxyphenyl acids only in the GADF group samples. These metabolites (e.g. Sulf-DHPhPA and Sulf-HPhPA, **Figure 4**) had not been detected from procyanidins before and may be produced in the liver from the colonic degradation products.

Again, as for the EC dimers and EC metabolites with intact catechol moieties, some of the phenolic acids existed in their non-conjugated form and may provide scavenging potential for several hours after GADF ingestion. However, it is evident that GADF polyphenols are extensively conjugated and the levels of free catechols may be too low to have any significant effect. In fact, many authors believe that the scavenging capacity of plant polyphenols is negligible “in vivo”. While we have to agree in part, we certainly do not think that this means that plant polyphenols, in particular procyanidins, exert no significant antioxidant effect. Apart from the observation that the circulating free catechol may not be negligible, the extent of metabolization shows how polyphenols are treated by the living organism as harmful. In fact, it is known that polyphenols may be pro-oxidant depending on their nature and concentration. Thus, they may stimulate the endogenous antioxidant systems by acting as mild pro-oxidants. Similarly, calorie restriction and moderate exercise are weak prooxidant insults that may result in antioxidant responses “in vivo” (*39, 40*). The antioxidant/pro-oxidant balance provided by polyphenols and their metabolites may result in an overall antioxidant effect of GADF, and of fruit and vegetables in general.

For several hours after GADF ingestion, a great variety of phenolic species are in contact with the gut tissues before, during and after metabolization, and many of them (mainly mono and polyconjugated epicatechin and free or conjugated smaller phenolic acids) are systemically bioavailable before being excreted. Some of these metabolites include the free catechol moiety. The metabolic outcome of GADF essentially corresponds to the degradation products of epicatechin oligomers and polymers

ACKNOWLEDGEMENT

GADF was a generous gift from Prof. Fulgencio Saura-Calixto, ICTAN-CSIC. The expert assistance of Dr. Olga Jáuregui from the *Servies Científico-Tècnics de la Universitat de Barcelona* is gratefully appreciated. Language revision by Christopher Evans is also appreciated.

Supporting Information Available: Tables summarizing the occurrence of the metabolites at the different times. This material is available free of charge via the Internet at http://pubs.acs.org

LITERATURE CITED

(1) Slavin, J. L., Dietary fiber and body weight. *Nutrition* **2005,** 21, (3), 411-418.
(2) Bingham, S. A.; Day, N. E.; Luben, R.; Ferrari, P.; Slimani, N.; Norat, T.; Clavel-Chapelon, F.; Kesse, E.; Nieters, A.; Boeing, H.; Tjnneland, A.; Overvad, K.; Martinez, C.; Dorronsoro, M.; Gonzalez, C. A.; Key, T. J.; Trichopoulou, A.; Naska, A.; Vineis, P.; Tumino, R.; Krogh, V.; Bueno-de-Mesquita, H. B.; Peeters, P. H. M.; Berglund, G.; Hallmans, G.; Lund, E.; Skeie, G.; Kaaks, R.; Riboli, E., Dietary fibre in food and protection against colorectal cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC): an observational study. *Lancet* **2003,** 361, (9368), 1496-1501.
(3) Dana, E. K., Dietary fiber, inflammation, and cardiovascular disease. *Mol. Nutr. Food Res.* **2005,** 49, (6), 594-600.
(4) Klenovv, S.; Glei, M.; Haber, B.; Owen, R.; Pool-Zobel, B. L., Carob fibre compounds modulate parameters of cell growth differently in human HT29 colon adenocarcinoma cells than in LT97 colon adenoma cells. *Food Chem. Toxicol.* **2008,** 46, (4), 1389-1397.
(5) Fardet, A.; Rock, E.; Rémésy, C., Is the in vitro antioxidant potential of whole-grain cereals and cereal products well reflected in vivo? *J. Cereal Sci.* **2008,** 48, (2), 258-276.
(6) Saura-Calixto, F.; Larrauri García, J. A. Concentrate of natural antioxidant dietetic fiber from grape, and preparation process, In CSIC: ES2130092 A1, 1999.
(7) Goñi, I.; Martín, N.; Saura-Calixto, F., In vitro digestibility and intestinal fermentation of grape seed and peel. *Food Chem.* **2005,** 90, (1-2), 281-286.
(8) Pérez-Jiménez, J.; Serrano, J.; Tabernero, M.; Arranz, S.; Díaz-Rubio, M. E.; García-Diz, L.; Goñi, I.; Saura-Calixto, F., Effects of grape antioxidant dietary fiber in cardiovascular disease risk factors. *Nutrition* **2008,** 24, (7-8), 646-653.
(9) Touriño, S.; Fuguet, E.; Jáuregui, O.; Saura-Calixto, F.; Cascante, M.; Torres, J. L., High-resolution liquid chromatography/electrospray ionization time-of-flight mass spectrometry combined with liquid chromatography/electrospray ionization tandem mass spectrometry to identify polyphenols from grape antioxidant dietary fiber. *Rapid Commun. Mass Spectrom.* **2008,** 22, (22), 3489-3500.
(10) Taubert, D.; Roesen, R.; Schomig, E., Effect of cocoa and tea intake on blood pressure: a meta-analysis. *Arch. Intern. Med.* **2007,** 167, (7), 626-634.
(11) Ward, N. C.; Croft, K. D.; Puddey, I. B.; Hodgson, J. M., Supplementation with grape seed polyphenols

results in increased urinary excretion of 3-hydroxyphenylpropionic acid, an important metabolite of proanthocyanidins in humans. *J. Agric. Food Chem.* **2004,** 52, (17), 5545-5549.
(12) Baba, S.; Osakabe, N.; Natsume, M.; Muto, Y.; Takizawa, T.; Terao, J., *In vivo* comparison of the bioavailability of (+)-Catechin, (–)-Epicatechin and their mixture in orally administered rats. *J. Nutr.* **2001,** 131, (11), 2885-2891.
(13) Tsang, C.; Auger, C.; Mullen, W.; Bornet, A.; Rouanet, J. M.; Crozier, A.; Teissedre, P. L., The absorption, metabolism and excretion of flavan-3-ols and procyanidins following the ingestion of a grape seed extract by rats. *Br. J. Nutr.* **2005,** 94, (2), 170-181.
(14) Appeldoorn, M. M.; Vincken, J.-P.; Aura, A.-M.; Hollman, P. C. H.; Gruppen, H., Procyanidin dimers are metabolized by human microbiota with 2-(3,4-Dihydroxyphenyl)acetic acid and 5-(3,4-Dihydroxyphenyl)-g-valerolactone as the major metabolites. *J. Agric. Food Chem.* **2009,** 57, (3), 1084-1092.
(15) Spencer, J. P. E., Metabolism of tea flavonoids in the gastrointestinal tract. *J. Nutr.* **2003,** 133, (10), 3255S-3261S.
(16) Walle, T., Absorption and metabolism of flavonoids. *Free Radical Biol. Med.* **2004,** 36, (7), 829-837.
(17) Rechner, A. R.; Kuhnle, G.; Bremner, P.; Hubbard, G. P.; Moore, K. P.; RiceEvans, C. A., The metabolic fate of dietary polyphenols in humans. *Free Radical Biol. Med.* **2002,** 33, (2), 220-235.
(18) Liu, D. Q.; Hop, C. E. C. A., Strategies for characterization of drug metabolites using liquid chromatography-tandem mass spectrometry in conjunction with chemical derivatization and on-line H/D exchange approaches. *J. Pharm. Biomed. Anal.* **2005,** 37, (1), 1-18.
(19) Groenewoud, G.; Hundt, H. K. L., The microbial metabolism of condensed (+)-catechins by the rat-cecal microflora. *Xenobiotica* **1986,** 16, (2), 99-107.
(20) Sang, S.; Lee, M. J.; Yang, I.; Buckley, B.; Yang, C. S., Human urinary metabolite profile of tea polyphenols analyzed by liquid chromatography/electrospray ionization tandem mass spectrometry with data-dependent acquisition. *Rapid Commun. Mass Spectrom.* **2008,** 22, (10), 1567-1578.
(21) Levsen, K.; Schiebel, H.-M.; Behnke, B.; Dötzer, R.; Dreher, W.; Elend, M.; Thiele, H., Structure elucidation of phase II metabolites by tandem mass spectrometry: an overview. *J. Chromatogr. A* **2005,** 1067, (1-2), 55-72.
(22) Natsume, M.; Osakabe, N.; Oyama, M.; Sasaki, M.; Baba, S.; Nakamura, Y.; Osawa, T.; Terao, J., Structures of (–)-epicatechin glucuronide identified from plasma and urine after oral ingestion of (–)-epicatechin: differences between human and rat. *Free Radical Biol. Med.* **2003,** 34, (7), 840-849.
(23) Gu, L.; Kelm, M. A.; Hammerstone, J. F.; Beecher, G.; Holden, J.; Haytowitz, D.; Prior, R. L., Screening of foods containing proanthocyanidins and their structural characterization using LC-MS/MS and thiolytic degradation. *J. Agric. Food Chem.* **2003,** 51, (25), 7513-7521.
(24) Cren-Olivé, C.; Déprez, S.; Lebrun, S.; Coddeville, B.; Rolando, C., Characterization of methylation site of monomethylflavan-3-ols by liquid chromatography/electrospray ionization tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2000,** 14, (23), 2312-2319.
(25) Kuhnle, G.; Spencer, J. P. E.; Schroeter, H.; Shenoy, B.; Debnam, E. S.; Srai, S. K. S.; Rice-Evans, C.; Hahn, U., Epicatechin and catechin are O-methylated and glucuronidated in the small intestine. *Biochem. Biophys. Res. Commun.* **2000,** 277, (2), 507-512.
(26) Okushio, K.; Suzuki, M.; Matsumoto, N.; Nanjo, F.; Hara, Y., Identification of (–)-Epicatechin metabolites and their metabolic fate in the rat. *Drug Metab. Dispos.* **1999,** 27, (2), 309-316.
(27) Vaidyanathan, J. B.; Walle, T., Glucuronidation and sulfation of the tea flavonoid (–)-Epicatechin by the human and rat enzymes. *Drug Metab. Dispos.* **2002,** 30, (8), 897-903.
(28) Poquet, L.; Clifford, M. N.; Williamson, G., Investigation of the metabolic fate of dihydrocaffeic acid. *Biochem. Pharmacol.* **2008,** 75, (5), 1218-1229.
(29) Matsui, Y.; Nakamura, S.; Kondou, N.; Takasu, Y.; Ochiai, R.; Masukawa, Y., Liquid chromatography-electrospray ionization-tandem mass spectrometry for simultaneous analysis of chlorogenic acids and their metabolites in human plasma. *J. Chromatogr. B* **2007,** 858, (1-2), 96-105.
(30) Gonthier, M. P.; Donovan, J. L.; Texier, O.; Felgines, C.; Rémésy, C.; Scalbert, A., Metabolism of dietary procyanidins in rats. *Free Radical Biol. Med.* **2003,** 35, (8), 837-844.
(31) Scalbert, A.; Williamson, G., Dietary intake and bioavailability of polyphenols. *J. Nutr.* **2000,** 130, (8), 2073S-2085S.
(32) Prasain, J. K.; Barnes, S., Metabolism and bioavailability of flavonoids in chemoprevention: current analytical strategies and future prospectus. *Mol. Pharm.* **2007,** 4, (6), 846-864.
(33) Shoji, T.; Masumoto, S.; Moriichi, N.; Akiyama, H.; Kanda, T.; Ohtake, Y.; Goda, Y., Apple procyanidin oligomers absorption in rats after oral administration: analysis of procyanidins in plasma using the porter method and high-performance liquid chromatography/tandem mass spectrometry. *J. Agric. Food Chem.* **2006,** 54, (3), 884-892.
(34) Spencer, J. P. E.; Chaudry, F.; Pannala, A. S.; Srai, S. K.; Debnam, E.; Rice-Evans, C., Decomposition of cocoa procyanidins in the gastric milieu. *Biochem. Biophys. Res. Commun.* **2000,** 272, (1), 236-241.
(35) Déprez, S.; Brezillon, C.; Rabot, S.; Philippe, C.; Mila, I.; Lapierre, C.; Scalbert, A., Polymeric proanthocyanidins are catabolized by human colonic microflora into low-molecular-weight phenolic acids. *J. Nutr.* **2000,** 130, (11), 2733-2738.
(36) Sarni-Manchado, P.; Cheynier, V.; Moutounet, M., Interactions of grape seed tannins with salivary proteins. *J. Agric. Food Chem.* **1999,** 47, (1), 42-47.
(37) Rios, L. Y.; Gonthier, M. P.; Rémésy, C.; Mila, I.; Lapierre, C.; Lazarus, S. A.; Williamson, G.; Scalbert, A., Chocolate intake increases urinary excretion of polyphenol-derived phenolic acids in healthy human subjects. *Am. J. Clin. Nutr.* **2003,** 77, (4), 912-918.
(38) Konishi, Y.; Hitomi, Y.; Yoshioka, E., Intestinal absorption of *p*-coumaric and gallic acids in rats after oral administration. *J. Agric. Food Chem.* **2004,** 52, (9), 2527-2532.
(39) Schulz, T. J.; Zarse, K.; Voigt, A.; Urban, N.; Birringer, M.; Ristow, M., Glucose restriction extends Caenorhabditis elegans life span by inducing mitochondrial respiration and increasing oxidative stress. *Cell Metab.* **2007,** 6, (4), 280-293.
(40) Ascensao, A.; Magalhaes, J. F.; Soares, J. M.; Ferreira, R. M.; Neuparth, M. J.; Appell, H. J.; Duarte, J. A., Cardiac mitochondrial respiratory function and oxidative stress: The role of exercise. *Int. J. Sports Med.* **2005,** 26, (4), 258-267.

This work was supported by the Spanish Ministry of Education and Science (research grants AGL2004-

07579-C04-02, -03; AGL2006-12210-C03-02/ALI; SAF2008-00164); doctoral fellowship to S.T. and post-doctoral contract to E.F.); the European Commission (Etherpaths project KBBE-grant agreement nº222639); ISCIII-RTICC (RD06/0020/0046); and Generalitat de Catalunya (2005SGR00204).

5. DISCUSIÓN GENERAL

5.1. Relación estructura / efecto de proantocianidinas

La discusión que se expone a continuación integra las publicaciones troncales generas de los estudios con las fracciones polifenólicas de corteza de pino y hamamelis (**publicación 1**; apartado 4.1.1 y **publicación 2**; apartado 4.1.2) y otras realizadas en colaboración (**Anexos; Apartado 8.1**) que han permitido llegar a una idea global de la influencia de la estructura de las proantocianidinas, (grado de polimerización y porcentaje de galoización) sobre sus efectos químicos y biológicos.

La actividad antioxidante de las (epi)catequinas y proantocianidinas ha sido atribuida en parte a la presencia del grupo catecol o pirogalol en el anillo B, capaz de reducir con elevada eficacia radicales libres del medio *(Rice-Evans et al., 1996)* mediante donación de hidrógenos y/o transferencia electrónica. Por otro lado la capacidad de transferencia electrónica puede resultar en una actividad pro-oxidante, especialmente en compuestos más reductores como (epi)galocatequinas *(Kondo et al., 1999)*, cuando son capaces de transferir un electrón al oxígeno y formar radical anión superóxido que a su vez puede generar el radical hidroxilo. Los mecanismos por los cuales actúan las proantocianidinas para ejercer posibles efectos beneficiosos sobre la salud todavía no son claros. Cada vez son más los estudios que descartan la idea clásica de que los polifenoles actúan exclusivamente como agentes captadores de radicales libres. Primero los polifenoles pueden ser tanto antioxidantes como pro-oxidantes. Segundo, los compuestos fenólicos son ampliamente metabolizados *in vivo* alterando sus potenciales redox.

La presente tesis pretende ser una aportación a la discusión sobre los efectos de los polifenoles, principalmente de las proantocianidinas. El grado de polimerización y el porcentaje de galoización son factores que influyen en los efectos de las proantocianidinas, es por ello que en el primer bloque de la tesis y con el fin de evaluar la influencia de estos dos factores sobre sus efectos químicos y biológicos se generaron fracciones de proantocianidinas con diferente composición en cuanto a grado de polimerización y porcentaje de galoización. Fracciones de corteza de pino (*Pinus pinaster*) constituidas por procianidinas, (epi)catequinas exentas en su estructura de ésteres de galato *(Touriño et al., 2005)*, fracciones de bagazo de uva (*Vitis vinifera*) en las que se incluían mayoritariamente procianidinas con un porcentaje en ésteres de galato intermedio *(Torres et al., 2002)* y fracciones de hamamelis (*Hamamelis virginiana*) con un elevado porcentaje de galoización gracias a que su composición incluye una mezcla rica de (epi)galocatequinas (prodelfinidinas), (epi)catequinas (procianidinas) y galotaninos (unidades de ácido gálico unidos a una glucosa)*(Touriño et al., 2008a) (Lizárraga et al., 2008)*.

5.1.1 Evaluación de la influencia del grado de polimerización

La influencia del grado de polimerización sobre la actividad antioxidante parece evidente, según los resultados de nuestros estudios *(Touriño et al., 2005)* y en los de muchos otros

autores *(Burda et al., 2001;Cao et al., 1997;Heim et al., 2002)*. Un aumento en el número de grupos catecol del anillo B es de esperar que resulte en un incremento de la capacidad antioxidante de las fracciones. Sin embargo, los resultados de las fracciones de bagazo de uva *(Torres et al., 2002)* en los distintos ensayos antioxidantes mostraron que fracciones de similar grado de galoización con un grado de polimerización en torno a 2 (Fracción X) y 4 (Fracción XI) mostraron similar eficacia antioxidante. Es decir, existe un punto a partir del cual la capacidad antioxidante de las proantocianidinas deja de aumentar aún cuando el grado de polimerización vaya en incremento. Este resultado coincidió con el obtenido por Gaulejac y colaboradores *(De Gaulejac et al., 1999)* en un estudio realizado igualmente con proantocianidinas de uva. Los autores sugirieron que la proporción directa entre grado de polimerización y actividad antioxidante deja de existir a partir de un grado de polimerización entre 2 y 3 posiblemente porque se produce un plegamiento de los oligómeros que esconde los grupos hidroxilos activos.

En el caso de las fracciones de corteza de pino, exentas de galato, se observó el mismo efecto para un grado de polimerización en torno a 4 (**publicación 1**; apartado 4.1.1) *(Touriño et al., 2005)*. En contraste con los resultados con procianidinas de uva, se comprobó, gracias a un estudio realizado en colaboración con el grupo de la Escuela Técnica Superior de Ingeniería de la Universidad de Santiago (**Anexo 8.1 III**) *(Jerez et al., 2007)*, que la capacidad antioxidante fracciones obtenidas de corteza de pino (*Pinus pinaster* y *Pinus radiata*) seguía luego aumentando hasta un grado de polimerización en torno a 7. La causa más plausible en la diferencia de los resultados, entre las fracciones de bagazo de uva y pino, es la presencia/ausencia de ésteres de galato en la estructura de las proantocianidinas que podrían contribuir a un plegamiento más compacto de la molécula.

Los métodos más utilizados (principalmente DPPH) para medir la capacidad reductora de radicales libres miden la transferencia de átomos de hidrógeno aunque se cree que el mecanismo redox principal es la transferencia electrónica. Además, radicales libres estables como el DPPH son parcialmente reactivos frente a solventes como el metanol. El radical HNTTM sintetizado en nuestro laboratorio *(Torres et al., 2003)* es exclusivamente sensible a la transferencia de electrones debido a la imposibilidad de captar hidrógenos por impedimento estérico del radical por lo que permite asegurar que un polifenol actúe por transferencia electrónica minimizando reacciones con el solvente.

Al poner en contacto las fracciones de bagazo de uva y hamamelis con el radical HNTTM se observó que las fracciones que mostraban mayor actividad antioxidante medida por DPPH o TEAC, mostraron una mayor transferencia de electrones. Los resultados de las fracciones de pino (exentas de galato) mostraron un incremento de la capacidad de donación de hidrógenos ligada al incremento en el grado de polimerización y mayor que el incremento en la capacidad de transferencia electrónica. Este resultado podría ser particularmente importante si se tiene en cuenta que una elevada capacidad de transferencia electrónica, como se ha comentado anteriormente, podría estar relacionada

con efectos pro-oxidantes. Las fracciones oligoméricas de pino mostraron una buena eficacia antioxidante (método DPPH) manteniendo una baja transferencia de electrones (método HNTTM) *(Touriño et al., 2005)*.

Uno de los parámetros más importantes para la conservación de gran parte de los alimentos procesados que se encuentran en forma de emulsión, desde zumos (<1%) hasta mayonesas (>80%), es la inhibición del enranciamiento lipídico. Además, los sistemas biológicos se asemejan más a emulsiones que a soluciones concentradas. Por ello, se evaluó la capacidad de inhibición de la peroxidación lipídica en emulsiones aceite/agua *(Torres et al., 2002;Touriño et al., 2005)*. Los resultados mostraron que los oligómeros son más eficientes que los monómeros en emulsiones. Dicho resultado concordó con la observación de que los compuestos hidrófobos tienden a ser acumulados en la interfase aceite/agua, mientras que los compuestos hidrófilos son menos activos debido a que se disuelven en la fase acuosa desde la cual es difícil de acceder a los radicales lipídicos *(Frankel, 2005)*. El carácter hidrófobo de las proantocianidinas es fácilmente deducible por su comportamiento en RP-HPLC, es decir, cuanto mayor es el tiempo de retención más hidrofobicidad presentan. La naturaleza hidrofóbica de las proantocianidinas en combinación con los grupos hidroxilos hidrófilos permite la exposición de las proantocianidinas en ambas fases de la emulsión. Entre las fracciones oligoméricas de diferentes grados de polimerización no se observaron diferencias significativas en los resultados, aunque sí se observó una tendencia a un incremento de la inhibición de la oxidación lipídica con respecto a un aumento en el grado de polimerización *(Pazos et al., 2005;Torres et al., 2002;Touriño et al., 2005)*. La similar eficacia observada entre las fracciones podría ser debida a la posibilidad que tienen las proantocianidinas de establecer interacciones hidrófobas y/o hidrofílicas en función del medio, lo cual corroboraría la hipótesis de que la distribución en las diferentes fases puede llegar a ser más importante que la actividad antioxidante en sí *(Plumb et al., 1998)*. En los estudios de inhibición de oxidación lipídica de membranas celulares realizados con estás fracciones se obtuvieron resultados muy similares a los obtenidos en emulsiones, las fracciones monoméricas fueron muy poco activas mientras que entre las fracciones oligoméricas se observo un ligero incremento de la actividad captadora del radical peroxilo con relación a un aumento en el grado de polimerización (**Anexo 8.1 V**) *(Ugartondo et al., 2007)*.

En los ensayos de evaluación de inhibición de la proliferación realizados con cultivos de células tumorales de melanoma humano (SK-MEL-28) se observó que las fracciones monoméricas tanto de pino como de uva mostraron menor actividad antiproliferativa que las fracciones oligoméricas. Entre las fracciones oligoméricas de ambos extractos (bagazo de uva y corteza de pino) no se observó un incremento significativo en función del grado de polimerización. En cambio, el grado de polimerización mostró ser más importante en los ensayos realizados sobre inhibición del ciclo celular, donde las fracciones tanto de corteza de pino como de bagazo de uva aumentaron a medida que aumentaba su grado de polimerización (**Anexo 8.1 IV**)*(Lizárraga et al., 2007)*.

5.1.2 Evaluación de la influencia del porcentaje de galoización (grupos galato) y de galo(epi)catequinas (pirogalol en el anillo B)

El efecto de los grupos galato en la estructura de las proantocianidinas se evidenció en el estudio con fracciones de hamamelis (**publicación 2;** apartado 4.1.2) *(Touriño et al., 2008a)*. El incremento en el porcentaje de galoización que supone un mayor número de grupos hidroxilo resultó en un aumento de la capacidad de donación de átomos de hidrógeno. Las fracciones de hamamelis presentaron un elevado porcentaje de galoización procedente de procianidinas (epicatequíngalato), prodelfinidinas (epigalocatequíngalato) y de los taninos hidrolizables (ésteres de ácido gálico). Las fracciones de hamamelis mostraron una capacidad antioxidante 1,5 y 3 veces mayor que las fracciones homologas de bagazo de uva (porcentaje de galoización medio) y corteza de pino (exentas de galatos) respectivamente. Un resultado a remarcar es la similar capacidad de transferencia de electrones que presentaron una de las fracciones compuestas fundamentalmente de taninos condensados (IVH) y otra de las fracciones compuesta mayoritariamente de taninos hidrolizables (VIIIH). La presencia de estructuras trihidroxibencénicas (ácido gálico / grupo pirogalol en el anillo B), común en ambas fracciones, parece ser responsable de la elevada transferencia electrónica.

Sabiendo que catequinas con presencia de un grupo pirogalol en el anillo B, tales como (epi)galocatequinas, son más reactivas que aquellas que presentan un grupo catecol ((epi)catequinas) o incluyen un galato en su estructura *(Kondo et al., 1999)* y, teniendo en cuenta que la transferencia electrónica puede derivar en un efecto pro-oxidante, se sintetizó en nuestro laboratorio el radical TNPTM, similar al HNTTM, capaz de ser reducido únicamente por un grupo pirogalol (1,2,3 trihidroxibenzeno)*(Torres et al., 2007)*. El uso de este radical permitió clasificar a los polifenoles o mezclas de polifenoles en función de su reactividad como agentes capaces de transferir electrones. Las fracciones IVH (proantocianidinas) y VIIIH (taninos hidrolizables), de similar eficacia en la transferencia electrónica frente al radical HNTTM, mostraron un efecto similar frente el radical TNPTM, probablemente debido a las galocatequinas o a compuestos minoritarios reactivos*(Touriño et al., 2008a)*. Fracciones de bagazo de uva y corteza de pino no reaccionaron con el radical TNPTM con lo que se comprueba que el grupo galato no es tan reactivo como el pirogalol del anillo B.

Los estudios realizados de bloqueo de ciclo celular, inducción de apoptosis, inducción de necrosis y captación de radicales hidroxilo y superóxido en células, mostraron una clara relación entre una elevada galoización y una alta eficacia en los ensayos (**Anexo 8.1 VI**) *(Lizárraga et al., 2008)*. La fracción VIIIH fue un potente agente desregulador del ciclo celular en células tumorales de colon (HT29), induciendo cambios significativos en todas las fases del ciclo celular (G1, S y G2), especialmente bloqueando la fase S de las células tumorales y posteriormente inhibiendo la síntesis de DNA. Curiosamente esta misma fracción fue capaz de inducir eficazmente la apoptosis y necrosis en las células tumorales. La apoptosis parece estar relacionada con el porcentaje de grupos galato y la necrosis podría estar relacionada con un efecto pro-oxidante. Los resultados obtenidos

para las fracciones de bagazo de uva (galoización media) y fracciones de corteza de pino (exentas de galoización) en los mismos ensayos mostraron una eficacia entre 10 y 15 veces menor respectivamente *(Lizárraga et al., 2007;Lizárraga et al., 2008)*. En el estudio de inhibición de proliferación de células de melanoma (SK-MEL-28) las fracciones mostraron un efecto similar al observado en células tumorales HT29. Existió una considerable diferencia en los resultados entre las fracciones de hamamelis y las fracciones de bagazo de uva y corteza de pino *(Touriño et al., 2008a)*. La gran diferencia entre el efecto sobre el ciclo celular de las fracciones de hamamelis con respecto a las de bagazo de uva y corteza de pino y la poca diferencia entre las de bagazo de uva y pino concordaría con otro de los resultados obtenidos en nuestro laboratorio tras realizar un estudio de la influencia de la transferencia electrónica de derivados de catequinas sobre el ciclo celular y la apoptosis (**Anexo 8.1 I**) *(Lozano et al., 2006)*. Los resultados de dicho estudio sugirieron que la apoptosis está ligada a la capacidad de transferencia electrónica y a la presencia de ésteres de galato. De entre todas las fracciones estudiadas, aquellas que presentan mayor capacidad de reducir al radical TNPTM parecen ser las que provocan más apoptosis tardía y/o necrosis, seguramente por un efecto pro-oxidante directo.

Los estudios de citotoxicidad *in vitro* realizados mediante ensayos de viabilidad celular en queratinocitos humanos (HaCaT) y fibroblastos de ratón de origen embrionario (3T3) *(Ugartondo et al., 2007)* mostraron que las fracciones más antioxidantes y con mayor capacidad de transferencia electrónica son las más citotóxicas. En general, las fracciones ensayadas de bagazo de uva, corteza de pino y hamamelis presentaban una eficacia antioxidante entre 1,3-2,5 veces mayor que la eficacia citotóxica, es decir que en las diferentes líneas celulares ensayadas las fracciones presentaron una actividad antioxidante efectiva en un rango no citotóxico. Las fracciones de pino presentaron la mejor relación actividad antioxidante–citotoxicidad en los cultivos de queratinocitos y fibroblastos.

En general y en concordancia con otros estudios publicados *(Alanko et al., 1999;Lee et al., 2006)* el orden de influencia de los factores estudiados es de menor a mayor: Grado de polimerización, porcentaje de esteres de galato, porcentaje de grupos pirogalol. Los resultados expuestos muestran una relación directa entre la capacidad antioxidante/ antirradicalaria, la capacidad antiproliferativa, la citotoxicidad y el porcentaje de grupos galato más pirogalol en el anillo B. Cuanto más antioxidantes fueron las fracciones, más capacidad antiproliferativa presentaron y más citotóxicas se mostraron. Esta relación podría ser explicada debido al efecto dual antioxidante/pro-oxidante de los polifenoles, o dicho de otra forma la capacidad de generar radicales o frustrar la actividad de los mismos dependiendo del medio en el que se encuentren, la concentración y/o la naturaleza del extracto polifenólico.

5.1.3 Posibles aplicaciones de las fracciones de proantocianidinas estudiadas.

Se ha demostrado con los resultados expuestos en esta tesis que las fracciones fenólicas con un grado de polimerización entre 2 y 4 son eficaces antioxidantes. Fracciones de pino con baja transferencia electrónica podrían ser utilizadas para aplicaciones cosméticas, especialmente la fracción XIP, que en nuestra consideración, sería la mejor opción para aplicaciones donde se requiera de un buen captador de radicales del medio sin afectar a las funciones celulares. Las fracciones de bagazo, de uva debido a su buen coeficiente actividad antioxidante-baja citotoxicidad, podrían utilizarse como antioxidantes alimentarios, en concreto la Fracción IVG ha sido considerada como un buen antioxidante para emulsiones y filetes de caballa congelados *(Pazos et al., 2005)* e incluso la misma fracción la hemos propuesto como ingrediente alimentario en pescado graso combinando los efectos beneficiosos de los ácidos poliinsaturados con las propiedades antioxidantes y ligeramente proapoptóticas de las procianidinas de extractos de uva (**Anexo 8.1 II**) *(Medina et al., 2006)*. Las fracciones de hamamelis debido a su elevada transferencia electrónica y a una elevada capacidad apoptótica/necrótica podrían ser agentes quimiopreventivos más potentes.

5.2 Absorción / metabolismo de proantocianidinas.

El segundo bloque de la tesis (**publicaciones 3 y 4**; apartados: 4.2.1 y 4.2.2) tuvo como objetivo el estudio de la biodisponibilidad de la fracción polifenólica de fibra dietética antioxidante de uva (GADF, *grape antioxidant dietary fiber*), o más concretamente de las proantocianidinas que forman parte de su composición. Frutas y verduras son una fuente de proantocianidinas y también de fibra dietética. El término de fibra dietética incluye a los polisacáridos, lignina y otras substancias asociadas a paredes celulares de los alimentos de origen vegetal resistentes a la hidrólisis de las enzimas digestivas humanas, que pueden ser sustrato de la fermentación de las bacterias colónicas. Los efectos benecifiosos de una dieta rica en fibra son numerosos. En la boca fibra desencadena un aumento de la salivación porque necesita más tiempo de masticación y causa posteriormente un retraso en el vaciado gástrico. En el intestino delgado disminuye o retrasa la absorción de materias orgánicas e inorgánicas y finalmente cuando llega al intestino grueso, la fibra acelera el tránsito intestinal aumentando el volumen de la masa fecal y una vez en el colón es fermentada por las bacterias, generando ácidos grasos volátiles de cadena corta (AGCC) como el acético, el propiónico y el butírico, los cuales son absorbidos a nivel del colon (85%) y son reutilizados por el organismo para proporcionar energía en el Ciclo de Krebs, aportando aproximadamente el 3% del toda de la energía obtenida de la dieta.

La fibra dietética antioxidante *(Saura-Calixto et al., 1999)*, es obtenida a partir de un concentrado de subproductos de la vinificación que combina los efectos de la fibra dietética con el de los polifenoles presentes en un elevado porcentaje (20%). Estos polifenoles presentan una alta capacidad antioxidante in *vitro (Saura-Calixto, 1998)* y recientemente se ha observado que GADF presenta efectos beneficiosos sobre la presión sanguínea y el colesterol en humanos *(Pérez-Jiménez et al., 2008)*. Otros estudios clínicos *(Taubert et al., 2007)* han sugerido que la ingesta de proantocianidinas podría derivar en una disminución de la presión sanguínea. Por ello, los efectos sobre la salud cardiovascular de GADF podrían ser debidos en parte a los efectos de los polifenoles en la fibra dietética de uva o más concretamente a sus metabolitos.

Aproximadamente entre un 5-10 % de los polifenoles incluidos en frutas y verduras están asociados a los polisacáridos celulósicos y hemicelulósicos que forman parte de las paredes vegetales *(Pinelo et al., 2006;Saura-Calixto, 1998)*. Estos polifenoles son difíciles de separar de la matriz que los envuelve *(Ishizu et al., 1999;Le Bourvellec et al., 2004)* y por eso, los polifenoles de la dieta se dividen en extraíbles y no extraíbles.

GADF al ser un concentrado de subproducto de la vinificación (básicamente pieles y pepitas) presentó polifenoles en ambas fracciones (extraíble y no extraíble). Los compuestos fenólicos de la fracción extraíble son fundamentalmente monómeros de flavonoides y oligómeros de proantocianidinas *(Pérez-Jiménez et al., 2008)*.

No obstante y, teniendo en cuenta que uno de los objetivos de la tesis es estudiar la biotransformación o metabolismo de los compuestos de la fracción polifenólica de GADF, se realizó un estudió minucioso de identificación de los compuestos fenólicos de la fracción extraíble de GADF (**publicación 3**; apartado 4.2.1) *(Touriño et al., 2008b)*. La identificación de estos compuestos facilitó posteriormente la búsqueda de los posibles metabolitos de la ingesta de GADF.

Un primer análisis por HPLC-DAD de los compuestos fenólicos de la fracción extraíble de GADF, mostró una mezcla compleja de diferentes familias de compuestos fenólicos. Con el fin de mejorar la identificación de los compuestos se utilizó una combinación de técnicas cromatográficas *(Sun et al., 2006)* que permitió separar los compuestos según su naturaleza en cuatro fracciones: (i) La fracción monomérica rica en ácidos fenólicos y derivados, (epi)catequinas y flavonoles; (ii) la fracción oligomérica compuesta fundamentalmente por oligómeros de proantocianidinas y derivados de flavonoles; (iii) la fracción antocianidínica que incluyó en su composición antocianinas conjugadas con azúcar u otros ácidos; (iv) la fracción polimérica formada por proantocianidinas. La obtención de estas cuatro fracciones minimizó el número de compuestos fenólicos que previamente co-eluían por RP-HPLC y simplificó el análisis e identificación.

La combinación de técnicas de espectrometría de masas ESI-TOF y ESI-MS/MS y ESI-TOF-MS acopladas a un RP-HPLC permitieron identificar de manera precisa los compuestos mayoritarios de la fracción soluble de GADF. Experimentos en tándem (barrido de neutros (NL), barrido de ión precursor (PI), barrido de ión producto) realizados por LC-MS/MS se utilizaron para determinar las estructuras en función de su fragmentación, mientras que ESI-TOF-MS posibilitó confirmar con masas exactas más de 100 compuestos fenólicos presentes en la fracción extraíble de GADF. La fracción polimérica, debido a que es difícil de separar por RP-HPLC, fue determinada por inyección directa al espectrómetro de masas (ESI-TOF-MS) con un barrido entre 300-3000 unidades de masa, encontrando proantocianidinas hasta un grado de polimerización en torno a 12, entre las cuales se observaron proantocianidinas con esteres de galato en su composición (mono-, di- y tri- galoizadas).

Pocos trabajos de identificación de compuestos fenólicos en matrices naturales han sido tan minuciosos como el aquí expuesto. Los resultados que se obtenidos en la **publicación 3** (apartado 4.2.1) *(Touriño et al., 2008b)* demuestran que la combinación de técnicas de espectrometría de masas permite resolver con mayor facilidad y fiabilidad, que cada uno de ellos por separado, la identidad de los compuestos, además se observó que la técnica de ESI-TOF-MS es particularmente útil debido que permite discriminar entre estructuras que presentan la misma fragmentación en ESI-MS/MS.

Los polifenoles de GADF son en términos generales una mezcla de ácidos hidroxicinámicos, antocianinas, flavonoides y fundamentalmente proantocianidinas y una fracción no extraíble compuesta fundamentalmente por proantocianidinas poliméricas *(Le*

Bourvellec et al., 2004). Aunque sin demasiado éxito, técnicas de extracción consideradas como eficaces para la liberación de compuestos embebidos en matrices celulósicas como la despolimerización en medio ácido *(Matthews et al., 1997)* y la "steam explosion" *(Fernández-Bolaños et al., 2001)* fueron probadas en nuestro laboratorio con el fin de determinar la composición fenólica de la fracción no extraíble pero los resultados obtenidos mostraron rendimientos bajos y de elevada variabilidad (información no adjunta).

Es de notable importancia conocer los metabolitos de los compuestos fenólicos de GADF para avanzar en la comprensión de los mecanismos de acción de la fibra antioxidante. Numerosos estudios han demostrado que los flavonoides monoméricos tipo catequinas *(Tsang et al., 2005)* y ácidos fenólicos *(Poquet et al., 2008)* son metabolizados en los enterocitos del intestino delgado alcanzando el corriente sanguíneo para llegar al hígado donde son nuevamente metabolizados aumentando su polaridad (metilados, sulfatados y glucuronidados) con el fin de ser eliminados vía orina. Las metabolizaciones que sufren los flavonoides disminuyen la citotoxicidad y parecen también disminuir la actividad biológica tal y como sugieren algunos estudios *(Cren-Olivé et al., 2003)*. Sin embargo, recientes investigaciones han demostrado que algunos metabolitos de epicatequina y otros flavonoides en condiciones fisiológicas, son mejores antioxidantes que el α-tocoferol *(Dueñas et al.)* y algunos sugieren que los metabolitos formados de flavonoides siguen ejerciendo la misma actividad biológica que los compuestos de los que provienen, tal es el caso del glucuronido de quercetina que aunque no ejerce efectos protectores en condiciones de equilibrio celular, cuando se forma un estado de estrés oxidativo protege, al menos parcialmente, de los radicales que desrregulan la función endotelial *(Lodi et al., 2009)*.

Los estudios de biodisponibilidad de flavonoides monoméricos aportan importante información de la reacción del organismo al ingerir dichos compuestos. No obstante, no son un modelo fiel de lo que realmente sucede cuando ingerimos frutas y verduras, o suplementos de la dieta como extractos polifenólicos o fibras dietéticas puesto que la mayoría de los polifenoles dietéticos están presentes en forma polimérica *(Santos-Buelga et al., 2000)*. Por ello, se realizó el estudio de la metabolización de los compuestos fenólicos de GADF en ratas (**publicación 4**; apartado 4.2.2) *(Touriño et al., 2009)*

En los pocos estudios realizados de biodisponibilidad de proantocianidinas no parece haber un consenso sobre los procesos de absorción y metabolización. Algunos estudios sugieren que las proantocianidinas debido a que presentan una absorción muy limitada en el intestino delgado llegan intactas al colon donde son degradadas por la fermentación bacteriana *(Gonthier et al., 2003;Scalbert et al., 2000)*. Sin embargo, otros han observado que dímeros y trímeros pueden ser absorbidos y excretados vía orina *(Tsang et al., 2005)* e incluso conjugados como tales *(Shoji et al., 2006)*. Otros sugieren que los oligómeros son previamente hidrolizados en sus unidades monoméricas que son posteriormente metabolizadas *(Spencer et al., 2000)*. Nuestros resultados (**publicación**

4; apartado 4.2.2)*(Touriño et al., 2009)* sugieren que por un lado, oligómeros de pequeño tamaño pueden ser absorbidos y seguramente metabolizados y por otro lado, el gran número de metabolitos de epicatequina monomérica identificados en orina de rata, nos hace pensar que los oligómeros con grado de polimerización baja han sido previamente hidrolizados, mientras que los polímeros de mayor grado de polimerización alcanzan el colon asociados a proteínas *(Sarni-Manchado et al., 1999)* y con la matriz de lignina *(Matthews et al., 1997)* donde son degradados por la microbiota. Nuestra hipótesis se basa en la gran cantidad de metabolitos de epicatequina identificados y la baja concentración de monómeros libres de epicatequina en GADF (<0. 01%). Más de 18 metabolitos de epicatequina (metilos, sulfatos y glucurónidos) fueron identificados en las muestras de orina después de la ingesta de GADF. La identificación de los compuestos realizada por técnicas de MS/MS permitió determinar la posición de conjugación de gran parte de los metabolitos. Curiosamente algunos presentaron el grupo catecol del anillo B libre, este resultado es particularmente interesante si se tiene en cuenta que la presencia del grupo catecol es una condición necesaria para presentar capacidad antioxidante (captadora de radicales libres) *(Rice-Evans et al., 1999)*. Por otro lado, la identificación de un mayor número de metabolitos colónicos en las muestras de orina después de haber ingerido GADF que en las muestras del grupo de EC indican que las proantocianidinas que no son absorbidas en el intestino delgado y que llegan al colón son directamente degradadas a ácidos hidroxicinámicos y fenólicos, concordando con los resultados obtenidos por Appeldoorn y colaboradores *(Appeldoorn et al., 2009)*. De nuevo, algunos de los metabolitos identificados en las muestras de orina de rata tras la ingesta de GADF mostraron el grupo catecol libre (ácido 3,4–di-hidroxifenilacético y ácido 3,4-dihidroxifenilpropiónico), aunque algunos fueron identificados con uno o los dos grupos hidroxilos conjugados por sulfatos o glucurónidos. La extensa metabolización sugiere que los polifenoles, en concreto las proantocianidinas, son considerados por el organismo como agentes tóxicos. Ésto podría estar relacionado con la actividad pro-oxidante que pueden ejercer algunos flavonoides dependiendo de su naturaleza y concentración. De forma aparentemente paradójica tal actividad pro-oxidante puede resultar en un efecto antioxidante final. De hecho, se ha comprobado que estímulos pro-oxidantes, como restricción calórica *(Schulz et al., 2007)* y ejercicio físico *(Ascensão et al., 2005)*, resultan en una activación de los sistemas endógenos de defensa y consecuentemente un efecto antioxidante, siempre y cuando los sistemas antioxidantes no se vean desbordados por un elevada concentración de ROS.

Existe un balance homeostático en el organismo sobre el cual podrían ejercer alguna influencia los polifenoles, o concretamente las proantocianidinas. La metabolización disminuye tanto la citotoxicidad como las actividades biológicas de los flavonoides, pero tal y como nos indican nuestros resultados, grupos catecol responsables de la capacidad antioxidante siguen biodisponibles en el organismo. Los resultados obtenidos en la presente tesis tanto con fracciones polifenólicas de uva, pino y hamamelis como con fibra antioxidante de uva muestran que el efecto de los polifenoles, particularmente de las proantocianidinas, seguramente depende del balance antioxidante/pro-oxidante y de cómo este carácter dual incide en el equilibrio redox del organismo. La consecuencia

puede ser reforzar: (i) un estado reducido del organismo, relacionado con un estado general de salud; (ii) un estado levemente oxidado relacionado con procesos como la apoptosis o; (iii) un estado oxidado relacionado con procesos de necrosis y pérdida de salud en general.

Posiblemente, los polifenoles incluidos en frutas y verduras faciliten el estado inicial reducido mediante una combinación de efecto captador de radicales libres y estimulador de los sistemas endógenos de defensa. Éste sería también el efecto de extractos menos agresivos como los de pino o uva. Otros extractos más reactivos como los de hamamelis o té, ejercerían este mismo efecto o un efecto pro-oxidante neto en función de la cantidad ingerida.

6. CONCLUSIONES

Las conclusiones parciales de cada trabajo se pueden encontrar al final de las correspondientes discusiones. A continuación se exponen las conclusiones globales más significativas:

- Los efectos químicos y biológicos en los que están implicados las proantocianidinas están directamente relacionadas con el grado de polimerización, porcentaje de galatos y porcentaje de grupos pirogalol en el anillo B.

- En las fracciones de proantocianidinas se observa una clara relación directa entre capacidad antioxidante, efecto antiproliferativo, efecto apoptótico y citotoxicidad.

- Las fracciones más eficaces como captadoras de radicales libres (antioxidantes) son también las más potencialmente pro-oxidantes.

- Ensayos con los radicales HNTTM y TNPTM permiten evaluar la reactivad de los polifenoles y predecir sus efectos antioxidantes/pro-oxidantes.

- La combinación de técnicas de espectrometría de masas (LC-ESI-MS/MS y LC-ESI-TOF) permite identificar con mayor fiabilidad los compuestos fenólicos en mezclas naturales complejas.

- Los polifenoles de GADF son biodisponibles como productos de despolimerización en el intestino delgado y de degradación en el colón, conjugados con grupos glucuronato, metilo y sulfato.

- La extensa metabolización de GADF sugiere que el organismo considera las proantocianidinas como compuestos tóxicos, tal toxicidad podría estar relacionada con la actividad pro-oxidante que pueden ejercer en algunas situaciones las proantocianidinas.

- Una parte de los metabolitos de GADF presentan el grupo catecol libre, indicando que la metabolización no siempre resulta en una supresión de la actividad reductora (antioxidante) de radicales libres de las proantocianidinas.

- Tanto las fracciones polifenólicas purificadas como la fibra antioxidante de uva presentan una dualidad antioxidante/pro-oxidante. De forma aparentemente paradójica la actividad pro-oxidante puede resultar en un efecto antioxidante final.

7. BIBLIOGRAFÍA

Abrahamse, S. L.; Kloots, W. J., van Amelsvoort, J. M. M.; Absorption, distribution, and secretion of epicatechin and quercetin in the rat. *Nutrition Research.* ***2005***, *25 (3)*,305-317.

Adamson, G. E.; Lazarus, S. A.; Mitchell, A. E.; Prior, R. L.; Cao, G.; Jacobs, P. H.; Kremers, B. G.; Hammerstone, J. F.; Rucker, R. B.; Ritter, K. A., Schmitz, H. H.; HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. *Journal of Agricultural and Food Chemistry.* ***1999***, *47 (10)*,4184-4188.

Akagawa, M.; Sasaki, D.; Ishii, Y.; Kurota, Y.; Yotsu-Yamashita, M.; Uchida, K., Suyama, K.; New method for the quantitative determination of major protein carbonyls, a-aminoadipic and d-glutamic semialdehydes investigation of the formation mechanism and chemical nature *in vitro* and *in vivo*. *Chemical Research in Toxicology.* ***2006***, *19 (8)*,1059-1065.

Alanko, J.; Riutta, A.; Holm, P.; Mucha, I.; Vapaatalo, H., Metsa-Ketela, T.; Modulation of arachidonic acid metabolism by phenols: Relation to their structure and antioxidant/prooxidant properties. *Free Radical Biology and Medicine.* ***1999***, *26 (1-2)*,193-201.

Albertini, R.; Rindi, S.; Passi, A.; Bardoni, A.; Salvini, R.; Pallavicini, G., De Luca, G.; The effect of cornea proteoglycans on liposome peroxidation. *Archives of Biochemistry and Biophysics.* ***1996***, *327 (2)*,209-214.

Appeldoorn, M. M.; Vincken, J. P.; Aura, A.M.; Hollman, P. C. H., Gruppen, H.; Procyanidin dimers are metabolized by human microbiota with 2-(3,4-Dihydroxyphenyl)acetic acid and 5-(3,4-Dihydroxyphenyl)-δ-valerolactone as the major metabolites. *Journal of Agricultural and Food Chemistry.* ***2009***, *57 (3)*,1084-1092.

Aruoma, O. I.; Halliwell, B.; David, B., Paul, G. W., *Immunopharmacology of Free Radical Species*, Academic Press, London **1995**, pp. 199-214.

Ascensão, A.; Soares, J. M.; Ferreira, R. M.; Neuparth, M. J. H.; Appell, J., Duarte, A. J.; Cardiac mitochondrial respiratory function and oxidative stress: the role of exercise. *International Journal of Sports Medicine.* ***2005***, *26 (4)*,258-267.

Aura, A. M.; O'Leary, K. A.; Williamson, G.; Ojala, M.; Bailey, M.; Puupponen-Pimia, R.; Nuutila, A. M.; Oksman-Caldentey, K. M., Poutanen, K.; Quercetin derivatives are deconjugated and converted to hydroxyphenylacetic acids but not methylated by human fecal flora *in vitro*. *Journal of Agricultural and Food Chemistry.* ***2002***, *50 (6)*,1725-1730.

Autier, P., Gandini, S.; Vitamin D supplementation and total mortality: A meta-analysis of randomized controlled trials. *Archives of Internal Medicine.* ***2007***, *167 (16)*,1730-1737.

Awika, J. M.; Rooney, L. W.; Wu, X.; Prior, R. L., Cisneros-Zevallos, L.; Screening methods to measure antioxidant activity of sorghum (Sorghum bicolor) and sorghum products. *Journal of Agricultural and Food Chemistry.* ***2003***, *51 (23)*,6657-6662.

Babior, B. M.; Phagocytes and oxidative stress. *The American Journal of Medicine.* ***2000***, *109 (1)*,33-44.

Bader, A. N.; Pivovarenko, V. G.; Demchenko, A. P.; Ariese, F., Gooijer, C.; Excited state and ground state proton transfer rates of 3-hydroxyflavone and its derivatives studied by Shpol'skii Spectroscopy: The influence of redistribution of electron density. *The Journal of Physical Chemistry B.* ***2004***, *108 (29)*,10589-10595.

Bagchi, D.; Bagchi, M.; Hassoun, E. A., Stohs, S. J.; *In vitro* and *in vivo* generation of reactive oxygen species, DNA damage and lactate dehydrogenase leakage by selected pesticides. *Toxicology.* ***1995***, *104 (1-3)*,129-140.

Bagchi, D.; Balmoori, J.; Bagchi, M.; Ye, X.; Williams, C. B., Stohs, S. J.; Comparative effects of TCDD, endrin, naphthalene and chromium (VI) on oxidative stress and tissue damage in the liver and brain tissues of mice. *Toxicology.* ***2002***, *175 (1-3)*,73-82.

Bagchi, M.; Zafra-Stone, S.; Bagchi, D.; Patel, S.; Ramesh, C. G.; Dvm; Mvsc; Phd; Dabt, Fact, *Veterinary Toxicology*, Academic Press, Oxford **2007**, pp. 313-334.

Bairati, I.; Meyer, F.; Gelinas, M.; Fortin, A.; Nabid, A.; Brochet, F.; Mercier, J.-P.; Tetu, B.; Harel, F.; Masse, B.; Vigneault, E.; Vass, S.; del Vecchio, P., Roy, J.; A randomized trial of antioxidant vitamins to prevent second primary cancers in head and neck cancer patients. *Journal of the National Cancer Institute.* ***2005***, *97 (7)*,481-488.

Balazy, M., Chemtob, S.; Trans-arachidonic acids: new mediators of nitro-oxidative stress. *Pharmacology & Therapeutics.* ***2008***, *119 (3)*,275-290.

Banerjee, B. D.; Seth, V., Ahmed, R. S.; Pesticide-induced oxidative stress: Perspectives and trends. *Reviews on Environmental Health.* ***2001***, *16 (1)*,1-40.

Bardullas, U.; Limón-Pacheco, J. H.; Giordano, M.; Carrizales, L.; Mendoza-Trejo, M. S., Rodríguez, V. M.; Chronic low-level arsenic exposure causes gender-specific alterations in locomotor activity, dopaminergic systems, and thioredoxin expression in mice. *Toxicology and Applied Pharmacology. In Press, Corrected Proof*.

Bartosz, G.; Janaszewska, A.; Ertel, D., Bartosz, M.; Simple determination of peroxyl radical-trapping capacity. *IUBMB Life.* ***1998***, *46 (3)*,519 - 528.

Beazley, D.; Ahokas, R.; Livingston, J.; Griggs, M., Sibai, B. M.; Vitamin C and E supplementation in women at high risk for preeclampsia: A double-blind, placebo-controlled trial. *American Journal of Obstetrics and Gynecology.* ***2005***, *192 (2)*,520-521.

Beecher, G. R.; Overview of dietary flavonoids: nomenclature, occurrence and intake. *The Journal of Nutrition.* ***2003***, *133 (10)*,3248S-3254.

Begonja, A. J.; Teichmann, L.; Geiger, J.; Gambaryan, S., Walter, U.; Platelet regulation by NO/cGMP signaling and NAD(P)H oxidase-generated ROS. *Blood Cells, Molecules, and Diseases.* ***2006***, *36 (2)*,166-170.

Behl, C.; Alzheimer's disease and oxidative stress: implications for novel therapeutic approaches. *Progress in Neurobiology.* ***1999***, *57 (3)*,301-323.

Benetou, V.; Trichopoulou, A.; Orfanos, P.; Naska, A.; Lagiou, P.; Boffetta, P., Trichopoulos, D.; Conformity to traditional Mediterranean diet and cancer incidence: the Greek EPIC cohort. *British Journal of Cancer. 99 (1)*,191-195.

Bickers, D. R., Athar, M.; Oxidative Stress in the Pathogenesis of Skin Disease. *Journal of Investigative Dermatology.* ***2006***, *126 (12)*,2565-2575.

Bloomer, R. J., Gregory, S. M., *Advances in Clinical Chemistry*, Elsevier **2008**, pp. 1-50.

Bocco, A.; Cuvelier, M. E.; Richard, H., Berset, C.; Antioxidant activity and phenolic composition of citrus peel and seed extracts. *Journal of Agricultural and Food Chemistry.* ***1998***, *46 (6)*,2123-2129.

Bommareddy, A.; Hora, J.; Cornish, B., Dwivedi, C.; Chemoprevention by a-santalol on UVB radiation-induced skin tumor development in mice. *Anticancer Research.* ***2007***, *27 (4 B)*,2185-2188.

Boots, A. W.; Haenen, G. R. M. M., Bast, A.; Health effects of quercetin: From antioxidant to nutraceutical. *European Journal of Pharmacology.* ***2008***, *585 (2-3)*,325-337.

Bors, W.; Heller, W.; Michel, C., Saran, M.; Flavonoids as antioxidants: determination of radical-scavenging efficiencies. *Methods in Enzymology.* ***1990***, *186* 343-355.

Braconi, D.; Possenti, S.; Laschi, M.; Geminiani, M.; Lusini, P.; Bernardini, G., Santucci, A.; Oxidative damage mediated by herbicides on yeast cells. *Journal of Agricultural and Food Chemistry.* ***2008***, *56 (10)*,3836-3845.

Brenner-Lavie, H.; Klein, E.; Zuk, R.; Gazawi, H.; Ljubuncic, P., Ben-Shachar, D.; Dopamine modulates mitochondrial function in viable SH-SY5Y cells possibly via its interaction with complex I: Relevance to dopamine pathology in schizophrenia. *Biochimica et Biophysica Acta (BBA) - Bioenergetics.* ***2008***, *1777 (2)*,173-185.

Brewster, A. M.; El-Zein, R.; Hajek, R.; Hernandez, M. S.; Gor, B., Jones, L. A.; Low fat diet and oxidative damage. *AACR Meeting Abstracts.* ***2006***, *2006 (3)*,B13.

Burda, S., Oleszek, W.; Antioxidant and antiradical activities of flavonoids. *Journal of Agricultural and Food Chemistry.* ***2001***, *49 (6)*, 2774-2779.

Cao, G.; Alessio, H., Cutler, R.; Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Biology and Medicine.* ***1994***, *16 (1)*, 135-137.

Cao, G.; Sofic, E., Prior, R. L.; Antioxidant and prooxidant behavior of flavonoids: structure-Activity relationships. *Free Radical Biology and Medicine.* ***1997***, *22 (5)*, 749-760.

Caro, A. A., Cederbaum, A. I.; Role of cytochrome P450 in phospholipase A2 and arachidonic acid-mediated cytotoxicity. *Free Radical Biology and Medicine.* ***2006***, *40 (3)*, 364-375.

Catalá, A.; Lipid peroxidation of membrane phospholipids generates hydroxy-alkenals and oxidized phospholipids active in physiological and/or pathological conditions. *Chemistry and Physics of Lipids.* ***2009***, *157 (1)*, 1-11.

Chalmers, A. H.; Blake-Mortimer, J. S., Winefield, A. H.; The prooxidant state and psychologic stress. *Environmental health perspectives.* ***2003***, *111 (1)*.

Cheeseman, K. H.; David, B., Paul, G. W., *Immunopharmacology of free radical species*, Academic Press, London **1995**, pp. 233-245.

Chen, H. P.; Zhang, L.; Xu, B. H.; Kang, L. M.; Yang, S. L.; Zeng, H. H., Shi, X. Y.; Nitric oxide stimulates embryonic somatotroph differentiation and growth hormone mRNA and protein expression through a cyclic guanosine monophosphate-independent mechanism. *Tissue and Cell.* ***2009***, *41 (2)*,133-140.

Cheng, I. F., Breen, K.; On the ability of four flavonoids, baicilein, luteolin, naringenin, and quercetin, to suppress the fenton reaction of the iron-ATP complex. *BioMetals.* ***2000***, *13 (1)*,77-83.

Cheng, T. O.; The Mediterranean diet revisited. *An International Journal of Medicine.* ***2001***, *94 (3)*,174-175.

Christen, W. G.; Glynn, R. J.; Chew, E. Y.; Albert, C. M., Manson, J. E.; Folic acid, pyridoxine, and cyanocobalamin combination treatment and age-related macular degeneration in women: The women's antioxidant and folic acid cardiovascular study. *Archives of Internal Medicine.* ***2009***, *169 (4)*,335-341.

Church, D. F., Pryor, W. A.; Free radical chemistry of cigarettes smoke and its toxicological implications . *Environmental Health Perspectives.* ***1985***, *64* 111-126.

Coleman, M. P.; Alexe, D.-M.; Albreht, T., McKee, M. (Eds.), *Responding to the challenge of cancer in Europe*, Institute of Public Health of the Republic of Slovenia, Ljubljana **2008**.

Conde, E.; Cadahía, E.; García-Vallejo, M. C., Fernández de Simón, B.; Polyphenolic Composition of Quercus suber Cork from Different Spanish Provenances. *Journal of Agricultural and Food Chemistry.* ***1998***, *46 (8)*,3166-3171.

Cook, N. R.; Albert, C. M.; Gaziano, J. M.; Zaharris, E.; MacFadyen, J.; Danielson, E.; Buring, J. E., Manson, J. E.; A randomized factorial trial of vitamins C and E and beta carotene in the secondary prevention of cardiovascular events in women: results from the women's antioxidant cardiovascular study. *Archives of Internal Medicine.* ***2007***, *167 (15)*,1610-1618.

Cren-Olivé, C. é.; Teissier, E.; Duriez, P., Rolando, C.; Effect of catechin *O*-methylated metabolites and analogues on human LDL oxidation. *Free Radical Biology and Medicine.* ***2003***, *34 (7)*,850-855.

Cuyckens, F., Claeys, M.; Mass spectrometry in the structural analysis of flavonoids. *Journal of Mass Spectrometry.* ***2004***, *39 (1)*,1-15.

Daigle, D. J., Conkerton, E. J.; Analysis of Flavonoids by HPLC. *Journal of Liquid Chromatography & Related Technologies.* ***1983***, *6 (2 supp 1)*,105 - 118.

Davies, K. J. A.; Intracellular proteolytic systems may function as secondary antioxidant defenses: An hypothesis. *Journal of Free Radicals in Biology & Medicine.* ***1986***, *2 (3)*,155-173.

De Gaulejac, N. S. C.; Vivas, N.; de Freitas, V., Bourgeois, G.; The influence of various phenolic compounds on scavenging activity assessed by an enzymatic method. *Journal of the Science of Food and Agriculture.* ***1999***, *79 (8)*,1081-1090.

De Lederkremer, R. M.; Marino, C., Horton, D., *Advances in Carbohydrate Chemistry and Biochemistry*, Academic Press **2003**, pp. 199-306.

De Rijke, E.; Zafra-Gómez, A.; Ariese, F.; Brinkman, U. A. T., Gooijer, C.; Determination of isoflavone glucoside malonates in Trifolium pratense L. (red clover) extracts: quantification and stability studies. *Journal of Chromatography A.* ***2001***, *932 (1-2)*,55-64.

De Rijke, E.; Joshi, H. C.; Sanderse, H. R.; Ariese, F.; Brinkman, U. A. T., Gooijer, C.; Natively fluorescent isoflavones exhibiting anomalous Stokes' shifts. *Analytica Chimica Acta.* ***2002***, *468 (1)*,3-11.

De Villiers, A.; Lestremau, F.; Szucs, R.; Gélébart, S.; David, F., Sandra, P.; Evaluation of ultra performance liquid chromatography: Part I. Possibilities and limitations. *Journal of Chromatography A.* ***2006***, *1127 (1-2)*,60-69.

De Zwart, L. L.; Meerman, J. H. N.; Commandeur, J. N. M., Vermeulen, N. P. E.; Biomarkers of free radical damage : Applications in experimental animals and in humans. *Free Radical Biology and Medicine.* ***1999***, *26 (1-2)*,202-226.

Del Río, L. A.; Sandalio, L. M.; Palma, J.; Bueno, P., Corpas, F. J.; Metabolism of oxygen radicals in peroxisomes and cellular implications. *Free Radical Biology and Medicine.* ***1992***, *13 (5)*,557-580.

DeLange, R. J., Glazer, A. N.; Phycoerythrin fluorescence-based assay for peroxy radicals: A screen for biologically relevant protective agents. *Analytical Biochemistry.* ***1989***, *177 (2)*,300-306.

Deng, F., Zito, S. W.; Development and validation of a gas chromatographic-mass spectrometric method for simultaneous identification and quantification of marker compounds including bilobalide, ginkgolides and flavonoids in Ginkgo biloba L. extract and pharmaceutical preparations. *Journal of Chromatography A.* ***2003***, *986 (1)*,121-127.

Déprez, S.; Brezillon, C.; Rabot, S.; Philippe, C.; Mila, I.; Lapierre, C., Scalbert, A.; Polymeric proanthocyanidins are catabolized by human colonic microflora into low-molecular-weight phenolic acids. *The Journal of Nutrition.* ***2000***, *130 (11)*,2733-2738.

Dey, G.; Sachan, A.; Ghosh, S., Mitra, A.; Detection of major phenolic acids from dried mesocarpic husk of mature coconut by thin layer chromatography. *Industrial Crops and Products.* ***2003***, *18 (2)*,171-176.

Dicker, E., Cederbaum, A. I.; Increased oxygen radical-dependent inactivation of metabolic enzymes by liver microsomes after chronic ethanol consumption. *The FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology.* ***1988***, *2 (13)*,2901-2906.

Dixon, R. A.; Lamb, C. J.; Masoud, S.; Sewalt, V. J. H., Paiva, N. L.; Metabolic engineering: prospects for crop improvement through the genetic manipulation of phenylpropanoid biosynthesis and defense responses - a review. *Gene.* ***1996***, *179 (1)*,61-71.

Dizdaroglu, M.; Jaruga, P.; Birincioglu, M., Rodriguez, H.; Free radical-induced damage to DNA: mechanisms and measurement. *Free Radical Biology and Medicine.* ***2002***, *32 (11)*,1102-1115.

Dreher, D., Junod, A. F.; Role of oxygen free radicals in cancer development. *European Journal of Cancer.* ***1996***, *32 (1)*,30-38.

Droge, W.; Free Radicals in the Physiological Control of Cell Function. *Physiological Reviews.* ***2002***, *82 (1)*,47-95.

Drost, E.; Skwarski, K.; Sauleda, J.; Soler, N.; Roca, J.; Agusti, A., MacNee, W.; Oxidative stress and airway inflammation in severe exacerbations of COPD. *Thorax.* ***2005***, *60 (4)*,2943-2300.

Dueñas, M.; González-Manzano, S.; González-Paramás, A., Santos-Buelga, C.; Antioxidant evaluation of *O*-methylated metabolites of catechin, epicatechin and quercetin. *Journal of Pharmaceutical and Biomedical Analysis. In Press, Corrected Proof*.

Duflo, F.; Debon, R.; Goudable, J.; Chassard, D., Allaouchiche, B.; Alveolar and serum oxidative stress in ventilator-associated pneumonia. *British Journal of Anaesthesia.* ***2002***, *89 (2)*,231-236.

Durak, L.; Elgün, S. N.; Bingöl, N. K.; Cimen, M. Y. B.; Kacmaz, M.; BüyüKKocaK, S., Öztürk, S.; Effects of cigarette smoking with different tar content on erythrocyte oxidant/antioxidant status. *Addiction Biology.* ***2002***, *7 (2)*,255-258.

Duthie, G. G.; Arthur, J. R., Duthie, S. J.; in: Sen, C. K., Packer, L., Hiinninen, O. (Eds.), *Handbook of Oxidants and Antioxidants in Exercise*, Elsevier Science B.V., Amsterdam **2000**, pp. 977-993.

Duthie, G. G.; Gardner, P. T., Kyle, J. A. M.; Plant polyphenols: are they the new magic bullet? *Proceedings of the Nutrition Society.* ***2003***, *62 (03)*,599-603.

Duthie, S.; Collins, A.; Duthie, G., Dobson, V.; Quercetin and myricetin protect against hydrogen peroxide-induced DNA damage (strand breaks and oxidised pyrimidines) in human lymphocytes. *Mutation Research.* ***1997a***, *393 (3)*,223-231.

Duthie, S.; Johnson, W., Dobson, V.; The effect of dietary flavonoids on DNA damage (strand breaks and oxidised pyrimdines) and growth in human cells. *Mutation Research.* ***1997b***, *390 (1-2)*,141-151.

Dvorakova, M.; Moreira, M. M.; Dostalek, P.; Skulilova, Z.; Guido, L. F., Barros, A. A.; Characterization of monomeric and oligomeric flavan-3-ols from barley and malt by liquid chromatography-ultraviolet detection-electrospray ionization mass spectrometry. *Journal of Chromatography A.* ***2008***, *1189 (1-2)*,398-405.

Ebadi, M.; Srinivasan, S. K., Baxi, M. D.; Oxidative stress and antioxidant therapy in Parkinson's disease. *Progress in Neurobiology.* ***1996***, *48 (1)*,1-19.

Esposito, F.; Arlotti, G.; Maria Bonifati, A.; Napolitano, A.; Vitale, D., Fogliano, V.; Antioxidant activity and dietary fibre in durum wheat bran by-products. *Food Research International.* ***2005***, *38 (10)*,1167-1173.

EUFIC, www.eufic.org 2009.

Farouque, H. M. O.; Leung, M.; Hope, S. A.; Baldi, M.; Schechter, C.; Cameron, J. D., Meredith, I. T.; Acute and chronic effects of flavanol-rich cocoa on vascular function in subjects with coronary artery disease: A randomized double-blind placebo-controlled study. *Clinical Science.* ***2006***, *111 (1)*,71-80.

Felgines, C.; Talavera, S.; Gonthier, M. P.; Texier, O.; Scalbert, A.; Lamaison, J. L., Rémésy, C.; Strawberry Anthocyanins are recovered in urine as glucuro- and sulfoconjugates in humans. *The Journal of Nutrition.* ***2003***, *133 (5)*,1296-1301.

Ferguson, L. R.; Philpott, M., Karunasinghe, N.; Oxidative DNA damage and repair: significance and biomarkers. *The Journal of Nutrition.* ***2006***, *136 (10)*,2687S-2689.

Fernández-Bolaños, J.; Felizón, B.; Heredia, A.; Rodríguez, R.; Guillén, R., Jiménez, A.; Steam-explosion of olive stones: hemicellulose solubilization and enhancement of enzymatic hydrolysis of cellulose. *Bioresource Technology.* ***2001***, *79 (1)*,53-61.

Fiamegos, Y. C.; Nanos, C. G.; Vervoort, J., Stalikas, C. D.; Analytical procedure for the *in vial derivatization* extraction of phenolic acids and flavonoids in methanolic and aqueous plant extracts followed by gas chromatography with mass-selective detection. *Journal of Chromatography A.* ***2004***, *1041 (1-2)*,11-18.

Frankel, E. N.; *Lipid oxidation*, The Oily Press & Lipid Library **2005**.

Frei, B.; Reactive oxygen species and antioxidant vitamins: mechanisms of action. . *The American Journal of Medicine.* ***1994***, *97 (3A)*,5s-13s.

Frenkel, K.; Carcinogen-mediated oxidant formation and oxidative DNA damage. *Pharmacology & Therapeutics.* ***1992***, *53 (1)*,127-166.

Frison-Norrie, S., Sporns, P.; Identification and quantification of flavonol glycosides in almond seedcoats using MALDI-TOF MS. *Journal of Agricultural and Food Chemistry.* ***2002***, *50 (10)*,2782-2787.

Fuentes-Alventosa, J. M.; Rodríguez-Gutiérrez, G.; Jaramillo-Carmona, S.; Espejo-Calvo, J. A.; Rodríguez-Arcos, R.; Fernández-Bolaños, J.; Guillén-Bejarano, R., Jiménez-Araujo, A.; Effect of extraction method on chemical composition and functional characteristics of high dietary fibre powders obtained from asparagus by-products. *Food Chemistry.* ***2009***, *113 (2)*,665-671.

Fulcrand, H.; Benabdeljalil, C.; Rigaud, J.; Cheynier, V., Moutounet, M.; A new class of wine pigments generated by reaction between pyruvic acid and grape anthocyanins. *Phytochemistry.* ***1998***, *47 (7)*,1401-1407.

Fung, T. T.; Rexrode, K. M.; Mantzoros, C. S.; Manson, J. E.; Willett, W. C., Hu, F. B.; Mediterranean diet and incidence of and mortality from coronary heart disease and stroke in women. *Circulation.* ***2009***, *119 (8)*,1093-1100.

Galati, G.; Sabzevari, O.; Wilson, J. X., O'Brien, P. J.; Prooxidant activity and cellular effects of the phenoxyl radicals of dietary flavonoids and other polyphenolics. *Toxicology.* ***2002***, *177 (1)*,91-104.

García, F. J.,Evaluación *in vitro* e *in vivo* de la funcionalidad de un producto rico en antioxidantes.(Tesis), Nutrición y Bromatología, Universidad de Murcia, **2005**

Gil, M. I.; Tomás-Barberán, F. A.; Hess-Pierce, B.; Holcroft, D. M., Kader, A. A.; Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *Journal of Agricultural and Food Chemistry.* ***2000***, *48 (10)*,4581-4589.

Göksel Şener, A.; Özer, Ş., Gül, A. l.-D.; Melatonin protects against mercury (II)-induced oxidative tissue damage in rats. *Pharmacology & Toxicology.* ***2003***, *93 (6)*,290-296.

Goñi, I.; Martín, N., Saura-Calixto, F.; In vitro digestibility and intestinal fermentation of grape seed and peel. *Food Chemistry.* ***2005***, *90 (1-2)*,281-286.

Goñi, I.; Díaz-Rubio, M. E.; Pérez-Jiménez, J., Saura-Calixto, F.; Towards an updated methodology for measurement of dietary fiber, including associated polyphenols, in food and beverages. *Food Research International.* ***2009***, *In Press, Corrected Proof*.

Gonthier, M.-P.; Donovan, J. L.; Texier, O.; Felgines, C.; Remesy, C., Scalbert, A.; Metabolism of dietary procyanidins in rats. *Free Radical Biology and Medicine.* ***2003a***, *35 (8)*,837-844.

Gonthier, M. P.; Cheynier, V.; Donovan, J. L.; Manach, C.; Morand, C.; Mila, I.; Lapierre, C.; Rémésy, C., Scalbert, A.; Microbial aromatic acid metabolites formed in the gut account for a major fraction of the polyphenols excreted in urine of rats fed red wine polyphenols. *The Journal of Nutrition.* ***2003b***, *133 (2)*,461-467.

Gorinstein, S.; Martín-Belloso, O.; Park, Y.-S.; Haruenkit, R.; Lojek, A.; Cíz, M.; Caspi, A.; Libman, I., Trakhtenberg, S.; Comparison of some biochemical characteristics of different citrus fruits. *Food Chemistry.* ***2001***, *74 (3)*,309-315.

Graf, B. A.; Mullen, W.; Caldwell, S. T.; Hartley, R. C.; Duthie, G. G.; Lean, M. E. J.; Crozier, A., Edwards, C. A.; Disposition and metabolism of [$^{2}{}_{14}$C]quercetin-4'-glucoside in rats. *Drug Metabolism and Disposition: The Biological Fate of Chemicals.* ***2005***, *33 (7)*,1036-1043.

Graham, L. P. (Ed.), *An introduction to medicinal chemistry*, Oxford University Press, New York **2005**.

Griendling, K. K.; Minieri, C. A.; Ollerenshaw, J. D., Alexander, R. W.; Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circulation Research.* ***1994***, *74 (6)*,1141-1148.

Griendling, K. K.; Sorescu, D.; Lassègue, B., Ushio-Fukai, M.; Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. *Arteriosclerosis, Thrombosis, and Vascular Biology.* ***2000***, *20 (10)*,2175-2183.

Guillon, F., Champ, M.; Structural and physical properties of dietary fibres, and consequences of processing on human physiology. *Food Research International.* ***2000***, *33 (3-4)*,233-245.

Guldberg, H. C., Marsden, C. A.; Catechol-*O*-methyl transferase: Pharmacological aspects and physiological role. *Pharmacol Rev.* ***1975***, *27 (2)*,135-206.

Gupte, A., Mumper, R. J.; Elevated copper and oxidative stress in cancer cells as a target for cancer treatment. *Cancer Treatment Reviews.* ***2009***, *35 (1)*,32-46.

Halliwell, B.; Gutteridge, J.; Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Archives of Biochemistry and Biophysics.* ***1986***, *246 (2)*,501-514.

Halliwell, B.; Gutteridge, J. M. C.; The antioxidants of human extracellular fluids. *Archives of Biochemistry and Biophysics.* ***1990***, *280 (1)*,1-8.

Halliwell, B., Gutteridge, J. M. C.; The definition and measurement of antioxidants in biological systems. *Free Radical Biology and Medicine.* ***1995a***, *18 (1)*,125-126.

Halliwell, B.; Henderson, B.; Edwards, J. C. W., Pettipher, E. R., *Mechanisms and Models in Rheumatoid Arthritis*, Academic Press, London **1995b**, pp. 301-316.

Halliwell, B.; *Free Radicals in Byology and Medicine*, Oxford University **1999**.

Halliwell, B., Whiteman, M.; Measuring reactive species and oxidative damage *in vivo* and in cell culture: how should you do it and what do the results mean? *British Journal of Pharmacology.* ***2004***, *142 (2)*,231-255.

Han, D.; Loukianoff, S.; McLaughlin, L.; Chandan, K. S.; Ph.D; Facsm; Lester, P.; Ph.D; Fos; Osmo, O. P. H.; M.D, Ph.D, *Handbook of Oxidants and Antioxidants in Exercise*, Elsevier Science B.V., Amsterdam **2000**, pp. 433-483.

Harborne, J. B.; *The flavonoids: advances in research since 1986*, Chapman and Hall, London **1993**.

Haslam, E.; Vegetable tannins - Lessons of a phytochemical lifetime. *Phytochemistry.* ***2007***, *68 (22-24)*,2713-2721.

Heim, K. E.; Tagliaferro, A. R., Bobilya, D. J.; Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *The Journal of Nutritional Biochemistry.* ***2002***, *13 (10)*,572-584.

Hercberg, S.; Galan, P.; Preziosi, P.; Bertrais, S.; Mennen, L.; Malvy, D.; Roussel, A.-M.; Favier, A., Briancon, S.; The SU.VI.MAX Study: A Randomized, Placebo-Controlled Trial of the Health Effects of Antioxidant Vitamins and Minerals. *Archives of Internal Medicine.* ***2004***, *164 (21)*,2335-2342.

Herdener, M.; Heigold, S.; Saran, M., Bauer, G.; Target cell-derived superoxide anions cause efficiency and selectivity of intercellular induction of apoptosis. *Free Radical Biology and Medicine.* ***2000***, *29 (12)*,1260-1271.

Himmelfarb, J.; Stenvinkel, P.; Ikizler, T. A., Hakim, R. M.; The elephant in uremia: Oxidant stress as a unifying concept of cardiovascular disease in uremia. *Kidney International.* ***2002***, *62 (5)*,1524-1538.

Hodnick, W. F.; Kung, F. S.; Roettger, W. J.; Bohmont, C. W., Pardini, R. S.; Inhibition of mitochondrial respiration and production of toxic oxygen radicals by flavonoids : A structure-activity study. *Biochemical Pharmacology.* ***1986***, *35 (14)*,2345-2357.

Holst, B., Williamson, G.; Nutrients and phytochemicals: from bioavailability to bioefficacy beyond antioxidants. *Current Opinion in Biotechnology.* ***2008***, *19 (2)*,73-82.

Horváthová, K.; Chalupa, I.; Sebová, L.; Tóthová, D., Vachálková, A.; Protective effect of quercetin and luteolin in human melanoma HMB-2 cells. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis.* ***2005***, *565 (2)*,105-112.

Hsu, I. C.; Metcalf, R. A.; Sun, T.; Welsh, J. A.; Wang, N. J., Harris, C. C.; Mutational hot spot in the p53 gene in human hepatocellular carcinomas. *Nature.* ***1991***, *350 (6317)*,427-428.

Huang, D.; Ou, B.; Hampsch-Woodill, M.; Flanagan, J. A., Deemer, E. K.; Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated b-cyclodextrin as the solubility enhancer. *Journal of Agricultural and Food Chemistry.* ***2002***, *50 (7)*,1815-1821.

Ishida, Y.; Kitagawa, K.; Goto, K., Ohtani, H.; Solid sampling technique for direct detection of condensed tannins in bark by matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Communications in Mass Spectrometry.* ***2005***, *19 (5)*,706-710.

Ishizu, T.; Kintsu, K., Yamamoto, H.; NMR Study of the Solution Structures of the Inclusion Complexes of -b-Cyclodextrin with (+)-Catechin and (-)-Epicatechin. *The Journal of Physical Chemistry B.* ***1999***, *103 (42)*,8992-8997.

Jacobi, J.; Kristal, B.; Chezar, J.; Shaul, S. M., Sela, S.; Exogenous superoxide mediates pro-oxidative, proinflammatory, and procoagulatory changes in primary endothelial cell cultures. *Free Radical Biology and Medicine.* ***2005***, *39 (9)*,1238-1248.

Jarrett, S. G.; Lin, H.; Godley, B. F., Boulton, M. E.; Mitochondrial DNA damage and its potential role in retinal degeneration. *Progress in Retinal and Eye Research.* ***2008***, *27 (6)*,596-607.

Jaruga, P., Dizdaroglu, M.; Repair of products of oxidative DNA base damage in human cells. *Nucleic Acids Research.* ***1996***, *24 (8)*,1389-1394.

Jerez, M.; Touriño, S.; Sineiro, J.; Torres, J. L., Núñez, M. J.; Procyanidins from pine bark: Relationships between structure, composition and antiradical activity. *Food Chemistry.* ***2007***, *104 (2)*,518-527.

Ji, L. L.; in: Sen, C. K., Packer, L., Hiinninen, O. (Eds.), *Handbook of Oxidants and Antioxidants in Exercise*, Elsevier Science B.V., Amsterdam **2000**, pp. 689-712.

Jiménez, A.; Selga, A.; Torres, J. L., Juliá, L.; Reducing activity of polyphenols with stable radicals of the TTM series. Electron transfer versus H-abstraction reactions in flavan-3-ols. *Organic Letters.* ***2004***, *6 (24)*,4583-4586.

Johnson, M. K., Loo, G.; Effects of epigallocatechin gallate and quercetin on oxidative damage to cellular DNA. *Mutation Research/DNA Repair.* ***2000***, *459 (3)*,211-218.

Jussi-Pekka Rauha, H. V. R. K.; Effect of eluent on the ionization efficiency of flavonoids by ion spray, atmospheric pressure chemical ionization, and atmospheric pressure photoionization mass spectrometry. *Journal of Mass Spectrometry.* ***2001***, *36 (12)*,1269-1280.

Kadiiska, M. B.; Gladen, B. C.; Baird, D. D.; Graham, L. B.; Parker, C. E.; Ames, B. N.; Basu, S.; FitzGerald, G. A.; Lawson, J. A.; Marnett, L. J.; Morrow, J. D.; Murray, D. M.; Plastaras, J.; Roberts Ii, L. J.; Rokach, J.; Shigenaga, M. K.; Sun, J.; Walter, P. B.; Tomer, K. B.; Barrett, J. C., Mason, R. P.; Biomarkers of oxidative stress study: III. Effects of the nonsteroidal anti-inflammatory agents indomethacin and meclofenamic acid on measurements of oxidative products of lipids in CCl4 poisoning. *Free Radical Biology and Medicine.* ***2005***, *38 (6)*,711-718.

Kanupriya; Dipti, P.; Sharma, S. K.; Sairam, M.; Ilavazhagan, G.; Sawhney, R. C., Banerjee, P. K.; Flavonoids protect U-937 macrophages against tert-butylhydroperoxide induced oxidative injury. *Food and Chemical Toxicology.* ***2006***, *44 (7)*,1024-1030.

Kaul, N.; Siveski-Iliskovic, N.; Hill, M.; Slezak, J., Singal, P. K.; Free radicals and the heart. *Journal of Pharmacological and Toxicological Methods.* ***1993***, *30 (2)*,55-67.

Keith, M. E.; Jeejeebhoy, K. N.; Langer, A.; Kurian, R.; Barr, A.; O'Kelly, B., Sole, M. J.; A controlled clinical trial of vitamin E supplementation in patients with congestive heart failure. *Am J Clin Nutr.* ***2001***, *73 (2)*,219-224.

Kelm, M. A.; Johnson, J. C.; Robbins, R. J.; Hammerstone, J. F., Schmitz, H. H.; High-performance liquid chromatography separation and purification of cacao (Theobroma cacao L.) procyanidins according to degree of polymerization using a diol stationary phase. *Journal of Agricultural and Food Chemistry.* ***2006***, *54 (5)*,1571-1576.

Kondo, K.; Kurihara, M.; Miyata, N.; Suzuki, T., Toyoda, M.; Mechanistic studies of catechins as antioxidants against radical oxidation. *Archiv. Biochem. Biophys.* ***1999a***, *362 (1)*,79-86.

Kondo, K.; Kurihara, M.; Miyata, N.; Suzuki, T., Toyoda, M.; Mechanistic studies of catechins as antioxidants against radical oxidation. *Archives of Biochemistry and Biophysics.* ***1999b***, *362 (1)*,79-86.

Kostyuk, V. A.; Potapovich, A. I.; Vladykovskaya, E. N.; Korkina, L. G., Afanas'ev, I. B. A.; Influence of metal ions on flavonoid protection against asbestos-induced cell injury. *Archives of Biochemistry and Biophysics.* ***2001***, *385 (1)*,129-137.

Kucuk, O.; Sarkar, F. H.; Sakr, W.; Djuric, Z.; Pollak, M. N.; Khachik, F.; Li, Y.-W.; Banerjee, M.; Grignon, D.; Bertram, J. S.; Crissman, J. D.; Pontes, E. J., Wood, D. P., Jr.; Phase II randomized clinical trial of lycopene supplementation before radical prostatectomy. *Cancer Epidemiology, Biomarkers & Prevention : a Publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology.* ***2001***, *10 (8)*,861-868.

Lario, Y.; Sendra, E.; García-Pérez, J.; Fuentes, C.; Sayas-Barberá, E.; Fernández-López, J., Pérez-Alvarez, J. A.; Preparation of high dietary fiber powder from lemon juice by-products. *Innovative Food Science & Emerging Technologies.* ***2004***, *5 (1)*,113-117.

Lavandera, J.; Batlle, A., Buzaleh, A.; Metabolization of porphyrinogenic agents in brain: Involvement of the Phase I drug metabolizing system. A comparative study in liver and kidney. *Cellular and Molecular Neurobiology.* ***2007***, *27 (6)*,717-729.

Lazarus, S. A.; Adamson, G. E.; Hammerstone, J. F., Schmitz, H. H.; High-Performance liquid chromatography/mass spectrometry analysis of proanthocyanidins in foods and beverages. *Journal of Agricultural and Food Chemistry.* ***1999***, *47 (9)*,3693-3701.

Le Bourvellec, C.; Guyot, S., Renard, C. M. G. C.; Non-covalent interaction between procyanidins and apple cell wall material: Part I. Effect of some environmental parameters. *Biochimica et Biophysica Acta (BBA) - General Subjects.* ***2004***, *1672 (3)*,192-202.

Lecomte, E.; Artur, Y.; Chancerelle, Y.; Herbeth, B.; Galteau, M.-M.; Jeandel, C., Siest, G.; Malondialdehyde adducts to, and fragmentation of, apolipoprotein B from human plasma. *Clinica Chimica Acta.* ***1993***, *218 (1)*,39-46.

Lee, J.; Koo, N., Min, D. B.; Reactive oxygen species, aging, and antioxidative nutraceuticals. *Comprehensive Reviews in Food Science and Food Safety.* ***2004***, *3 (1)*,21-33.

Lee, K. W., Lee, H. J.; Biphasic effects of dietary antioxidants on oxidative stress-mediated carcinogenesis. *Mechanisms of Ageing and Development.* ***2006***, *127 (5)*,424-431.

Lenzi, P.; Frenzilli, G.; Gesi, M.; Ferrucci, M.; Lazzeri, G.; Fornai, F., Nigro, M.; DNA damage associated with ultrastructural alterations in rat myocardium after loud noise exposure. *Environmental Health Perspectives.* ***2003***, *111 (4)*,467-471.

Lieber, C. S.; Baraona, E.; Hernández-Muñoz, R.; Kubota, S.; Sato, N.; Kawano, S.; Matsumura, T., Inatomi, N.; Impaired oxygen utilization. A new mechanism for the hepatotoxicity of ethanol in sub-human primates. *The Journal of Clinical Investigation.* ***1989***, *83 (5)*,1682-1690.

Lieber, C. S.; Chandan, K. S.; Ph.D; Facsm; Lester, P.; Ph.D; Fos; Osmo, O. P. H.; M.D, Ph.D, *Handbook of Oxidants and Antioxidants in Exercise*, Elsevier Science B.V., Amsterdam **2000**, pp. 951-975.

Limón-Pacheco, J., Gonsebatt, M. E.; The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis.* ***2009***, *674 (1-2)*,137-147.

Liochev, S., Fridovich, I.; The role of $O_2^{\cdot -}$ in the production of $HO^{\cdot}$: *in vitro* and *in vivo. Free Radical Biology and Medicine.* ***1994***, *16 (1)*,29-33.

Liska, D.; Lyon, M., Jones, D. S.; Detoxification and biotransformational imbalances. *Explore:The Journal of Science and Healing.* ***2006***, *2 (2)*,122-140.

Litwinienko, G., Kasprzycka-Guttman, T.; A DSC study on thermoxidation kinetics of mustard oil. *Thermochimica Acta.* ***1998***, *319 (1-2)*,185-191.

Litwinienko, G.; Kasprzycka-Guttman, T., Jamanek, D.; DSC study of antioxidant properties of dihydroxyphenols. *Thermochimica Acta.* ***1999***, *331 (1)*,79-86.

Liu, R.; Ye, M.; Guo, H.; Bi, K., Guo, D.; Liquid chromatography/electrospray ionization mass spectrometry for the characterization of twenty-three flavonoids in the extract of *Dalbergia odorifera*. *Rapid Communications in Mass Spectrometry.* ***2005a***, *19 (11)*,1557-1565.

Liu, R. H., Finley, J.; Potential Cell Culture Models for Antioxidant Research. *Journal of Agricultural and Food Chemistry.* ***2005b***, *53 (10)*,4311-4314.

Lizárraga, D.; Lozano, C.; Briedé, J. J.; Van Delft, J. H.; Touriño, S.; Centelles, J. J.; Torres, J. L., Cascante, M.; The importance of polymerization and galloylation for the antiproliferative properties of procyanidin-rich natural extracts. *FEBS Journal.* ***2007***, *274 (18)*,4802-4811.

Lizárraga, D.; Touriño, S.; Reyes-Zurita, F. J.; De Kok, T. M.; Van Delft, J. H.; Maas, L. M.; Briedé, J. J.; Centelles, J. J.; Torres, J. L., Cascante, M.; Witch Hazel (Hamamelis virginiana) fractions and the importance of gallate moieties - Electron transfer capacities in their antitumoral properties. *Journal of Agricultural and Food Chemistry.* ***2008***, *56 (24)*,11675-11682.

Llorach, R.; Espín, J. C.; Tomás-Barberán, F. A., Ferreres, F.; Artichoke (Cynara scolymus L.) Byproducts as a Potential Source of Health-Promoting Antioxidant Phenolics. *Journal of Agricultural and Food Chemistry.* ***2002***, *50 (12)*,3458-3464.

Lodi, F.; Jiménez, R.; Moreno, L.; Kroon, P. A.; Needs, P. W.; Hughes, D. A.; Santos-Buelga, C.; González-Paramas, A.; Cogolludo, A.; López-Sepulveda, R.; Duarte, J., Pérez-Vizcaino, F.; Glucuronidated and sulfated metabolites of the flavonoid quercetin prevent endothelial dysfunction but lack direct vasorelaxant effects in rat aorta. *Atherosclerosis.* ***2009***, *204 (1)*,34-39.

Loft, S.; Vistisen, K.; Ewertz, M.; Tjonneland, A.; Overvad, K., Poulsen, H. E.; Oxidative DNA damage estimated by 8-hydroxydeoxyguanosine excretion in humans: influence of smoking, gender and body mass index. *Carcinogenesis.* ***1992***, *13 (12)*,2241-2247.

Long, L. H.; Clement, M. V., Halliwell, B.; Artifacts in cell culture: Rapid generation of hydrogen peroxide on addition of (-)-epigallocatechin, (-)- epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media. *Biochemical and Biophysical Research Communications.* ***2000***, *273 (1)*,50-53.

López, M. E.; Zainal, T. A.; Chung, S. S.; Aiken, J. M.; Weindruch, R.; Chandan, K. S.; Ph.D; Facsm; Lester, P.; Ph.D; Fos; Osmo, O. P. H.; M.D, Ph.D, *Handbook of Oxidants and Antioxidants in Exercise*, Elsevier Science B.V., Amsterdam **2000**, pp. 831-880.

Lozano, C.; Juliá, L.; Jiménez, A.; Touriño, S.; Centelles, J. J.; Cascante, M., Torres, J.; Electron-transfer capacity of catechin derivatives and influence on the cell cycle and apoptosis in HT29 cells. *FEBS Journal.* ***2006***, *273 (11)*,2475-2486.

Määttä-Riihinen, K. R.; Kamal-Eldin, A., Törrönen, A. R.; Identification and quantification of phenolic compounds in berries of Fragaria and Rubus species (family rosaceae). *Journal of Agricultural and Food Chemistry.* ***2004***, *52 (20)*,6178-6187.

MacFarlane, P. M.; Wilkerson, J. E. R.; Lovett-Barr, M. R., Mitchell, G. S.; Reactive oxygen species and respiratory plasticity following intermittent hypoxia. *Respiratory Physiology & Neurobiology.* ***2008***, *164 (1-2)*,263-271.

Manach, C.; Scalbert, A.; Morand, C.; Rémésy, C., Jiménez, L.; Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition.* ***2004***, *79 (5)*,727-747.

Mannisto, P. T., Kaakkola, S.; Catechol-O-methyltransferase (COMT): Biochemistry, Molecular Biology, Pharmacology, and Clinical Efficacy of the New Selective COMT Inhibitors. *Pharmacol Rev.* ***1999***, *51 (4)*,593-628.

Marín, F. R.; Soler-Rivas, C.; Benavente-García, O.; Castillo, J., Pérez-Alvarez, J. A.; By-products from different citrus processes as a source of customized functional fibres. *Food Chemistry.* ***2007***, *100 (2)*,736-741.

Maritim, A. C.; Sanders, R. A., Watkins, J. B.; Diabetes, oxidative stress, and antioxidants: A review. *Journal of Biochemical and Molecular Toxicology.* ***2003***, *17 (1)*,24-38.

Martín-Carron, N.; García-Alonso, A.; Goñi, I., Saura-Calixto, F.; Nutritional and physiological properties of grape pomace as a potential food ingredient. *American Journal of Enology and Viticulture.* ***1997***, *48 (3)*,328-332.

Martínez-Cayuela, M.; Oxygen free radicals and human disease. *Biochimie.* ***1995***, *77 (3)*,147-161.

Martínez-González, M. A.; de la Fuente-Arrillaga, C.; Nunez-Cordoba, J. M.; Basterra-Gortari, F. J.; Beunza, J. J.; Vazquez, Z.; Benito, S.; Tortosa, A., Bes-Rastrollo, M.; Adherence to Mediterranean diet and risk of developing diabetes: prospective cohort study. *British Medical Journal.* ***2008***, *336 (7657)*,1348-1351.

Mashayekhi F, A.-h. F., Rezaie A, Zamani MJ, Khorasani R, Abdollahi M.; Alteration of cyclic nucleotides levels and oxidative stress in saliva of human subjects with periodontitis. *The Journal of Contemporary Dental Practice.* ***2005***, *6 (4)*,46-53.

Maslen, S. L.; Goubet, F.; Adam, A.; Dupree, P., Stephens, E.; Structure elucidation of arabinoxylan isomers by normal phase HPLC-MALDI-TOF/TOF-MS/MS. *Carbohydrate Research.* ***2007***, *342 (5)*,724-735.

Masquelier, J., Société Civile d'Investigations Pharmacologiques d'Aquitanie, Horpah Overseas Ltd., International Nutrition Company, US 4698360 **1987**.

Masters, C. J.; On the role of the peroxisome in the metabolism of drugs and xenobiotics. *Biochemical Pharmacology.* ***1998***, *56 (6)*,667-673.

Matthews, S.; Mila, I.; Scalbert, A., Donnelly, D. M. X.; Extractable and non-extractable proanthocyanidins in barks. *Phytochemistry.* ***1997***, *45 (2)*,405-410.

Medina, I.; Lois, S.; Lizárraga, D.; Pazos, M.; Touriño, S.; Cascante, M., Torres, J. L.; Functional fatty fish supplemented with grape procyanidins. Antioxidant and proapoptotic properties on colon cell lines. *Journal of Agricultural and Food Chemistry.* ***2006***, *54 (10)*,3598-3603.

Mehta, J. L.; Rasouli, N.; Sinha, A. K., Molavi, B.; Oxidative stress in diabetes: A mechanistic overview of its effects on atherogenesis and myocardial dysfunction. *The International Journal of Biochemistry & Cell Biology.* ***2006***, *38 (5-6)*,794-803.

Méndez, M. A.; Popkin, B. M.; Jakszyn, P.; Berenguer, A.; Tormo, M. J.; Sanchéz, M. J.; Quiros, J. R.; Pera, G.; Navarro, C.; Martínez, C.; Larrañaga, N.; Dorronsoro, M.; Chirlaque, M. D.; Barricarte, A.; Ardanaz, E.; Amiano, P.; Agudo, A., Gonzalez, C. A.; Adherence to a Mediterranean Diet Is Associated with Reduced 3-Year Incidence of Obesity. *The Journal of Nutrition.* ***2006***, *136 (11)*,2934-2938.

Meng, X.; Lee, M.-J.; Li, C.; Sheng, S.; Zhu, N.; Sang, S.; Ho, C.-T., Yang, C. S.; Formation and Identification of 4'-O-Methyl-(-)-Epigallocatechin in Humans. *Drug metabolism and disposition: the biological fate of chemicals.* ***2001***, *29 (6)*,789-793.

Merken, H. M., Beecher, G. R.; Measurement of food flavonoids by High-performance liquid chromatography: A Review. *Journal of Agricultural and Food Chemistry.* ***2000***, *48 (3)*,577-599.

Ministerio de Sanidad y Consumo, 2006.

Misra, U. K.; Bradford, B. U.; Handier, J. A., Thurman, R. G.; Chronic Ethanol Treatment Induces H_2O_2 Production Selectively in Pericentral Regions of the Liver Lobule. *Alcoholism: Clinical and Experimental Research.* ***1992***, *16 (5)*,839-842.

Monagas, M.; Quintanilla-López, J. E.; Gómez-Cordovés, C.; Bartolomé, B., Lebrón-Aguilar, R.; MALDI-TOF MS analysis of plant proanthocyanidins. *Journal of Pharmaceutical and Biomedical Analysis. In Press, Corrected Proof*.

Morrow, J. D.; Awad, J. A.; Boss, H. J.; Blair, I. A., Roberts Lj, II; Non-Cyclooxygenase-Derived Prostanoids (F2-Isoprostanes) are Formed In situ on Phospholipids. *Proceedings of the National Academy of Sciences of the United States of America.* ***1992***, *89 (22)*,10721-10725.

Morrow, J. D., Roberts, L. J.; The isoprostanes: Unique bioactive products of lipid peroxidation. *Progress in Lipid Research.* ***1997***, *36 (1)*,1-21.

Moure, A.; Cruz, J. M.; Franco, D.; Domínguez, J. M.; Sineiro, J.; Domínguez, H.; José Núñez, M., Parajó, J. C.; Natural antioxidants from residual sources. *Food Chemistry.* ***2001***, *72 (2)*,145-171.

Nakamura, A.; Nakajima, M.; Yamanaka, H.; Fujiwara, R., Yokoi, T.; Expression of UGT1A and UGT2B mRNA in human normal tissues and various cell lines. *Drug metabolism and disposition: the biological fate of chemicals.* ***2008***, *36 (8)*,1461-1464.

Nakayama, T.; Kaneko, M.; Kodama, M., Nagata, C.; Cigarette smoke induces DNA single-strand breaks in human cells. *Nature.* ***1985***, *314 (6010)*,462-464.

Nemmiche, S.; Chabane-Sari, D., Guiraud, P.; Role of a-tocopherol in cadmium-induced oxidative stress in Wistar rat's blood, liver and brain. *Chemico-Biological Interactions.* ***2007***, *170 (3)*,221-230.

Nguyen, T. D.; Maquart, F.-X., Monboisse, J.-C.; Ionizing radiations and collagen metabolism: from oxygen free radicals to radio-induced late fibrosis. *Radiation Physics and Chemistry.* ***2005***, *72 (2-3)*,381-386.

Nishikawa, M.; Reactive oxygen species in tumor metastasis. *Cancer Letters.* ***2008***, *266 (1)*,53-59.

Nishikawa, M.; Hashida, M., Takakura, Y.; Catalase delivery for inhibiting ROS-mediated tissue injury and tumor metastasis. *Advanced Drug Delivery Reviews.* ***2009***, *61 (4)*,319-326.

Nomura, T.; Kikuchi, M.; Kubodera, A., Kawakami, Y.; Proton-donative antioxidant activity of fucoxanthin with 1,1-Diphenyl-2-Picrylhydrazyl (DPPH). *IUBMB Life.* ***1997***, *42 (2)*,361-370.

Ohshima, H.; Yoshie, Y.; Auriol, S., Gilibert, I.; Antioxidant and pro-oxidant actions of flavonoids: effects on DNA damage induced by nitric oxide, peroxynitrite and nitroxyl anion. *Free Radical Biology and Medicine.* ***1998***, *25 (9)*,1057-1065.

Omata, N.; Tsukahara, H.; Ito, S.; Ohshima, Y.; Yasutomi, M.; Yamada, A.; Jiang, M.; Hiraoka, M.; Nambu, M.; Deguchi, Y., Mayumi, M.; Increased oxidative stress in childhood atopic dermatitis. *Life Sciences.* ***2001***, *69 (2)*,223-228.

Ou, B.; Huang, D.; Hampsch-Woodill, M.; Flanagan, J. A., Deemer, E. K.; Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: A comparative study. *Journal of Agricultural and Food Chemistry.* ***2002***, *50 (11)*,3122-3128.

Ozer Arican, E. B. K., and Sezai Sasmaz; Oxidative stress in patients with acne vulgaris. *Mediators of Inflammation.* ***2005***, *2005 (6)*,380-384.

Packer, L.; Rimbach, G., Virgili, F.; Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (*Pinus maritima*) bark, pycnogenol. *Free Radical Biology and Medicine.* ***1999***, *27 (5-6)*,704-724.

Pai, T. G.; Suiko, M.; Sakakibara, Y., Liu, M.-C.; Sulfation of Flavonoids and Other Phenolic Dietary Compounds by the Human Cytosolic Sulfotransferases. *Biochemical and Biophysical Research Communications.* ***2001***, *285 (5)*,1175-1179.

Pasco, J.; Nicholson, G. C.; Ng, F.; Henry, M. J.; Williams, L.; Kotowicz, M. A.; Hodge, J. M.; Dodd, S.; Kapczinski, F.; Gama, C., Berk, M.; Oxidative stress may be a common mechanism linking major depression and osteoporosis. *Acta Neuropsychiatrica.* ***2008***, *20 (3)*,112-116.

Pastore, A.; Federici, G.; Bertini, E., Piemonte, F.; Analysis of glutathione: implication in redox and detoxification. *Clinica Chimica Acta.* ***2003***, *333 (1)*,19-39.

Pazos, M.; Gallardo, J. M.; Torres, J. L., Medina, I.; Activity of grape polyphenols as inhibitors of the oxidation of fish lipids and frozen fish muscle. *Food Chemistry.* ***2005***, *92 (3)*,547-557.

Pérez-Jiménez, J.; Serrano, J.; Tabernero, M.; Arranz, S.; Díaz-Rubio, M. E.; García-Diz, L.; Goñi, I., Saura-Calixto, F.; Effects of grape antioxidant dietary fiber in cardiovascular disease risk factors. *Nutrition.* ***2008***, *24 (7-8)*,646-653.

Pinelo, M.; Arnous, A., Meyer, A. S.; Upgrading of grape skins: Significance of plant cell-wall structural components and extraction techniques for phenol release. *Trends in Food Science & Technology.* ***2006***, *17 (11)*,579-590.

Plumb, G. W.; De Pascual-Teresa, S.; Santos-Buelga, C.; Cheynier, V., Williamson, G.; Antioxidant properties of catechins and proanthocyanidins: effect of polymerisation, galloylation and glycosylation. *Free Radical Research.* ***1998***, *29 (4)*,351-358.

Poquet, L.; Clifford, M. N., Williamson, G.; Investigation of the metabolic fate of dihydrocaffeic acid. *Biochemical Pharmacology.* ***2008***, *75 (5)*,1218-1229.

Prior, R. L.; Wu, X., Schaich, K.; Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry.* ***2005***, *53 (10)*,4290-4302.

Proestos, C.; Sereli, D., Komaitis, M.; Determination of phenolic compounds in aromatic plants by RP-HPLC and GC-MS. *Food Chemistry.* ***2006***, *95 (1)*,44-52.

Pryor, W., Dooley, M.; Inactivation of human alpha 1-proteinase inhibitor by cigarette smoke: effect of smoke phase and buffer. *The American Review of Respiratory Disease.* ***1985***, *131 (6)*,941-943.

Quintela, O.; Cruz, A.; Concheiro, M.; De Castro, A., López-Rivadulla, M.; Metodología LC-MS. Aspectos generales de la técnica y sus aplicaciones en el campo de la toxicología. *Revista de Toxicología.* ***2005***, *22* 7-14.

Rahman, I., *Asthma and COPD (Second Edition)*, Academic Press, Oxford **2009**, pp. 293-312.

Ratty, A., Das, N.; Effects of flavonoids on nonenzymatic lipid peroxidation: structure-activity relationship. *Biochemical Medicine and Metabolic Biology.* ***1988***, *39 (1)*,69-79.

Rechner, A. R.; Smith, M. A.; Kuhnle, G.; Gibson, G. R.; Debnam, E. S.; Srai, S. K. S.; Moore, K. P., Rice-Evans, C. A.; Colonic metabolism of dietary polyphenols: influence of structure on microbial fermentation products. *Free Radical Biology and Medicine.* ***2004***, *36 (2)*,212-225.

Redondo-Cuenca, A.; Villanueva-Suárez, M. J., Mateos-Aparicio, I.; Soybean seeds and its by-product okara as sources of dietary fibre. Measurement by AOAC and Englyst methods. *Food Chemistry.* ***2008***, *108 (3)*,1099-1105.

Regoli, F., Winston, G. W.; Quantification of total oxidant scavenging capacity of antioxidants for peroxynitrite, peroxyl radicals, and hydroxyl radicals. *Toxicology and Applied Pharmacology.* ***1999***, *156 (2)*,96-105.

Rice-Evans, C.; Lester, P.; Midori, H., Toshikazu, Y., *Antioxidant Food Supplements in Human Health*, Academic Press, San Diego **1999**, pp. 239-253.

Rice-Evans, C. A.; Miller, N. J., Paganga, G.; Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine.* ***1996***, *20 (7)*,933-956.

Richardson, P.; Phytochemical methods. Second edition. By J. B. Harborne. *Brittonia.* ***1985***, *37 (3)*,309-309.

Rigaud, J.; Escribano-Bailón, M. T.; Prieur, C.; Souquet, J. M., Cheynier, V.; Normal-phase high-performance liquid chromatographic separation of procyanidins from cacao beans and grape seeds. *Journal of Chromatography A.* ***1993***, *654 (2)*,255-260.

Robards, K., Antolovich, M.; Analytical Chemistry of Fruit Bioflavonoids: A Review. *Analyst.* ***1997****(122)*.

Roberts Ii, L. J., Morrow, J. D.; The generation and actions of isoprostanes. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism.* ***1997***, *1345 (2)*,121-135.

Roberts Ii, L. J.; Montine, T. J.; Markesbery, W. R.; Tapper, A. R.; Hardy, P.; Chemtob, S.; Dettbarn, W. D., Morrow, J. D.; Formation of Isoprostane-like Compounds (Neuroprostanes) in Vivo from Docosahexaenoic Acid. *Journal of Biological Chemistry.* ***1998***, *273 (22)*,13605-13612.

Sánchez-Moreno, C.; Review: Methods used to evaluate the free radical scavenging activity in foods and biological systems. *Food Science and Technology International.* ***2002***, *8 (3)*,121-137.

Sánchez-Rabaneda, F.; Jáuregui, O.; Lamuela-Raventós, R. M.; Viladomat, F.; Bastida, J., Codina, C.; Qualitative analysis of phenolic compounds in apple pomace using liquid chromatography coupled to mass spectrometry in tandem mode. *Rapid Communications in Mass Spectrometry.* ***2004***, *18 (5)*,553-563.

Sang, S.; Lee, M. J.; Yang, I.; Buckley, B., Yang, C. S.; Human urinary metabolite profile of tea polyphenols analyzed by liquid chromatography/electrospray ionization tandem mass spectrometry with data-dependent acquisition. *Rapid Communications in Mass Spectrometry.* ***2008***, *22 (10)*,1567-1578.

Santos-Buelga, C. and Scalbert, A.; Proanthocyanidins and tannin-like compounds - nature, occurrence, dietary intake and effects on nutrition and health. *Journal of the Science of Food and Agriculture.* ***2000***, *80 (7)*,1094-1117.

Sarni-Manchado, P.; Cheynier, V., Moutounet, M.; Interactions of grape seed tannins with salivary proteins. *Journal of Agricultural and Food Chemistry.* ***1999***, *47 (1)*,42-47.

Saulnier, L., Thibault, J. F.; Ferulic acid and diferulic acids as components of sugar-beet pectins and maize bran heteroxylans. *Journal of the Science of Food and Agriculture.* ***1999***, *79 (3)*,396-402.

Saura-Calixto, F.; Antioxidant Dietary Fiber Product: A New Concept and a Potential Food Ingredient. *Journal of Agricultural and Food Chemistry.* ***1998***, *46 (10)*,4303-4306.

Saura-Calixto, F., Larrauri García, J. A., Concentrate of natural antioxidant dietetic fiber from grape, and preparation process. CSIC (Ed.) **1999**.

Scalbert, A., Williamson, G.; Dietary intake and bioavailability of polyphenols. *The Journal of Nutrition.* ***2000***, *130 (8)*,2073S-2085S.

Schermann, J.-P., *Spectroscopy and modeling of biomolecular building blocks*, Elsevier, Amsterdam **2008**, pp. 129-207.

Schieber, A.; Stintzing, F. C., Carle, R.; By-products of plant food processing as a source of functional compounds : recent developments. *Trends in Food Science & Technology.* ***2001***, *12 (11)*,401-413.

Schieber, A.; Hilt, P.; Streker, P.; Endreß, H.-U.; Rentschler, C., Carle, R.; A new process for the combined recovery of pectin and phenolic compounds from apple pomace. *Innovative Food Science & Emerging Technologies.* ***2003***, *4 (1)*,99-107.

Schoenberg, M. H.; Birk, D., Beger, H. G.; Oxidative stress in acute and chronic pancreatitis. *The American Journal of Clinical Nutrition.* ***1995***, *62 (6)*,1306S-1314.

Scholz, S., Williamson, G.; Interactions affecting the bioavailability of dietary polyphenols in vivo. *International Journal for Vitamin and Nutrition Research.* ***2007***, *77 (3)*,224-235.

Schrader, M., Fahimi, H. D.; Peroxisomes and oxidative stress. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research.* ***2006***, *1763 (12)*,1755-1766.

Schreck R; Albermann K, Baeuerle, P.; Nuclear factor kappa B: an oxidative stress-responsive transcription factor of eukaryotic cells (a review). *Free radical research communications.* ***1992***, *17 (4)*,221-237.

Schulz, T. J.; Zarse, K.; Voigt, A.; Urban, N.; Birringer, M., Ristow, M.; Glucose restriction extends caenorhabditis elegans life span by inducing mitochondrial respiration and increasing oxidative stress. *Cell Metabolism.* ***2007***, *6 (4)*,280-293.

Schwarz, K.; Huang, S.; German, J. B.; Tiersch, B.; Hartmann, J., Frankel, E. N.; Activities of antioxidants are affected by colloidal properties of oil-in-water and water-in-oil emulsions and bulk oils. *Journal of Agricultural and Food Chemistry.* ***2000***, *48* 4874-4882.

Sengupta, P. K., Kasha, M.; Excited state proton-transfer spectroscopy of 3-hytdroxyflavone and quercetin. *Chemical Physics Letters.* ***1979***, *68 (2-3)*,382-385.

Sesink, A. L. A.; Arts, I. C. W.; Faassen-Peters, M., Hollman, P. C. H.; Intestinal uptake of quercetin-3-glucoside in rats involves hydrolysis by lactase phlorizin hydrolase. *The Journal of Nutrition.* ***2003***, *133 (3)*,773-776.

Shamat, M. A.; The role of the gastrointestinal microflora in the metabolism of drugs. *International Journal of Pharmaceutics.* ***1993***, *97 (1-3)*,1-13.

Shi, H.; Hudson, L. G., Liu, K. J.; Oxidative stress and apoptosis in metal ion-induced carcinogenesis. *Free Radical Biology and Medicine.* ***2004***, *37 (5)*,582-593.

Shilov, V., Sergienko, V.; Oxidative stress in keratinocytes as an etiopathogenetic factor of psoriasis. *Bulletin of Experimental Biology and Medicine.* ***2000***, *129 (4)*,309-313.

Shoji, T.; Masumoto, S.; Moriichi, N.; Akiyama, H.; Kanda, T.; Ohtake, Y., Goda, Y.; Apple procyanidin oligomers absorption in rats after oral administration: analysis of procyanidins in plasma using the porter method and high-performance liquid chromatography/tandem mass spectrometry. *Journal of Agricultural and Food Chemistry.* ***2006***, *54 (3)*,884-892.

Shou, W. Z., Naidong, W.; Simple means to alleviate sensitivity loss by trifluoroacetic acid (TFA) mobile phases in the hydrophilic interaction chromatography-electrospray tandem mass spectrometric (HC-ESI/MS/MS) bioanalysis of basic compounds. *Journal of Chromatography B.* ***2005***, *825 (2)*,186-192.

Simmonds, N. J.; David, B., Paul, G. W., *Immunopharmacology of Free Radical Species*, Academic Press, London **1995**, pp. 143-174.

Singh, U., Jialal, I.; Oxidative stress and atherosclerosis. *Pathophysiology.* ***2006***, *13 (3)*,129-142.

Somogyi, Á.; Károly, V.; András, T., Akos, V., *Medical Applications of Mass Spectrometry*, Elsevier, Amsterdam **2008**, pp. 93-140.

Spácil, Z.; Nováková, L., Solich, P.; Analysis of phenolic compounds by high performance liquid chromatography and ultra performance liquid chromatography. *Talanta.* ***2008***, *76 (1)*,189-199.

Spector, A.; Oxidative stress-induced cataract: mechanism of action. *The FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology.* ***1995***, *9 (12)*,1173-1182.

Spencer, J. P. E.; Chaudry, F.; Pannala, A. S.; Srai, S. K.; Debnam, E., Rice-Evans, C.; Decomposition of cocoa procyanidins in the gastric milieu. *Biochemical and Biophysical Research Communications.* ***2000***, *272 (1)*,236-241.

Spencer, J. P. E.; Schroeter, H.; Shenoy, B.; S. Srai, S. K.; Debnam, E. S., Rice-Evans, C.; Epicatechin is the primary bioavailable form of the procyanidin dimers B2 and B5 after transfer across the small intestine. *Biochemical and Biophysical Research Communications.* ***2001***, *285 (3)*,588-593.

Spigno, G.; Tramelli, L., De Faveri, D. M.; Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics. *Journal of Food Engineering.* ***2007***, *81 (1)*,200-208.

Spiteller, G.; Peroxyl radicals: Inductors of neurodegenerative and other inflammatory diseases. Their origin and how they transform cholesterol, phospholipids, plasmalogens, polyunsaturated fatty acids, sugars, and proteins into deleterious products. *Free Radical Biology and Medicine.* ***2006***, *41 (3)*,362-387.

Stern, R.; Kogan, G.; Jedrzejas, M. J., Soltés, L.; The many ways to cleave hyaluronan. *Biotechnology Advances.* ***2007***, *25 (6)*,537-557.

Stief, T. W.; The blood fibrinolysis/deep-sea analogy: A hypothesis on the cell signals singlet oxygen/photons as natural antithrombotics. *Thrombosis Research.* ***2000***, *99 (1)*,1-20.

Stojceska, V.; Ainsworth, P.; Plunkett, A.; Ibanoglu, E., Ibanoglu, S.; Cauliflower by-products as a new source of dietary fibre, antioxidants and proteins in cereal based ready-to-eat expanded snacks. *Journal of Food Engineering.* ***2008***, *87 (4)*,554-563.

Stults, J. T.; Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). *Current Opinion in Structural Biology.* ***1995***, *5 (5)*,691-698.

Sudjaroen, Y.; Haubner, R.; Würtele, G.; Hull, W. E.; Erben, G.; Spiegelhalder, B.; Changbumrung, S.; Bartsch, H., Owen, R. W.; Isolation and structure elucidation of phenolic antioxidants from Tamarind (Tamarindus indica L.) seeds and pericarp. *Food and Chemical Toxicology.* ***2005***, *43 (11)*,1673-1682.

Sun, B.; Leandro, C.; Ricardo da Silva, J. M., Spranger, I.; Separation of Grape and Wine Proanthocyanidins According to Their Degree of Polymerization. *Journal of Agricultural and Food Chemistry.* ***1998***, *46 (4)*,1390-1396.

Sun, B.; Leandro, M. C.; de Freitas, V., Spranger, M. I.; Fractionation of red wine polyphenols by solid-phase extraction and liquid chromatography. *Journal of Chromatography A.* ***2006***, *1128 (1-2)*,27-38.

Takeoka, G. R., Dao, L. T.; Antioxidant Constituents of Almond [Prunus dulcis (Mill.) D.A. Webb] Hulls. *Journal of Agricultural and Food Chemistry.* ***2003***, *51 (2)*,496-501.

Taubert, D.; Roesen, R., Schomig, E.; Effect of Cocoa and Tea Intake on Blood Pressure: A Meta-analysis. *Archives of Internal Medicine.* ***2007***, *167 (7)*,626-634.

Thannickal, V. J., Fanburg, B. L.; Activation of an H_2O_2-generating NADH oxidase in human lung fibroblasts by transforming growth factor b-1. *Journal of Biological Chemistry.* ***1995***, *270 (51)*,30334-30338.

Torres, J.; Varela, B.; Brillas, E., Juliá, L.; Tris(2,4,6-trichloro-3,5-dinitrophenyl)methyl radical: a new stable coloured magnetic species as a chemosensor for natural polyphenols. *Chemical Communications.* ***2003***, *1* 74-75.

Torres, J. L., Bobet, R.; New flavanol derivatives from grape (*Vitis vinifera*) by-products. Antioxidant Aminoethylthio-flavan-3-ol conjugates from a polymeric waste fraction used as a source of flavanols. *Journal of Agricultural and Food Chemistry.* ***2001a***, *49 (10)*,4627-4634.

Torres, J. L., Lozano, C.; Chromatographic characterization of proanthocyanidins after thiolysis with cysteamine. *Chromatographia.* ***2001b***, *54 (7-8)*,523-526.

Torres, J. L.; Varela, B.; García, M. T.; Carilla, J.; Matito, C.; Centelles, J. J.; Cascante, M.; Sort, X., Bobet, R.; Valorization of grape (Vitis vinifera) byproducts. Antioxidant and biological properties of polyphenolic fractions differing in procyanidin composition and flavonol content. *Journal of Agricultural and Food Chemistry.* ***2002***, *50 (26)*,7548-7555.

Torres, J. L.; Carreras, A.; Jiménez, A.; Brillas, E.; Torrelles, X.; Rius, J., Juliá, L.; Reducing Power of Simple Polyphenols by Electron-Transfer Reactions Using a New Stable Radical of the PTM Series, Tris(2,3,5,6-tetrachloro-4-nitrophenyl)methyl Radical. *The Journal of Organic Chemistry.* ***2007***, *72 (10)*,3750-3756.

Touriño, S.; Selga, A.; Jiménez, A.; Juliá, L.; Lozano, C.; Lizárraga, D.; Cascante, M., Torres, J. L.; Procyanidin fractions from pine (Pinus pinaster) bark: Radical scavenging power in solution, antioxidant activity in emulsion, and Antiproliferative effect in melanoma cells. *Journal of Agricultural and Food Chemistry.* ***2005***, *53 (12)*,4728-4735.

Touriño, S.; Lizárraga, D.; Carreras, A.; Lorenzo, S.; Ugartondo, V.; Mitjans, M.; Vinardell, M. P.; Juliá, L.; Cascante, M., Torres, J. L.; Highly galloylated tannin fractions from witch hazel (Hamamelis virginiana) bark: Electron transfer capacity, in vitro antioxidant activity, and effects on skin-related cells. *Chemical Research in Toxicology.* ***2008a***, *21 (3)*,696-704.

Touriño, S.; Fuguet, E.; Jáuregui, O.; Saura-Calixto, F.; Cascante, M., Torres, J. L.; High-resolution liquid chromatography/electrospray ionization time-of-flight mass spectrometry combined with liquid chromatography/electrospray ionization tandem mass spectrometry to identify polyphenols from grape antioxidant dietary fiber. *Rapid Communications in Mass Spectrometry.* ***2008b***, *22 (22)*,3489-3500.

Touriño,S; Contribución al establecimiento de las bases científicas para el uso de fibra dietética antioxidante y fracciones polifenólicas en la prevención de cáncer.(Tesis), Departament de Química Biològica i Modelització molecular (QBM), Grup: Nutracèutics i Radicals lliures (IQAC-CSIC), In: University of Barcelona, **2009**

Touriño, S.; Fuguet, E.; Vinardell, M. P.; Cascante, M., Torres, J. L.; Phenolic metabolites of grape antioxidant dietary fiber in rat urine. *Journal of Agricultural and Food Chemistry.* ***2009*** *(Submitted)*.

Tsang, C.; Auger, C.; Mullen, W.; Bornet, A.; Rouanet, J. M.; Crozier, A., Teissedre, P. L.; The absorption, metabolism and excretion of flavan-3-ols and procyanidins following the ingestion of a grape seed extract by rats. *The British Journal of Nutrition.* ***2005***, *94 (2)*,170-181.

Ugartondo, V.; Mitjans, M.; Touriño, S.; Torres, J. L., Vinardell, M. P.; Comparative antioxidant and cytotoxic effect of procyanidin fractions from grape and pine. *Chemical Research in Toxicology.* ***2007***, *20 (10)*,1543-1548.

Valko, M.; Izakovic, M.; Mazur, M.; Rhodes, C. J., Telser, J.; Role of oxygen radicals in DNA damage and cancer incidence. *Molecular and Cellular Biochemistry.* ***2004***, *266 (1-2)*,37-56.

Valko, M.; Rhodes, C. J.; Moncol, J.; Izakovic, M., Mazur, M.; Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions.* ***2006***, *160 (1)*,1-40.

Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M. T. D.; Mazur, M., Telser, J.; Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology.* ***2007***, *39 (1)*,44-84.

van der Vliet, A., Bast, A.; Role of reactive oxygen species in intestinal diseases. *Free Radical Biology and Medicine.* ***1992***, *12 (6)*,499-513.

VanRollins, M.; Woltjer, R. L.; Yin, H.; Morrow, J. D., Montine, T. J.; F2-Dihomo-isoprostanes arise from free radical attack on adrenic acid. *Journal of Lipid Research.* ***2008***, *49 (5)*,995-1005.

Vékey, K.; Mass spectrometry and mass-selective detection in chromatography. *Journal of Chromatography A.* ***2001***, *921 (2)*,227-236.

Victor, V. M.; Rocha, M., De la Fuente, M.; Immune cells: free radicals and antioxidants in sepsis. *International Immunopharmacology.* ***2004***, *4 (3)*,327-347.

Vidavalur, R.; Otani, H.; Singal, P., Maulik, N.; Significance of wine and resveratrol in cardiovascular disease: French paradox revisited. *Experimental Cardiology.* ***2006***, *11 (3)*, 217-225.

Virág, L.; Szabó, É.; Gergely, P., Szabó, C.; Peroxynitrite-induced cytotoxicity: mechanism and opportunities for intervention. *Toxicology Letters.* ***2003***, *140-141* 113-124.

Virág, L.; Poly(ADP-ribosyl)ation in asthma and other lung diseases. *Pharmacological Research.* ***2005***, *52 (1)*,83-92.

Vivas, N.; Nonier, M. F.; V ivas De Gaulejac, N.; Absalon, C.; Bertrand, A., Mirabel, M.; Differentiation of proanthocyanidin tannins from seeds, skins and stems of grapes (*Vitis vinifera*) and heartwood of Quebracho (*Schinopsis balansae*) by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and thioacidolysis/liquid chromatography/electrospray ionization mass spectrometry. *Analytica Chimica Acta.* ***2004***, *513 (1)*,247-256.

Vorbach, C.; Harrison, R., Capecchi, M. R.; Xanthine oxidoreductase is central to the evolution and function of the innate immune system. *Trends in Immunology.* ***2003***, *24 (9)*,512-517.

Walgren, R. A.; Lin, J.-T.; Kinne, R. K. H., Walle, T.; Cellular uptake of dietary flavonoid quercetin 4'-b-glucoside by sodium-dependent glucose transporter SGLT1. *The Journal of Pharmacology and Experimental Therapeutics.* ***2000***, *294 (3)*,837-843.

Walle, T.; Browning, A. M.; Steed, L. L.; Reed, S. G., Walle, U. K.; Flavonoid Glucosides Are Hydrolyzed and Thus Activated in the Oral Cavity in Humans. *The Journal of Nutrition.* ***2005***, *135 (1)*,48-52.

Ward, N. C.; Croft, K. D.; Puddey, I. B., Hodgson, J. M.; Supplementation with grape seed polyphenols results in increased urinary excretion of 3-hydroxyphenylpropionic acid, an important metabolite of proanthocyanidins in humans. *Journal of Agricultural and Food Chemistry.* ***2004***, *52 (17)*,5545-5549.

Wasson, G. R.; McKelvey-Martin, V. J., Downes, C. S.; The use of the comet assay in the study of human nutrition and cancer. *Mutagenesis.* ***2008***, *23 (3)*,153-162.

Watson, D. G.; Atsriku, C., Oliveira, E. J.; Review role of liquid chromatography-mass spectrometry in the analysis of oxidation products and antioxidants in biological systems. *Analytica Chimica Acta.* ***2003***, *492 (1-2)*,17-47.

Wayner, D. D.; Burton, G. W.; Ingold, K.; Barclay, L. R., Locke, S. J.; The relative contributions of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma. . *Biochimica et Biophysica Acta (BBA) - General Subjects.* ***1987***, *924 (3)*,408-419.

Williams, V. M.; Porter, L. J., Hemingway, R. W.; Molecular weight profiles of proanthocyanidin polymers. *Phytochemistry.* ***1983***, *22 (2)*,569-572.

Winterbourn, C. C.; Toxicity of iron and hydrogen peroxide: the Fenton reaction. *Toxicology Letters.* ***1995***, *82-83* 969-974.

Wolfe, K. L., Liu, R. H.; Apple peels as a value-added food ingredient. *Journal of Agricultural and Food Chemistry.* ***2003***, *51 (6)*,1676-1683.

Wolfgang Hümmer, P. S.; Analysis of proanthocyanidins. *Molecular Nutrition & Food Research.* ***2008***, *52 (12)*,1381-1398.

Wood, L. G.; Gibson, P. G., Garg, M. L.; Biomarkers of lipid peroxidation, airway inflammation and asthma. *The European Respiratory Journal: Official Journal of the European Society for Clinical Respiratory Physiology.* ***2003***, *21 (1)*,177-186.

Wright, J. S.; Johnson, E. R., DiLabio, G. A.; Predicting the activity of phenolic antioxidants: theoretical method, analysis of substituent effects, and application to major families of antioxidants. *Journal of the American Chemical Society.* ***2001***, *123 (6)*,1173-1183.

Xu, M.; Guo, H.; Han, J.; Sun, S.-f.; Liu, A.-h.; Wang, B.-r.; Ma, X.-c.; Liu, P.; Qiao, X.; Zhang, Z.-c., Guo, D.-a.; Structural characterization of metabolites of salvianolic acid B from *Salvia miltiorrhiza* in normal and antibiotic-treated rats by liquid chromatography-mass spectrometry. *Journal of Chromatography B.* ***2007***, *858 (1-2)*,184-198.

Yanagida, A.; Kanda, T.; Shoji, T.; Ohnishi-Kameyama, M., Nagata, T.; Fractionation of apple procyanidins by size-exclusion chromatography. *Journal of Chromatography A.* ***1999***, *855 (1)*,181-190.

Yang, C. S.; Lee, M.-J., Chen, L.; Human salivary tea catechin levels and catechin esterase activities: Implication in human cancer prevention studies. *Cancer Epidemiology, Biomarkers & Prevention: a Publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology.* ***1999***, *8 (1)*,83-89.

Yen, G. C.; Chen, H. Y., Peng, H. H.; Antioxidant and pro-oxidant effects of various tea extracts. *Journal of Agricultural and Food Chemistry.* ***1997***, *45 (1)*,30-34.

Yoshino, M.; Haneda, M.; Naruse, M., Murakami, K.; Prooxidant activity of flavonoids: copper-dependent strand breaks and the formation of 8-Hydroxy-2'-deoxyguanosine in DNA. *Molecular Genetics and Metabolism.* ***1999***, *68 (4)*,468-472.

Zakhari, S., *Alcohol: Research & health* 2006, 245-254.

Zandi, P., Gordon, M. H.; Antioxidant activity of extracts from old tea leaves. *Journal of Agricultural and Food Chemistry.* ***1999***, *64* 285-288.

Zhang, L.; Zuo, Z., Lin, G.; Intestinal and hepatic glucuronidation of flavonoids. *Molecular Pharmaceutics.* ***2007***, *4 (6)*,833-845.

Zhao, W.; Spitz, D. R.; Oberley, L. W., Robbins, M. E. C.; Redox modulation of the pro-fibrogenic mediator plasminogen activator inhibitor-1 following ionizing radiation. *Cancer Research.* ***2001***, *61 (14)*,5537-5543.

Zhu, B. T.; Ezell, E. L., Liehr, J. G.; Catechol-O-methyltransferase-catalyzed rapid O-methylation of mutagenic flavonoids. Metabolic inactivation as a possible reason for their lack of carcinogenicity in vivo. *Journal of Biological Chemistry.* ***1994***, *269 (1)*,292-299.

8. ANEXOS

Los anexos constan de dos apartados. El apartado (**8.1**) incluye las publicaciones complementarias de la tesis, realizadas en colaboración, que han permitido llegar a una idea y a unas conclusiones más globales sobre el tema de estudio. También se incluye un trabajo de colaboración con el Departamento de Genética Molecular del CSIC.

Las publicaciones complementarias están colocadas por orden cronológico:

I. **Electron-transfer capacity of catechin derivatives and influence on the cell cycle and apoptosis in HT29 cells.** Lozano, C.; Juliá, L.; Jiménez, A.; Touriño, S.; Centelles, J.J.; Cascante, M. and Torres J.L; *FEBS Journal*; 273(11): 2475-86; 2006 *(ver pág. 173)*

II. **Functional fatty fish supplemented with grape procyanidins. Antioxidant and proapoptotic properties on colon cell lines.** Medina, I.; Lois, S.; Lizárraga, D.; Pazos, M.; Touriño, S.; Cascante, M. and Torres; J.L.; *J. Agric. Food Chem.*; 54 (10): 3598 -3603; 2006 *(ver pág. 187)*

III. **Procyanidins from pine bark: relationships between structure, composition and antiradical activity**. Jerez, M.; Touriño, S.; Sineiro, J.; Torres, J.L. and Núñez, M.J.; *Food Chem.*; 104 (2): 518-527; 2007 *(ver pág. 195)*

IV. **The importance of polymerization and galloylation for the antiproliferative properties of procyanidin-rich natural extracts.** Lizárraga, D.; Lozano, C.; Briede´, J. J.; Van Delft, J. H.; Touriño, S.; Centelles, J. J.; Torres J.L. and Cascante, M.; *FEBS Journal*; 274: 4802–4811; 2007 *(ver pág. 207)*

V. **Comparative antioxidant and cytotoxic effect of procyanidin fractions from grape and pine.** Ugartondo, V.; Mitjans, M.; Touriño, S.; Torres, J.L. and Vinardell M.P.; *Chem. Res. Toxicol.*; 20: 1543–1548; 2007 *(ver pág. 219)*

VI. **Witch Hazel (*Hamamelis virginiana*) fractions and the importance of gallate moieties electron transfer capacities in their antitumoral properties**. Lizárraga, D.; Touriño, S.; Reyes-Zurita, F.J.; De Kok T.M.; Van Delft J.H.; Maas, L.M.; Briedé J.J.; Centelles J.J.; Torres J.L. and Cascante M.; *J. Agric. Food Chem.;* 56(24):11675–11682, 2008 *(ver pág. 227)*

VII. **The maize ZmMYB42 represses the phenylpropanoid pathway and affects the cell wall structure, composition and degradability in *Arabidopsis thaliana*.** Sonbol, F-M.; Capellades, M.; Fornalé, S.; Ruel, K.; Touriño, S.; Torres, J.L.; Rovira, P.; Encina, A.; Puigdomènech, P.; Rigau, J. and Caparrós-Ruiz, D. *Plant Mol. Biol.*; 70(3): 283-296, 2009 *(ver pág. 287)*

El segundo apartado de los Anexos (**8.2**) incluye el material suplementario de las publicaciones troncales de la presente tesis.

8.2.1 Material suplementario publicación 1 *(ver pág. 255)*

8.1 Publicaciones complementarias

I. Electron-transfer capacity of catechin derivatives and influence on the cell cycle and apoptosis in HT29 cells.

Lozano, C.; Juliá, L.; Jiménez, A.; ***Touriño, S****.;* Centelles, J.J.; Cascante, M. and Torres J.L.; ***FEBS Journal***; 273(11): 2475-86; **2006**

the FEBS Journal

Electron-transfer capacity of catechin derivatives and influence on the cell cycle and apoptosis in HT29 cells

Carles Lozano[1,2], Lluís Juliá[1], Aurora Jiménez[1], Sonia Touriño[1], Josep J. Centelles[2], Marta Cascante[2] and Josep Lluís Torres[1]

1 Institute for Chemical and Environmental Research (IIQAB-CSIC), Barcelona, Spain
2 Department of Biochemistry and Molecular Biology, Associated Unit to CSIC, University of Barcelona, Spain

Keywords
apoptosis; catechins; electron transfer; free radicals; gallate ester

Correspondence
J. L. Torres, Institute for Chemical and Environmental Research (IIQAB-CSIC), Jordi Girona 18–26, 08034 Barcelona, Spain
Fax: +34 93 204 5904
Tel: +34 93 400 6112
E-mail: jltqbp@iiqab.csic.es

(Received 30 January 2006, revised 27 March 2006, accepted 31 March 2006)

doi:10.1111/j.1742-4658.2006.05255.x

Galloylated and nongalloylated catechin conjugates with cysteine derivatives have been synthesized and evaluated for their capacity to scavenge free radicals and to influence crucial functions (cell cycle, apoptosis) in HT29 colon carcinoma cells. We show that the nonphenolic part of the molecule modified the capacity of catechins to donate hydrogen atoms and to transfer electrons to free radicals. Nongalloylated derivatives did not significantly influence either the cell cycle or apoptosis. Among the galloylated species, 4β-[*S*-(*O*-ethyl-cysteinyl)]epicatechin 3-*O*-gallate, which showed a high electron-transfer capacity (5 e^- per molecule), arrested the cell cycle and induced apoptosis as expected for galloylated catechins such as tea (–)-epigallocatechin 3-*O*-gallate. 4β-[*S*-(*N*-Acetyl-*O*-methyl-cysteinyl)]epicatechin 3-*O*-gallate, which showed the highest hydrogen-donating capacity (10 H per molecule) while keeping the electron-transfer capacity low (2.9 e^- per molecule), did not trigger any significant apoptosis. The gallate moiety did not appear to be sufficient for the pro-apoptotic effect of the catechin derivatives in HT29 cells. Instead, a high electron-transfer capacity is more likely to be behind this effect. The use of stable radicals sensitive exclusively to electron transfer may help to design molecules with either preventive scavenging action (high hydrogen donation, low electron transfer) or therapeutic pro-apoptotic activity (high electron transfer).

Polyphenols of plant origin are potent free-radical scavengers [1,2] and are increasingly appreciated as chemopreventive agents against conditions such as cancer and cardiovascular diseases [3,4]. They appear to minimize the number of oxidative DNA mutations and protein modifications by scavenging harmful reactive oxygen species (ROS) [5]. Moreover, some polyphenols of the flavonoid type show antiproliferative and pro-apoptotic activities [6]. In particular, flavanols (catechins) from tea, grape and other sources may exert their beneficial action by a combination of prophylactic and therapeutic effects related to both their radical-scavenging capacity and their influence on the cell machinery [7,8]. The gallate moiety appears to be behind the influence of some catechins on the cell cycle and the induction of apoptosis in tumour cells [9], probably via enzyme–ligand interactions with some key protein domains [10,11]. Another line of evidence suggests that some catechins induce apoptosis via the formation of the superoxide radical from molecular oxygen by electron transfer [12]. The superoxide anion may participate directly in the apoptotic toxic response

Abbreviations
AMCys-Cat, 4β-[*S*-(*N*-acetyl-*O*-methyl-cysteinyl)]catechin; AMCys-Ec, 4β-[*S*-(*N*-acetyl-*O*-methyl-cysteinyl)]epicatechin; AMCys-EcG, 4β-[*S*-(*N*-acetyl-*O*-methyl-cysteinyl)]epicatechin 3-*O*-gallate; ARP, antiradical power; Cys-Ec, 4β-(S-cysteinyl)epicatechin; DPPH, 1,1-diphenyl-2-picrylhydrazyl free radical; Ec, (–)-epicatechin; ECys-Cat, 4β-[*S*-(*O*-ethyl-cysteinyl)]catechin; ECys-Ec, 4β-[*S*-(*O*-ethyl-cysteinyl)]epicatechin; ECys-EcG, 4β-[*S*-(*O*-ethyl-cysteinyl)]epicatechin 3-*O*-gallate; EgcG, (–)-epigallocatechin 3-*O*-gallate; HNTTM, tris(2,4,6-trichloro-3,5-dinitrophenyl)methyl radical; PI, propidium iodide; ROS, reactive oxygen species.

or be involved in the regulation of apoptotic pathways [13]. Pro-apoptotic tea (–)-epigallocatechin 3-*O*-gallate (EgcG) which includes two trihydroxybenzene moieties (ring B and gallate ester) appears to be a particularly efficient reducing (electron donating) agent [14].

Catechin conjugates with thiols have been described [15–17]. The derivatives are obtained by acid depolymerization of grape polymeric procyanidins in the presence of the thiol and show higher antiradical capacity than their underivatized counterparts in the 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) assay [16,17]. Interestingly, the nonphenolic part of the molecule appears to influence the capacity of the conjugates to penetrate biological membranes, particularly the skin layers [18]. We present evidence that these nonphenolic moieties may also modulate the redox behaviour of molecules and their capacity to induce apoptosis by a mechanism involving electron transfer, whereas the gallate moiety may be a necessary, but not sufficient, condition to explain the pro-apoptotic effect.

Results

Synthesis and purification

Catechin conjugates with cysteine derivatives (Fig. 1) were generated by acid depolymerization of grape procyanidins and purified essentially as described

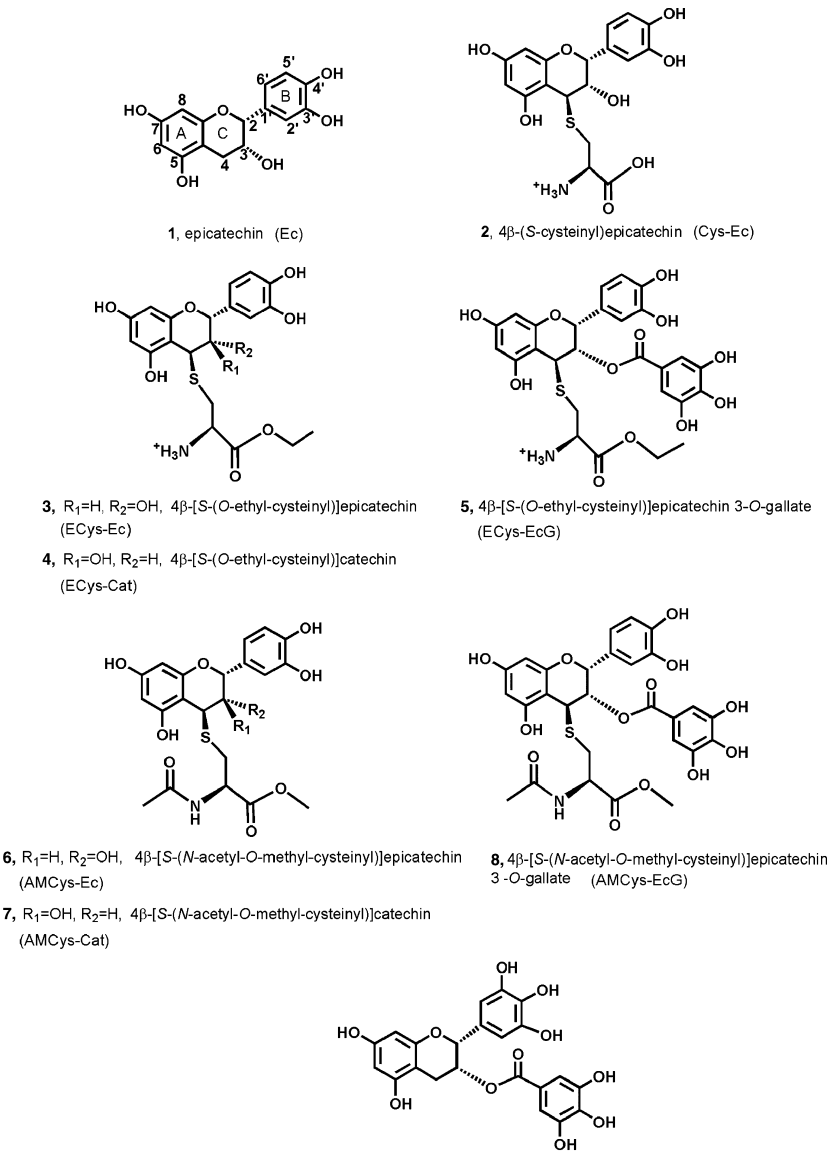


Fig. 1. Structures of the catechin thioconjugates.

previously [17]. 4β-[*S*-(*O*-Ethyl-cysteinyl)]epicatechin (ECys-Ec), 4β-[*S*-(*O*-ethyl-cysteinyl)]catechin (ECys-Cat) and 4β-[*S*-(*O*-ethyl-cysteinyl)]epicatechin 3-*O*-gallate (ECys-EcG) (**3**–**5**) were obtained from the ethyl ester of cysteine and separated from the crude depolymerization mixture using a strong cation-exchange resin (MacroPrep™ High S 50 μm) by taking advantage of the free amino function on the cysteinyl moiety. 4β-[*S*-(*N*-Acetyl-*O*-methyl-cysteinyl)]epicatechin (AMCys-Ec), 4β-[*S*-(*N*-acetyl-*O*-methyl-cysteinyl)]catechin (AMCys-Cat) and 4β-[*S*-(*N*-acetyl-*O*-methyl-cysteinyl)]epicatechin 3-*O*-gallate (AMCys-EcG) (**6**–**8**) were obtained from *N*-acetyl-cysteine. Under the depolimerization conditions (60 °C, HCl, methanol, 15 min) the methyl ester was readily obtained from the free carboxylic acid.

The stereochemistry at C-2, C-3 and C-4 of compounds **3**–**8** was assigned from the hydrogen-coupling constants measured using ^{1}H NMR and following Thompson *et al.* [19]. In agreement with the literature, the 4β derivatives were the major isomers obtained irrespective of the 2,3-stereochemistry [19,20].

Free-radical scavenging activity

The new cysteinyl catechin derivatives were potent free-radical scavenging agents in the DPPH assay and moderate scavengers in the tris(2,4,6-trichloro-3,5-dinitrophenyl)methyl radical (HNTTM) assay. DPPH is a widely used stable free radical that is converted to the reduced form by incorporating a hydrogen atom via a mechanism that may involve direct hydrogen donation and/or electron transfer with subsequent proton incorporation [21,22]. HNTTM is a newly introduced stable radical that is exclusively quenched by electron transfer to give a stable anion with subsequent slow proton incorporation [23]. By comparing the results from both assays, information is obtained about the differential capacity of a given molecule to donate hydrogen atoms and transfer electrons.

Table 1 summarizes the values obtained for the new compounds **3**–**8** with both free radicals in comparison with the unprotected cysteine derivative Cys-Ec (**2**) and underivatized Ec (**1**) and EgcG (**9**). The results are expressed as antiradical power (ARP) or the inverse of ED_{50} (μmoles of product able to consume half the amount of free radical divided by μmoles of initial DPPH or HNTTM). By multiplying the ED_{50} value by two the stoichiometric value (theoretical moles of antioxidant able to reduce 1 mole of radical) is obtained. The inverse of this value represents the moles of radical reduced by 1 mole of antioxidant and gives an estimate of the number of hydrogen atoms or electrons per mole of antioxidant involved in the processes. Although the number of hydrogen atoms donated (DPPH assay) and electrons transferred (HNTTM assay) to the corresponding free radical were similar in the case of Ec (**1**), the conjugates were able to transfer more hydrogen atoms than electrons. Differences between the ability to donate hydrogen atoms and to transfer electrons for a given compound may be expressed as the ratio between hydrogen atoms and electrons per molecule of scavenger (H/e^-, Table 1 right-hand column). The hydrophobic uncharged derivatives **6**–**8** were less efficient than their positively charged counterparts **3**–**5** as electron donors. The outcome of the DPPH assay was more complex. The cationic catechin conjugate ECys-Cat (**4**) was almost equipotent to the gallate-containing epicatechin cationic conjugate ECys-EcG (**5**) and the gallate-containing hydrophobic uncharged AMCys-EcG (**8**)

Table 1. Free radical scavenging power and stoichiometry.

	DPPH (hydrogen donation)			HNTTM (electron transfer)			
Compound	ARP ($1/ED_{50}$)	Stoichiometric value	H atoms per molecule[a]	ARP ($1/ED_{50}$)	Stoichiometric value	Electrons per molecule[b]	H/e^- ratio
Trolox	3.9	0.52	1.9	5.4	0.37	2.7	0.7
Ec, **1**	5.5	0.36	2.8	4.8	0.42	2.4	1.2
Cys-Ec, **2**	8.3	0.24	4.2	4.8	0.42	2.4	1.7
ECys-Ec, **3**	7.1	0.28	3.6	4.3	0.47	2.1	1.7
ECys-Cat, **4**	10.0	0.20	5.0	4.1	0.48	2.1	2.4
ECys-EcG, **5**	11.1	0.18	5.6	10.1	0.20	5.0	1.1
AMCys-Ec, **6**	6.7	0.30	3.3	2.8	0.71	1.4	2.3
AMCys-Cat, **7**	5.0	0.40	2.5	3.1	0.64	1.6	1.6
AMCys-EcG, **8**	20.0	0.10	10.0	5.7	0.35	2.9	3.4
EgcG, **9**	21.3	0.09	10.6	11.3	0.17	5.9	1.8

[a] Moles reduced DPPH per mole antioxidant. [b] Moles of reduced HNTTM per mole antioxidant. Standard deviation ($n = 3$): $\leqslant 0.3$ (ARP), $\leqslant 0.04$ (stoichiometric value), $\leqslant 0.2$ (H or e^- per molecule).

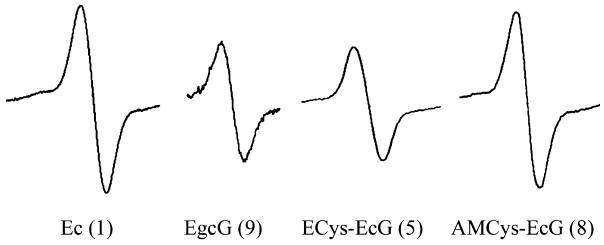


Fig. 2. EPR spectra of HNTTM radical solutions (60 μM initial concentration) in $CHCl_3$/MeOH (2:1) after treatment with the non-galloylated compound Ec (**1**) and the gallate compounds EgcG (**9**), ECys-EcG (**5**) and AMCys-EcG (**8**) at 10 μM. Microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 3.2. All these signals have approximately the same line width, AHpp = 3.2 ± 0.2 G.

was by far the most potent of the compounds tested. The latter was as efficient a hydrogen donor as the most potent tea catechin EgcG. Interestingly, AMCys-EcG (**8**) transferred only 2.9 electrons to HNTTM ($H/e^- = 3.4$), much like compound **1**, resulting in similar EPR spectra for the HNTTM radical (Fig. 2, left and right). The other gallate derivative ECys-EcG (**5**) transferred 5.0 electrons ($H/e^- = 1.1$) and EgcG (**9**) transferred 5.9 electrons ($H/e^- = 1.8$) resulting in significant reduction in the EPR signal of HNTTM (Fig. 2, centre).

Effect on HT29 cell viability

The effect of compounds **3**–**8** on the viability of a human carcinoma cell line (HT29) was examined using an MTT assay. The results showed a dose-dependent decrease in cell viability after treatment with catechins at the reported concentrations for 72 h (Fig. 3). Table 2 shows the mean IC_{50} values obtained and the concentrations at which the compounds were scavengers of 60 μM radical. IC_{50} (the product concentration that diminished viability by 50%) was calculated with respect to the total number of control cells after 72 h of incubation. All the new conjugates were more efficient than Ec (**1**) and Cys-Ec (**2**). For the new compounds **3**–**8**, the presence of the thiol derivatization at C-4 resulted in, at least, a threefold decrease in cell viability compared with the flavanol **1**. Interestingly, the derivative ECys-Cat (**4**) was more potent than the gallate-containing ECys-EcG (**5**), a tendency roughly in accordance with the antioxidant activity. The cationic compound ECys-Cat (**4**) was equipotent (68 μM) to the gallate-containing hydrophobic compound AMCys-EcG (**8**). The latter was the most potent agent of the new compounds in both the DPPH assay and the cell-viability assay. The catechin and epicatechin

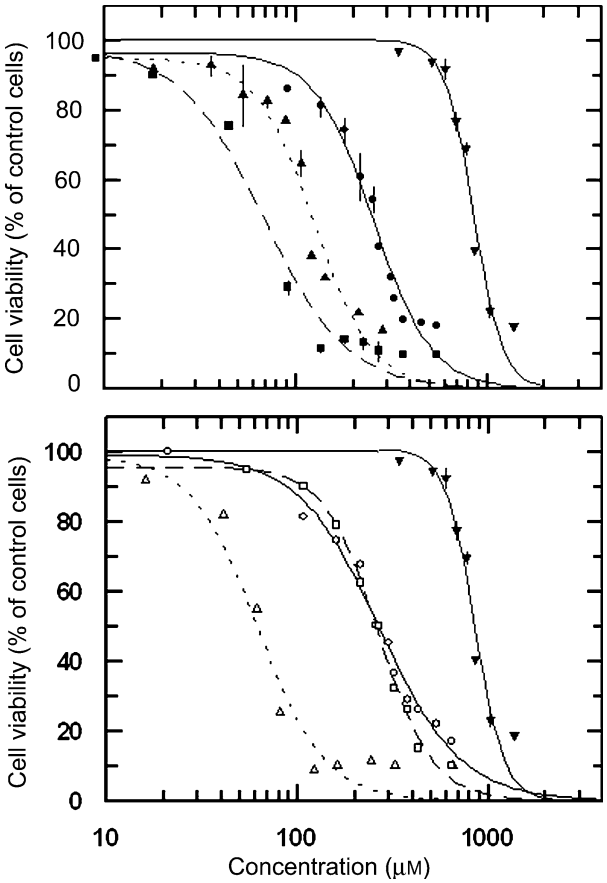


Fig. 3. HT29 colorectal adenocarcinoma cell viability in response to treatment with increasing doses (*x* axis) of catechin thio-derivatives ▼ (Ec, **1**), ● (ECys-Ec, **3**), ■ (ECys-Cat, **4**), ▲ (ECys-EcG, **5**), □ (AMCys-Ec, **6**), ○ (AMCys-Cat, **7**) and △ (AMCys-EcG, **8**). Viability is expressed as per cent of control cells (Ct, mean ± SD, $n = 4$).

derivatives reported here were more efficient in decreasing cell viability than the previously described cysteine derivatives of the same flavanols [17].

Cell cycle and apoptosis

To examine the effects of Ec (**1**) and derivatives **3**–**8** on the cell-cycle pattern at concentrations equal to their IC_{50}, HT29 cells were treated with each compound for 72 h, and cells were analysed using FACS (Fig. 4). Compound **1** and the cationic derivatives ECys-Ec (**3**) and ECys-Cat (**4**) did not influence the normal cell-cycle distribution. The galloylated cysteinyl compound ECys-EcG (**5**) induced a major arrest in the S phase. Neutral derivatives **6**–**8** did not induce any significant arrest in the S phase.

Because cell-cycle arrest may lead to the induction of apoptosis [24], in the next series of experiments we used FITC-FACS analysis to establish the apoptosis-

Table 2. Growth inhibitory potency against human colorectal adenocarcinoma HT29 cells compared with antiradical concentration. N, number of experiments performed; SD, standard deviation.

Compound	*n*	Mean ± SD IC_{50} (μM)	DPPH (μM)[a]	HNTTM (μM)[a]
Ec, **1**	4	826 ± 12	21.8	25.0
Cys-Ec, **2**	6	407 ± 21	14.4	25.0
ECys-Ec, **3**	4	255 ± 13	17.0	28.0
ECys-Cat, **4**	7	68 ± 10	12.0	29.2
ECys-EcG, **5**	7	126 ± 8	10.8	12.0
AMCys-Ec, **6**	5	268 ± 15	18.0	42.8
AMCys-Cat, **7**	5	267 ± 7	24.0	38.6
AMCys-EcG, **8**	4	62 ± 12	6.0	21.0

[a] Calculated theoretical concentration able to scavenge 100% of the 60 μM radical.

induction capacity of each compound. HT29 cells were treated with Ec (**1**) and compounds **3**–**8** at their IC_{50} values. Compound **1** and ECys-Cat (**4**) did not affect apoptosis. The gallate-containing compound ECys-EcG (**5**) induced a fourfold increase in the number of early and late apoptotic cells (27.3%) with respect to control cells (6.8%) (Fig. 5, left and right upper quadrants, respectively). The apoptosis induced by compounds **6**–**8** was low (Fig. 5, left and right upper quadrants).

Discussion

We are interested in the putative preventive activity of catechin derivatives, primarily against epithelial cancer, and skin and colon carcinomas in particular. This activity may result from the prevention of DNA damage by scavenging free radicals and/or an effect on the cell's replicating functions and apoptosis. The two actions may or may not be related. Polyphenol derivatives such as those described may be active via either or both of the two mechanisms.

Redox properties and viability

Conjugates with cysteine derivatives were potent scavengers of the DPPH radical. All except AMCys-Ec (**6**) and AMCys-Cat (**7**) were clearly more efficient than Ec (**1**), suggesting that most of the conjugates were better hydrogen donors than the corresponding underivatized flavanol. Interestingly, the ability of the new conjugates to transfer electrons, as measured by the recently introduced HNTTM radical [23], did not differ much from that of **1**. The conjugates showed an increased capacity to exchange hydrogen atoms while keeping the electron-transfer capacity low. This may have an important biological significance because electron transfer is sometimes regarded as an undesired effect [25]. Under certain conditions, flavonoids such as the pyrogallol-containing (–)-epigallocatechin (EgC) and (–)-epigallocatechin-gallate (EgcG) may participate in redox cycling via production of the active superoxide radical anion ($O_2^{\cdot-}$) and subsequently hydrogen peroxide [26–28]. Although this is less likely to occur with (–)-epicatechin or (+)-catechin, redox cycling has also been described for catechols [28]. The H/e^- value might be regarded as an estimate of how safe an antioxidant would be in terms of putative participation in redox cycling by electron transfer: the higher the H/e^- value, with the lower number of electrons involved, the better.

Compounds ECys-Cat (**4**) and AMCys-EcG (**8**) gave the highest H/e^- values (Table 1). H/e^- was also high for AMCys-Ec (**6**), but its absolute ARP was lower than that of compound **4**. Cationic catechin conjugate **4** was almost twice as potent as **1** as a hydrogen donor although equipotent as electron donor. More strikingly, compound **8** showed extraordinary capacity as a hydrogen donor, as high as that of the most potent tea catechin, EgcG (**9**), while

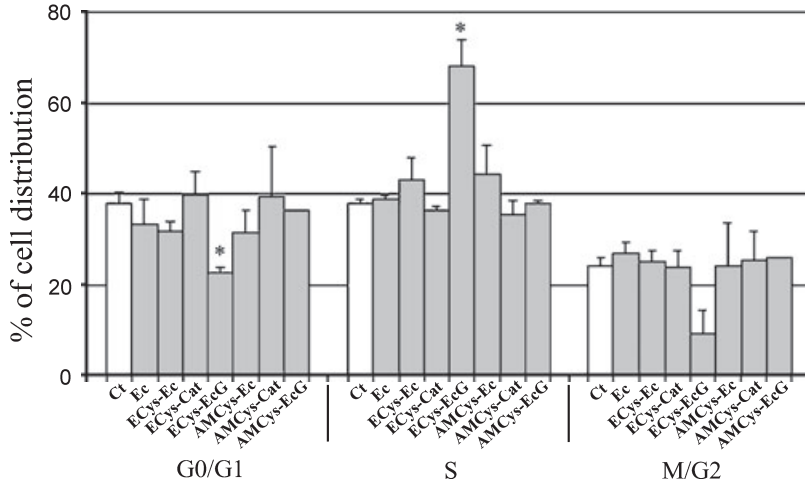


Fig. 4. Influence of the flavanols on HT29 cell cycle after 72 h of treatment. Control cells (Ct) are indicated by open bars. Doses in HT29 cells: Ec (**1**), 826 μM; ECys-Ec (**3**), 255 μM; ECys-Cat (**4**), 68 μM; ECys-EcG (**5**), 126 μM; AMCys-Ec (**6**), 268 μM; AMCys-Cat (**7**), 267 μM and AMCys-EcG (**8**), 62 μM. The results are the mean ± SD of three independent experiments. $*P < 0.05$, significant difference compared with Ct.

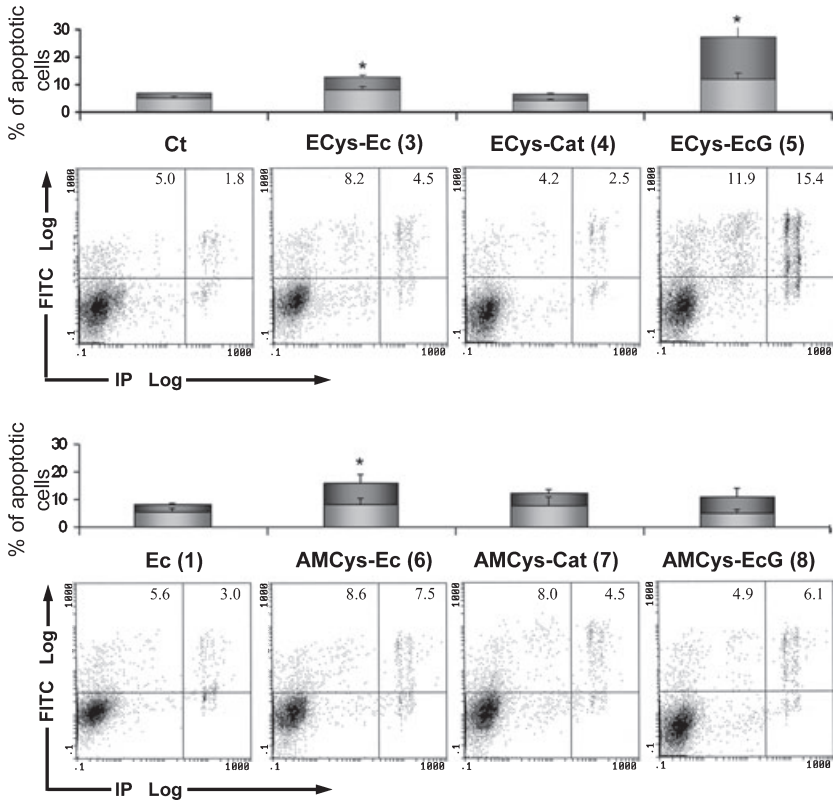


Fig. 5. Induction of apoptosis in HT29 cells. Representative dot plots of the flow cytometric quantification of intact, apoptotic and necrotic cells after 72 h of incubation with the indicated concentrations of compounds Ec (**1**, 826 μM), ECys-Ec (**3**, 255 μM), ECys-Cat (**4**, 68 μM), ECys-EcG (**5**, 126 μM), AMCys-Ec (**6**, 268 μM), AMCys-Cat (**7**, 267 μM) and AMCys-EcG (**8**, 62 μM). Live cells (annexin-FITC and PI double negative) occupy the lower left quadrant, early apoptotic cells (FITC-annexin V positive and PI negative) occupy the upper left quadrant and late apoptotic or necrotic cells (FITC-annexin V and PI double positive) occupy the upper right quadrant. The percentage of early (light grey bar) and late (dark grey bar) apoptotic cells in each condition is represented as a bars diagram, calculated from dot plots. The results are the mean ± SD of three separate experiments. $*P < 0.05$, significant difference compared to control cells (Ct).

keeping the electron-transfer capacity low (2.9 e^- per molecule, $H/e^- = 3.4$) compared with EgcG (5.9 e^- per molecule, $H/e^- = 1.8$).

Our previous results in the cell-viability assay using cysteamine and cysteine conjugates of catechins on HT29 epithelial colon carcinoma cells suggested that there was some coincidence between the free-radical scavenging capacity (DPPH assay, hydrogen donation) and the cell viability reduction potency [17]. Although there is no evidence that both effects are related, the results presented here show the same tendency. The three most potent scavengers in the DPPH assay (ECys-Cat **4**, ECys-EcG **5** and AMCys-EcG **8**) were also the most efficient compounds in the cell-viability assay (Table 2). These new results suggested that some relationship might exist between the two effects. Because the concentrations at which the compounds affected viability were much higher than those at which they were antioxidants (Table 2) the effect on cells might be attributed to an unspecific toxic pro-oxidant action. Regardless of the fact that the high concentration of hydroxyl groups may contribute to the overall activity, such an unspecific effect would not satisfactorily account for the differences recorded among the compounds, particularly those related to the cell cycle and apoptosis.

Electron transfer, the cell cycle and apoptosis

ROS, in addition to being potentially harmful, are important regulators of cell functions, including apoptosis. Small amounts of ROS, including hydroxyl radicals ($HO^{\cdot}$), superoxide radical anion ($O_2^{\cdot -}$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), are constantly generated in living cells and homeostasis in an organism very much depends on the right redox balance [29,30]. ROS may mediate apoptosis by directly taking part in the toxic warfare of the cell against itself or by regulating one or more apoptotic pathways [31,32]. Among ROS, the $O_2^{\cdot -}$, and not $HO^{\cdot}$, is apparently responsible for this effect, particularly in HT29 cells [32]. Once superoxide is generated it can work either as an apoptotic signal itself, providing a fail-safe mechanism that complements cyt-c-dependent caspase activation for the execution of cell death [13], or dismutate into hydrogen peroxide. The latter is a major mediator of oxidative stress and can also cause genomic damage indirectly [33]. Excessive generation of superoxide and hydrogen peroxide invariably accompanies molecular damage and appears to be a critical event in drug-induced apoptosis [31].

In the case of polyphenols, trihydroxybenzene-containing catechins such as EgcG are more efficient

producers of the superoxide radical anion than catechol-containing catechins such as Ec (**1**) [14,29,34], and they inhibit cancer cell growth via cell-cycle arrest and apoptosis induction by mechanisms involving the gallate moiety [12,35–37]. In agreement with the literature, our results show that the gallate-containing compound ECys-EcG (**5**) induced apoptosis (threefold compared with control cells, see Fig. 5) and triggered a significant arrest in the S phase of the cell cycle (Fig. 4). Several authors have reported that the gallate group decreases cyclin-dependent kinase 2 (Cdk2) expression and activity, and upregulates the expression of p21, a Cdk inhibitor [38–40] essential for progression from the G1 to the S phase of the cell cycle. Alternatively, the catechin gallates, acting as pro-oxidants, may damage the DNA directly through ROS [41]. All these events lead to stoppage of the cell-cycle progression at the S phase and may potentially result in inhibition of proliferation, cytostasis and possibly apoptosis in human tumours. It seems increasingly evident that the duality antioxidant/pro-oxidant of catechins may play a crucial role in their interactions with the cell machinery, most probably via formation of the superoxide anion radical by electron transfer.

ECys-EcG (**5**), together with EgcG (**9**), possessed a high electron-transfer capacity (five and six electrons per molecule, respectively). Compounds **1**–**4** and **6**, **7**, all lacking the gallate moiety and showing low electron-transfer capacity, did not induce any significant cell-cycle arrest or apoptosis. Surprisingly, the gallate-containing compound AMCys-EcG (**8**) did not induce cell-cycle arrest or apoptosis. Curiously, the electron-transfer capacity of **8** (2.9 electrons per molecule) as measured by the stable radical HNTTM was lower than expected, similar to those of the nongalloylated species (approximately 2 electrons per molecule, see Fig. 2). This finding appears to corroborate the observation from other authors that links the induction of apoptosis to the electron-transfer capacity and formation of the superoxide radical anion. The presence of the gallate moiety does not appear to be a sufficient condition for the induction of apoptosis in HT29 cells. Whether cell-cycle arrest and apoptosis are due to redox cycling with the production of hydrogen peroxide (pro-oxidant effect) or other ROS-mediated events is something that must be explored further.

Conclusions

The conjugation of catechins with both cationic and neutral cysteine derivatives produced compounds with an improved capacity to donate hydrogen atoms while keeping their capacity to participate in electron-transfer reactions low. One of the new molecules, AMCys-EcG (**8**), the most efficient DPPH scavenger of the flavanol thio-conjugates described to date, was the most effective derivative against colon carcinoma cell viability. Despite including a gallate moiety, this compound showed a low electron-transfer capacity and neither arrested the cell cycle nor induced apoptosis. This result, together with the observation that pro-apoptotic ECys-EcG (**5**) and EgcG (**9**) possessed higher electron-transfer capacity is suggesting that the gallate moiety may not be a sufficient condition to trigger apoptosis, which would be more directly related to the ability of the flavanol derivatives to transfer electrons. Our newly introduced scavenging assay using the stable radical HNTTM, which is exclusively sensitive to electron transfer, may be a valuable tool for predicting the pro-apoptotic activity of polyphenols and other putative drugs. The electron-transfer capacity of exogenous plant phenolics and its influence on the delicate balance between the antioxidant and pro-oxidant events governing cell functions may help to explain the putative cancer-preventive properties of catechins and their derivatives.

Experimental procedures

Materials

Analytical grade methanol (MeOH, Panreac, Montcada i Reixac, Spain) was used for the acid cleavage reaction and DPPH assay, deionized water and bulk ethanol (EtOH, Momplet y Esteban, Barcelona, Spain) for semipreparative and preparative cation-exchange chromatography, Milli-Q® water and HPLC grade acetonitrile (CH_3CN, Merck, Darmstadt, Germany) for analytical RP-HPLC, and deionized water and preparative grade CH_3CN (Scharlau, Barcelona, Spain) for preparative and semipreparative RP-HPLC. Deuterated solvents for NMR were from SDS (Peypin, France). Cysteine hydrochloride, L-cysteine ethyl ester hydrochloride and *N*-acetyl-L-cysteine (Aldrich, Steinheim, Germany) were of synthesis grade. (–)-Epicatechin (Ec, **1**) (–)-epigallocatechin 3-*O*-gallate (EgcG, **9**), MTT, dimethylsulfoxide, Trypan Blue solution 0.4%, propidium iodide (PI), the nonionic surfactant Igepal CA-630, α,α,α-tris(hydroxymethyl)aminomethane and NaCl/P_i were from Sigma (Steinheim, Germany). Acetic acid, 37% HCl (Merck) and NaCl (Carlo Erba, Milan, Italy) were of analytical grade. Triethylamine (Merck) was of buffer grade. Trifluoroacetic acid (Fluorochem, Glossop, UK) biotech grade was distilled in-house. DPPH (95%) was from Aldrich (Gillingham, UK), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) (97%) was from Aldrich (Milwaukee, MN). Fetal bovine serum was purchased from Gibco (Invitrogen, Carlsbad, CA). Trypsin–EDTA solution

C (0.05% trypsin and EDTA 1:5000 in NaCl/P_i) was from Biological Industries (Beit Haemek, Israel). RNase was from Roche Diagnostics (Mannheim, Germany). FITC–annexin V kit and binding buffer 4× for apoptosis assay were purchased from Bender MedSystems (MedSystems Diagnostics GmbH, Vienna, Austria).

Chromatographic equipment and columns

Analytical RP-HPLC was performed on a Kontron Analytical system (Kontron Instruments, Basel, Switzerland) fitted with a VYDAC^{TM} (The Separations Group, Hesperia, USA) C_{18}, 300 Å pore size, 5 μm particle size, 250 × 4.6 mm i.d. column. Cation-exchange chromatography was performed on a flash chromatography-type glass column (21 × 2.5 cm i.d., ~ 105 mL bed volume) packed in-house with MacroPrep^{TM} High S 50 μm (Bio-Rad Laboratories, Hercules, CA). Preparative RP-HPLC chromatography was performed on a Waters (Milford, USA) Prep LC 4000 pumping system with a Waters PrepPack® 1000 module fitted with a PrepPack® Waters cartridge (30 × 4.7 cm i.d) filled with VYDAC^{TM} (The Separations Group) C_{18}, 300 Å pore size, 15–20 μm particle size stationary phase. Detection was carried out using an analytical Merck-Hitachi (Darmstadt, Germany) L-4000 UV detector.

MS, NMR and EPR measurements

ES-MS analyses were recorded on a VG-Quattro® system from Fisons Instruments (Altrincham, UK). The carrier solution was Milli-Q water/CH_3CN (1:1) containing 1% (v/v) formic acid. ^{1}H NMR spectra were acquired on a Varian (Palo Alto, CA) Unity 300 spectrometer in the deuterated solvents $(CD_3)_2CO$ and D_2O.

EPR measurements were performed on a Varian E-109 spectrometer working in the X-band (microwave power, 20 mW; modulation amplitude, 3.2 G).

Preparation of the conjugates

Conjugates were obtained by acid depolymerization of plant procyanidins essentially as described previously [16]. To obtain the thio-conjugates **3**–**8** (Fig. 1) the solvent was eliminated from an aqueous fraction (400 mL, 6 g estimated polyphenols by mass, from 3.2 kg of grape byproduct) of polymeric procyanidins. The pellet was then dissolved in MeOH (400 mL) and dried. The resulting syrupy residue was dissolved in MeOH (400 mL) and a solution of the appropriate cysteine derivative (20 g) and 37% HCl (10 mL) in MeOH (400 mL) was added. The mixture was kept at 65 °C for 20 min under stirring. The reaction was then quenched with cold water (3.2 L).

Conjugates were separated from the whole mixture using the MacroPrep^{TM} High S resin. The eluents were: (A) 20 mM sodium phosphate, pH 2.3 buffer/EtOH (13:7, v/v) and (B) 20 mM sodium phosphate, pH 2.3 buffer/EtOH (3:2, v/v), 100 mM NaCl. The column was equilibrated with eluent (A), loaded with the quenched depolymerized mixture (500 mL) and washed with (A) (500 mL, 4.75 bed volumes). The retained catechin derivatives were released with 500 mL (4.75 bed volumes) of eluent (B). The operation was repeated until the whole mixture was consumed. The separation process was monitored by analytical RP-HPLC on a VYDAC^{TM} C_{18} column eluted with a binary system: (C) 0.10% (v/v) aqueous trifluoroacetic acid, (D) 0.09% (v/v) trifluoroacetic acid in water/CH_3CN (1:4, v/v) under isocratic conditions 19% (D) at a flow rate of 1.5 mL·min^{-1} and detection at 214 nm. The eluates containing the corresponding conjugate were pooled (3.5 L).

The mixture containing the *O*-ethyl-cysteinyl conjugates **3**–**5** was fractionated on a preparative RP-HPLC cartridge filled with VYDAC^{TM} C_{18} stationary phase by a CH_3CN gradient in 0.10% (v/v) aqueous trifluoroacetic acid (4–20% CH_3CN over 45 min). Fractions enriched in each of the three compounds were obtained: fraction **I**, 9–11% CH_3CN, compound ECys-Cat (**4**); fraction **II**, 12–16% CH_3CN, compound ECys-Ec (**3**); fraction **III**, 17–19% CH_3CN, compound ECys-EcG (**5**).

4β-[*S*-(*O*-ethyl-cysteinyl)]epicatechin (ECys-Ec, 3)

Fraction **II** from reversed-phase fractionation was concentrated, loaded onto the preparative cartridge and the target compound purified by CH_3CN gradient in triethylamine phosphate buffer and aqueous trifluoroacetic acid. Analysis of the fractions was accomplished under isocratic conditions in 0.10% (v/v) aqueous trifluoroacetic acid/CH_3CN using the VYDAC^{TM} C_{18} column, solvent system, flow rate and detection as described above. ECys-Ec (**3**) (354 mg) was obtained as the trifluoroacetate by lyophilization. δ_H(300 MHz; $(CD_3)_2CO$ + 3 drops D_2O) 1.24 (3 H, t, *J* 7.2 Hz, O-CH_2-C*H*$_3$), 3.93 (1 H, d, $J_{3,4}$ 2.1 Hz, 4-H 3,4-*trans* configuration), 4.06 (1 H, dd, *J* 2.4 and 0.9 Hz, 3-H), 4.26 (2 H, q, *J* 7.2 and 1.5 Hz, O-C*H*$_2$-CH_3), 4.71 (1 H, m, S-CH_2-C*H* <), 5.09 (1 H, s, 2-H 2,3-*cis* configuration), 5.90 (1 H, d, *J* 2.4 Hz, 8-H), 6.09 (1 H, d, *J* 2.4 Hz, 6-H), 6.80–6.81 (2 H, m, 5′-H, 6′-H), 7.04 (1 H, d, *J* 1.8 Hz, 2′-H). *m/z* 438.1 $(M + 1)^+$, calculated for $C_{20}H_{24}N_1O_8S_1$ $(M + H)^+$ 438.1. Purity (> 95%) was ascertained by RP-HPLC.

4β-[*S*-(*O*-ethyl-cysteinyl)]catechin (ECys-Cat, 4)

Fraction **I** from reversed-phase fractionation was concentrated, loaded onto the preparative cartridge, purified and characterized as stated for compound **3**. ECys-Cat (**4**) (68 mg) was obtained as the trifluoroacetate. δ_H(300 MHz; $(CD_3)_2CO$ + 3 drops D_2O) 1.24 (3 H, t, *J* 7.0 Hz, O-CH_2-

CH_3), 4.06 (1 H, 2d, $J_{2,3}$ 9.6 and 2.4 Hz, 3-H 2,3-*trans* configuration), 4.23 (1 H, d, $J_{3,4}$ 2.4 Hz, 4-H 3,4-*cis* configuration), 4.26 (2 H, q, *J* 7.0 and 2.4 Hz, O-CH_2-CH_3), 4.68–4.72 (1 H, m, S-CH_2-CH <), 4,78 (1 H, d, *J* 8.6 Hz; 2-H), 5.89 (1 H, d, *J* 2.4 Hz, 8-H), 6.10 (1 H, d, *J* 2.4 Hz, 6-H), 6.62 (2 H, m, 5′-H, 6′-H), 6.91 (1 H, s, 2′-H). *m/z* 438.1 $(M + 1)^+$, calculated for $C_{20}H_{24}N_1O_8S_1$ $(M + H)^+$ 438.1. Purity (> 93%) was ascertained by RP-HPLC.

4β-[*S*-(*O*-ethyl-cysteinyl)]epicatechin 3-*O*-gallate (ECys-EcG, 5)

Fraction **III** from reversed-phase fractionation was concentrated, loaded onto the preparative cartridge, purified and characterized as stated for compound **3**. ECys-EcG (**5**) (33 mg) was obtained as the trifluoroacetate. δ_H(300 MHz; $(CD_3)_2CO$ +3 drops D_2O) 1.28 (3 H, t, *J* 7.0 Hz, O-CH_2-CH_3), 4.15 (1 H, d, $J_{3,4}$ 1.8 Hz, 4-H 3,4-*trans* configuration), 4.29 (2H, q, *J* 7.0 and 1.8 Hz, O-CH_2-CH_3), 4.77 (1 H, m, S-CH_2-CH <), 5.28 (1 H, m, 3-H), 5.36 (1 H, bs, 2-H 2,3-*cis* configuration), 6.01 (1 H, d, *J* 2.1 Hz, 6-H), 6.13 (1 H, d, *J* 2.1 Hz, 8-H), 6.79 (1 H, d, *J* 8.1 Hz, 5′-H), 6.88 (1 H, dd, *J* 8.4 and 2.1 Hz, 6′-H), 6.96 (2 H, s, galloyl-H), 7.10 (1 H, d, *J* 1.8 Hz, 2′-H). *m/z* 590.1 $(M + 1)^+$ calculated for $C_{27}H_{28}N_1O_{12}S_1$ $(M + H)^+$ 590.1. Purity (> 96%) was ascertained by RP-HPLC.

The preparative RP-HPLC fractionation of the *N*-acetyl-*O*-methyl-cysteinyl conjugates **6**–**8** was performed directly from the depolymerized mixture under chromatographic conditions (6–20% CH_3CN over 54 min) similar to the conditions described for the ethyl-cysteine conjugates. Fractions of interest: fraction **IV**, 13–14% CH_3CN, compound AMCys-Cat (**7**); fraction **V**, 15–18% CH_3CN, compound AMCys-Ec (**6**); fraction **VI**, 18–19% CH_3CN, compound AMCys-EcG (**8**).

4β-[*S*-(*N*-Acetyl-*O*-methyl-cysteinyl)]epicatechin (AMCys-Ec, 6)

Fraction **V** from reversed-phase fractionation was concentrated, loaded onto the preparative cartridge and the target compound purified by CH_3CN gradient in triethylamine phosphate buffer and aqueous trifluoroacetic acid. Analysis of the fractions was accomplished as described above. AMCys-Ec (**6**) (818 mg) was obtained by lyophilization. δ_H(300 MHz; $(CD_3)_2CO$ +3 drops D_2O) 2.05 (3 H, s, CO-CH_3), 3.69 (3 H, s, O-CH_3), 4.02 (1 H, dd, $J_{2,3}$ 2.4 and 1.2 Hz, 3-H 2,3-*cis* configuration), 4.06 (1 H, d, $J_{3,4}$ 2.4 Hz, 4-H 3,4-*trans* configuration), 4.94 (1 H, m, S-CH_2-CH <), 5.22 (1 H, s, 2-H), 5.89 (1 H, d, *J* 2.4 Hz, 8-H), 6.06 (1 H, d, *J* 2.4 Hz, 6-H), 6.81–6.83 (2 H, m, 5′-H, 6′-H), 7.06 (1 H, d, *J* 2.1 Hz, 2′-H). *m/z* 464.7 $(M - 1)^-$, calculated for $C_{21}H_{23}N_1O_9S_1$ $(M - H)^-$ 464.5. Purity (> 99%) was ascertained by RP-HPLC.

4β-[*S*-(*N*-Acetyl-*O*-methyl-cysteinyl)]catechin (AMCys-Cat, 7)

Fraction **IV** from reversed-phase fractionation was concentrated, loaded onto the preparative cartridge, purified and characterized as stated for compound **6**. After lyophilization, AMCys-Cat (**7**) (64 mg) was obtained. δ_H(300 MHz; $(CD_3)_2CO$ +3 drops D_2O) 2.11 (3 H, s, CO-CH_3), 3.65 (3 H, s, O-CH_3), 4.15 (1 H, 2d, *J* 9.6 and 3.9 Hz, 3-H), 4.38 (1 H, d, $J_{3,4}$ 3.9 Hz, 4-H 3,4-*cis* configuration), 4.82 (1 H, m, S-CH_2-CH <), 4.95 (1 H, d, $J_{2,3}$ 9.6 Hz, 2-H 2,3-*trans* configuration), 5.78 (1 H, d, *J* 2.4 Hz, 8-H), 6.06 (1 H, d, *J* 2.4 Hz, 6-H), 6.78 (2 H, m, 5′-H, 6′-H), 6.92 (1 H, s, 2′-H). *m/z* 464.9 $(M - 1)^-$, calculated for $C_{21}H_{23}N_1O_9S_1$ $(M\text{-}H)^-$ 464.5. Purity (99%) was ascertained by RP-HPLC.

4β-[*S*-(*N*-Acetyl-*O*-methyl-cysteinyl)]epicatechin 3-*O*-gallate (AMCys-EcG, 8)

Fraction **VI** from reversed-phase fractionation was concentrated, loaded onto the preparative cartridge, purified and characterized as stated for compound **6**. After lyophilization, AMCys-EcG (**8**) (88 mg) was obtained. δ_H (300 MHz; $(CD_3)_2CO$ +3 drops D_2O) 2.09 (3H s, CO-CH_3), 3.71 (3 H, s, O-CH_3), 4.26 (1 H, d, $J_{3,4}$ 2.4 Hz, 4-H 3,4-*trans* configuration), 5.01 (1 H, m, S-CH_2-CH <), 5.21 (1 H, m, 3-H), 5.48 (1 H, bs, 2-H), 6.01 (1 H, d, *J* 2.4 Hz, 8-H), 6.07 (1 H, d, *J* 2.4 Hz, 6-H), 6.78 (1 H, d, *J* 8.1 Hz, 5′-H), 6.89 (1 H, dd, *J* 8.1 and 2.1 Hz, 6′-H), 6.96 (2 H, s, galloyl-H), 7.08 (1 H, d, *J* 2.1 Hz, 2′-H). *m/z* (EI) 616.3 $(M - 1)^-$ calculated for $C_{28}H_{27}N_1O_{13}S_1$ $(M - H)^-$ 616.6. Purity (95%) was ascertained by RP-HPLC.

Free-radical scavenging

Hydrogen atom donation capacity was evaluated using the DPPH assay [42,43]. The samples (0.1 mL) were added to aliquots (3.9 mL) of a solution made up with 4.8 mg DPPH in 200 mL of MeOH and the mixture incubated for 1 h at room temperature. The initial concentration of DPPH, ~ 60 μM, was calculated for every experiment from a calibration curve made by measuring the absorbance at 517 nm of standard samples of DPPH at different concentrations. The equation of the curve was $A_{517} = 11345 \times C_{DPPH}$ as determined by linear regression. The electron-transfer capacity was evaluated using the HNTTM assay. The radical scavengers were dissolved in $CHCl_3$/MeOH (2:1, v/v) at different concentrations. Aliquots (1 mL) were added to a solution (1 mL) of HNTTM (120 μM in $CHCl_3$/MeOH 2:1, v/v) [23] and the mixture incubated for 30 min. The initial concentration of radical, ~ 60 μM, was calculated for every experiment from calibration curves made by measuring the intensity (I_0) of the EPR signal (peak-to-peak line distance)

of standard samples of the radical at different concentrations. The equations of the curves were $I = 1980 \times C_{radical}$ or $I = 2262 \times C_{radical}$ depending on the experiment. For both assays the initial concentration of the scavengers ranged from 1 to 40 μM. The results were plotted as the degree of disappearance of absorbance ($[(1 - A/A_0) \times 100]$, DPPH assay) or signal intensity ($[(1 - I/I_0) \times 100]$, HNTTM assay) against μmoles of the sample divided by the initial μmoles of the radical. Each point was acquired in triplicate. A dose–response curve was obtained for every product. The results were expressed as the efficient dose ED_{50} given as μmoles of product able to consume half the amount of free radical divided by μmoles of initial free radical.

Cell culture

HT29 cells (colorectal adenocarcinoma) were purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine and antibiotics: 100 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin. Cells were grown in an isolated 37 °C–5% CO_2 tissue incubator compartment and the medium was changed every 3 days.

Cell-viability assay

Cell growth was determined using a variation of the MTT assay described by Mosmann [44]. HT29 cells were counted using Trypan Blue solution 4% in a Neubauer cell counter chamber (Brand, Wertheim, Germany) by observing viable (nonstained) and nonviable (stained) cells under a microscope [45]. Cells were seeded into 96-well plates at 2.5×10^3 cells per well and incubated for 24 h prior to addition of the compounds (dissolved in NaCl/P_i). After 3 days of culture, the supernatant was aspirated and 100 μL of filtered MTT (0.5 $mg \cdot mL^{-1}$ in cell culture medium) was added. The cell plates were incubated during 1 h and metabolically active cells reduced the dye to purple formazan. The supernatant was removed, and the dark blue MTT formazan precipitated was dissolved in dimethylsulfoxide (100 μL) and optical density (OD) measured at 550 nm on a multiwell reader (Merck ELISA System MIOS®).

The IC_{50} or compound concentration causing a 50% reduction in the mean OD value relative to the control was estimated using GRAFIT 3.00 (Data Analysis and Graphics Program, Erithacus Software Ltd. Microsoft Corp., Surrey, UK) curve option: IC_{50} curve – start at 0.

Cell-cycle analysis

Cell-cycle analysis and apoptosis quantification were conducted by flow cytometry. HT29 cells were seeded into six-well plates at 8×10^4 cells per well and incubated for 24 h prior to addition of the compounds. The IC_{50} concentration values of each sample were measured after 3 days of subculturing. Both treated and untreated cells were resuspended in ice-cold TBS 1× buffer (1 mL of 10 mM Tris and 150 mM NaCl, pH 7.4). PI (50 μL, 50 μg) and 1 mL Vindelov buffer at pH 7.4 containing 10 mM Tris, 10 mM NaCl, PI (50 μL, 50 μg), RNase (1 μL, 10 μg) and Igepal CA-630 (1 μL) were added to each sample, and cells were incubated for 1 h at 4 °C in the dark [46]. Cell-cycle distribution was analysed by flow cytometry using a FACS system. DNA histograms were collected with an Epics XL flow cytometer (Coulter Corporation, Miami, FL) and analysed using MULTICYCLE software (Phoenix Flow Systems, San Diego, CA).

Assessment of apoptosis

After 3 days of subculturing the cell plates as described for the cell-cycle treatment, cells were washed once in ice-cold binding buffer (10 mM Hepes sodium hydroxide pH 7.4, 140 mM NaCl, 2.5 mM calcium chloride) and resuspended in the same buffer (95 μL) at a maximum of 0.8×10^6 cells·mL^{-1} in the presence of FITC–annexin V binding (3 μL). After 30 min of incubation at room temperature in the dark, PI (20 μL, 20 μg) was added [47]. Cells, double-stained with PI and FITC–annexin V were processed by flow cytometry and laser-scanning cytometry, which collected green (525 nm) fluorescence for FITC-conjugated antibody and red (675 nm) fluorescence for PI, under 488 nm excitation.

Statistics

For statistical analysis Student's *t*-test was used. For each compound, a minimum of four duplicate experiments for the growth inhibition test and a minimum of three experiments for both cell-cycle analysis and assessment of apoptosis were conducted. Data are given as the mean ± SD. *P*-values of < 0.05 were considered significant.

Acknowledgements

Financial support (research grants PPQ2003-06602-C04-01 and -04, BQU2002-0485-C02-01 and doctoral fellowships to CL and ST from the Spanish Ministry of Education and Science; grant 2001SGR00048 and ITT program of the Work Community of Pyrenees from Generalitat de Catalalunya) is acknowledged. We thank Dr Irene Fernández from the Servei d'Espectrometria de Masses of the University of Barcelona for the mass spectrometry analyses, Ms Avencia Díez from the Servei d'Espectrometria de Ressonància

Paramagnètica Electrònica at IIQAB-CSIC for the EPR analyses and Dr Francisco José Sánchez-Baeza and Ms Montserrat Sindreu from the Servei d'Espectrometria de Ressonància Magnètica Nuclear at IIQAB-CSIC for the NMR analyses.

References

1 Rice-Evans CA, Miller NJ & Paganga G (1996) Structure–antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol Med* **20**, 933–956.
2 Yokozawa T, Chen CP, Dong E, Tanaka T, Nonaka GI & Nishioka I (1998) Study on the inhibitory effect of tannins and flavonoids against the 1,1-diphenyl-2-picrylhydrazyl radical. *Biochem Pharmacol* **56**, 213–222.
3 Katiyar SK & Mukhtar H (1997) Tea antioxidants in cancer chemoprevention. *J Cell Biochem (Suppl)* **27**, 59–67.
4 Yang CS, Landau JM, Huang MT & Newmark HL (2001) Inhibition of carcinogenesis by dietary polyphenolic compounds. *Ann Rev Nutr* **21**, 381–406.
5 Diplock AT, Charleux JL, Crozier-Willi G, Kok FJ, Rice-Evans C, Roberfroid M, Stahl W & Viña-Ribes J (1998) Functional food science and defence against reactive oxidative species. *Br J Nutr* **80** (Suppl. 1), S77–S112.
6 Mertens-Talcott SU, Talcott ST & Percival SS (2003) Low concentrations of quercetin and ellagic acid synergistically influence proliferation, cytotoxicity and apoptosis in MOLT-4 human leukemia cells. *J Nutr* **133**, 2669–2674.
7 Chung FL, Schwartz J, Herzog CR & Yang YM (2003) Tea and cancer prevention: studies in animals and humans. *J Nutr* **133**, 3268S–3274S.
8 Hsu S, Lewis J, Singh B, Schoenlein P, Osaki T, Athar M, Porter AG & Schuster G (2003) Green tea polyphenol targets the mitochondria in tumor cells inducing caspase 3-dependent apoptosis. *Anticancer Res* **23**, 1533–1539.
9 Gupta S, Hussain T & Mukhtar H (2003) Molecular pathway for (–)-epigallocatechin-3-gallate-induced cell cycle arrest and apoptosis of human prostate carcinoma cells. *Arch Biochem Biophys* **410**, 177–185.
10 Ahmad N, Gali H, Javed S & Agarwal R (1998) Skin cancer chemopreventive effects of a flavonoid antioxidant silymarin are mediated via impairment of receptor tyrosine kinase signaling and perturbation in cell cycle progression. *Biochem Biophys Res Commun* **247**, 294–301.
11 Liang YC, Lin-shiau SY, Chen CF & Lin JK (1997) Suppression of extracellular signals and cell proliferation through EGF receptor binding by (–)-epigallocatechin gallate in human A431 epidermoid carcinoma cells. *J Cell Biochem* **67**, 55–65.
12 Alanko J, Riutta A, Holm P, Mucha I, Vapaatalo H & Metsa-Ketela T (1999) Modulation of arachidonic acid metabolism by phenols: relation to their structure and antioxidant/prooxidant properties. *Free Radical Biol Med* **26**, 193–201.
13 Cai J & Jones DP (1998) Superoxide in apoptosis. Mitochondrial generation triggered by cytochrome *c* loss. *J Biol Chem* **273**, 11401–11404.
14 Azam S, Hadi N, Khan NU & Hadi SM (2004) Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: implications for anticancer properties. *Toxicol Vitro* **18**, 555–561.
15 Tanaka T, Kusano R & Kouno I (1998) Synthesis and antioxidant activity of novel amphipathic derivatives of tea polyphenol. *Bioorg Med Chem Lett* **8**, 1801–1806.
16 Torres JL & Bobet R (2001) New flavanol derivatives from grape (*Vitis vinifera*) byproducts. Antioxidant aminoethylthio-flavan-3-ol conjugates from a polymeric waste fraction used as a source of flavanols. *J Agric Food Chem* **49**, 4627–4634.
17 Torres JL, Lozano C, Juliá L, Sánchez-Baeza FJ, Anglada JM, Centelles JJ & Cascante M (2002) Cysteinyl-flavan-3-ol conjugates from grape procyanidins. Antioxidant and antiproliferative properties. *Bioorg Med Chem* **10**, 2497–2509.
18 Alonso C, Ramon E, Lozano C, Parra JL, Torres JL & Coderch L (2004) Percutaneous absorption of flavan-3-ol conjugates from plant procyanidins. *Drugs Exp Clin Res* **30**, 1–10.
19 Thompson RS, Jacques D & Haslam E (1972) Plant proanthocyanidins. Part I. Introduction: the isolation, structure, and distribution in nature of plant procyanidins. *J Chem Soc Perkin Trans* **I**, 1387–1399.
20 Kolodziej H (1990) Thiolysis of birch bark procyanidins: structural dependence in formation of 2,3-*cis*-3,4-*cis*-flavan-4-benzylthioethers from procyanidins. *Phytochemistry* **29**, 1671–1674.
21 Dangles O, Fargeix G & Dufour C (2000) Antioxidant properties of anthocyanins and tannins: a mechanistic investigation with catechin and the 3',4',7-trihydroxyflavylium ion. *J Chem Soc Perkin Trans* **2**, 1653–1663.
22 Foti MC, Daquino C & Geraci C (2004) Electron-transfer reaction of cinnamic acids and their methyl esters with the DPPH center dot radical in alcoholic solutions. *J Org Chem* **69**, 2309–2314.
23 Torres JL, Varela B, Brillas E & Juliá L (2003) Tris(2,4,6-trichloro-3,5-dinitrophenyl) methyl radical: a new stable coloured magnetic species as a chemosensor for natural polyphenols. *Chem Commun* **1**, 74–75.
24 Pucci B, Kasten M & Giordano A (2000) Cell cycle and apoptosis. *Neoplasia* **2**, 291–299.
25 Kovacic P, Sacman A & Wu-Weis M (2002) Nephrotoxins: widespread role of oxidative stress and electron transfer. *Curr Med Chem* **9**, 823–847.

26 Kondo K, Kurihara M, Miyata N, Suzuki T & Toyoda M (1999) Scavenging mechanisms of (–)-epigallocatechin gallate and (–)-epicatechin gallate on peroxyl radicals and formation of superoxide during the inhibitory action. *Free Radical Biol Med* **27**, 855–863.
27 Kondo K, Kurihara M, Miyata N, Suzuki T & Toyoda M (1999) Mechanistic studies of catechins as antioxidants against radical oxidation. *Arch Biochem Biophys* **362**, 79–86.
28 Long LH, Clement MV & Halliwell B (2000) Artifacts in cell culture: rapid generation of hydrogen peroxide on addition of (–)-epigallocatechin, (–)-epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media. *Biochem Biophys Res Commun* **273**, 50–53.
29 Kovacic P & Jacintho JD (2001) Mechanisms of carcinogenesis: focus on oxidative stress and electron transfer. *Curr Med Chem* **8**, 773–796.
30 Pelicano H, Feng L, Zhou Y, Carew JS, Hileman EO, Plunkett W, Keating MJ & Huang P (2003) Inhibition of mitochondrial respiration: a novel strategy to enhance drug-induced apoptosis in human leukemia cells by a reactive oxygen species-mediated mechanism. *J Biol Chem* **278**, 37832–37839.
31 Wardman P (2001) Electron transfer and oxidative stress as key factors in the design of drugs selectively active in hypoxia. *Curr Med Chem* **8**, 739–761.
32 Wenzel U, Nickel A, Kuntz S & Daniel H (2004) Ascorbic acid suppresses drug-induced apoptosis in human colon cancer cells by scavenging mitochondrial superoxide anions. *Carcinogenesis* **25**, 703–712.
33 Konat GW & Cochrane CG (2003) H_2O_2-induced higher order chromatin degradation: a novel mechanism of oxidative genotoxicity. *J Biosci* **28**, 57–60.
34 Khan AU & Wilson T (1995) Reactive oxygen species as cellular messengers. *Chem Biol* **2**, 437–445.
35 Ahmad N, Cheng P & Mukhtar H (2000) Cell cycle dysregulation by green tea polyphenol epigallocatechin-3-gallate. *Biochem Biophys Res Commun* **275**, 328–334.
36 Jung YD & Ellis LM (2001) Inhibition of tumour invasion and angiogenesis by epigallocatechin gallate (EGCG), a major component of green tea. *Int J Exp Pathol* **82**, 309–316.
37 Valcic S, Timmermann BN, Alberts DS, Wachter GA, Krutzsch M, Wymer J & Guillen JM (1996) Inhibitory effect of six green tea catechins and caffeine on the growth of four selected human tumor cell lines. *Anticancer Drugs* **7**, 461–468.
38 Kim CH & Moon SK (2005) Epigallocatechin-3-gallate causes the p2l/WAF1-mediated G(1)-phase arrest of cell cycle and inhibits matrix metalloproteinase-9 expression in TNF-alpha-induced vascular smooth muscle cells. *Arch Biochem Biophys* **435**, 264–272.
39 Ahmad N, Adhami VM, Gupta S, Cheng PY & Mukhtar H (2002) Role of the retinoblastoma (pRb)-E2F/DP pathway in cancer chemopreventive effects of green tea polyphenol epigallocatechin-3-gallate. *Arch Biochem Biophys* **398**, 125–131.
40 Liberto M & Cobrinik D (2000) Growth factor-dependent induction of p21 (CIP1) by the green tea polyphenol, epigallocatechin gallate. *Cancer Lett* **154**, 151–161.
41 Shackelford RE, Kaufmann WK & Paules RS (2000) Oxidative stress and cell cycle checkpoint function. *Free Radical Biol Med* **28**, 1387–1404.
42 Blois MS (1958) Antioxidant determinations by the use of a stable free radical. *Nature* **181**, 1199–1200.
43 Brand-Williams W, Cuvelier ME & Berset C (1995) Use of a free-radical method to evaluate antioxidant activity. *Lebensm-Wiss Technol* **28**, 25–30.
44 Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**, 55–63.
45 Freshney R (1987) *Culture of Animal Cells: A Manual of Basic Technique*. Liss, New York.
46 Comín-Anduix B, Agell N, Bachs O, Ovadi J & Cascante M (2001) A new bis-indole, KARs, induces selective M arrest with specific spindle aberration in neuroblastoma cell line SH-SY5Y. *Mol Pharmacol* **60**, 1235–1242.
47 Comín-Anduix B, Boros LG, Marín S, Boren J, Callol-Massot C, Centelles JJ, Torres JL, Agell N, Bassilian S & Cascante M (2002) Fermented wheat germ extract inhibits glycolysis/pentose cycle enzymes and induces apoptosis through poly(ADP-ribose) polymerase activation in Jurkat T-cell leukemia tumor cells. *J Biol Chem* **277**, 46408–46414.

II. Functional fatty fish supplemented with grape procyanidins. Antioxidant and proapoptotic properties on colon cell lines.

Medina, I.; Lois, S.; Lizárraga, D.; Pazos, M.; ***Touriño, S.***; Cascante, M. and Torres; J.L.; ***J Agric. Food Chem.***; 54 (10): 3598 -3603; **2006**

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Functional Fatty Fish Supplemented with Grape Procyanidins. Antioxidant and Proapoptotic Properties on Colon Cell Lines

I. MEDINA,*,† S. LOIS,† D. LIZÁRRAGA,‡ M. PAZOS,† S. TOURIÑO,§ M. CASCANTE,‡ AND J. L. TORRES§

Instituto de Investigaciones Marinas del CSIC, Eduardo Cabello 6, E-36208 Vigo, Spain, Department of Biochemistry and Molecular Biology, CSIC, University of Barcelona, Martí i Franquès 1, 08028 Barcelona, Spain, and Instituto de Investigaciones Químicas y Ambientales de Barcelona, IIQAB−CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain

This work shows the properties of grape procyanidins with additional anticarcinogenic properties for increasing the shelf life of functional seafood preparations. Galloylated procyanidins (100 ppm, 2.7 mean degree of polymerization, 25% galloylation) extended the shelf life of minced horse mackerel muscle stored at 4 °C more than 8 days compared to controls without addition of polyphenols. The levels of endogenous α-tocopherol, EPA, and DHA of fish muscle were also preserved after 10 days at 4 °C. Therefore, the presence of procyanidins increased the stability of a product based on minced fish muscle during cold storage and maintained its functionality associated with the presence of polyunsaturated fatty acids and α-tocopherol. In addition, grape procyanidins showed a significant capacity to induce apoptosis in colon cancer cells (HT29 cell line) while being inactive in noncancer control cells (IEC-6). Thus, the product based on fatty fish muscle supplemented with grape procyanidins appears to be a stable functional food offering the combined action of ω-3 fatty acids and natural polyphenols.

KEYWORDS: Fish lipids; procyanidins; functional seafood; anticarcinogenic

INTRODUCTION

Functional foods is a term used to refer to foods and isolated food ingredients that provide an additional physiological benefit beyond that of meeting basic nutritional needs (*1*). Within the past decade, there has been an increment of consumer and industrial interest in the health-enhancing role of specific foods or physiologically active food components (*2*). Marine bioactive lipids are a main goal of food companies which already have products in the market claiming to be stable ω-3 oils from marine sources. This fact has a special relevance for fatty and semifatty fish species such as horse mackerel, mackerel, herring, etc. Their muscles contain high amounts of ω-3 polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA, 20:5ω-3) and docosahexaenoic acid (DHA, 22:6ω-3), which have shown potential benefits to human health, particularly in preventing cardiovascular diseases (*3*, *4*). Fish is also an excellent source of other compounds with potential activities for human health such as ascorbic acid and α-tocopherol (*1*), involved in the stabilization of the high content of PUFA and rapidly degraded during the oxidative postmortem processes (*5*).

However, the oxidative development of off-flavors and rancidity continues to be the main objection in the exploitation and commercialization of products based on fish muscle. Moreover, the biological functionality of their lipids and other compounds is lost even at low storage temperatures (−18 °C) (*5*, *6*). Grape seeds and skin are a rich source of oligomeric catechins (procyanidins, **Figure 1**) which have been recently employed as natural antioxidants for stabilizing marine lipids (*7*). Such polyphenols are potent free radical scavengers (*8*) increasingly appreciated as chemopreventive agents against health conditions such as cancer and cardiovascular diseases (*9*, *10*). Apart from their scavenging activity, catechins, particularly those including a gallate ester moiety, appear to interact with the replicating machinery of tumor cells, probably through enzyme−ligand interactions with some key protein domains (*11*, *12*). They also trigger apoptosis (programmed cell death) by mechanisms that are currently under investigation (*13*, *14*).

This work is aimed at studying the functional properties of grape procyanidins with additional anticarcinogenic properties, as an effective additive against oxidation of fish functional compounds. For such a purpose, a seafood product based on minced horse mackerel muscle was supplemented with galloylated procyanidins and the oxidation of molecules associated with fish functionality such as PUFA and α-tocopherol was studied. The polyphenolic mixture will be, at least in part, bioavailable in the colon after ingestion of the supplemented seafood (*15*). Since procyanidins are potential cancer cell inhibitors reported to exert actions on cancer cells (*16*), their effect on colon cancer cells has been evaluated.

* To whom correspondence should be addressed. Fax +34 986 292762. E-mail: medina@iim.csic.es.
† Instituto de Investigaciones Marinas del CSIC.
‡ University of Barcelona.
§ IIQAB−CSIC.

10.1021/jf0527145 CCC: $33.50
Published on Web 04/22/2006

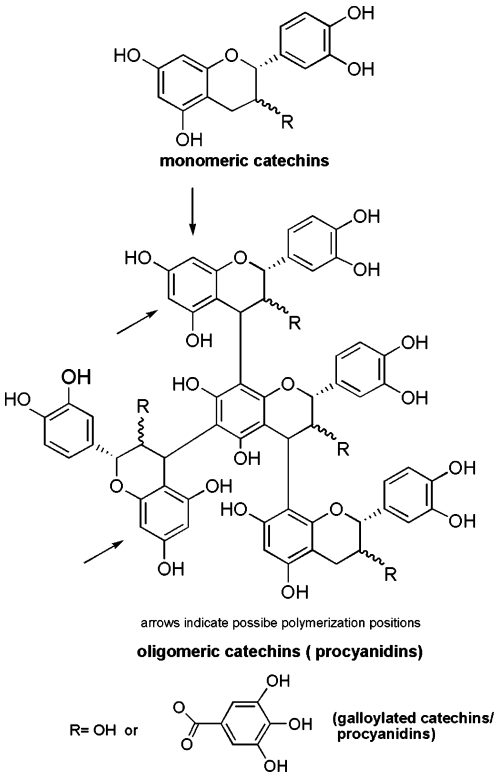


Figure 1. Structures of monomeric and oligomeric catechins.

MATERIALS AND METHODS

Materials. Fresh Atlantic horse mackerel (*Trachurus trachurus*) was supplied by a local market. Grape procyanidins were isolated from a total phenolic fraction extracted from grape pomace (*Vitis vinifera*) essentially as described by Torres et al. (*17*). The fraction employed in this work was a mixture of procyanidins (**Figure 1**), labeled as procyanidins **IVB**. They were selected after previous experiments on fish lipids and minced muscle (*7*) and consisted of oligomers with a mean degree of polymerization of 2.7 and 25% galloylation, both estimated from HPLC analysis after depolymerization with cysteamine as described by Torres and Selga (*18*). All chemicals and solvents used for procyanidin and fish analysis were either analytical or HPLC grade. Dulbecco's Modified Eagle's Medium (DMEM) and Dulbecco's phosphate-buffered saline (PBS) were obtained from Sigma Chemical. Co (St. Louis, MO), antibiotic (10 000 U/mL penicillin, 10 000 μg mL streptomycin) from Gibco-BRL (Eggenstein, Germany), Fetal calf serum (FCS) was obtained from Nalgen. Trypsin EDTA solution C (0.05% trypsin−0.02% EDTA) was purchased from Biological Industries (Kibbutz Beit Haemet, Israel). Trypan Blue solution 0.2%, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), propidium iodide (PI), and Igepal CA-630 were from Sigma Chemical Co (St. Louis, MO); α,α,α-tris(hydroxymethyl)-aminomethane was from Aldrich-Chemie (Steinheim, Germany), and Annexin V/FITC kit was from Bender Med Systems (Med Systems Diagnostics GmbH, Vienna, Austria).

Preparation and Storage of Horse Mackerel Muscle. Fifty different fish, 16 kg, of fresh Atlantic horse mackerel (*Trauchurus trauchurus*) were deboned and eviscerated, and the white muscle was separated and minced to obtain a muscle homogenate. Streptomycin sulfate (200 ppm) was added for inhibiting microbial growth. Procyanidins **IVB** were added at concentrations of 50 and 100 ppm (w/w). Portions of 10 g of minced muscle were placed into plastic air bags and then sealed. Controls and samples with procyanidins **IVB** were kept refrigerated at 4 °C for 13 days. After that, samples showed microbial growth. Duplicate samples were taken every day. Ninety samples were prepared and analyzed. The experiment was performed in duplicate.

Sensory Analysis. A total of four panelists trained in descriptive analysis of fishy off-flavors sniffed the same raw samples that were used for chemical determinations. Approximately 10 g of muscle was placed in separate sterile polystyrene Petri dishes and put on a tray of ice. Panelists concentrated on detecting rancidity/painty odors using a hedonic scale from 7 to ≤1; 7 showed absolutely fresh and ≤1 was putrid (*19*).

Lipid Extraction. Lipids were extracted from fish muscle according to Bligh and Dyer (*20*) and quantified gravimetrically (*21*).

Peroxide Value and Thiobarbituric Acid Reactive Substances (TBARS) Analyses. The peroxide value of fish muscle was determined by the ferric thiocyanate method (*22*) and was expressed as milliequivalents of oxygen molecule per kilogram of oil (mequiv of O_2/kg of oil). Analyses were performed in duplicate. TBARS, expressed as millimoles of malonaldialdehyde per kilogram of muscle (mmol of MDA/kg of muscle), was determined according to Vyncke (*23*). Analyses were performed in duplicate.

Inhibition of formation of peroxides and TBARS was calculated during the propagation period of controls according to Frankel (*5*). Induction periods were calculated as the time (in days) required for a sudden change in the rate of oxidation by the method of tangents to the two parts of the kinetic curve (*24*).

Determination of α-Tocopherol. α-Tocopherol was extracted by adaptation of the Burton et al. (*25*) procedure as described by Pazos et al. (*26*). The analysis of α-tocopherol was performed by HPLC according to Cabrini et al. (*27*).

Fatty Acid Analysis. Fatty acid composition of lipids extracted from horse mackerel muscle was determined by gas chromatography (*28*).

Cell Culture. Human colorectal adenocarcinoma HT29 cells (ATCC HTB-38) and two noncancer rat intestinal cell lines IEC-6 (ECCAC No. 88071401) and IEC-18 (ECCAC No. 88011801) were used. The cells have epithelial morphology and adherent growth properties. HT29, IEC-6, and IEC-18 cells were maintained as monolayer culture at 95% humidity, 5% CO_2, and 37 °C. Cells were passaged at preconfluent densities by the use of trypsin EDTA solution C. Cells were cultured and passaged in DMEM supplemented with 10% heat-inactivated fetal calf serum and 0.01% streptomycin/penicillin.

Cell Growth Inhibition. HT29, IEC-6, and IEC-18 were seeded into 96-well flat-bottomed microtiter plates; the samples contained 200 μL of cell suspension at 15×10^3, 25×10^3, and 5×10^3 cells/mL, respectively. After the adherence of cells for 24 h of incubation at 37 °C, the procyanidins **IVB** were dissolved in fresh medium, aliquoted, and added to the cells to obtain final concentrations from 1 to 300 μg/mL. The cultures were incubated for 72 h in a incubator with 95% humidity, 5% CO_2 at 37 °C. After incubation, the medium was removed, 50 μL of MTT (5 mg/mL in phosphate-buffered saline (PBS)) and 50 μL of medium were added to each well, and the mixtures were incubated for 1 h. The blue MTT formazan precipitate was dissolved in 100 μL of DMSO, and the absorbance values at 550 nm were recorded on an ELISA plate reader (Tecan Sunrise MR20-301, TECAN Australia). Absorbance was proportional to the number of living cells. The growth inhibition curves and the concentrations that caused 50% of inhibition cell growth (IC_{50}) were calculated using Grafit 3.0 software. The assay was performed by a variation of the MTT assay (*29*).

Cell Cycle Analysis. The assay was carried out by flow cytometry by using a fluorescence-activated cell sorter (FACS). HT29, IEC-6, and IEC-18 cells were plated in six-well flat-bottomed microtiter plates, at a density of 87.3×10^3, 146×10^3, and 29.1×10^3 cells/well, respectively. The number of cells was estimated using Trypan Blue and a Neubauer cell counter chamber (Brand, Wertheim, Germany). The cultures were incubated for 72 h, in the absence or presence of the polyphenolic mixture at their respective IC_{50} values. Thereafter, the cells were trypsinized, pelleted by centrifugation, and stained in Tris-buffered saline (TBS) containing 50 μg/mL PI, 10 μg/mL RNase free of DNase, and 0.1% Igepal CA-630 for 1 h at 4 °C in the dark. Cell cycle analysis was performed by FACS (Epics XL flow cytometer, Coulter Corporation, Hialeah, FL) at 488 nm. All experiments were performed in triplicate.

Apoptosis. The occurrence of apoptosis (programmed cell death) was determined by measuring Annexin V/FITC and propidium iodide (PI) staining by FACS. Cells were seeded, treated, and collected as described above. Following centrifugation, cells were washed in binding

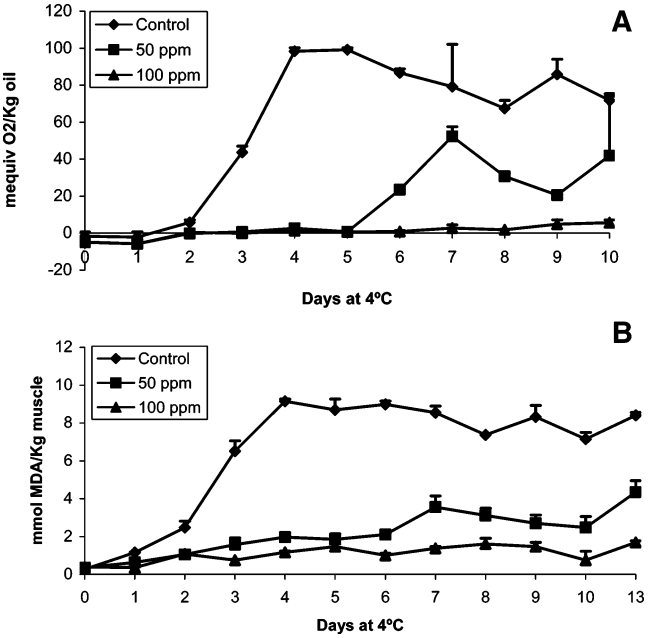


Figure 2. Hydroperoxide **(A)** and TBARS **(B)** formation during chilled storage of minced fish muscle at 4 °C (mean ± standard deviation of experiments performed in duplicate).

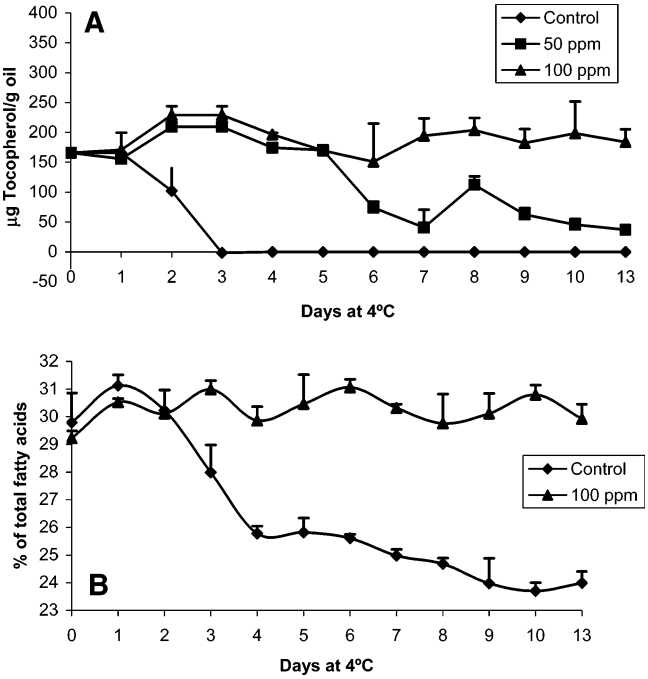


Figure 3. Concentration of α-tocopherol **(A)** and DHA **(B)** during chilled storage of minced fish muscle at 4 °C (mean ± standard deviation of experiments performed in duplicate).

buffer (10 mM Hepes/sodium hydroxide pH 7.4, 140 mM sodium chloride, 2.5 mM calcium chloride) and resuspended in the same buffer, Annexin V/FITC was added according to the Annexin V/FITC kit. After 30 min of incubation at room temperature and darkness, propidium iodide was added 1 min before FACS analysis at 20 μg/mL. Experiments were performed in triplicate. Assessment of apoptosis for HT29, IEC-6, and IEC-18 cells was done after 72 h of treatment with procyanidins **IVB** at their respective IC_{50} concentrations.

Statistical Analysis. The data from fish experiments were compared by one-way analysis of variance (ANOVA) (*30*), and the means were compared by a least squares difference method (*31*). Significance was declared at $p < 0.01$. Cell growth, cell cycle, and apoptosis assays were analyzed by the Student's *t*-test and were considered statistically significant at $p < 0.05$ or $p < 0.001$. Data given are representative of three independent experiments.

RESULTS AND DISCUSSION

Preservation of Fish Lipids from Oxidation. Procyanidins **IVB** supplemented to minced fish muscle promoted a significant preservation of fish lipids from oxidation (**Figure 2** and **Table 1**). As the sensory and chemical data demonstrate, the overall quality of the minced fish product was maintained longer in the samples containing procyanidins **IVB** than in controls. Sensory analysis revealed that control samples lost sensory quality by the third day in which panelists first detected a clear rancid odor. Minced fish muscle supplemented with procyanidins **IVB** maintained a very fresh odor for 7 days. By the seventh day, a slightly rancid odor was detected in samples supplemented with 50 ppm. After 10 days at 4 °C, rancid odors were not detected in fish muscle supplemented with 100 ppm. Additionally, the panelists were not able to appreciate significant changes in the taste of the fish homogenate supplemented with 100 ppm of procyanidins **IVB**.

In agreement with the sensory analysis, the formation of peroxides and TBARS in controls was significant by the third day and was retarded in samples containing procyanidins **IVB** (**Figure 2**). The induction periods of peroxide and aldehyde formation were 1.8 and 1.9 days for controls, 4.9 and 5.8 days for samples with 50 ppm procyanidins **IVB**, and longer than 10 days in samples with 100 ppm procyanidins **IVB**. The amount of oxidation products, peroxides, and MDA equivalents formed was significantly lower in fish muscle supplemented with procyanidins **IVB** than in controls.

These data are in agreement with the contents of α-tocopherol (**Figure 3A**). The oxidation of α-tocopherol can be significantly related to the oxidation produced in postmortem fish (*6*). The content of α-tocopherol in controls dropped significantly by the second day, whereas such a decrease was observed only by the sixth day in samples with 50 ppm **IVB** and was not observed in samples with 100 ppm after 10 days. Recent studies have described that α-tocopherol is the last defense of fish muscle to inhibit oxidation and its reduction below a critical level leads lipid oxidation (*6*). These data demonstrated that 100 ppm

Table 1. Development of Rancid Odors, Percentage Inhibition[a] of the Formation of Peroxides and TBARS, and Proportion of Original α-Tocopherol Remaining in Minced Fish Muscle during Storage at 4 °C by the Supplementation of 50 and 100 ppm Procyanidins (Mean ± SD)[b]

	sensory analysis		hydroperoxides		TBARS		α-tocopherol	
procyanidins IV	day 3	day 7	day 3	day 6	day 3	day 7	day 3	day 6
control	rancid odor	rancid odor	0.0 ± 0.2 a	0.7 ± 0.5 a	0.1 ± 0.1 a	0.1 ± 0.1 a	0.0 ± 0.5 a	0.1 ± 0.1 a
50 ppm	fresh odor	rancid odor	98.6 ± 2.8 b	72.9 ± 2.7 b	75.8 ± 5.5 b	58.4 ± 7.0 b	100.2 ± 1.5 b	55.3 ± 2.2 b
100 ppm	fresh odor	fresh odor	100.1 ± 0.2 b	99.0 ± 0.8 c	88.4 ± 0.7 c	83.9 ± 1.0 c	105.3 ± 3.4 b	99.8 ± 2.0 c

[a] % inhibition = $[(C - S)/C] \times 100$, where C = oxidation product formed and S = oxidation product formed in sample. [b] Values in each column with the same letter were not significantly different ($p < 0.01$). Number of samples analyzed in the experiment: 90.

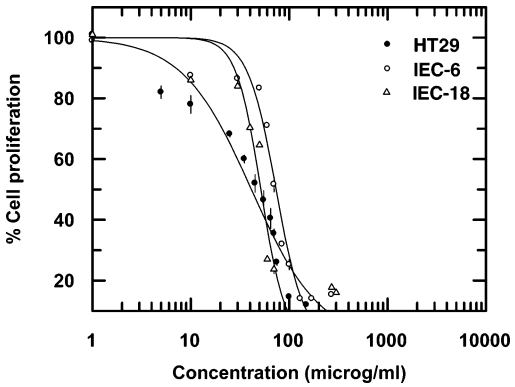


Figure 4. Percentage of proliferation of HT29 (●), IEC-6 (○), and IEC-18 (△) cells as a function of procyanidin concentration. Cells were treated for 72 h. Data are shown as the mean ± standard deviation of experiments performed in triplicate.

procyanidins **IVB** maintained the levels of endogenous α-tocopherol in minced horse mackerel muscle for 13 days at 4 °C.

Regarding the stability of fish bioactive PUFA, a significant reduction of the amount of DHA in control samples during chilling storage was recorded (**Figure 3B**). The DHA content of control samples decreased from an initial proportion of 29.8% total fatty acids to 25.8% by the fourth day and to 23.7% by the tenth day. Fish minced muscle supplemented with 100 ppm procyanidins **IVB** conserved its original levels of DHA for 10 days at 4 °C.

The data of this study revealed that the supplementation of procyanidins **IVB** with 25% galloylation stabilized the fish product based on minced muscle and maintained its functionality associated with the presence of PUFA and α-tocopherol. The supplementation of 100 ppm procyanidins **IVB** extended the shelf life of minced horse mackerel muscle for more than 8 days comparative to controls. Other polyphenolic antioxidants of natural origin such as olive oil phenolics or tea catechins have provided evidence to inhibit lipid oxidation of fish muscle (*32*, *33*). However, the effectiveness reported seems to be lower than that achieved by the supplementation of procyanidins **IVB**. Mackerel muscle containing 300 ppm tea catechins showed 60% inhibition of the formation of TBARS during the first 4 days at 4 °C and 20% after 6 days (*32*). The effectiveness of procyanidins **IVB** was also higher than that of hydroxytyrosol for preserving frozen fish fillets from oxidation (*33*). In other fish products such as sterilized tuna, 100 ppm olive oil phenolics slightly inhibited thermal oxidation of muscle after 4 days (*34*).

Regarding the stability and biological activity of grape procyanidins during chilled storage, some studies have found that grape procyanidins and their antioxidant activity were stable for more than 1 year at 4 °C (*35*). Additionally, the fish homogenate supplemented with 100 ppm procyanidins **IVB** did not show significant oxidation for more than 15 days (data not shown), meaning that the procyanidins were still active even when the fish product was not proper for consumption (microbiological growth).

Functional Properties of Grape Procyanidins. The procyanidins **IVB** described so far as food antioxidants may also exert a beneficial action on the humans consuming the supplemented fish product. Because procyanidins appear to be poorly absorbed in the gut and reach the end of the intestinal tract mostly intact (*15*), their putative preventive effect is likely to be more evident in the colon. The effect of 25% galloylated procyanidins **IVB** on the cell viability, cell cycle, and apoptosis

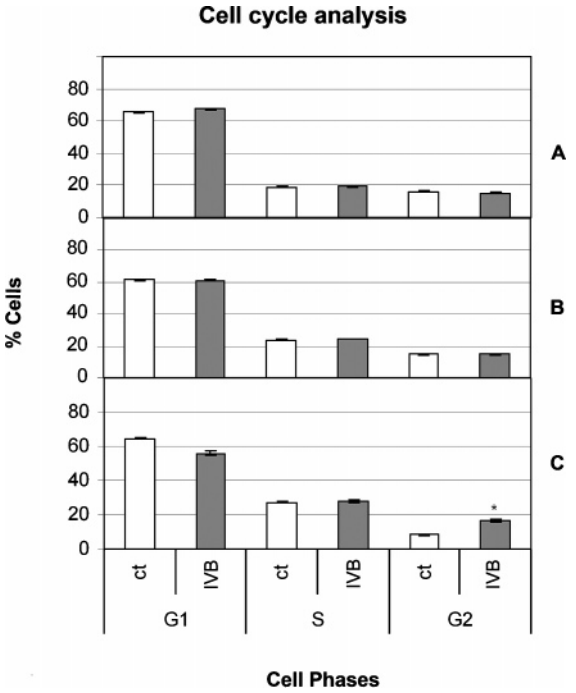


Figure 5. Cell cycle analysis by PI staining. Cells treated with procyanidins **IVB** at their IC_{50} values: **(A)** 75 μg/mL, IEC-6; **(B)** 56 μg/mL, IEC-18; and **(C)** 59 μg/mL, HT29. Percentages of cells at different cell stages (G1, S, and G2) are represented. % cells ± standard error of the mean (SEM) (*, $p < 0.05$; **, $p < 0.001$). ct, untreated control. Experiments were performed in triplicate.

in colon cancer cells was investigated using the HT29 epithelial colon carcinoma immortalized cell line. Two other cell lines (IEC-6 and IEC-18) were used as control models of noncancer colon cells.

Cell Viability. Cells were treated with different concentrations of procyanidins **IVB**, and the results showed that the decrease in cell number was dose-dependent (**Figure 4**). The calculated mean IC_{50} values were for HT29, 59 ± 3 μg/mL; for IEC-6, 74 ± 4 μg/mL; and for IEC-18, 52 ± 8 μg/mL. The procyanidins affected very little the cell viability in all three cell lines.

Cell Cycle. There was a moderate arrest of the cell cycle in the G2 phase in cancer HT29 cells (**Figure 5C**). The cell cycle distribution in noncancer IEC-6 and IEC-18 rat intestinal cell lines was not affected by procyanidins (**Figure 5A**,**B**). An arrest in G2 has also been described for other galloylated and nongalloylated polyphenols possibly by a mechanism involving enzyme inhibition (*36*).

Apoptosis. The procyanidins **IVB** induced the appearance of 16% apoptotic HT29 cells with respect to the untreated control (**Figure 5**). Interestingly the proapoptotic effect was selective for cancer cells. No induction of apoptosis was recorded for any of the noncancer cell lines. In addition, these two control cell lines presented a small percentage of necrotic cells (6% in IEC-6 and 5% in IEC-18) after treatment with the procyanidins (**Figure 6**). The proapoptotic effect of catechins, particularly tea (−)-epigallocatechin gallate, has also been described for different cell lines (*13*, *37*). Since the galloyl moiety of some catechins appears to be crucial for their proapoptotic effect (*13*, *14*), the presence of galloylated procyanidins **IVB** ($G = 25\%$) may explain, at least in part, the apoptosis elicited on HT29 cells. The influence of procyanidins **IVB** on the cell cycle (arrest in G2) may be attributed to other components of the mixture because the galloylated catechins appear to arrest the cell cycle in the G0/G1 phase in most of the cells tested so far (*12*, *14*).

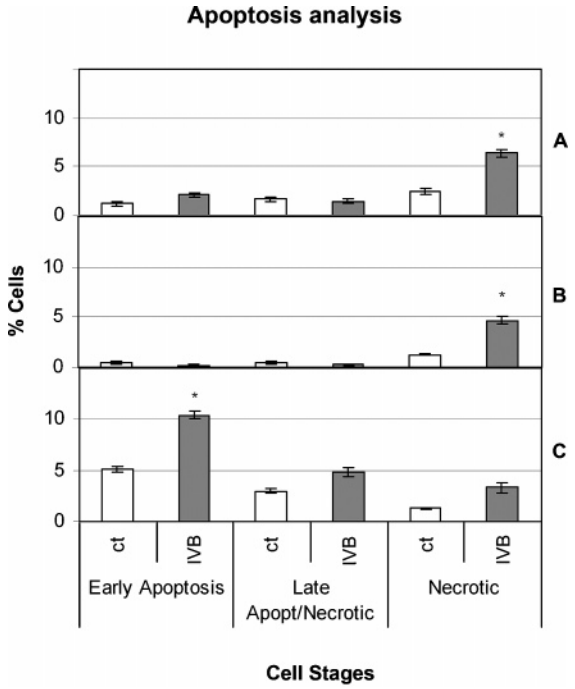


Figure 6. Analysis of apoptosis by annexin V/FITC and PI staining. Cells treated with procyanidins **IVB** at their IC_{50} values: **(A)** 75 μg/mL, IEC-6; **(B)** 56 μg/mL, IEC-18; and **(C)** 59 μg/mL, HT29. Percentages of cells at different cell stages are represented. Cell stages: early apoptosis (Annexin V^+PI^-); late apoptosis/necrotic (Annexin V^+PI^-); necrotic (Annexin V^-PI^+). % cells ± SEM (*, $p < 0.05$; **, $p < 0.001$). Experiments were performed in triplicate.

In any case, and most interestingly, the effects recorded for the antioxidant mixture on cell cycle and apoptosis was selective for cancer cells.

Briefly, data from the cellular studies show that the procyanidins **IVB** exerted little effect on the cell viability and cell cycle integrity of epithelial colon cells of both cancer and noncancer phenotype. The procyanidins exert a mild and selective proapoptotic effect on HT29 colon carcinoma cells. The healthy noncancer cells are not significantly affected by the procyanidins.

Conclusions. Functional ω-3 unsaturated fatty acids as well as α-tocopherol in minced horse mackerel muscle were stabilized by addition of an antioxidant extract composed mainly of procyanidins. The results with cell lines suggest that grape procyanidins **IVB** may also exert a direct preventive effect on colon epithelial cells by acting as an antioxidant and a selective proapoptotic agent. Thus, the product based on fatty fish muscle supplemented with grape procyanidins appears to be an interesting and stable functional food offering the combined action of ω-3 fatty acids and natural polyphenols.

ACKNOWLEDGMENT

M. Jesús Gonzalez and Elsa Silva are gratefully acknowledged for their technical assistance.

LITERATURE CITED

(1) Mazza, G. *Functional Food, Biochemical and Processing aspects;* Mazza, G., Ed.; Technomic Publishing Co., Inc.: Lancaster, PA, 1998.
(2) Hasler, C. M. Functional foods: Their role in disease prevention and health promotion. *Food Technol.* **1998**, *52*, 63−70.
(3) Nilsen, D. W.; Albrektsen, G.; Landmark, K. Effects of a high-dose concentrate of n-3 fatty acids or corn oil introduced early after an acute myocardial infarction on serum triacylglycerol and HDL cholesterol. *Am. J. Clin. Nutr.* **2001**, *74* (1), 50−56.
(4) Lee, K. W.; Lip, G. Y. H. The role of omega-3 fatty acids in the secondary prevention of cardiovascular disease. *QJM* **2003**, *96* (7), 465−480.
(5) Frankel, E. N. *Lipid Oxidation*; The Oily Press: Dundee, Scotland, 1998.
(6) Pazos, M.; Sánchez, L.; Medina, I. Alpha-tocopherol oxidation in fish muscle during chilling and frozen storage. *J. Agric. Food Chem.* **2005**, *53*, 4000−4005.
(7) Pazos, M.; Gallardo, J. M.; Torres, J. L.; Medina, I. Activity of grape polyphenols as inhibitors of the oxidation of fish lipids and frozen fish muscle. *Food Chem.* **2005**, *92*, 547−557.
(8) Yokozawa, T.; Chen, C. P.; Dong, E.; Tanaka, T.; Nonaka, G. I.; Nishioka, I. Study on the inhibitory effect of tannins and flavonoids against the 1,1-diphenyl-2-picrylhydrazyl radical. *Biochem. Pharmacol.* **1998**, *56*, 213−222.
(9) Yang, C. S.; Landau, J. M.; Huang, M. T.; Newmark, H. L. Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annu. Rev. Nutr.* **2001**, *21*, 381−406.
(10) Murphy, K. J.; Chronopoulos, A. K.; Singh, I.; Francis, M. A.; Moriarty, H.; Pike, M. J.; Turner, A. H.; Mann, N. J.; Sinclair, A. J. Dietary flavanols and procyanidin oligomers from cocoa (*Theobroma cacao*) inhibit platelet function. *Am. J. Clin. Nutr.* **2003**, *77* (6), 1466−1473.
(11) Liang, Y. C.; Lin-Shiau, S. Y.; Chen, C. F.; Lin, J. K. Suppression of extracellular signals and cell proliferation through EGF receptor binding by (−)-epigallocatechin gallate in human A431 epidermoid carcinoma cells. *J. Cell Biochem.* **1997**, *67* (1), 55−65.
(12) Ahmad, N.; Cheng, P. Y.; Mukhtar, H. Cell cycle dysregulation by green tea polyphenol epigallocatechin-3-gallate. *Biochem. Biophys. Res. Commun.* **2000**, *275* (2), 328−334.
(13) Tan, X. H.; Hu, D. R.; Li, S. R.; Han, Y.; Zhang, Y. L.; Zhou, D. Y. Differences of four catechins in cell cycle arrest and induction of apoptosis in LoVo cells. *Cancer Lett.* **2000**, *158* (1), 1−6.
(14) Gupta, S.; Hussain, T.; Mukhtar, H. Molecular pathway for (−)-epigallocatechin-3-gallate-induced cell cycle arrest and apoptosis of human prostate carcinoma cells. *Archiv. Biochem. Biophys.* **2003**, *410* (1), 177−185.
(15) Gonthier, M. P.; Donovan, J. L.; Texier, O.; Felgines, C.; Remesy, C.; Scalbert, A. Metabolism of dietary procyanidins in rats. *Free Radical Biol. Med.* **2003**, *35* (8), 837−844.
(16) Matito, C.; Mastorakou, F.; Centelles, J. J.; Torres, J. L.; Cascante, M. 2003. Antiproliferative effect of antioxidant polyphenols from grape in murine Hepa-1c1c7. *Eur. J. Nutr.* **2003**, *42* (1), 43−49.
(17) Torres, J. L.; Varela, B.; García, M. T.; Carilla, J.; Matito, C.; Centelles, J. J.; Cascante, C.; Sort, X.; Bobet, R. Valorization of grape (*Vitis vinifera*) byproducts. Antioxidant and biological properties of polyphenolic fractions differing in procyanidin composition and flavonol content. *J. Agric. Food Chem.* **2002**, *50*, 7548−7555.
(18) Torres, J. L.; Selga, A. Procyanidin size and composition by thiolysis with cysteamine hydrochloride and chromatography. *Chromatographia* **2003**, *57*, 441−445.
(19) Kelleher, S. D.; Silva, L. A.; Hultin, H. O.; Wilhelm. K. A. Inhibition of lipid oxidation during processing of washed, minced Atlantic mackerel. *J. Food Sci.* **1992**, *57*, 1103−1108.
(20) Bligh, E.; Dyer, W. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911−917.
(21) Herbes, S.; Allen, C. Lipid quantification of freshwater invertebrates: method modification for microquantitation. *Can. J. Fish Aquat. Sci.* **1983**, *40*, 1315−1317.
(22) Chapman, R. H.; McKay, J. The estimation of peroxides in fats and oils by the ferric thiocyanate method. *J. Am. Oil Chem. Soc.* **1949**, *26*, 360−363.

(23) Vyncke, W. Direct determination of the thiobarbituric acid value in trichloracetic acid extracts of fish as a measure of oxidative rancidity. *Fette Seifen Anstrichm.* **1970**, *72*, 1084−1087.
(24) Alaiz, M.; Hidalgo, F. J.; Zamora, R. Comparative antioxidant activity of Maillard- and oxidized lipid-damage bovine serum albumin. *J. Agric. Food Chem.* **1997**, *45*, 3250−3254.
(25) Burton, G. W.; Webb, A.; Ingold, K. U. A mild, rapid, and efficient method of lipid extraction for use in determining vitamin E/lipid ratios. *Lipids* **1985**, *20*, 29−39.
(26) Pazos, M.; González, M. J.; Gallardo, J. M.; Torres, J. L.; Medina, I. Preservation of the endogenous antioxidant system of fish muscle by grape polypenols during frozen storage. *Eur. Food Res. Technol.* **2005**, *220*, 514−519.
(27) Cabrini, L.; Stefanelli, C.; Fiorentini, D.; Landi, L. Ubiquinol prevents α-tocopherol consumption during liposome peroxidation. *Biochem. Int.* **1991**, *23*, 743−750.
(28) Christie, W. W. *Lipid Analysis*; Pergamon Press: Oxford, UK, 1982.
(29) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65* (1), 55−63.
(30) Sokal, R.; Rohlf, F. *Biometry*; Freeman and Co.: San Francisco, CA, 1981.
(31) Statsoft. *Statistica for Macintosh*; Statsoft and its Licensors: Tulsa, OK, 1994.
(32) Tang, S. Z.; Sheehan, D.; Buckley, D. J.; Morrissey, P. A.; Kerry, J. P. Anti-oxidant activity of added tea catechins on lipid oxidation of raw minced red meat, poultry and fish muscle. *Int. J. Food Sci. Technol.* **2001**, *36* (6), 685−692.
(33) Pazos, M.; Alonso, A.; Fernández-Bolaños, J.; Torres, J. L.; Medina, I. Physicochemical properties of natural phenolics from grapes and olive oil by-products and their antioxidant activity in frozen horse mackerel fillets. *J. Agric. Food Chem.* **2006**, *54* (2), 366−373.
(34) Medina, I.; Satué-Gracia, M. T.; German, J. B.; Frankel, E. N. Comparison of natural polyphenols antioxidant from extra virgin olive oil with synthetic antioxidants in tuna lipids during thermal oxidation. *J. Agric. Food. Chem.* **1999**, *47*, 4873−4879.
(35) Pinelo, M. Extraction of antioxidants from grape byproducts. Influence of the operation and process. Doctoral Thesis, University of Santiago de Compostela, Spain, 2004.
(36) Chen, C.; Shen, G. X.; Hebbar, V.; Hu, R.; Owuor, E. D.; Kong, A. N. T. Epigallocatechin-3-gallate-induced stress signals in HT-29 human colon adenocarcinoma cells. *Carcinogenesis* **2003**, *24* (8), 1369−1378.
(37) Nihal, M.; Ahmad, N.; Mukhtar, H.; Wood, G. S. Anti-proliferative and proapoptotic effects of (−)-epigallocatechin-3-gallate on human melanoma: Possible implications for the chemoprevention of melanoma. *Int. J. Cancer* **2005**, *114* (4), 513−521.

Received for review November 2, 2005. Revised manuscript received March 22, 2006. Accepted March 23, 2006. This work was performed within Research Projects PPQ2003-06602-C04-01, -03, and -04 and AGL2004-07579-C04-02 and -03 financed by the Spanish Ministry of Education and Science. We thank Xunta de Galicia for a postgraduate grant to S.L. and the Spanish Ministry of Education and Science for postgraduate grants to D.L. and S.T.

JF0527145

III. Procyanidins from pine bark: relationships between structure, composition and antiradical activity.

Jerez, M.; ***Touriño, S.***; Sineiro, J.; Torres, J.L. and Núñez, M.J.; ***Food Chem***.; 104 (2): 518-527; **2007**

Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 104 (2007) 518–527
Food Chemistry
www.elsevier.com/locate/foodchem

Procyanidins from pine bark: Relationships between structure, composition and antiradical activity

María Jerez [a], Sonia Touriño [b], Jorge Sineiro [a], Josep Lluís Torres [b], María José Núñez [a,*]

[a] Escuela Técnica Superior de Ingeniería, Universidad de Santiago de Compostela, Lope Gómez de Marzoa, 15782 Santiago de Compostela, Spain
[b] Instituto de Investigaciones Químicas y Ambientales, CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain

Received 16 June 2006; received in revised form 5 October 2006; accepted 28 November 2006

Abstract

Barks of *Pinus pinaster* and *Pinus radiata* were studied as source of procyanidins; these raw materials, considered a byproduct of forestal industry, were extracted with ethanol. The extract was partially fractionated to obtain an aqueous fraction (FA) containing a great part of the procyanidins from barks and with potential application to both food and medical fields. FAs were rich in polyphenols, 65–87% of which were procyanidins; the mean degree of polymerization (mDP) was 7.9 for radiata (rFA) and 10.6 for pinaster (pFA) varieties.

The aqueous fractions were chromatographed on Sephadex LH-20 to obtain specific fractions differing in DP and composition. These fractions were analysed by thiolysis with cysteamine, followed by RP-HPLC. Results showed that (+)-catechin was the main terminal unit for both barks and also the main extension unit for radiata. In contrast (−)-epicatechin was predominant as extension unit in pinaster and this could have implications for applications in oils, emulsions and biological systems.

In terms of antiradical activity, expressed as specific antiradical units, the entire rFA gave the best results, together with rF5. For *P. pinaster* bark, the best results were achieved for fractions F5–F8, with DP 7–22. The whole fraction from radiata represents an economic alternative of great interest because the fractionation costs can be avoided.

Keywords: *Pinus pinaster*; *Pinus radiata*; Bark; Antiradical power; Procyanidins; Aqueous fraction; Fractionation

1. Introduction

Great interest is currently centred on potential benefits of plant polyphenols as complements to the organism's antioxidant defence system. Polyphenols are potent free radical-scavengers and preventive against cardiovascular diseases, cancer and other disorders; they are increasingly used as natural food additives, acting as flavouring, colouring and antioxidant agents (Plumb, de Pascual-Teresa, Santos-Buelga, Cheynier, & Williamson, 1998). Among the different phenolics, flavonoids, phenolic acids, stilbenes and tannins, especially condensed tannins (proanthocyanidins), are particularly important (Hagerman et al., 1998). Proanthocyanidins are the most abundant polyphenols in plants after lignins, and they may represent up to 50% in several barks (Matthews et al., 1997); they can be divided into procyanidins and prodelphynidins. Pycnogenol, basically a procyanidin mixture from maritime pine, is probably the most studied phenolic from trees.

Procyanidins are composed of oligomers and polymers, consisting of (+)-catechin and/or (−)-epicatechin units linked mainly through C4 → C8 and/or C4 → C6 bonds (B-type). These flavan-3-ol units can be doubly linked by a C4 → C8 bond and an additional ether bond from O7 → C2 (A-type). Most of the activities of procyanidins, including the free radical-scavenging capacity, largely depend on their structure, particularly their degree of polymerization (DP) (Gaulejac, Vivas, Freitas, & Bourgeois, 1999; Touriño et al., 2005). Moreover, the presence of gallate esters seems decisive for the regulation of the cell cycle

* Corresponding author. Tel.: +34 981 563100x16792; fax: +34 981 528050.
E-mail address: eqmajose@usc.es (M.J. Núñez).

0308-8146/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2006.11.071

by catechins (Liberto & Cobrinik, 2000). In food systems, partition phenomena of procyanidins play an important role in the preservant action (Frankel, 2001). The main difficulty in studies on procyanidins is probably that of obtaining them in an individual molecular form. The complete purification of a procyanidin with a DP above five is almost impossible. Therefore, for studying their structures and properties, mixtures more or less polymerized are often employed (Guyot, Marnet, & Drilleau, 2001). Moreover, synergistic effects of active mixtures make plant extracts and fractions more interesting than pure compounds for functional food applications.

Many methods have been proposed for separating procyanidins according to their DP. For analytical purposes, a suitable separation can be achieved by normal-phase HPLC, TLC or reversed-phase HPLC. On a preparative scale, gel chromatography with different packings, such as Sephadex G-25, LH-20 and Toyopearl TSK HW 40, has been used (Kantz & Singleton, 1990; Karonen, Loponen, Ossipov, & Pihlaja, 2004). Normal-phase HPLC can separate procyanidins according to their degree of polymerization up to decamers, but this method is specific for monomers and oligomers and results in a severe underestimation of procyanidins, considering the prevalence of the polymers in nature (Czochanska, Foo, Newman, & Porter, 1980). Reversed-phase HPLC is the method commonly used for the separation of flavan-3-ol monomers and some small oligomers, especially dimers and trimers. However, there is difficulty in determining the DP using reversed-phase methods since oligomers elute in non-sequential order. Furthermore, analysis of higher oligomeric procyanidins is not feasible, since the number of isomers increases with increasing degrees of polymerization. This effect results in a retention time overlap of isomers of differing DP, causing the higher oligomers to co-elute as a large unresolved peak (Santos-Buelga & Williamson, 2003). The technique of thin-layer chromatography (TLC), with a silica phase, permits the separation of oligomeric proanthocyanidins up to heptamers (Lea, 1978). This method can be used only for qualitative analysis. Sephadex LH-20 and Toyopearl TSK HW-40 or TSK HW-50 columns are classically used to fractionate proanthocyanidins on the basis of molecular size; these are eluted with a mobile phase of alcohol–water or acetone–water. Large molecular weight procyanidins are then recovered with 60–70% acetone in water, with no further separation (Taylor, Barofsky, Kennedy, & Deinzer, 2003; Touriño et al., 2005).

Depolymerization by thiolysis has been proved as an efficient method for determining the nature of the flavan-3-ol units within procyanidins and for determining the average degree of polymerization. Thiolysis is performed in the presence of acid and a nucleophilic reagent, such as toluene-α-thiol (Rigaud, Perez-Ilzarbe, Da Silva, & Cheynier, 1991), cysteamine hydrochloride (Torres & Selga, 2003) or phloroglucinol (Kennedy & Jones, 2001). The extension subunits of procyanidins are attacked by the nucleophilic reagent to form the corresponding thio-derivatives and the terminal unit is released as the free flavan-3-ol. Degradation products can be analysed by reversed-phase HPLC, and the results will provide information on the nature of the extension and terminal units and on the average degree of polymerization.

Pine is one of the plants with the highest content of procyanidins. The phenolics of pine bark are (+)-catechin, (−)-epicatechin, dihydroquercetin, phenolic acids and, most of all procyanidin dimers, trimers, oligomers and polymers (Wood, Senthilmohan, & Peskin, 2002). Pine bark procyanidins have diverse biomedical applications (Packer, Rimbach, & Virgili, 1999; Rohdewald, 2002). Several constituents of pine bark extracts, such as gallic, protocatechuic acids and catechin, are readily adsorbed by human skin and make the preparations useful for topical application (Sarikaki et al., 2004). Besides, because pine procyanidins are devoid of gallate esters, which appear to interfere with crucial cell functions, they may be innocuous chemopreventive agents of choice for many applications (Touriño et al., 2005). The use of polyphenols as food antioxidants is also frequent, especially in fatty foods, such as fish, making it possible to prolong the storage time of several species both fresh and frozen products (Pazos, Alonso, Fernández-Bolaños, Torres, & Medina, 2006). Another dietetic application has recently reported (in a patent) by Degre (2003) who found that polymeric procyanidins (DP > 10) were strong inhibitors of intestinal α-amylase, so they may be useful in dietetics to suppress α-amylase activities, and/or the caloric values of food.

Working with procyanidins from different pine species, we have detected structural variations (Jerez, Pinelo, Sineiro, & Nuñez, 2006) which may be related to different physicochemical and biological properties. The aim of this work is to achieve the fractionation of structurally different procyanidins contained in the aqueous fractions from crude ethanolic extracts of bark of two varieties of pine, *Pinus pinaster* and *Pinus radiata*, and to characterize the different fractions for procyanidin content, degree of polymerization and antiradical activity. The information gained about the properties of the mixtures will help to define their possible applications as food antioxidants and/or as functional components .

2. Materials and methods

2.1. Plant material

Pine (*P. pinaster* and *P. radiata*) barks provided by M. Bouzas Garrido, S.A. (Vedra, A Coruña, Spain) were dried at room temperature for a week. They were then ground in an analytical mill MF 10 IKA-WERKE (Staufen, Germany) to less than 1 mm.

2.2. Reagents

Ethanol, methanol, acetone, toluene, formic acid, hydrochloric acid, sulphuric acid and vanillin were

obtained from Panreac (Moncada i Reixac, Spain). Lipophilic Sephadex LH-20 and 2,2-diphenyl-1-picrylhydrazyl were purchased from Sigma–Aldrich Química, S.A. (Madrid, Spain). Water, solvents and reagents for analytical RP-HPLC were MilliQ® water, HPLC grade CH_3CN (E. Merck, Darmstadt, Germany) and trifluoroacetic acid (TFA, Fluorochem, Derbyshire, UK), biotech grade distilled in-house. Analytical grade MeOH, cysteamine hydrochloride (Sigma–Aldrich, Steinheim, Germany) and fuming 37% hydrochloric acid (HCl, Merck, Darmstadt, Germany) were used for fraction depolymerisation. RP-HPLC standards: (+)-catechin (Cat), (−)-epicatechin (Ec) and procyanidins (B2) were purchased from Sigma–Aldrich. 4β-(2-Aminoethylthio)- catechin (Cya–Cat), and 4β-(2-aminoethylthio) epicatechin (Cya–Ec) were prepared as described by Torres and Bobet (2001).

2.3. Extraction and fractionation of extracts

Pine bark was extracted in an immersion extractor of 10 × 4.5 cm i.d. The extractor was kept at 37.5 °C by a thermostatted external water bath, ethanol being the solvent. A condenser was fitted to avoid solvent losses. Extraction was accomplished by continuously upward pumping of fresh solvent (17 ml/min) through the cake bed.

The bark extracts obtained were solvent-fractionated, essentially as described by Torres and Bobet (2001). Briefly, the total extract was ethanol-evaporated. The resulting solid was resuspended in water and lyophilized to yield the crude extract, which was defatted with petroleum ether. The residue was dried, suspended in water, acidified with acetic acid and extracted with ethyl acetate. Two fractions were obtained: an organic fraction and an aqueous fraction which contained mainly polymers. The solvent (water saturated with ethyl acetate) was eliminated from the aqueous fraction. The pellet was them resuspended in water and lyophilized to yield fraction FA.

2.4. Separation of the fraction FA on Sephadex LH-20 column

A sample of fraction FA (400 mg/4 ml of 50% methanol) was applied to a 50 × 2.5 cm i.d. Sephadex LH-20 column. Sephadex LH-20 (50 g) was suspended in 50% methanol in water and it was allowed to swell for 24 h before the column was manually packed by elution with the same solvent. The column was eluted with the sequence of solvents mixture shown in Table 1 at a flow rate of 3 ml/min. Samples were collected every 2 min using a fraction collector (Model 2110, BIO-RAD) and their absorbances were measured at 280 nm and 400 nm and only at the latter when the mobile phase contained acetone. Fractions were collected and evaporated under vacuum to remove organic solvents, and dissolved in methanol (4 ml). The column was washed exhaustively with 70% acetone/water (v/v) to obtain fraction F8, containing compounds with a higher DP. The elution of procyanidins was monitored using thin-layer chromatography (TLC).

Table 1
Solvent mixture for the fractionation of FA

P. pinaster vol (ml)	*P. radiata* vol (ml)	Acetone (% vol)	Methanol (% vol)	Water (% vol)	
575	540	0	60	40	(A)
300	240	0	75	25	(B)
360	390	0	90	10	(C)
300	360	10	80	10	(D)
300	300	20	65	15	(E)
300	300	30	40	30	(F)

2.5. TLC

Identification of procyanidins according to their degree of polymerization was effected by TLC under the conditions used by Lea (1978). TLC of fractions was performed on 20 cm × 20 cm silica gel 60 F_{254} plates (E. Merck, Darmstadt, Germany) eluted with toluene–acetone–formic acid (3:6:1). Separated components were visualized by spraying with 5% vanillin dissolved in ethanol acidified with 10% HCl, followed by heating the plate with a hot air blower. The flavans and procyanidins were revealed as orange to reddish spots.

2.6. Antiradical activity

The antiradical activity of the fractions was evaluated by using the method described by Brand-Williams, Cuvelier, and Berset (1995). A 6.1×10^{-5} M solution of DPPH$^{\cdot}$ in methanol was prepared daily and 980 μl of this solution were mixed with 20 μl of each sample. The initial concentration of DPPH was calculated for every experiment from a calibration curve made by measuring the absorbance at 515 nm of standard samples of DPPH at different concentrations. The equation of the curve was $Abs_{515\,nm} = 11{,}223\ C_{DPPH}$, as determined by linear regression. The results were plotted as the inhibition percentage at 515 nm, defined as $((A_0 - A/A_0) \times 100)$ against the amount of sample divided by the initial amount (μmol) of DPPH. Each point was repeated in triplicate. A dose–response curve was obtained for every extract and fraction. ED_{50} corresponds to microlitres of fraction able to consume half the amount of free radical divided by micromoles of initial DPPH. The antiradical activity (AR) unit was defined as the amount of sample able to consume half the amount of free radical. The results are also expressed as specific antiradical (sAR) activity (units divided by mass of procyanidins).

2.7. Vanillin assay

The analytical method normally employed to estimate the total amount of catechin and procyanidins is the colorimetric measurement after reaction with aromatic aldehydes

such as dimethylamino-cinnamaldehyde (DMACA) or vanillin. Quantification of total flavan-3-ols in each fraction obtained from a Sephadex LH-20 column was performed according to the method described by Sun, Ricardo-da-Silva, and Spranger (1998).

2.5 ml of 25% H_2SO_4 in methanol and 2.5 ml of 1% (w/v) vanillin in methanol were added to 1 ml of each fraction diluted in methanol. The mixture was allowed to stand for 15 min at room temperature, and the absorbance was then measured at 500 nm. The blank was the same as the reaction medium but without vanillin. The total amount of procyanidins in each fraction was expressed as procyanidin B2 equivalents.

2.8. Thiolysis conditions

Fractions from the Sephadex LH-20 column were diluted to 1 mg/ml procyanidin concentration, following the results of vanillin assay.

An aliquot (200 μl) of the fraction was added to the thiolysis mixture (200 μl) which consisted of cysteamine hydrochloride (500 mg) and 37% HCl (200 μl) dissolved in methanol (930 μl). The mixture (400 μl) was kept at 65 °C in a water bath for 15 min.

2.9. Analytical RP-HPLC and estimation of mean procyanidin composition

The size and composition of the procyanidins were estimated from the RP-HPLC analysis of the depolymerised fractions, as described Torres and Selga (2003). Briefly, the terminal flavan-3-ols units were released as such by acid cleavage in the presence of cysteamine whereas the extension moieties were released as the C4 cysteamine derivatives. Thiolysis reaction media (20 μl) were analysed by RP-HPLC on a Smart system (Amersham–Pharmacia Biotech, Uppsala, Sweden) equipped with a μ peak monitor (Amersham–Pharmacia Biotech) and fitted with a 100 × 2.1 mm i.d. μRPC C2/C18 SC 2.1/10 column. Elution: [A] 0.10% (v/v) aqueous TFA, [B] 0.08% (v/v) TFA in water/CH_3CN (1:4), gradient 8–23% [B] over 45 min. The flow rate was 100 μl/min. The detection was done at 214, 280 and 320 nm. The parameters calculated were: mean degree of polymerization (mDP) = total nmol/nmol terminal units, mean molecular weight (mMW) = total mass/nmol terminal units.

3. Results and discussion

The starting-point of this work was the processing of crude extracts, obtained in previous works from the ethanolic extraction of *P. pinaster* and *P. radiata* barks. The mean degrees of polymerisation for these extracts of *P. pinaster* and *P. radiata* were 7.0 and 5.4, respectively, the specific antiradical powers being 34.3 and 31 AR(antiradical) units/mg polyphenols (Jerez et al., 2006). Crude extracts were partially separated, essentially as previously described (Torres & Bobet, 2001), rendering a fraction, FOW, with compounds soluble in both ethyl acetate and water, and another fraction, FA, containing species soluble in water but not in organic solvents. This fraction, named aqueous fraction from now on, is analysed in this paper.

Fig. 1 shows the HPLC chromatogram for the aqueous fraction of *P. pinaster*, pFA; it basically consists of a broad hump, which indicates the presence of a mixture of procyanidins with different DPs (profile for *P. radiata* is similar). When pFA was depolymerised by thiolysis with toluene-α-thiol (Jerez, 2003), the resolution of this peak showed the existence of (+)-catechin, (−)-epicatechin and the corresponding benzyl-thioethers. As the entire FAs are complex, more homogeneous procyanidin fractions (with different degrees of polymerisation suitable for different applications) can be obtained by fractionating FAs on a Sephadex LH-20 column. Resulting fractions will be later

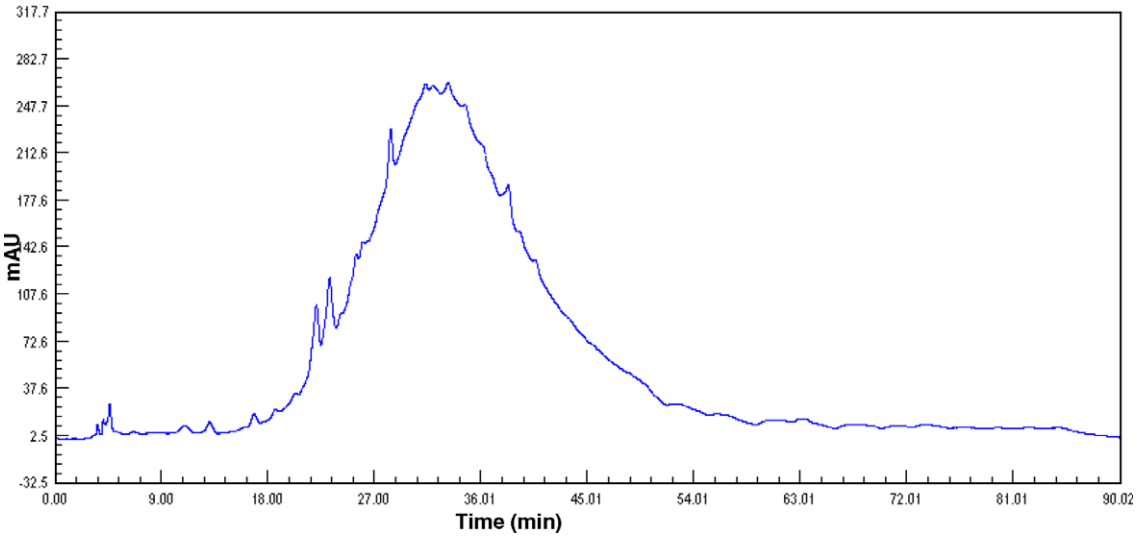


Fig. 1. Chromatogram of the aqueous fraction FA from *P. pinaster*.

depolymerised for characterization. Table 1 summarises the elution conditions used. This mixture was previously used by other authors (Taylor et al., 2003) for fractioning hop procyanidins. Under these conditions, low molecular weight (MW) compounds are eluted first and bulkier procyanidins are recovered last.

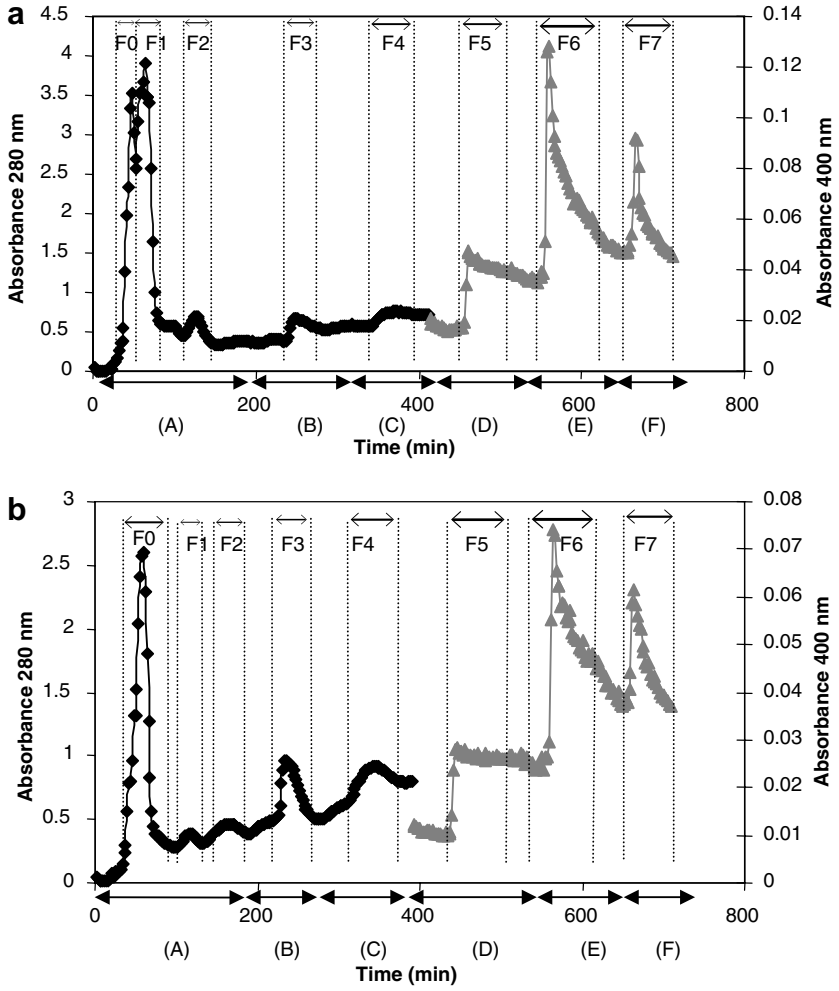


Fig. 2. Chromatographic profile obtained by fractionation of fraction FA on Sephadex LH-20 column: (a) *P. pinaster* and (b) *P. radiata*.

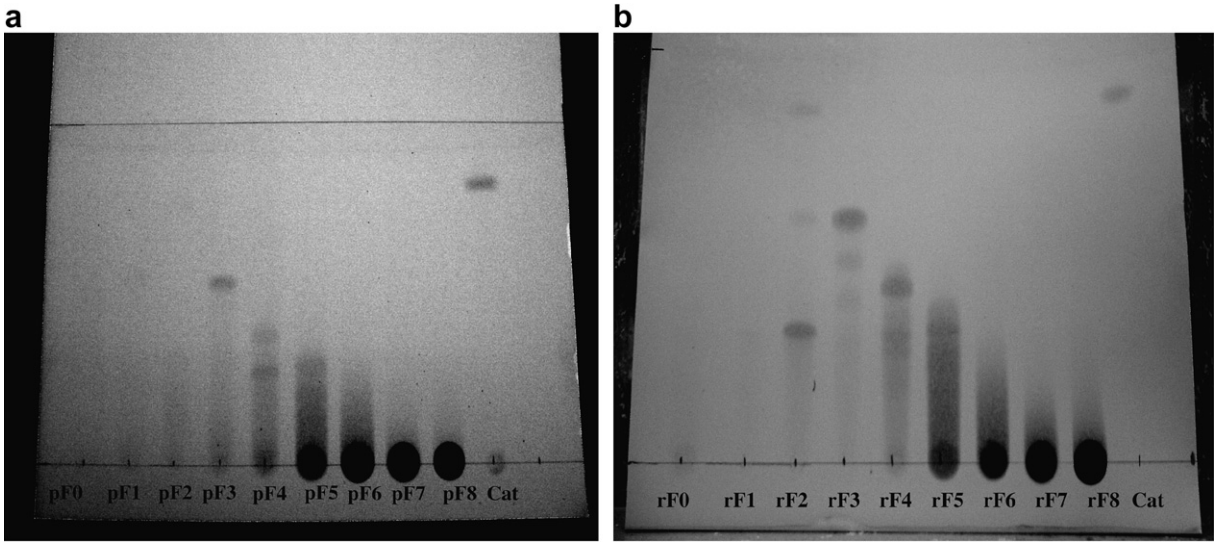


Fig. 3. TLC of fractions from Sephadex LH-20 column runs in one dimension with (toluene/acetone/formic acid) as solvent: (a) *P. pinaster* and (b) *P. radiata*.

Fig. 2a and b shows the elution profiles which were obtained by measuring the absorbance of the fractions collected at intervals of 2 min in an UV-V Jasco V-530 spectrophotometer. Fractions under each peak were pooled into 8 fractions, F0–F7. Procyanidins of higher MW remained tightly adsorbed at the top of the column. Because the chromatographic mode is adsorption rather than exclusion, the separation can be incomplete (Yanagida et al., 2000). Washing with 70% acetone resulted in the recovery of high mDP procyanidins. This washing was carried out, instigating the recovery of fraction F8. Acetone was chosen because the carbonyl oxygen serves as a strong H-bond acceptor and allows displacement of bound polymeric phenols from Sephadex LH-20.

TLC of each fraction was performed to obtain preliminary information concerning their procyanidin content (DP), following the vanillin assay (Fig. 3a and b). In the first fractions, pF0, pF2 for *P. pinaster* and rF0, rF1 for *P. radiata*, no spots were observed, indicating the absence of flavan-3-ols, either as monomers or as their derivatives, and these fractions were not included in the subsequent analyses. In any case these fractions may contain phenolic acids and taxifolin (dihydroquercetin, present basically in fractions soluble in both ethyl acetate and water), frequent in pine bark (Wood et al., 2002). (+)-catechin was only observed, as traces, in rF2. The developed spots in fractions pF3–pF4 and rF2–rF4 corresponded to procyanidins of low molecular weight. For the other fractions, the spots remained on the base-line, indicating that they included bulky hydrophilic high molecular weight compounds.

Each one of the obtained fractions containing procyanidins was thiolysed with cysteamine, which was preferred to toluene-α-thiol for being more user-friendly and much less toxic (Torres & Selga, 2003). Figs. 4a and b and 5a and b

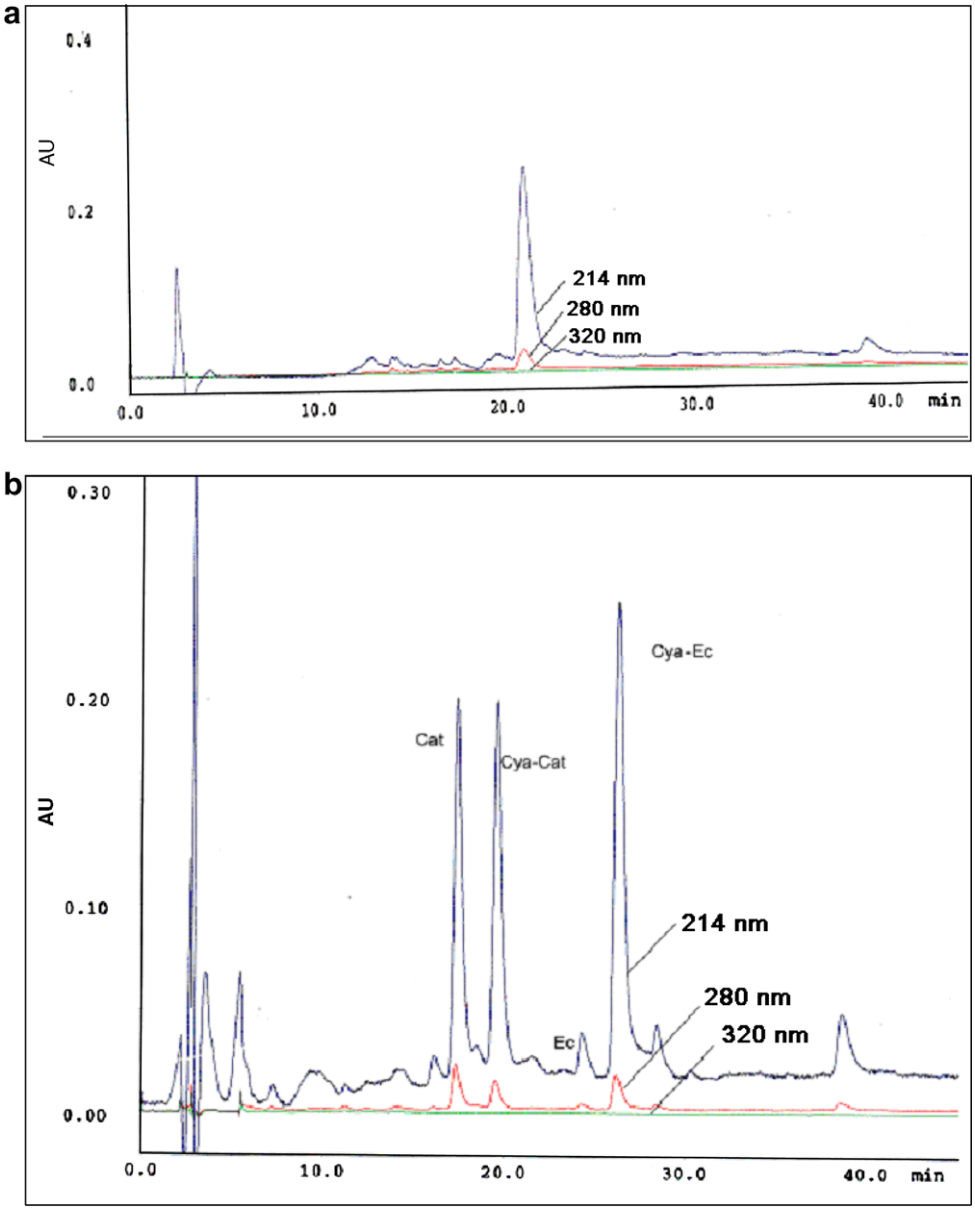


Fig. 4. RP-HPLC chromatograms of fraction F3 from *P. pinaster* (pF3), obtained by fractionating FA: (a) raw fraction and (b) thiolysed fraction.

show the chromatogram of F3 of the barks from the two pine varieties. In agreement with our previous results, *P. radiata* contained more procyanidins than *P. pinaster*. The profile after thiolysis indicated that (+)-catechin was the main terminal unit in both cases. Regarding the extension units, the situation is different. *P. radiata* contained almost exclusively catechin units whereas *P. Pinaster* included both epicatechin and catechin extension units (Fig. 4b).

In Tables 2 and 3, the structural compositions of the different fractions are indicated. For all *P. pinaster* fractions and whole pFA, epicatechin was the main extension unit, with a ratio of 3–6 with respect to catechin, except for pF3, for which the ratio was 1.2.

P. radiata fractions were also consistent with whole rFA, with a proportion catechin/epicatechin extension units of 1.2. Again, rF3 was the exception to the rule, the ratio being 10.8. In this case, the mean composition of rF2 was also different, because epicatechin predominated as the extension unit. The presence of catechin as an abundant extension unit in *P. radiata* may influence the physicochemical properties of procyanidins, which will be more hydrophobic than those from *P. pinaster*. This fact can be of interest for the possible antioxidant activity in oils, emulsions and biological systems.

Tables 4 and 5 summarise the mean structural parameters and activity of the entire FAs and eluted fractions (procyanidin content, antiradical power, mDP and

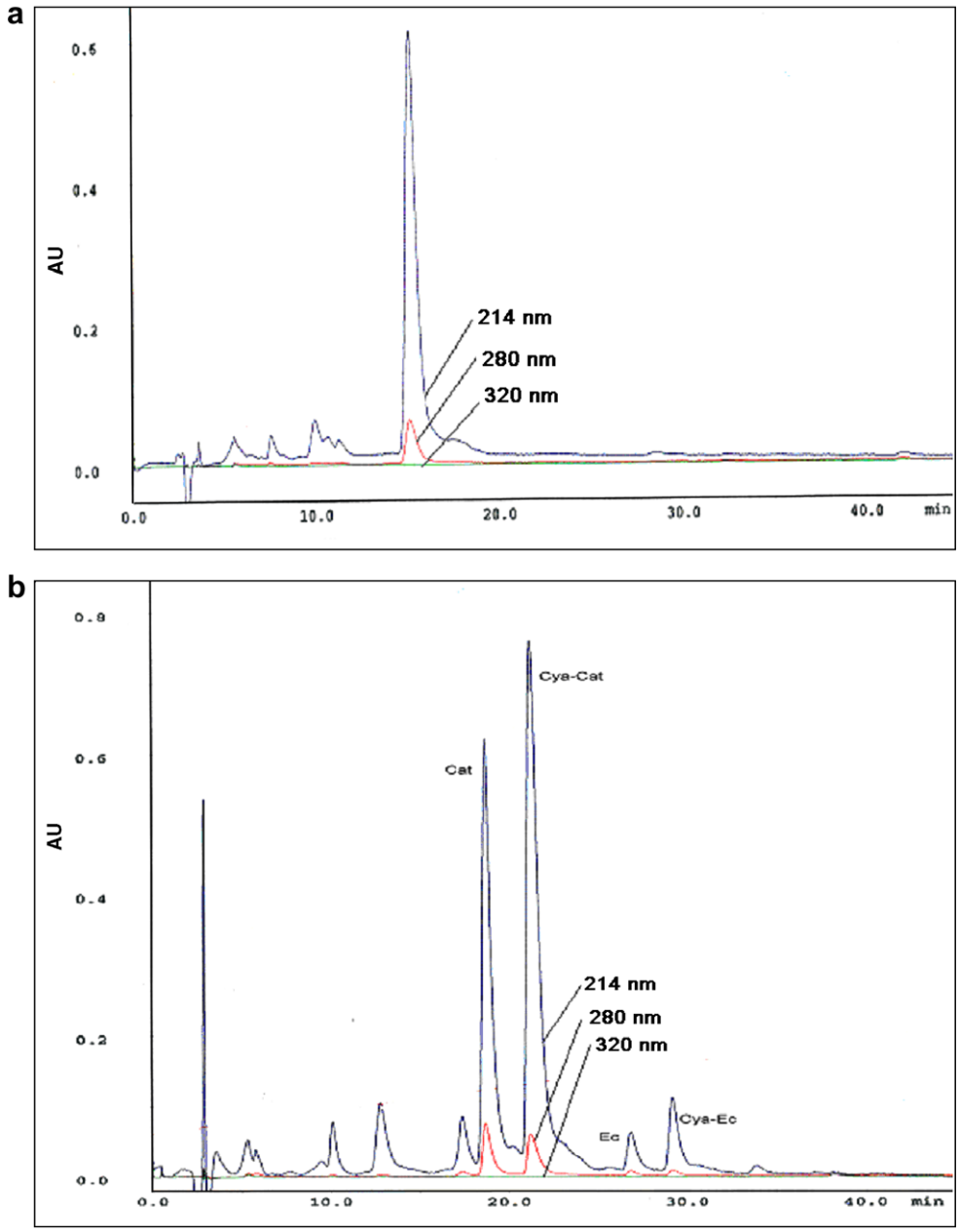


Fig. 5. RP-HPLC chromatogram of fraction F3 from *P. radiata* (rF3), obtained by fractionating FA: (a) raw fraction and (b) thiolysed fraction.

Table 2
Structural composition of fractions from FA of *P. pinaster*, determined by HPLC following thiolysis degradation

Fraction	Proportion of terminal units (%)		Proportion of extension units (%)	
	Catechin	Epicatechin	Aminoethylthio catechin	Aminoethylthio epicatechin
pFA	8.1 ± 0.1	1.4 ± 0.2	13.5 ± 0.4	77.0 ± 0
pF3	35.9 ± 0.3	3.8 ± 0	26.9 ± 1.0	33.5 ± 0.7
pF4	20.5 ± 0.6	1.6 ± 0.3	18.0 ± 0.3	60.0 ± 0
pF5	13.1 ± 0.3	0.8 ± 0	14.5 ± 0.7	71.7 ± 0.9
pF6	7.8 ± 0.4	nd	12.4 ± 0.2	79.6 ± 0.4
pF7	5.5 ± 0.1	nd	12.6 ± 0.1	81.9 ± 0.1
pF8	4.6 ± 0.2	nd	12.9 ± 0.5	82.7 ± 0.2

Table 3
Structural composition of fractions from FA of *P. radiata*, determined by HPLC following thiolysis degradation

Fraction	Proportion of terminal units (%)		Proportion of extension units (%)	
	Catechin	Epicatechin	Aminoethylthio catechin	Aminoethylthio epicatechin
FA	10.4 ± 0.6	2.3 ± 0.2	44.2 ± 0.4	43.4 ± 0.17
F2	40.8 ± 3.2	7.9 ± 0.9	21.7 ± 1.2	29.8 ± 1.1
F3	44.7 ± 1.5	3.4 ± 0.7	47.5 ± 0.4	4.4 ± 0.4
F4	25.8 ± 0.9	2.4 ± 0.3	48.3 ± 1.0	23.6 ± 0.2
F5	15.2 ± 1.2	0.4 ±0.5	45.8 ± 0.3	38.7 ± 0.4
F6	9.9 ± 0.1	1.1 ± 0.1	49.1 ± 0.1	39.9 ± 0.2
F7	8.0 ± 0.2	nd	50.4 ± 0.4	41.5 ± 0.4
F8	6.3 ± 0.3	0.6 ± 0.1	53 ± 0	40 ± 0

mMW), which were calculated from the thiolysis data. Procyanidins for FAs represent 65% of total polyphenols in pinaster, increasing to 87% for radiata. Up to fraction 4, eluted with water–methanol, mDP were similar for the two varieties; *P. radiata* was richer in procyanidins and was more active. The antiradical activity per mass (specific antiradical activity, sAR) is more suitable for interpreting the effect of polymerisation on the scavenging activity. According to this index, values are similar for pF3, pF4, and not very different from that of the total pFA. In contrast, sAR for the whole rFA was much higher than sAR for rF2–rF4. From F5 on, fractions eluted with acetone, the situation is different; pF5 and rF5, with mDP about 7, showed the highest sAR, and the subsequent fractions from *P.radiata* presented a gradual decrease in this parameter, down to values similar to those of the first fractions. In *P. pinaster*, the behaviour did not follow exactly the same trend; pF6–pF8 showed similar sAR values, around 56. This different pattern may be related to the differences detected in the extension units for both varieties (Tables 2 and 3). Fractions with mDP above 7 from *P. pinaster* contained mostly epicatechin as extension unit while *P. radiata* oligomers contained both catechin and epicatechin in similar proportions. This fact may influence the antiradical effectiveness, because the stereoposition of the phenolic OH group in C3 is involved in the oxidation mechanism, especially by radicals.

The increase of antiradical power of flavanols with mDP up to 7 can be explained by the electron delocalisation through intramolecular links among monomers, because C4–C8 confers a very tight conformation but, at a given point, with new units the steric hindrance can be decisive (Gaulejac et al., 1999). Gaulejac et al. (1999) found an increase in activity for procyanidins from 1 to 4 units. In the present work, we have recorded an increase up to 6–7 units and from there, either a stabilisation (*P. pinaster* 12–22 mDP) or a drop in sAR (*P. radiata* 9–15 mDP).

When procyanidins incorporate gallate, tendencies are diverse. The trend is an increase of antiradical power until DP = 3; if we compare values for F3 (Tables 3, 4) with an analogous fraction of grape pomace with 25% galloylation (Pazos, 2005), antiradical power for grape is 1.5 times

Table 4
Characteristics of fractions from FA of *P. pinaster*

Fractions	mDP	mMW	Procyanidins (mg)	Total AR activity units	AR activity units/mg procyanidins (sAR)
pFA (400 mg)	10.6 ± 0.4	3067 ± 119	139 ± 3.0	5488	39.5
pF3	2.5 ± 0	731 ± 6	1.1 ± 0.1	41.9	38.2
pF4	4.6 ± 0.1	1313 ± 18	2.9 ± 0.3	106.7	36.3
pF5	7.2 ± 0.1	2090 ± 44	8.8 ± 0.2	561.8	63.5
pF6	12.8 ± 0.4	3713 ± 110	24 ± 0.4	1360.5	56.7
pF7	18.2 ± 0.3	5258 ± 91	10.5 ± 0.2	580.6	55.2
pF8	22.2 ± 1.1	6425 ± 303	22.4 ± 0.5	1261.8	56.3

Table 5
Characteristics of fractions from FA of *P. radiata*

Fractions	mDP	mMW	Procyanidins (mg)	Total AR activity units	AR activity units/mg procyanidins (sAR)
rFA (400 mg)	7.9 ± 0.2	2278 ± 56	166 ± 4	9650	58.1
rF2	2.1 ± 0.1	598 ± 28	2.0 ± 0.3	84.6	41.2
rF3	2.1 ± 0.1	603 ± 9	2.2 ± 0.1	99.5	45.8
rF4	3.6 ± 0.2	1033 ± 45	3.8 ± 0.2	187.8	49.9
rF5	6.5 ± 0.3	1875 ±88	13.0 ± 0.4	851.1	65.4
rF6	9.2 ± 0.1	2647 ± 13	26.9 ± 0.1	1428.6	53.1
rF7	12.6 ± 0.4	3651 ± 110	11.9 ± 0.3	571.4	48.0
rF8	14.6 ± 0.5	4226 ± 137	50.6 ± 0.6	2353	46.5

higher than that for rF3, and 3 times higher than pF3. In the literature, we have not found references to procyanidins with high DP and galloylated, although Gaulejac et al. (1999) pointed out that, from trimers on, gallate may induce the adoption of a very compact "box structure", which lowers ability to scavenge free radicals. In agreement with the literature, we did not detect the presence of any gallate ester in pine bark extracts. None of the procyanidins absorbed at 320 nm and the thiolysis did not yield any galloylated monomer or conjugate (e.g. Figs. 4 and 5). In any case, since galloylation does not appear to be relevant for protection against oxidation in emulsions (Touriño et al., 2005), pine bark may become an excellent source of antioxidants, rich in procyanidins and easy to handle. Moreover, because aqueous fractions are often discarded, this paper may also be a new contribution towards the integral use of plants.

Acknowledgements

We thank Spanish Ministry MCyT for Project PPQ2003-06602-C04-01 y 02.

References

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *Lebensmittel-Wissenschaft & Technologie, 28*, 25–30.

Czochanska, Z., Foo, L. Y., Newman, R. H., & Porter, L. J. (1980). Polymeric proanthocyanidins. Stereochemistry, structural units and molecular weight. *Journal of the Chemical Society – Perkin Transactions, I*, 2278–2286.

Degre, M. F. (2003). Polymeric procyanidins as α-amylase inhibitors for dietetic composition. Fr. Demande FR 2002-2571 20020228.

Frankel, E. N. (2001). Interfacial lipid oxidation and antioxidation. *Journal of Oleo Science, 50*, 387–391.

Gaulejac, N., Vivas, N., Freitas, V., & Bourgeois, G. (1999). The influence of various phenolic compounds on scavenging activity assessed by an enzymatic method. *Journal of the Science of Food and Agriculture, 79*, 1081–1090.

Guyot, S., Marnet, N., & Drilleau, J-F. (2001). Thiolysis-HPLC characterization of apple procyanidins covering a large range of polymerization states. *Journal of Agricultural and Food Chemistry, 49*, 14–20.

Hagerman, A. E., Riedl, K. M., Jones, A., Sovik, K. N., Ritchard, N. T., Hartfeld, P. W., et al. (1998). High molecular weight plant poliphenolics (tannins) as biological antioxidants. *Journal of Agricultural and Food Chemistry, 46*, 1887–1892.

Jerez, M. (2003). Extracción de sustancias Bioactivas a partir de corteza de Pino. Ms. Thesis, University Santiago de Compostela, Spain.

Jerez, M., Pinelo, M., Sineiro, J., & Nuñez, M. J. (2006). Influence of extraction conditions on phenolic yields from pine bark: assessment of procyanidins polymerization degree by thiolysis. *Food Chemistry, 94*, 406–414.

Kantz, K., & Singleton, V. L. (1990). Isolation and determination of polymeric polyphenols using Sephadex LH-20 and analysis of grape tissue extracts. *American Journal of Enology and Viticulture, 41*(3), 223–228.

Karonen, M., Loponen, J., Ossipov, V., & Pihlaja, K. (2004). Analysis of procyanidins in pine bark with reversed-phase and normal-phase high-performance liquid chromatography-electrospray ionization mass spectrometry. *Analytica Chimica Acta, 522*, 105–112.

Kennedy, J. A., & Jones, G. P. (2001). Analysis of proanthocyanidin cleavage products following acid-catalysis in the presence of excess phloroglucinol. *Journal of Agricultural and Food Chemistry, 49*, 1740–1746.

Lea, A. G. H. (1978). The phenolics of ciders: oligomeric and polymeric procyanidins. *Journal of the Science of Food and Agriculture, 29*, 471–477.

Liberto, M., & Cobrinik, D. (2000). Growth factor-dependent induction of p21 (CIP1) by green tea by polyphenol, epigallocatechin gallate. *Cancer Letters, 154*, 151–161.

Matthews, S., Mila, I., Scalbert, A., Pollet, B., Lapierre, C., Hervé du Penhoat, C., et al. (1997). Method for estimation of proanthocyanidins based on their acid depolymerization in presence of nucleophiles. *Journal of Agricultural and Food Chemistry, 45*, 1195–1201.

Packer, L., Rimbach, G., & Virgili, F. (1999). Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (*Pinus maritima*) bark, pycnogenol. *Free Radical Biology and Medicine, 27*, 704–724.

Pazos, M. (2005). Inhibición de la oxidación lipídica en el músculo de pescado mediante la utilización de polifenoles obtenidos a partir de bagazo de uva Ph.D. Thesis, USC.

Pazos, M., Alonso, A., Fernández-Bolaños, J., Torres, J. L., & Medina, I. (2006). Physicochemical properties of natural phenolics obtained from grape and olive oil by-products and their antioxidant activity in frozen fish fillets. *Journal of Agricultural and Food Chemistry, 54*, 366–373.

Plumb, G. W., de Pascual-Teresa, S., Santos-Buelga, C., Cheynier, V., & Williamson, G. (1998). Antioxidant properties of catechins and proanthocyanidins: effect of polymerisation, galloylation and glycosilation. *Free Radical Research, 29*, 351–358.

Rigaud, J., Perez-Ilzarbe, J., Da Silva, J. M. R., & Cheynier, V. (1991). Micro method for the identification of proanthocyanidin using thiolysis monitored by high-performance liquid chromatography. *Journal of Chromatography A, 540*, 401–405.

Rohdewald, P. (2002). A review of the French maritime pine bark extract (Pycnogenol (R)), a herbal medication with a diverse clinical pharmacology. *International Journal of Clinical Pharmacology and Therapeutics, 40*, 158–168.

Santos-Buelga, C., & Williamson, G. (2003). *Methods in polyphenol analysis*. Cambridge, UK: Royal Society of Chemistry Pub..

Sarikaki, V., Rallis, M., Tanojo, H., Panten, I., Dotsiva, Y., Laukas, Y. L., et al. (2004). In vitro percutaneous absorption of pine bark extract (PYC) in human skin. *Journal of Toxicology – Cutaneous and Ocular Toxicology, 23*, 149–158.

Sun, B., Ricardo-da-Silva, J. M., & Spranger, I. (1998). Critical factors of vanillin assay for catechins and proanthocyanidins. *Journal of Agricultural and Food Chemistry, 46*, 4267–4274.

Taylor, A. W., Barofsky, E., Kennedy, J. A., & Deinzer, M. L. (2003). Hop (*Humulus lupulus* L.) Proanthocyanidins characterized by mass spectrometry, acid catalysis, and gel permeation chromatography. *Journal of Agricultural and Food Chemistry, 51*, 4101–4110.

Torres, J. L., & Bobet, R. (2001). New flavanol-derivatives from grape (*Vitis vinifera*) byproducts. Antioxidant aminoethylthio-flavan-3-ol conjugates from a polymeric waste fraction used as a source of flavanols. *Journal of Agricultural and Food Chemistry, 49*, 4627–4634.

Torres, J. L., & Selga, A. (2003). Procyanidin size and composition by thiolysis with cysteamine hydrochloride and chromatography. *Chromatographia, 57*, 441–445.

Touriño, S., Selga, A., Jiménez, A., Juliá, L., Lozano, C., Lizárraga, D., et al. (2005). Procyanidin fractions from *Pinus pinaster* bark: radical scavenging power in solution, antioxidant activity in emulsion and antiproliferative effect in mellanoma cells. *Journal of Agricultural and Food Chemistry, 53*, 4728–4735.

Wood, J. E., Senthilmohan, S. T., & Peskin, A. V. (2002). Antioxidant activity of procyanidin-containing plant extracts at different pHs. *Food Chemistry, 77*, 155–161.

Yanagida, A., Kanda, T., Takahashi, T., Kamimura, A., Hamazono, T., & Honda, S. (2000). Fractionation of apple procyanidins according to their degree of polymerization by normal-phase high-performance liquid chromatography. *Journal of Chromatography A, 890*, 251–259.

IV. The importance of polymerization and galloylation for the antiproliferative properties of procyanidin-rich natural extracts.

Lizárraga, D.; Lozano, C.; Briede´, J. J.; Van Delft, J. H.; ***Touriño, S.***; Centelles, J. J.; Torres J.L. and Cascante, M.; ***FEBS Journal***; 274: 4802–4811; **2007**

the FEBS Journal

The importance of polymerization and galloylation for the antiproliferative properties of procyanidin-rich natural extracts

D. Lizarraga[1], C. Lozano[2], J. J. Briedé[3], J. H. van Delft[3], S. Touriño[2], J. J. Centelles[1], J. L. Torres[2] and M. Cascante[1,2]

1 Biochemistry and Molecular Biology Department, Biology Faculty, University of Barcelona, Biomedicine Institute from University of Barcelona (IBUB) and Centre for Research in Theoretical Chemistry, Scientific Park of Barcelona (CeRQT-PCB), Associated Unit to CSIC, Spain
2 Institute for Chemical and Environmental Research (IIQAB-CSIC), Barcelona, Spain
3 Department of Health Risk Analysis and Toxicology, Maastricht University, the Netherlands

Keywords
antiproliferative; apoptosis; cell cycle; colon cancer; scavenger capacity

Correspondence
M. Cascante Serratosa, Department of Biochemistry and Molecular Biology, University of Barcelona, Biology Faculty, Av. Diagonal 645, 08028 Barcelona, Spain
Fax: +34 934021219
Tel: +34 934021593
E-mail: martacascante@ub.edu

(Received 2 May 2007, revised 3 July 2007, accepted 18 July 2007)

doi:10.1111/j.1742-4658.2007.06010.x

Grape (*Vitis vinifera*) and pine (*Pinus pinaster*) bark extracts are widely used as nutritional supplements. Procyanidin-rich fractions from grape and pine bark extract showing different mean degrees of polymerization, percentage of galloylation (percentage of gallate esters) and reactive oxygen species-scavenging capacity were tested on HT29 human colon cancer cells. We observed that the most efficient fractions in inhibiting cell proliferation, arresting the cell cycle in G_2 phase and inducing apoptosis were the grape fractions with the highest percentage of galloylation and mean degree of polymerization. Additionally, the antiproliferative effects of grape fractions were consistent with their oxygen radical-scavenging capacity and their ability to trigger DNA condensation–fragmentation.

Colorectal cancer is the third most commonly diagnosed cancer in the world and is one of the major causes of cancer-associated mortality in the USA [1,2]. Epidemiological studies indicate that colon cancer incidence is inversely related to the consumption of fruit, vegetables and green tea [3,4]. Specifically, the imbalance between high-level oxidant exposure and antioxidant capacity in the colon has been linked to increased cancer risk and is strongly influenced by dietary antioxidants [5–7]. Several studies have demonstrated that polyphenolic compounds are capable of providing protection against cancer initiation and its subsequent development [8–11].

A variety of health-promoting products obtained from grape seeds and skins, tea leaves, pine and other plant byproducts are currently available and a great deal of research is being devoted to testing the putative beneficial effect of these products in relation to their polyphenolic content [12–16]. Catechins and their polymeric forms (proanthocyanidins) are being studied in particular depth. The composition of monomeric catechins and their oligomers and polymers (proanthocyanidins), as well as the percentage of galloylated species in these natural extracts, differs between tea, grape and pine bark.

The antiproliferative activity of catechins and proanthocyanidins is associated with their ability to inhibit cell proliferation and to induce cell cycle arrest and apoptosis [17,18]. Most of the polyphenols in tea are monomers of gallocatechins and their gallates [19], whereas grape contains monomers and oligomers of

Abbreviations
DMPO, 5,5-dimethyl-1-pyrolline-*N*-oxide; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide; PI, propidium iodide.

catechins with some galloylation and mainly polymerized procyanidins [20]. In contrast, procyanidin fractions from pine bark extracts do not contain gallocatechins or gallates.

The influence of polyphenolic structure on antioxidant activity, protective capacity and, particularly, on the mechanism of action remains open to debate and further study is required. Research with different cell lines has shown that the most widely studied natural polyphenol, epigallocatechin-3-gallate from green tea, is a potent antioxidant and chemopreventive agent [21,22]. These and other results suggest that the galloylation of catechins and the presence of gallocatechin moieties in natural extracts could be important chemical characteristics. They may be useful indicators in evaluating the potential of natural plant extracts for colon cancer prevention or treatment and the degree of polymerization related to the bioavailability in the colon.

Procyanidins and monomeric catechins (Fig. 1) are the main active polyphenols in grape and pine bark. The difference between grape and pine catechins and procyanidins is found in the presence of gallate esters in position 3 (galloylation). Whereas grape flavanols are galloylated to some extent [23,24], pine bark appears to be devoid of gallate esters [25,26]. It has been reported that oligomeric procyanidins are not significantly absorbed in the intestinal tract, and reach the colon mainly intact [27]. They are therefore bioavailable to the epithelial cells in the intestinal wall, where procyanidins and other phenolics are extensively degraded, metabolized and absorbed. In a first stage, the oligomers are depolymerized and the constitutive catechin units are partially absorbed as glucuronates, sulfates and methyl esthers, as described for the small intestine [28]. They are also, in part, extensively metabolized to phenolic acids such as 3-hydroxyphenylvaleric acid and 3-hydroxyphenylpropionic acid, which are then absorbed as glucuronates and sulfates [27,29]. The gallate esters are more stable than the simple catechins upon being metabolized [30] and may be more bioavailable in the colon. Gallates have been reported to inhibit cell growth, trigger cell cycle arrest in tumor cell lines and induce apoptosis [31,32]. Furthermore, studies have shown that they also offer protection by scavenging reactive oxygen species such as superoxide anion, hydrogen peroxide and hydroxyl radicals, which cause destruction of biochemical components that are important in physiological metabolism [33,34]. This capacity to prevent the imbalance between high-level oxidant exposure and antioxidant capacity, which leads to several pathological processes, may contribute to the chemopreventive effect of the gallic acid derivatives. Because grape is a rich source of procyanidins and contains some galloylation, procyanidin fractions from grape could be potential antiproliferative compounds of interest in the prevention of colon cancer.

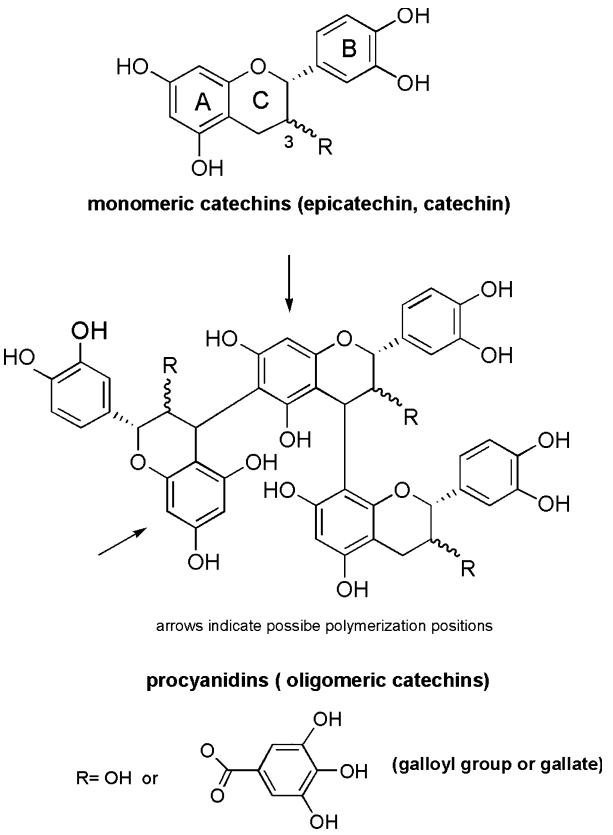


Fig. 1. Structure of the major polyphenols found in white grape pomace.

In the present study, we investigated the relationship of different structural factors of procyanidins, such as the mean degree of polymerization and percentage of galloylation, with their antiproliferative potential and their scavenging capacity for hydroxyl and superoxide anion radicals.

Results and Discussion

Growth inhibition capacity

Table 1 shows that pine bark extracts containing oligomers (XIP, VIIIP, IVP, VIP and OWP) reduced proliferation of the carcinoma cell line HT29 dose-dependently with IC_{50} values between 100 and 200 μM and IC_{80} values between 200 and 300 μM, whereas the IC_{50} and IC_{80} values of fraction VP containing monomers were almost one order of magnitude higher (1551 and 2335 μM, respectively). If we consider that the pine

Table 1. Comparative chemical characteristics and HT29 cell growth inhibition of grape and pine fractions. Percentage of galloylation (%G), mean degree of polymerization (mDP) and mean relative molecular mass (mM_r) from Torres *et al.* [50] and Touriño *et al.* [26].

	Fraction	%G	mDP	mM_r	IC_{50} (μM)	IC_{80} (μM)
Grape	VIIIG	34	3.4	1160	55 ± 3	76 ± 3
	IVG	25	2.7	880	67 ± 3	100 ± 3
	VIG	16	2.4	751	56 ± 7	113 ± 7
	OWG	15	1.7	552	99 ± 18	134 ± 18
	VG	0	1	290	410 ± 10	483 ± 10
Pine	XIP	0	3.4	999	108 ± 4	308 ± 4
	VIIIP	0	3	876	123 ± 6	199 ± 6
	IVP	0	2.9	833	127 ± 6	204 ± 6
	VIP	0	2.7	777	143 ± 7	230 ± 7
	OWP	0	2.1	601	190 ± 5	305 ± 5
	VP	0	1	290	1551 ± 14	2335 ± 14

fractions are not galloylated, it can clearly be concluded that oligomers are much more efficient than monomers at inhibiting colon carcinoma cell proliferation.

Under the same experimental conditions, the grape polyphenolic fractions with an equivalent degree of polymerization but also with a percentage of galloylation ≥15% (VIIIG, IVG, VIG and OWG) produced IC_{50} and IC_{80} values that were approximately half those of the homologous pine fractions. Moreover, as was observed for pine fractions, the grape oligomers were much more efficient than the monomers.

These results clearly show that both polymerization and galloylation enhance the antiproliferative capacity of polyphenolic fractions, which suggests that natural polyphenolic extracts with a high degree of galloylation and containing oligomers are more suitable as potential antiproliferative agents than those containing monomers.

Cell cycle analysis

To examine the effects of grape and pine fractions on the cell cycle pattern at concentrations equal to their IC_{50} and IC_{80} values (Table 1), HT29 cells were treated with each fraction for 72 h and then analyzed with a fluorescence-activated cell sorter (FACS) (Fig. 2). The cell cycle distribution pattern induced after grape polyphenolic treatments showed that, at IC_{50}, the fractions with the highest mean degree of polymerization and percentage of galloylation (VIIIG and IVG) induced a G_2-phase cell cycle arrest, whereas the rest of the fractions did not have a significant effect on the cell cycle distribution. At IC_{80}, the G_2-phase arrest induced by fractions VIIIG and IVG was enhanced, and fraction VIG displayed a significant effect (Fig. 2A). Fraction VIG is chemically classified in Table 1 as having the third highest mean degree of polymerization and galloylation, situated below fractions VIIIG and IVG, respectively.

To determine whether galloylation was required to induce the G_2-phase arrest, we also examined the non-galloylated pine fractions with high mean degrees of polymerization (VIIIP and IVP) and observed that they also induced a G_2-phase arrest at their respective IC_{50} values (Fig. 2B). These results showed that procyanidin polymerization plays a more important role than galloylation in cell cycle arrest.

Apoptosis induction

HT29 cell incubations with polyphenolic fractions were performed at the concentrations described in Experimental procedures. As show in Fig. 3A, at IC_{50}, the grape polyphenolic fractions VIIIG and IVG induced significant percentages of apoptosis in HT29 cells (approximately 25% and 17%, respectively) as measured by FACS analysis. Fraction VIIIG also induced a significant percentage of necrosis (approximately 5%), which could be due to a prooxidant effect at high concentration [35,36]. Moreover, this percentage is negligible in comparison to the apoptotic effect induced by fraction VIIIG on HT29 cells. At a concentration equal to IC_{80}, fractions VIIIG and IVG induced significant percentages of apoptosis in HT29 cells (approximately 24% and 18%, respectively) and fraction VIG also displayed a significant effect (approximately 22%) (Fig. 3A). Fraction VIG is chemically classified in Table 1 as having the third highest mean degree of polymerization and galloylation, situated below fractions VIIIG and IVG, respectively.

The pine fractions VIIIP and IVP were analyzed to determine whether galloylation enhanced the apoptotic induction observed; a significant percentage of apoptosis was induced, but the percentages were

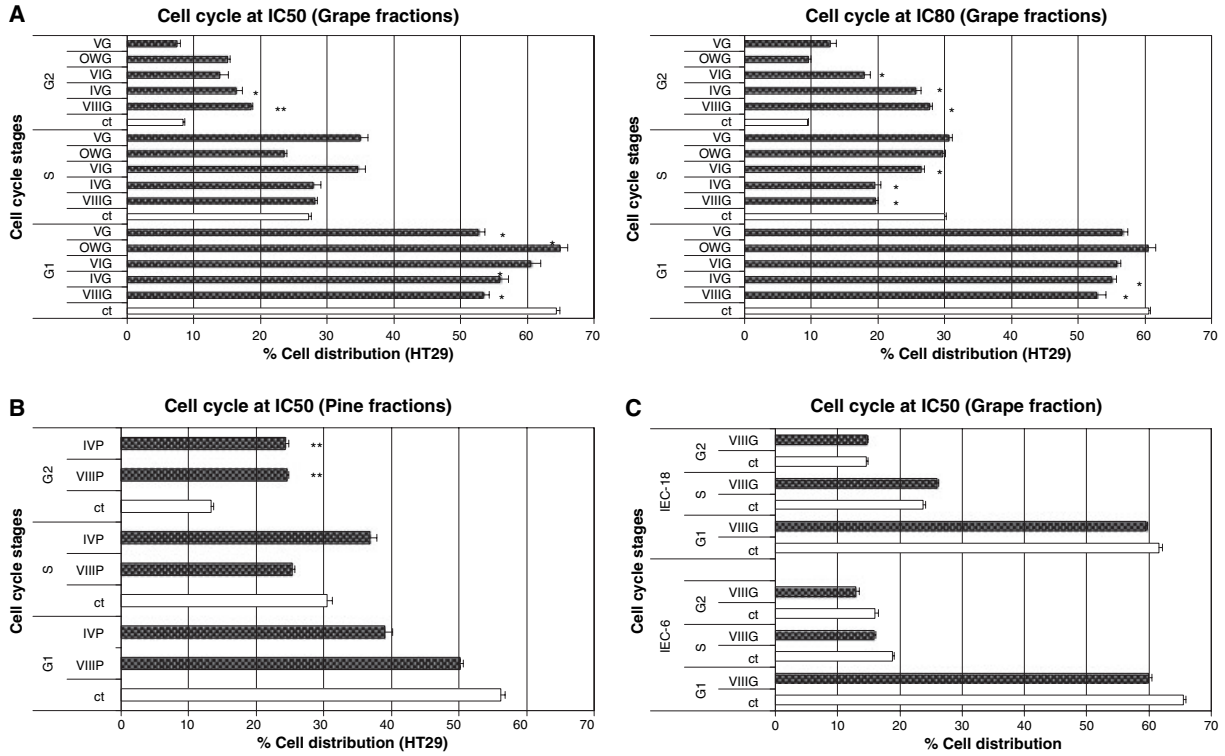


Fig. 2. Cell cycle analysis of HT29, IEC-6 and IEC-18 cells treated with grape and pine polyphenolic fractions. (A) HT29 cells at their respective grape IC_{50} and IC_{80} values. (B) HT29 cells at pine IC_{50}. (C) IEC-6 and IEC-18 cells treated with grape fraction VIIIG at HT29 IC_{50}. Percentages of cells in different cell stages are shown. Cell phases analyzed: G_1, S and G_2 (% cells ± SEM, $*P < 0.05$, $**P < 0.001$). Experiments were performed in triplicate.

lower than those induced by the grape fractions (Fig. 3B).

These results show that galloylation plays a more important role than polymerization in apoptosis induction. Next, apoptosis induction by the two most highly galloylated and polymerized fractions (VIIIG and IVG) was analyzed by Hoescht staining, which revealed early membrane alterations at the beginning of the apoptotic process. Chromatin condensation was also seen, and confirmed the induction of apoptosis by fractions VIIIG and IVG (Fig. 4A). Finally, DNA fragmentation was detected as a late marker of apoptosis by observing the pattern of DNA laddering at IC_{50} and IC_{80} (Fig. 4B).

Oxygen radical scavenging activity as detected by ESR spectroscopy

The next series of experiments used ESR spectroscopy to test the radical-scavenging capacity of the fractions. The results show that the oligomeric fractions (VIIIG, IVG, VIIIP and IVP), which were the most effective in the previous assays using HT29 cells, were also the most efficient as hydroxyl radical and superoxide scavengers at 50 μM (Fig. 5A). Fraction VIIIG was the most potent radical scavenger, followed by fraction IVG and the pine fractions VIIIP and IVP. The same levels of efficiency were also observed in the induction of cell cycle arrest and apoptosis. When fractions were tested at their respective IC_{50} values, fractions VIIIG, IVG, VIIIP and IVP were again the most effective (Fig. 5B). There is a clear relationship between high scavenger capacity/lower IC_{50} and a high level of apoptosis induction. Grape fractions proved to be more potent scavengers than pine fractions in both radical generation systems. The apparent high efficiencies detected for the monomers (VG and VP) can be largely attributed to the high concentrations used (410 μM and 1551 μM, respectively).

Interestingly, the efficiencies observed for grape oligomeric fractions, which proved to be better apoptotic inducers and better ROS scavengers than pine oligomeric fractions, are apparently related to the degree of galloylation and are enhanced by the polymerization of the fractions. Hydroxyl radical (OH) is the most reactive product of reactive oxygen species formed by successive one-electron reductions of molecular oxygen (O_2) in cell metabolism, is primarily responsible for the

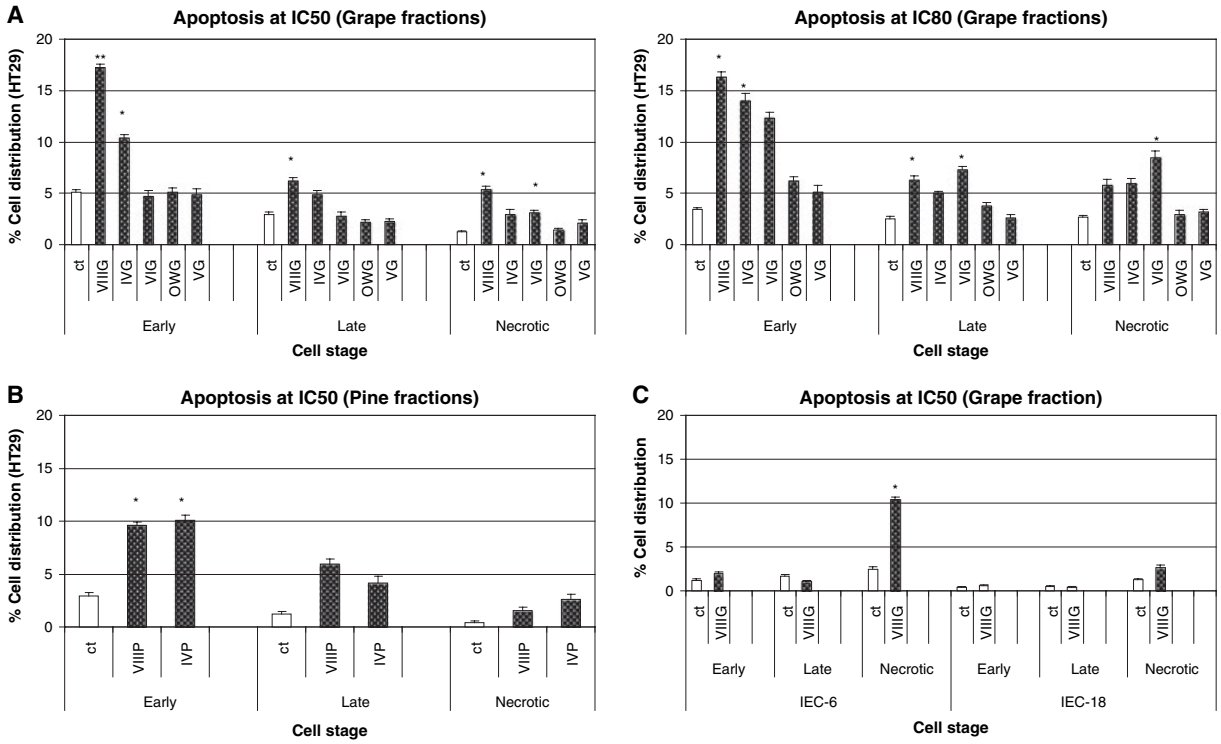


Fig. 3. Apoptosis was induced in HT29 tumor cells and did not affect normal epithelial cells. (A) HT29 cells after treatment with grape polyphenolic fractions at their respective IC_{50} and IC_{80} values. (B) HT29 cells after treatment with pine polyphenolic fractions at their respective IC_{50} values. (C) IEC-6 and IEC-18 cells treated with grape fraction VIIIG at HT29 IC_{50}. Percentages of cells in different cell stages are shown (cell stages shown on the *x*-axis). (% cells ± SEM, $*P < 0.05$, $**P < 0.001$). Experiments were performed in triplicate.

cytotoxic effects observed in aerobic organisms from bacteria to plants and animals, and has been identified as playing a role in the development of many human cancers [37,38].

Cancer chemoprevention conducted by administering chemical and dietary components to interrupt the initiation, promotion and progression of tumors is considered to be a new and promising approach in cancer prevention [39–41]. However, the development of effective and safe agents for the prevention and treatment of cancer remains inefficient and costly, and falls short of the requirements for primary prevention among the high-risk population and for prevention in cancer survivors [42].

In recent years, many popular, polyphenol-enriched dietary supplements have been commercialized, such as tea catechins, grape seed proanthocyanidins and other natural antioxidant extracts, each of which has been claimed to exert chemopreventive activity in cellular models of cancer [43,44]. Recent publications have stated that the antiproliferative activity of flavonoids is dependent on particular structure motifs, such as gallate groups and degree of polymerization [45,46].

Our results suggest that polymerization plays a greater role than galloylation in cell cycle arrest in HT29 cells. Interestingly, galloylation appears to be more influential than polymerization in the biological apoptosis activities tested and in the hydroxyl and superoxide anion radical-scavenging capacity of the fractions when compared at the same concentration of 50 μM (Fig. 5A). The galloylated and polymerized grape procyanidins were the most effective hydroxyl radical scavengers and also triggered cell cycle arrest and apoptosis, and although this does not necessarily indicate that both effects are mechanistically related, such as relationship cannot be ruled out. The present results are in general agreement with previously reported data for pure compounds [47]. Essentially, the induction of apoptosis seems to be related to the electron transfer capacity of the phenolic extracts. Other antioxidants with anti-inflammatory and anticancer activities have been reported, such as edaravone [48] and the flavonoid silydianin [49], both of which induce apoptosis and act as radical scavengers.

It was also observed that the most efficient procyanidin fraction, VIIIG, which induced approximately

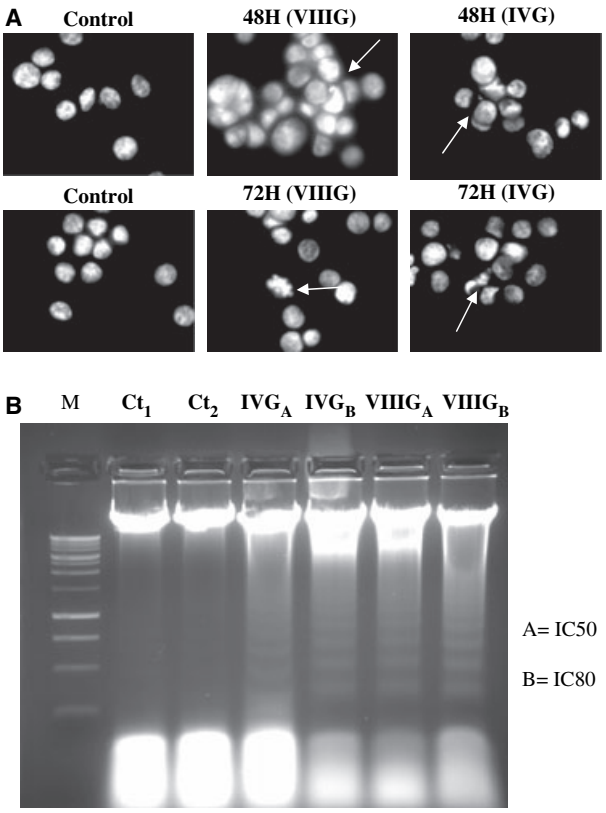


Fig. 4. Induction of apoptosis by grape fractions VIIIG and IVG in HT29 cells. (A) Nuclear condensation of HT29 cells. Arrows indicate the apoptotic cells with condensed and fragmented nuclei. (B) DNA laddering induced in both treatments.

30% apoptosis in HT29 cells, did not induce apoptosis or affect the cell cycle of the intestinal nontumoral cell lines IEC-18 and IEC-6, and even induced 10% necrosis in the IEC-6 cell line (Figs 2C and 3C). The results obtained provide information about the activities of procyanidin mixtures with different origins and structures on colon epithelial cells. These results should be useful in defining the putative benefits of plant polyphenols in nutritional supplements. Additionally, this study provides useful insights into the polyphenolic structure, which should help in the rational design of formulations for potent chemopreventive or antiproliferative natural vegetable products on the basis of apoptosis-inducing activity.

Experimental procedures

Materials

DMEM and Dulbecco's phosphate-buffered saline (NaCl/P_i) were obtained from Sigma Chemical Co. (St Louis, MO, USA), antibiotics (10 000 $U \cdot mL^{-1}$ penicillin, 10 000 $\mu g \cdot mL^{-1}$ streptomycin) were obtained from Gibco-BRL (Eggenstein, Germany), and fetal bovine serum was obtained from Invitrogen (Carlsbad, CA, USA). Trypsin/EDTA solution C (0.05% trypsin/0.02% EDTA) was purchased from Biological Industries (Kibbutz Beit Haemet, Israel). 3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide (MTT), dimethylsulfoxide, propidium iodide (PI) and Igepal CA-630 were obtained from Sigma Chemical Co. NADH disodium salt (grade I) was supplied by Boehringer (Mannheim, Germany). RNase and agarose MP were obtained from Roche Diagnostics (Mannheim, Germany). Iron(II) sulfate heptahydrate was obtained from Merck (Darmstadt, Germany) α-α-α-Tris(hydroxymethyl)aminomethane was obtained from Aldrich-Chemie (Steinheim, Germany) and moviol from Calbiochem (La Jolla, CA, USA). The annexin V/fluorescein isothiocyanate (FITC) kit was obtained from Bender System (Vienna, Austria), the Realpure DNA extraction kit, including proteinase K, was obtained from Durviz S.L. (Paterna, Spain), and Blue/Orange Loading dye and the 1 kb DNA ladder were purchased from Promega (Madison, WI, USA). 5,5-Dimethyl-1-pyrolline-*N*-oxide (DMPO), hydrogen peroxide, phenazine methosulfate and Hoescht were obtained from Sigma (St Louis, MO). DMPO was further purified by charcoal treatment.

Fractions

The polyphenolic mixtures were obtained previously in our laboratories [26,50] and contain mainly procyanidins. OWG and OWP are composed of species that are soluble in both ethyl acetate and water, and the rest of the fractions (G for grape, P for pine) were generated by a combination of preparative RP-HPLC and semipreparative chromatography on a Toyopearl TSK HW-40F column (TosoHass, Tokyo, Japan), which separated the components by size and hydrophobicity. The phenolics were eluted from the latter column with MeOH (fractions VG, VP) and water/acetone 1 : 1 (fractions IVG, VIG, VIIIG, IVP, VIP, VIIIP and XIP), evaporated almost to dryness, redissolved in Milli-Q water, and freeze-dried. The second and third columns of Table 1 show the average chemical composition of the fractions.

Cell culture

Human colorectal adenocarcinoma HT29 cells (ATCC HTB-38) and two nontumoral intestinal rat cell lines, IEC-6 (ECCAC no. 88071401) and IEC-18 (ECCAC no. 88011801), were used in all of the experiments. HT29, IEC-6 and IEC-18 cells were maintained in monolayer culture in an incubator with 95% humidity and 5% CO_2 at 37 °C. HT29, IEC-6 and IEC-18 cells were passaged at preconfluent densities using trypsin/EDTA solution C.

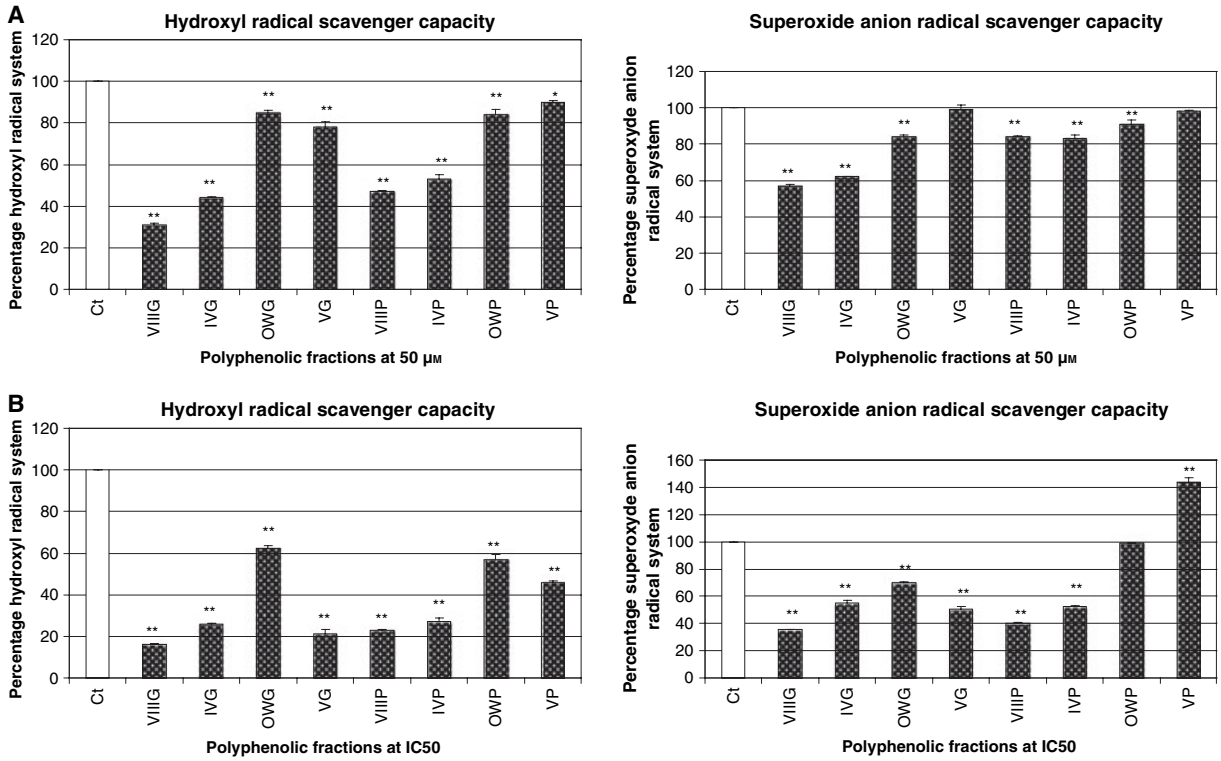


Fig. 5. Scavenging activity of OH and O_2^- analyzed by ESR. Grape and pine fractions were evaluated at: (A) 50 μM and (B) IC_{50} in HT29 cells in hydroxyl radical- and superoxide anion radical-generating systems, as described in Experimental procedures. Experiments were performed in duplicate (*$P < 0.05$, **$P < 0.001$).

Cells were cultured and passaged in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 0.1% streptomycin/penicillin.

Cell growth inhibition

HT29, IEC-6 and IEC-18 cells were seeded densities of 3×10^3 cells per well, 5×10^3 cells per well and 1×10^3 cells per well, respectively, in 96-well flat-bottomed plates. After 24 h of incubation at 37 °C, the polyphenolic mixtures were added to the cells at different concentrations from 5 μM to 2300 μM in fresh medium. The culture was incubated for 72 h, after which the medium was removed and 50 μL of MTT (5 mg·mL^{-1} in NaCl/P_i) with 50 μL of fresh medium was added to each well and incubated for 1 h. The blue MTT formazan precipitated was dissolved in 100 μL of dimethylsulfoxide, and the absorbance values at 550 nm were measured on an ELISA plate reader (Tecan Sunrise MR20-301; TECAN, Salzburg, Austria). Absorbance was proportional to the number of living cells. The growth inhibition concentrations that caused 50% (IC_{50}) and 80% (IC_{80}) cell growth inhibition were calculated using GRAFIT 3.0 software. The assay was performed using a variation of the MTT assay described by Mosmann [51].

Cell cycle analysis

The assay was carried out using flow cytometry with a FACS. HT29, IEC-6 and IEC-18 cells were plated in six-well flat-bottomed plates at densities of 87.3×10^3 cells per well, 146×10^3 cells per well and 29.1×10^3 cells per well, respectively. The number of cells was determined as cells per area of well, as used in the cell growth inhibition assay. The culture was incubated for 72 h in the absence or presence of the polyphenolic mixture at its respective IC_{50} values. The cells were then trypsinized, pelleted by centrifugation [371 ***g*** for 3 min at room temperature (RT) using a 5415D centrifuge (Eppendorf, Hamburg, Germany) and a 24-place fixed angle rotor] and stained in Tris-buffered saline (NaCl/Tris) containing 50 μg·mL^{-1} PI, 10 μg·mL^{-1} RNase free of DNase and 0.1% Igepal CA-630 in the dark for 1 h at 4 °C. Cell cycle analysis was performed with a FACS (Epics XL flow cytometer; Coulter Corporation, Hialeah, FL, USA) at 488 nm. All experiments were performed in triplicate, as described previously [47].

Apoptosis analysis by FACS

Annexin V/FITC and PI staining were measured by FACS. Cells were seeded, treated and collected as described in

the previous section. Following centrifugation [371 ***g*** for 3 min at RT using a 5415D centrifuge (Eppendorf) with 24-place fixed angle rotor], cells were washed in binding buffer (10 mM Hepes, pH 7.4, 140 mM sodium chloride, 2.5 mM calcium chloride) and resuspended in the same buffer. Annexin V/FITC was added using the annexin V/FITC kit. Following 30 min of incubation at room temperature and in the dark, PI was added 1 min before the FACS analysis at 20 $\mu g \cdot mL^{-1}$. Experiments were performed in triplicate.

Apoptosis detection by DNA laddering

DNA isolation and purification were performed after 72 h in the presence and absence of grape fractions VIIIG and IVG. The fractions were assayed at their respective IC_{50} and IC_{80} values. After treatment, cells were scraped off slides and collected by centrifugation at 14 000 ***g*** for 10 s at RT using a 5415D centrifuge (Eppendorf) and 24-place fixed angle rotor. Cells were then lysed by adding 600 μL of Realpure kit lysis buffer and 10 μL of proteinase K, and incubated for 1 h at 55 °C. RNA digestion was performed with 1.5 μL of RNase for 1 h at 37 °C, and this was followed by protein precipitation with 360 μL of Realpure kit buffer and centrifugation at 14 000 ***g*** for 10 min at RT using a 5415D centrifuge (Eppendorf) and 24-place fixed angle rotor. The DNA sample was extracted with isopropanol/ethanol, dried, and eluted in 100 μL of Realpure kit DNA hydration solution. Equal amounts of DNA (20 μg), estimated by measuring absorption at 260/280 nm, were electrophoretically separated on 1% TAE agarose gel and viewed under a UV transilluminator (Vilber Lourmat, Marne-la-Vallée, France).

Apoptosis detection by Hoescht staining

Apoptotic induction was also studied using Hoescht staining. Samples were incubated with grape fractions VIIIG and IVG at 0, 48 and 72 h. After incubation, cells were trypsinized and fixed with cold methanol for 1 h at − 20 °C. After being rinsed with $NaCl/P_i$ three times, cells were stained in the dark with Hoescht (50 $ng \cdot mL^{-1}$ in $NaCl/P_i$) for 50 min. Finally, cells were rinsed, suspended in $NaCl/P_i$ and diluted 1 : 2 with moviol. The samples were mounted on a slide and observed with a fluorescent microscope at an excitation wavelength of 334 nm and an emission wavelength of 365 nm.

ESR spectroscopy

ESR measurements were performed at concentrations that caused 50% cell growth inhibition (IC_{50}) and 50 μM grape and pine fractions (VIIIG, IVG, OWG, VG, VIIIP, IVP, OWP and VP). Molar concentrations were calculated from the mean molecular mass of the fractions estimated by thiolysis with cysteamine, as described in [52]. OH and O_2^- formation were detected by ESR spectroscopy using DMPO (100 mM) as a spin trap. ESR spectra were recorded at room temperature in glass capillaries (100 μL; Brand AG, Wertheim, Germany) on a Bruker EMX 1273 spectrometer (Bruker, Karlsruhe, Germany) equipped with an ER 4119HS high-sensitivity cavity and a 12 kW power supply operating at X-band frequencies. The modulation frequency of the spectrometer was 100 kHz. Instrumental conditions for the recorded spectra were: magnetic field, 3490 G; scan range, 60 G; modulation amplitude, 1 G; receiver gain, 1×10^5; microwave frequency, 9.85 GHz; power, 50 mW; time constant, 40.96 ms; scan time, 20.97 s; number of scans, 25. Spectra were quantified by peak surface measurements using the WIN-EPR spectrum manipulation program (Bruker).

All incubations were done at room temperature; the hydroxyl radical generation system used 500 μM $FeSO_4$ and 550 μM H_2O_2, and hydroxyl radicals generated in this system were trapped by DMPO, forming a spin adduct detected by the ESR spectrometer. The typical 1 : 2 : 2 : 1 ESR signal of DMPO-OH was observed. The superoxide radical generation system used performed using 50 μM of the reduced form of β-NADH and 3.3 μM phenazine methosulfate, and the superoxide radicals generated in this system were trapped by DMPO, forming a spin adduct detected by the ESR spectrometer. The typical ESR signal of DMPO-OOH/DMPO-OH was observed. The OH and O_2-scavenging activity was calculated on the basis of decreases in the DMPO-OH or DMPO-OOH/DMPO-OH signals, respectively, in which the coupling constant for DMPO-OH was 14.9 G.

Data presentation and statistical analysis

Assays were analyzed using the Student's *t*-test and were considered statistically significant at $P < 0.05$ and $P < 0.001$. The data shown are representative of three independent experiments, with the exception of ESR experiments, which were performed in duplicate. ESR experiments were analyzed separately by radicals, Two-way ANOVA was applied (day was a block factor; due to the nonsignificant effect of the day factor, we reanalyzed with a one-way ANOVA), and finally, a multicomparison between compounds with respect to the control was performed. ANOVA with Bonferroni and Scheffe *post hoc* test was performed in ESR experiments.

Acknowledgements

This work was supported by grants PPQ 2003-06602-C04-01, PPQ 2003-06602-C04-04, AGL2004-07579-C04-02 and AGL2004-07579-C04-03 from the Spanish Ministry of Education and Science, and ISCIII-RTICC (RD06/0020/0046) from the Spanish government and

the European Union FEDER funds. We thank Professor Francesc Oliva (Department of Statistics at the University of Barcelona) for his assistance with statistical analysis.

References

1 Parkin DM (2004) International variation. *Oncogene* **23**, 6329–6340.
2 Potter JD, Slattery ML, Bostick RM & Gapstur SM (1993) Colon cancer: a review of the epidemiology. *Epidemiol Rev* **15**, 499–545.
3 Steinmetz KA & Potter JD (1991) Vegetables, fruit, and cancer. I. Epidemiology. *Cancer Causes Control* **2**, 325–357.
4 Park OJ & Surh YJ (2004) Chemopreventive potential of epigallocatechin gallate and genistein: evidence from epidemiological and laboratory studies. *Toxicol Lett* **150**, 43–56.
5 Bruce WR, Giacca A & Medline A (2000) Possible mechanisms relating diet and risk of colon cancer. *Cancer Epidemiol Biomarkers Prev* **9**, 1271–1279.
6 Hietanen E, Bartsch H, Bereziat JC, Camus AM, McClinton S, Eremin O, Davidson L & Boyle P (1994) Diet and oxidative stress in breast, colon and prostate cancer patients: a case-control study. *Eur J Clin Nutr* **48**, 575–586.
7 Theodoratou E, Kyle J, Cetnarskyj R, Farrington SM, Tenesa A, Barnetson R, Porteous M, Dunlop M & Campbell H (2007) Dietary flavonoids and the risk of colorectal cancer. *Cancer Epidemiol Biomarkers Prev* **16**, 684–693.
8 Mukhtar H & Ahmad N (1999) Green tea in chemoprevention of cancer. *Toxicol Sci* **52**, 111–117.
9 Lee KW, Lee HJ & Lee CY (2004) Vitamins, phytochemicals, diets, and their implementation in cancer chemoprevention. *Crit Rev Food Sci Nutr* **44**, 437–452.
10 Witschi H, Espiritu I, Ly M, Uyeminami D, Morin D & Raabe OG (2004) Chemoprevention of tobacco smoke-induced lung tumors by inhalation of an epigallocatechin gallate (EGCG) aerosol: a pilot study. *Inhal Toxicol* **16**, 763–770.
11 Delmas D, Lancon A, Colin D, Jannin B & Latruffe N (2006) Resveratrol as a chemopreventive agent: a promising molecule for fighting cancer. *Curr Drug Targets* **7**, 423–442.
12 Hakimuddin F, Paliyath G & Meckling K (2006) Treatment of mcf-7 breast cancer cells with a red grape wine polyphenol fraction results in disruption of calcium homeostasis and cell cycle arrest causing selective cytotoxicity. *J Agric Food Chem* **54**, 7912–7923.
13 Sime S & Reeve VE (2004) Protection from inflammation, immunosuppression and carcinogenesis induced by UV radiation in mice by topical Pycnogenol. *Photochem Photobiol* **79**, 193–198.
14 Kumar N, Shibata D, Helm J, Coppola D & Malafa M (2007) Green tea polyphenols in the prevention of colon cancer. *Front Biosci* **12**, 2309–2315.
15 McKay DL & Blumberg JB (2007) A review of the bioactivity of south African herbal teas: rooibos (Aspalathus linearis) and honeybush (Cyclopia intermedia). *Phytother Res* **21**, 1–16.
16 Wright TI, Spencer JM & Flowers FP (2006) Chemoprevention of nonmelanoma skin cancer. *J Am Acad Dermatol* **54**, 933–946; quiz 947–950.
17 Tan XHD, Li S, Han Y, Zhang Y & Zhou D (2000) Differences of four catechins in cell cycle arrest and induction of apoptosis in LoVo cells. *Cancer Lett* **158**, 1–6.
18 Kozikowski AP, Tuckmantel W, Bottcher G & Romanczyk LJ Jr (2003) Studies in polyphenol chemistry and bioactivity. 4 (1) Synthesis of trimeric, tetrameric, pentameric, and higher oligomeric epicatechin-derived procyanidins having all-4beta, 8-interflavan connectivity and their inhibition of cancer cell growth through cell cycle arrest. *J Org Chem* **68**, 1641–1658.
19 Nakamuta M, Higashi N, Kohjima M, Fukushima M, Ohta S, Kotoh K, Kobayashi N & Enjoji M (2005) Epigallocatechin-3-gallate, a polyphenol component of green tea, suppresses both collagen production and collagenase activity in hepatic stellate cells. *Int J Mol Med* **16**, 677–681.
20 Shi J, Yu J, Pohorly JE & Kakuda Y (2003) Polyphenolics in grape seeds – biochemistry and functionality. *J Med Food* **6**, 291–299.
21 Siddiqui IA, Adhami VM, Saleem M & Mukhtar H (2006) Beneficial effects of tea and its polyphenols against prostate cancer. *Mol Nutr Food Res* **50**, 130–143.
22 Zhang Q, Tang X, Lu Q, Zhang Z, Rao J & Le AD (2006) Green tea extract and (–)-epigallocatechin-3-gallate inhibit hypoxia- and serum-induced HIF-1alpha protein accumulation and VEGF expression in human cervical carcinoma and hepatoma cells. *Mol Cancer Ther* **5**, 1227–1238.
23 Prieur CRJ, Cheynier V & Moutounet M (1994) Oligomeric and polymeric procyanidins from grape seeds. *Phytochemistry* **36**, 781–784.
24 Souquet J-MCV, Brossaud F & Moutounet M (1996) Polymeric proanthocyanidins from grape skins. *Phytochemistry* **43**, 509–512.
25 Rohdewald P (2002) A review of the French maritime pine bark extract (Pycnogenol), a herbal medication with a diverse clinical pharmacology. *Int J Clin Pharmacol Ther* **40**, 158–168.
26 Tourino S, Selga A, Jimenez A, Julia L, Lozano C, Lizarraga D, Cascante M & Torres JL (2005) Procyanidin fractions from pine (Pinus pinaster) bark: radical scavenging power in solution, antioxidant

activity in emulsion, and antiproliferative effect in melanoma cells. *J Agric Food Chem* **53**, 4728–4735.
27 Gonthier MP, Donovan JL, Texier O, Felgines C, Remesy C & Scalbert A (2003) Metabolism of dietary procyanidins in rats. *Free Radic Biol Med* **35**, 837–844.
28 Kuhnle G, Spencer JP, Schroeter H, Shenoy B, Debnam ES, Srai SK, Rice-Evans C & Hahn U (2000) Epicatechin and catechin are O-methylated and glucuronidated in the small intestine. *Biochem Biophys Res Commun* **277**, 507–512.
29 Rechner AR, Smith MA, Kuhnle G, Gibson GR, Debnam ES, Srai SK, Moore KP & Rice-Evans CA (2004) Colonic metabolism of dietary polyphenols: influence of structure on microbial fermentation products. *Free Radic Biol Med* **36**, 212–225.
30 Meselhy MR, Nakamura N & Hattori M (1997) Biotransformation of (–)-epicatechin 3-O-gallate by human intestinal bacteria. *Chem Pharm Bull (Tokyo)* **45**, 888–893.
31 Salucci M, Stivala LA, Maiani G, Bugianesi R & Vannini V (2002) Flavonoids uptake and their effect on cell cycle of human colon adenocarcinoma cells (Caco2). *Br J Cancer* **86**, 1645–1651.
32 Stagos D, Kazantzoglou G, Magiatis P, Mitaku S, Anagnostopoulos K & Kouretas D (2005) Effects of plant phenolics and grape extracts from Greek varieties of Vitis vinifera on mitomycin C and topoisomerase I-induced nicking of DNA. *Int J Mol Med* **15**, 1013–1022.
33 Subirade I, Fernandez Y, Periquet A & Mitjavila S (1995) Catechin protection of 3T3 Swiss fibroblasts in culture under oxidative stress. *Biol Trace Elem Res* **47**, 313–319.
34 Cao Z & Li Y (2004) Potent induction of cellular antioxidants and phase 2 enzymes by resveratrol in cardiomyocytes: protection against oxidative and electrophilic injury. *Eur J Pharmacol* **489**, 39–48.
35 Alanko J, Riutta A, Holm P, Mucha I, Vapaatalo H & Metsa-Ketela T (1999) Modulation of arachidonic acid metabolism by phenols: relation to their structure and antioxidant/prooxidant properties. *Free Radic Biol Med* **26**, 193–201.
36 Azam S, Hadi N, Khan NU & Hadi SM (2004) Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: implications for anticancer properties. *Toxicol In Vitro* **18**, 555–561.
37 Halliwell B & Gutteridge JM (1992) Biologically relevant metal ion-dependent hydroxyl radical generation. An update. *FEBS Lett* **307**, 108–112.
38 Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M & Telser J (2007) Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* **39**, 44–84.
39 Mathers JC (2002) Pulses and carcinogenesis: potential for the prevention of colon, breast and other cancers. *Br J Nutr* **88** (Suppl. 3), S273–S279.
40 Witte JS, Longnecker MP, Bird CL, Lee ER, Frankl HD & Haile RW (1996) Relation of vegetable, fruit, and grain consumption to colorectal adenomatous polyps. *Am J Epidemiol* **144**, 1015–1025.
41 Manju V & Nalini N (2005) Chemopreventive efficacy of ginger, a naturally occurring anticarcinogen during the initiation, post-initiation stages of 1,2-dimethylhydrazine-induced colon cancer. *Clin Chim Acta* **358**, 60–67.
42 Zou DM, Brewer M, Garcia F, Feugang JM, Wang J, Zang R, Liu H & Zou C (2005) Cactus pear: a natural product in cancer chemoprevention. *Nutr J* **4**, 25–37.
43 Joshi SS, Kuszynski CA & Bagchi D (2001) The cellular and molecular basis of health benefits of grape seed proanthocyanidin extract. *Curr Pharm Biotechnol* **2**, 187–200.
44 Depeint F, Gee JM, Williamson G & Johnson IT (2002) Evidence for consistent patterns between flavonoid structures and cellular activities. *Proc Nutr Soc* **61**, 97–103.
45 Fiuza SM, Gomes C, Teixeira LJ, Girao da Cruz MT, Cordeiro MN, Milhazes N, Borges F & Marques MP (2004) Phenolic acid derivatives with potential anticancer properties – a structure–activity relationship study. Part 1: methyl, propyl and octyl esters of caffeic and gallic acids. *Bioorg Med Chem* **12**, 3581–3589.
46 Brusselmans K, Vrolix R, Verhoeven G & Swinnen JV (2005) Induction of cancer cell apoptosis by flavonoids is associated with their ability to inhibit fatty acid synthase activity. *J Biol Chem* **280**, 5636–5645.
47 Lozano C, Torres JL, Julia L, Jimenez A, Centelles JJ & Cascante M (2005) Effect of new antioxidant cysteinyl-flavanol conjugates on skin cancer cells. *FEBS Lett* **579**, 4219–4225.
48 Kokura S, Yoshida N, Sakamoto N, Ishikawa T, Takagi T, Higashihara H, Nakabe N, Handa O, Naito Y & Yoshikawa T (2005) The radical scavenger edaravone enhances the anti-tumor effects of CPT-11 in murine colon cancer by increasing apoptosis via inhibition of NF-kappaB. *Cancer Lett* **229**, 223–233.
49 Zielinska-Przyjemska M & Wiktorowicz K (2006) An in vitro study of the protective effect of the flavonoid silydianin against reactive oxygen species. *Phytother Res* **20**, 115–119.
50 Torres JL, Varela B, Garcia MT, Carilla J, Matito C, Centelles JJ, Cascante M, Sort X & Bobet R (2002) Valorization of grape (Vitis vinifera) byproducts. Antioxidant and biological properties of polyphenolic fractions differing in procyanidin composition and flavonol content. *J Agric Food Chem* **50**, 7548–7555.
51 Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**, 55–63.
52 Selga A & Torres JL (2005) Efficient preparation of catechin thio conjugates by one step extraction/depolymerization of pine (Pinus pinaster) bark procyanidins. *J Agric Food Chem* **53**, 7760–7765.

V. Comparative antioxidant and cytotoxic effect of procyanidin fractions from grape and pine.

Ugartondo, V.; Mitjans, M.; ***Touriño, S.***; Torres, J.L. and Vinardell M.P.; ***Chem. Res. Toxicol.***; 20: 1543–1548; **2007**

Comparative Antioxidant and Cytotoxic Effect of Procyanidin Fractions from Grape and Pine

Vanessa Ugartondo,[†] Montserrat Mitjans,[†] Sonia Touriño,[‡] Josep Lluis Torres,[‡] and María Pilar Vinardell*,[†]

Department of Physiology, Faculty of Pharmacy, Universitat de Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain, and Institute for Chemical and Environmental Research, CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain

Received July 11, 2007

There is a great interest in characterizing the biological properties of natural compounds obtained from plants, especially polyphenols. We studied the structure–activity–cytotoxicity relationships of polyphenolic fractions obtained from grape pomace and pine bark. These fractions contained similar polymerised flavonoids but different percentages of pyrogallol groups that confer on them different biological properties. The human keratinocyte cell line HaCaT and the mouse fibroblast cell line 3T3 were used to study the cytotoxicity of the different fractions after 24, 48, and 72 h of exposure. Antioxidant activity of the fractions was evaluated by measuring the inhibition of hemolysis mediated by AAPH. Our results demonstrate that the polyphenolic fractions studied show high antioxidant capacity in a concentration range that is not harmful to normal human cells. Pine fractions presented slightly lower antioxidant activity than grape fractions but are less cytotoxic. This data provides useful information to help design safe antioxidant products that act without altering critical cell functions.

Introduction

The efficient use of natural resources is currently the focus of many efforts in both science and technology. The management of agriculture and forestry must be sustainable from both economic and environmental viewpoints. The agrifood industries produce a large volume of waste each year (*1*). Because of the high economic cost of disposal and the potential environmental risk associated with an excess of biomass, the possibility of recycling by finding new applications for these wastes has great potential.

Plant residues from food and forestry industries contain considerable amounts of potentially interesting compounds, but the value of the products obtained must compensate for the cost of their recovery. For this reason, it is essential both to improve the extraction processes and to substantiate the activity and safety claims (*2*) of the new products.

Among the biologically active species present in agricultural by-products, polyphenols and particularly flavonoids are widely appreciated for their putative health-promoting properties. The best-described property of flavonoids is their capacity to act as free radical scavengers (*3*). They also show other properties that may or may not be related to their scavenging potential. These include, but are not limited to, antiproliferation of carcinogenic cells, cell cycle regulation, induction of apoptosis, inhibition of platelet aggregation, and antibacterial and antiallergic properties (*4–8*). Therefore, it is assumed that flavonoids play a relevant role in the prevention of degenerative diseases such as cancer and cardiovascular diseases and that it may be wise to include in our diet vegetables, fruits, and moderate amounts of plant-derived products such as tea, wine, and chocolate, which are rich in polyphenols (*9, 10*).

Polyphenolic mixtures have already been proposed as food antioxidants and preventive agents against skin irritation and cancer (*4, 11*). In accordance with the scientific and market interest in polyphenols as chemopreventive agents, our group is investigating the possible applications of plant proanthocyanidins in the fields of food preservation, skin protection, and cancer with particular emphasis on their structure–activity relationships and safety profiles.

From white grape (*Vitis vinifera*) pomace and pine (*Pinus pinaster*) bark polyphenolic extracts, we generated fractions containing different amounts of monomeric catechins and oligomeric procyanidins (*12–14*). Figure 1 summarizes the structure of the procyanidins found in both sources. We previously carried out several assays to assess the structure/function relationships of these fractions. We determined their efficiency as antioxidants under different experimental setups, including free radical scavenging in solution and inhibition of lipid peroxidation in both pure oil and oil-in-water emulsion. Furthermore, we investigated the influence of these compounds on the proliferation of different tumoral cell lines and their capacity to induce apoptosis (*13, 14*).

The fractions from the two sources are highly homologous in terms of mean molecular size; they are mainly differentiated by their galloylation (presence of gallate esters). Grape pomance (skin, seeds, and a small amount of stems) are galloylated to some extent, but pine bark appears to contain only procyanidins with no measurable galloylation (*4, 15*). Because the gallate group both provides high antiradical power and appears to interfere with crucial cell functions, galloylation appears to be a crucial structural feature defining the activity and toxicity of phenolic mixtures.

The aim of this study is to take a step forward in the characterization of the biological properties of procyanidins by using a set of grape and pine fractions with different mean size and galloylation. We report the protective antioxidant potential

* Corresponding author: Phone: +34 934024505. Fax: +34934035901. E-mail: mpvinardellmh@ub.edu.
[†] Universitat de Barcelona.
[‡] Institute for Chemical and Environmental Research.

10.1021/tx700253y CCC: $37.00
Published on Web 09/08/2007

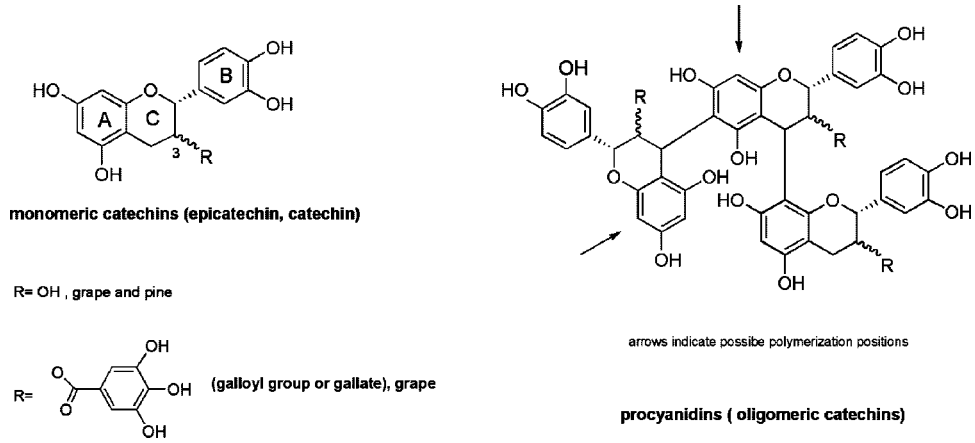


Figure 1. Structures of the procyanidins found in grape and pine fractions.

Table 1. Size and Composition of Polyphenolic Fractions from Parellada White Grape Pomace and Pine Bark (*11, 12*)

fraction	mDP	mMW	galloylation (%)
OWG	1.7	552	15
IVG	2.7	880	25
VIIIG	3.4	1160	34
XIG	3.7	1232	31
OWP	2.1	601	
IVP	2.9	833	
VIIIP	3.0	876	
XIP	3.4	999	
control	Mdp	Mmw	galloylation (%)
(−)-Epicatechin	1.0	290	0

in a biological system, namely, the inhibition of red blood cell lysis after the addition of AAPH (2,2′-azobis(amidinopropane)dihydrochloride), a peroxil radical initiator, and the estimation of possible toxic effects by using cell culture assays. We evaluated the relationship between the potential cytotoxic properties and the antioxidant activity of these polyphenolic fractions and how their structure (polymerization degree and percentage of galloylation) may influence their behaviour. The characterization of these biological properties will permit us to better define the possible applications of phenolics and to study their potential health benefits and risks in depth.

Experimental Procedures

Materials. 1. Grape Fractions. The total extract, **OWG**, was obtained from Parellada grape (*Vitis vinifera*) pomace following the procedure described by Torres and Bobet (*12*). **OWG** contained monomeric cathechins, oligomeric catechins (procyanidins), and, in lower proportion, flavonols, mainly glycosylated (*16*). Isolated procyanidins with variable galloylation, which we labeled **IVG**, **VIIIG**, **XIG**, were obtained by application of size-exclusion chromatography to **OWG**, as described previously (*13*). Procyanidin size and composition were estimated by thiolysis with cysteamine, and glycosylated flavonols were detected by analytical RP-HPLC at 365 nm. The qualitative composition of the fractions, the mean molecular weight (mMW), the degree of polymerization (mDP), and the percentage of galloylation previously described by our group (*13*) are summarized in Table 1. The fractions contained mostly procyanidins. Molar concentrations of these procyanidins were calculated using the mean molecular weight of the mixtures, which was estimated by thiolysis with cysteamine as described (*14*).

2. Pine Bark Fractions. The polyphenolic total extract, **OWP**, was obtained essentially as described for grape pomace (*12*), with some extraction modification (*14*)**OWP** contained monomeric and oligomeric catechins and other monomeric flavonoids. From this mixture, our group generated fractions homologous to those obtained from grape pomace, differing in composition and procyanidin structure (Table 1). The procyanidin oligomers **IVP**, **VIIIP**, and **XIP** were obtained using a combination of chromatographic techniques. 2,2′-Azobis(amidinopropane)dihydrochloride (AAPH) and (−)-Epicatechin (Ec) were purchased from Sigma (ST Louis, MO).

Blood Samples and Preparation of Red Blood Cells and AAPH. Blood samples were obtained from healthy donors by venipuncture (Blood Bank of Hospital Vall d'Hebrón, Barcelona, Spain), following the ethical guidelines of the Hospital, and collected in citrated tubes. Blood was centrifuged at 1000*g* for 10 min, and the plasma and buffy coat were removed. Red blood cells (RBCs) were washed three times in phosphate buffer isotonic saline (PBS) containing 22.2 mM Na2HPO4, 5.6 mM KH2PO4, 123.3 mM NaCl, and glucose 10.0 mM in distilled water (pH 7.4). The cells were then resuspended in isotonic saline solution to get the desired cellular density (8×10^9 cells/mL). An AAPH solution was prepared at the moment of its use using the same buffer and protected from the light.

Antioxidant Activity. We measured the hemolysis of RBCs mediated by AAPH using a modification of the method described previously (*17*). The addition of AAPH (a peroxyl radical initiator) to the suspension of RBCs induces the oxidation of cell membrane lipids and proteins, thereby resulting in hemolysis. The erythrocyte suspension (250 μL) was incubated in the presence of AAPH at a final concentration of 100 mM for 150 min in a shaker at 37 °C to achieve 100% hemolysis. Hemolysis was assessed by reading the absorbance of the hemoglobin released at 540 nm in a Shimadzu spectrophotometer.

The antihemolytic activity of fractions from different sources was tested by adding several concentrations of the compounds solved in PBS, ranging from 12.5 to 200 μg/mL, to the RBC suspension in the presence of 100 mM AAPH at 37 °C for 2.5 h. A blood sample incubated at the same conditions but without AAPH or fractions was included as a control for the spontaneous hemolysis. The IC_{50} or concentration inducing 50% inhibition of the hemolysis induced by **AAPH** was determined for each compound.

Culture of Cell Lines and Experimental Treatments. We used the spontaneously immortalized human keratinocyte cell line, HaCaT, and the mouse fibroblast cell line, 3T3 from "Banco de Células Eucariotas", Barcelona (Spain). Cells were grown in Dulbeccos's modified Eagle's medium (DMEM) (4.5 g/L glucose) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM Hepes buffer, and 1% penicillin (10,000 U/mL)/streptomycin (10,000 μg/mL) and maintained in a humidified atmosphere with 5% CO_2 at 37 °C. When 75 cm^2 culture flasks were approximately 80% confluent, the cells were seeded into the central 60 wells of 96-well plates as follows: for HaCaT, at densities of 10×10^4 cells/mL, 6.5×10^4 cells/mL, and 5.5×10^4 cells/mL for 24, 48, and 72 h of exposure, respectively, and for 3T3 at densities of 8.5×10^4 cells/mL, 2.5×10^4 cells/mL, and 1.5×10^4 cells/mL for 24, 48, and 72 h of exposure, respectively (*18*).

Plates were incubated at 37 °C, 5% CO_2 for 24 h. Triplicate runs were performed with different passage cells.

After 1 day of incubation, the growth medium was removed and replaced with exposure medium (DMEM medium supplemented with 5% FBS, 2 mM L-glutamine, 10 mM Hepes buffer, and 1% antibiotic mixture), with or without the polyphenolic fractions at concentrations ranging from 500 μg/mL to 7 μg/mL previously sterilized by filtration. Controls, containing culture medium only, were included in each plate. Cells were then incubated at 37 °C and 5% CO_2 for 24, 48, or 72 h.

NRU Assay. The NRU assay was performed as described by Borenfreund and Puerner (*19*) and modified to remove the use of formaldehyde (*20*). After the treatments, the medium was aspirated and replaced with 100 μL per well of NR solution (50 μg/mL in RPMI medium without phenol red and serum). After 3 h of incubation at 37 °C and 5% CO_2, the medium was aspirated, the cells were washed twice with PBS, and a solution containing 50% ethanol and absolute 1% acetic acid in distilled water was added (100 μL per well) to release into the supernatant the dye that had been absorbed into the viable cells. After 10 min on a microtitre-plate shaker, the absorbance of neutral red was measured at a wavelength of 550 nm in a Bio-Rad 550 microplate reader.

Statistical Analysis. Each experiment was performed at least three times using three replicates for each concentration assayed. Results were expressed as the mean ± SE.

The cytotoxicity of each fraction was expressed as the percentage of viability compared to control wells (the mean optical density of untreated cells was set to 100% viability) in terms of its IC_{50} (concentration of product that causes 50% inhibition of growth or death of the cell population); IC_{50} was calculated from the dose–response curves by linear regression analysis. NRU assay results were expressed as the percentage of uptake of neutral red dye by the lysosomes.

Statistical significance was determined by Student's *t*-test and one-way analysis of variance (ANOVA) using the SPSS software (SPSS Inc., Chicago, IL, USA). Statistical significance was considered $P < 0.05$.

Results and Discussion

Antioxidant Activity. Because of the basic chemical structure of their components (monomeric and oligomeric catechins), the most obvious feature of polyphenolic fraction mixtures is their strong antioxidant activity (*4*). By means of chemical methods (DPPH, HNTTM, and ABTS Assays), previous studies have demonstrated that extracts from pine and grape appear to be efficient antioxidant agents (*13, 14, 21*). It is known that flavonoids can display antioxidant activity in numerous biological systems; therefore, we considered it appropriate to evaluate the antioxidant potential of these fractions using a biological method. Because of their susceptibility to peroxidation, red blood cells (RBCs) have been used as a model to investigate oxidative damage in biomembranes. We therefore chose to investigate the oxidation of RBCs induced by AAPH, a well-known peroxyl radical initiator that causes hemolysis by means of membrane lipid and protein oxidation, and the extent of protection offered by the polyphenolic fractions in order to compare their efficacy as antioxidants.

Dose–response curves were analyzed, and IC_{50} values were obtained (concentration inducing 50% inhibition of hemolysis induced by AAPH). These values are represented in Figure 2, together with that for (−)-Epicatechin, a known antioxidant flavonoid present in grapes and tea (*22, 23*).

All of the fractions tested showed an inhibition of the in vitro AAPH-induced red blood cell hemolysis in a dose-dependent manner (data not shown), and all of them were more effective than (−)-Epicatechin, showing significant differences in all cases.

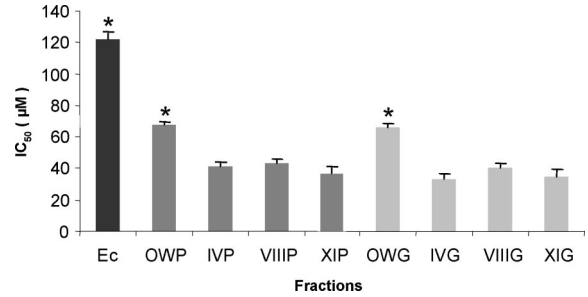


Figure 2. Antioxidant activity of the fractions from different sources and (−)-Epicatechin by the AAPH assay in red blood cells. Results are expressed as IC_{50} or the concentration inducing 50% inhibition of the hemolysis induced by AAPH (mean ± SE). (*) Marked compounds are statistically different to the rest. $P < 0.05$ was considered to denote statistically significant differences.

Among grape fractions, the highest antioxidant power corresponded to the mixtures of compounds with the highest degree of polymerization and galloylation and no glycosylated flavonols. The presence of glycosylated flavonols, which are less efficient scavengers than the aglycons, lowered the overall antiradical power of fractions such as the total fraction **OWG** (*13*).

The most efficient grape fraction was **IVG**, although **VIIIG** and **XIG** gave similar results. (No statistical differences were noted for IC_{50} values.) At equal galloylation (**VIIIG** and **XIG**) antioxidant capacity was proportional to mDP. These observations corroborate other studies in which it is described that antioxidant activity depends on polymerization and increases with galloylation (*24*).

Pine bark fractions also showed good antioxidant activity against oxidation of RBCs. In this case, the most potent antioxidant was fraction **XIP** with an antioxidant efficiency 3 times higher than that obtained for (−)-Epicatechin. We also found a very strong correlation between antioxidant activity and the degree of polymerization ($r = 0.967$) of pine bark fractions, i.e., the higher the mDP, the better the capacity to inhibit AAPH-induced oxidation. Total fraction **OWP** was the least effective, possibly because of its higher levels of monomeric catechins, which reduce antioxidant capacity.

When comparing homologous fractions, pine polyphenols were slightly less potent antioxidants than those from grape (although no statistically significant differences were recorded). These data agree with the results previously obtained by our group (*14*). This less effective antioxidant activity may be attributed to the absence of galloyl esters in their structure, which confer extra antioxidant capacity as reported (*25, 26*).

Studies have suggested that prehemolytic damage caused by AAPH is mediated mainly through lipid peroxidation and to a lesser extent by the oxidation of proteins located in the hydrophobic region of the membrane (*27*). Then, fractions according to their antihemolytic effect should prevent lipid peroxidation and protein oxidation.

Several studies have tried to discover a structure–activity relationship responsible for the biological activity of catechins and other flavonoids, but no conclusive evidence has been found so far (*28*). Several investigations have shown that flavonoids such as (−)-Epicatechin, (+)-catechin, and their related procyanidins can adsorb to membranes through associations with the polar headgroups of phospholipids, generating an environment rich in flavonoids. Such a flavonoid coat would provide protection against oxidants as well as other external aggressors by limiting the access of oxidants to the bilayer and/or controlling the rate of propagation of free radical chain reactions

occurring in the hydrophobic core membranes (*29*). Particularly, galloylated catechins could affect the membrane configuration by forming more compact structures that limit the access of pro-oxidants (*16*). This could be one of the reasons why the grape fractions were in general more active antioxidants. However, it is known that gallate groups influence intracellular events (cell cycle, apoptosis) as reported elsewhere (*13, 30–32*); therefore, it may be preferable, in some cases, to use fractions composed of nongalloylated catechins for applications related to food and skin protection.

In conclusion, all of these polyphenolic fractions are effective antioxidants that can protect human red blood cells from free radical induced oxidative hemolysis (*33*). We have demonstrated that pine fractions although slightly less potent than grape fractions showed effective antioxidant properties (especially those with high mDP (**XIP** and **IVP**)) and that for this reason they are an interesting option for the design of safe products that exert antioxidant protection without influencing normal cell functions.

Cytotoxicity Evaluation. The natural antioxidant properties shown by the polyphenolic fractions suggested that potential applications in different areas can be explored, but we have to guarantee that these new fractions are safe, that is, that the possible concentration range employed does not result in unacceptable damage to normal body cells (*34*). We think it is reasonable to use, as a primary screening stage, in vitro toxicity assays to select the least toxic compounds from among the most actives ones. Use of simple and reproducible in vitro tests consisting of cultures of submerged monolayers of epidermal keratinocytes and dermal fibroblast will allow us to predict adverse effects including potential toxicity and to define safe application concentrations for future formulations (*35*). In this study, we determined cytotoxicity through the neutral red uptake assay in human keratinocyte HaCaT and murine fibroblast 3T3 cell lines and clarified the in vitro cell toxicity effects of our new polyphenolic fractions. We selected the 3T3 cell line because 3T3 neutral red uptake assay is recommended by the U.S. National Institute of Environmental Health Science (NIEHS) Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). The use of the HaCaT, nontumorigenic, spontaneously immortalized keratinocyte cell line provides an almost unlimited supply of identical cells, ensuring high intra and interlaboratory reproducibility (*34*).

We exposed cell cultures to the test compounds for 24, 48, and 72 h, and typical concentration–response curves were recorded to calculate the IC_{50} or dose of compound that inhibits viability to 50%. These IC_{50} values are represented in Figure 3 and Figure 4 for HaCaT and 3T3.

All the fractions showed a certain degree of toxicity as indicated by the decrease in the rate of neutral red uptake. After 48 h of exposure, there was an increase in the cytotoxicity induced by all the fractions, but no significant differences were recorded as compared to the cytotoxicity after 72 h.

The responses of fibroblast and keratinocytes to the given fractions were different. The 3T3 cell line was in general the most sensitive to both pine and grape fractions, although grape fractions showed more significant effects. This different sensitivity of the cells has been previously reported (*36*), and is related to morphologic and physiologic differences between the cell types, especially the differing ability to resist oxidative stress.

Cytotoxicity of grape fractions showed a strong correlation with the degree of polymerization ($r = 0.968$ and 0.978 for 72 h to HaCaT and 3T3, respectively) and the percentage of galloylation ($r = 0.973$ and 0.966 for 72 h to HaCaT and 3T3, respectively) i.e., the fractions with the highest degree of polymerization and galloylation (**XIG** and **VIIIG**) exerted the most toxic effect on the cell cultures. This result is in agreement with those of other authors who also attribute the greater level of cytotoxicity to polyphenolic compounds with these characteristics (*32, 37*).

Pine fractions exhibited lower cytotoxicity, but toxicity increased with the degree of polymerization ($r = 0.897$ and

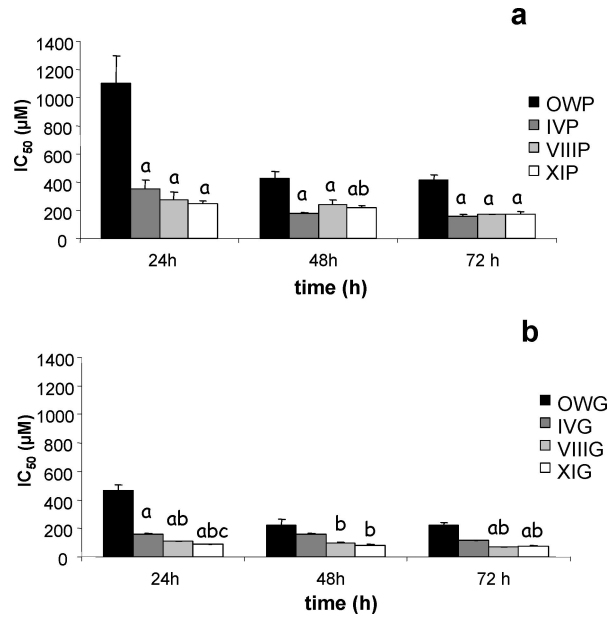


Figure 3. Comparative cytotoxicity of pine fractions (a) and grape fractions (b) toward proliferation of **HaCaT**cells after 24, 48, and 72 h of exposure. Data are presented as IC_{50} or dose inhibiting viability to 50% (mean ± SE). (a) a, statistically different from OWP; b, statistically different from IVP. (b) a, statistically different from OWG; b, statistically different from IVG; c, statistically different from VIIIG. $P < 0.05$ was considered to denote statistically significant differences.

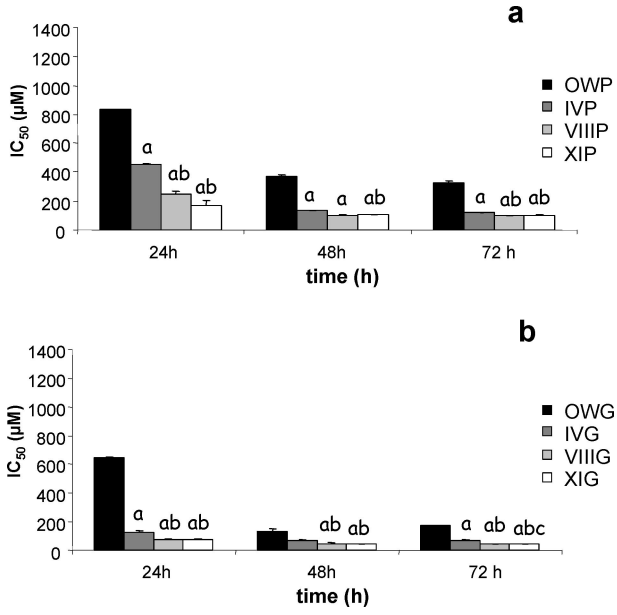


Figure 4. Comparative cytotoxicity of pine fractions (a) and grape fractions (b) toward proliferation of **3T3**cells after 24, 48, and 72 h of exposure. The data are presented as IC_{50} or dose inhibiting viability to 50% (mean ± SE). (a) a, statistically different from OWP; b, statistically different from IVP. (b) a, statistically different from OWG; b, statistically different from IVG; c, statistically different from VIIIG. $P < 0.05$ was considered to denote statistically significant differences.

$r = 0.932$ for 72 h to HaCaT and 3T3, respectively). The lower toxic effect of these fractions was not an unexpected result because their composition is devoid of galloyl esters.

When comparing homologous fractions, those from grape were more cytotoxic in all cases, except for the pair **IVG–IVP**, for which we did not find significant differences. As galloylation is the main difference between grape and pine fractions, these results confirmed the influence of gallate groups in cell viability, and their role in cell cycle regulation. Previous studies in melanoma cells have detected higher antiproliferative and apoptotic effects of galloylated catechins than nongalloylated ones (*26, 38*), but in some cases, especially for applications not related to anticancer drugs such as food or skin protection, it is preferable to use compounds that do not alter normal cell functions.

Although all the fractions tested in this study have shown more cytotoxicity than (−)-Epicatechin (data not shown) (*34*), they exhibited antioxidant activities at concentrations nontoxic to cells. We found a strong correlation between antioxidant and cytotoxic activities for all fractions and for all exposure conditions. The best antioxidant fraction was also the most toxic to cells. To find out if we can work in a safe range of concentrations with these fractions, we calculated the relationship between the cytotoxicity index (IC_{50}) at 72 h in 3T3 and the antioxidant potential. We found that while antioxidant concentration of the more effective pine fractions, **XIP** and **VIIIP**, was approximately 2.5-fold lower than the cytotoxic concentration, in the case of homologous grape fractions, **XIG** and **VIIIG**, it was only 1.3-fold lower. From all of this, we can conclude that an effective antioxidant activity of procyanidin mixtures can be obtained at a concentration range not toxic for the cell lines studied. This is especially true in the case of pine fractions, which present an effective antioxidant capacity with low cytotoxicity due to their lack of gallate groups.

Summary and Conclusions

Plant phenolics from agrifood byproducts are being increasingly used as nutraceuticals. To explore the structure–activity–toxicity relationships of antioxidant procyanidins present in plant extracts, we used a collection of polyphenolic fractions from two different sources (grape and pine). The results obtained in this study show that we can get effective antioxidant activity from these compounds in a concentration range that is safe for normal cells.

Although grape fractions presented slightly higher antioxidant capacity, the observation that pine fractions such as **VIIIP** and **XIP** that are rich in nongalloylated procyanidins with moderate mDP showed an efficient antihemolysis activity with relatively low cytotoxicity provides useful information for the design of safe antioxidant products that exert their protection without altering crucial cell functions.

Acknowledgment. This work was supported by grant AGL2006-12210-C03-02/ALI from Ministerio de Ciencia y Tecnología, Spain. Vanessa Ugartondo holds a doctoral grant from Generalitat de Catalunya, Spain. We are grateful to Robin Rycroft for language assistance.

References

(1) Laufenberg, G., Kunz, B., and Nystroem, M. (2003) Transformation of vegetable waste into value added products: (A) the upgrading concept; (B) practical implementations. *Bioresour. Technol. 87*, 167–198.

(2) Torres, J. L., Selga, A., and Cascante, M. (2003) Bioactive products from by-products and wastes. *Electron. J. Environ. Agric. Food. Chem. 2*, 211–214.

(3) Rice-Evans, C. (2001) Flavonoid antioxidants. *Curr. Med. Chem. 8*, 797–807.

(4) Packer, L., Rimbach, G., and Virgili, F. (1999) Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (Pinus maritima) bark, pycnogenol. *Free Radical Biol. Med. 27*, 704–724.

(5) Hara-Kudo, Y., Yamasaki, A., Sasaki, M., Okubo, T., Minai, Y., Haga, M., Kondo, K., and Sugita-Konishi, Y. (2005) Antibacterial action on pathogenic bacterial spore by green tea catechins. *J. Sci. Food Agric. 85*, 2354–2361.

(6) Toda, M., Okubo, S., Hiyoshi, R., and Shimamura, T. (1989) The Bactericidal Activity of Tea and Coffee. *Lett. Appl. Microbiol. 8*, 123–125.

(7) Hayakawa, S., Kimura, T., Saeki, K., Koyama, Y., Aoyagi, Y., Noro, T., Nakamura, Y., and Isemura, M. (2001) Apoptosis-inducing activity of high molecular weight fractions of tea extracts. *Biosci. Biotechnol. Biochem. 65*, 459–462.

(8) Ruf, J. C. (1999) Wine and polyphenols related to platelet aggregation and atherothrombosis. *Drugs Exp. Clin. Res. 25*, 125–131.

(9) Katiyar, S. K., and Mukhtar, H. (1997) Tea antioxidants in cancer chemoprevention. *J. Cell Biochem. Suppl. 27*, 59–67.

(10) Middlenton, E, Jr., Kandaswami, C., and Theoharis, C. (2000) The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol. Rev. 52*, 673–751.

(11) Medina, I., Lois, S., Lizarraga, D., Pazos, M., Touriño, S., Cascante, M., and Torres, J. L. (2006) Functional fatty fish supplemented with grape procyanidins. Antioxidant and proapoptotic properties on colon cell lines. *J. Agric. Food Chem. 54*, 3598–3603.

(12) Torres, J. L., and Bobet, R. (2001) New flavanol derivatives from grape (*Vitis vinifera*) byproducts. Antioxidant aminoethylthio-flavan-3-ol conjugates from a polymeric waste fraction used as a source of flavanols. *J. Agric. Food Chem. 49*, 4627–4634.

(13) Torres, J. L., Varela, B., García, M. T., Carilla, J., Matito, C., Centelles, J. J., Cascante, M., Sort, X., and Bobet, R. (2002) Valorization of grape (*Vitis vinifera*) byproducts. Antioxidant and biological properties of polyphenolic fractions differing in procyanidin composition and flavonol content. *J. Agric. Food. Chem. 50*, 7548–7555.

(14) Touriño, S., Selga, A., Jiménez, A., Julià, L., Lozano, C., Lizarraga, D., Cascante, M., and Torres, J. L. (2005) Procyanidin fractions from Pine (*Pinus pinaster*) bark: radical scavenging power in solution, antioxidant activity in emulsion, and antiproliferative effect in melanoma cells. *J. Agric. Food Chem. 53*, 4728–4735.

(15) Selga, A., Sort, X., Bobet, R., and Torres, J. L. (2004) Efficient one pot extraction and depolymerization of grape (*Vitis vinifera*) pomace procyanidins for the preparation of antioxidant thio-conjugates. *J. Agric. Food Chem. 52*, 467–473.

(16) Pazos, M., Lois, S., Torres, J. L., and Medina, I. (2006) Inhibition of hemoglobin- and iron-promoted oxidation in fish microsomes by natural phenolics. *J. Agric. Food. Chem. 54*, 4417–4423.

(17) Miki, M., Tamai, H., Mino, M., Yamamoto, Y., and Niké, E. (1987) Free radical chain oxidation of rar red blood cells by molecular oxygen and its inhibition by α-toxopherol. *Arch. Biochem. Biophys. 258*, 373–80.

(18) Babich, H., Krupka, M. E., Nissim, H. A., and Zuckerbraun, H. L. (2005) Differential in vitro cytotoxicity of (−)-epicatechin gallate (ECG) to cancer and normal cells from the human oral cavity. *Toxicol. In Vitro, 19*, 231–42.

(19) Borenfreund, E. P. J. A. (1983) Rapid colorimetric assay to cellular growth and survival: Application to proliferation and cytotoxicity assay. *Toxicol. Lett. 24*, 119–124.

(20) Riddell, R. J., Clothier, R. H., and Balls, M. (1986) An evaluation of three in vitro cytotoxicity assays. *Food Chem. Toxicol. 24*, 469–471.

(21) Vuorela, S., Kreander, K., Karonen, M., Nieminem, R., Hämäläinen, M., Galkin, A., Laitinen, L., Salminen, J., Moilanen, E., Pihlaja, K., Vuorela, H., Vuorela, P., and Heinonen, M. (2005) Preclinical evaluation of rapeseed, raspberry, and pine bark phenolics for health related effects. *J. Agric. Food Chem. 53*, 5922–5931.

(22) Mitjans, M., Del Campo, J., Abajo, C., Martinez, V., Selga, A., Lozano, C., Torres, J. L., and Vinardell, M. P. (2004) Immunomodulatory activity of a new family of antioxidants obtained from grape polyphenols. *J. Agric. Food Chem. 52*, 7297–7299.

(23) Kitawa, S., Sakamoto, H., and Tano, H. (2004) Inhibitory effects of flavonoids on free-radical-induced hemolysis and their oxidative effects on hemoglobin. *Chem. Pharm. Bull. 52*, 999–1001.

(24) Plumb, G. W., De Pascual-Teresa, S., Santos-Buelga, C., Cheynier, V., and Williamson, G. (1998) Antioxidant properties of catechins and proanthocyanidins: effect of polymerisation, galloylation and glycosylation. *Free Radical Res. 29*, 351–358.

(25) Sroka, Z. (2005) Antioxidative and antiradical properties of plant phenolics. *Z. Naturforsch., C: Biosci. 60*, 833–843.

(26) Torres, J. L., Lozano, C., Julià, L., Sánchez, F. J., Anglada, J., Centelles, J. J., and Cascante, M. (2002) Cysteiniyl-flavan-3-ol conjugates from grape procyanidins. Antioxidant and antiproliferative properties. *Bioorg. Med. Chem. 10*, 2497–2509.
(27) Simao, A., Suzukawa, A., Casado, M. F., Oliveira, R., Guarnier, F., and Cecchini, R. (2006) Genistein abrogates pre-hemolytic and oxidative stress damage induced by 2,2′-azobis(amidinopropane). *Life Sci. 78*, 1202–1210.
(28) Caturla, N., Vera-Samper, E., Villalaín, J., Mateo, C., and Micol, V. (2003) The relationship between the antioxidant and the antibacterial preperties of galloylated catechins and the structure of phospholipid model membranes. *Free Radical Biol. Med. 34*, 648–662.
(29) Erlejman, A.G., Verstraeten, S. V., Fraga, C. G., and Oteiza, P. I. (2004) The interaction of flavonoids with membranes: Potential determination of flavonoid antioxidant effects. *Free Radical Res. 38*, 1311–1320.
(30) Na, H. K., and Surh, Y. J. (2006) Intracellular signaling network as a prime chemopreventive target of (−)-epigallocatechin gallate. *Mol. Nutr. Food Res. 50*, 152–159.
(31) Tan, X. H., Hu, D. R., Li, S. R., Han, Y., Zhanc, Y. L., and Zhou, D. Y. (2000) Differences of four cathechins in cell cycle arrest and induction of apoptosis in LoVo cells. *Cancer Lett. 158*, 1–6.
(32) Matito, C., Mastorakou, F., Centelles, J. J., Torres, J. L., and Cascante, M. (2003) Antiproliferative effect of antioxidant polyphenols from grape in murine Hepa-1c1c7. *Eur. J. Nutr. 42*, 43–49.
(33) Dai, F., Miao, Q., Zhou, B., Yang, L., and Liu, Z. (2006) Protective effects of flavonols and theirglycosides against free radical-induced oxidative hemolysis of red blood cells. *Life Sci. 78*, 2488–2493.
(34) Ugartondo, V., Mitjans, M., Lozano, C., Torres, J. L., and Vinardell, M. P. (2006) Comparative study of the cytotoxicity induced by antioxidant epicatechin conjugates obtained from grape. *J. Agric. Food Chem. 54*, 6945–6950.
(35) Benavides, T., Mitjans, M., Martínez, V., Clapés, P., Infante, M. R., Clothier, R. H., and Vinardell, M. P. (2004) Assessment of primary eye and skin irritants by in vitro cytotoxicity and phototoxicity models: an in vitro approach of nex arginine-based surfactants-induced irritation. *Toxicology 197*, 229–237.
(36) Clothier, R., Willshaw, A., and Cox, H. (1999) The use of human keratynocytes in the EU/COLIPA international in vitro phototoxicity test validation study and the ECVAM/COLIPA study on UV filter chemicals. ATLA. *Altern. Lab. Anim. 27*, 247–259.
(37) Schmidt, M., Schmitz, H. J., Baumgart, A., Guédon, D., Netsch, M. I., Kreuter, M. H., Schmidlin, C. B., and Schrenk, D. (2005) Toxicity of green tea extracts and their constituents in rat hepatocytes in primary culture. *Food Chem. Toxicol, 43*, 307–314.
(38) Lozano, C., Torres, J., Julià, L., Jiménez, A., Centelles, J. J., and Cascante, M. (2005) Effect of new antioxidant cysteinyl-flavanol conjugates on skin cancer cells. *FEBS Lett. 579*, 4219–4225.

TX700253Y

VI. Witch Hazel (*Hamamelis virginiana*) fractions and the importance of gallate moieties electron transfer capacities in their antitumoral properties.

Lizárraga, D.; ***Touriño, S.***; Reyes-Zurita, F.J.; De Kok T.M.; Van Delft J.H.; Maas, L.M.; Briedé J.J.; Centelles J.J.; Torres J.L. and Cascante M.; ***J. Agric. Food Chem.***; 56(24):11675–11682, **2008**

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Witch Hazel (*Hamamelis virginiana*) Fractions and the Importance of Gallate Moieties—Electron Transfer Capacities in Their Antitumoral Properties

DANEIDA LIZÁRRAGA,[†,§] SONIA TOURIÑO,[#] FERNANDO J. REYES-ZURITA,[⊥] THEO M. DE KOK,[§] JOOST H. VAN DELFT,[§] LOU M. MAAS,[§] JACCO J. BRIEDÉ,[§] JOSEP J. CENTELLES,[†] JOSEP L. TORRES,[#] AND MARTA CASCANTE*[,†,#]

Department of Biochemistry and Molecular Biology, unit associated with CSIC, Faculty of Biology, University of Barcelona, Biomedicine Institute of the University of Barcelona (IBUB), Diagonal 645, E-08028 Barcelona, Spain; Institute for Chemical and Environmental Research (IIQAB-CSIC), Jordi Girona 18-26, 08034 Barcelona, Spain; Biochemistry and Molecular Biology Department, Science Faculty, University of Granada, Campus Fuentenueva s/n, 18071 Granada, Spain; and Department of Health Risk Analysis and Toxicology, Maastricht University, P.O. Box 616, 6200 MD, Maastricht, The Netherlands

Witch hazel (*Hamamelis virginia*) extracts are used in traditional medicine. They are particularly rich in gallate esters included in proanthocyanidins, hydrolyzable tannins (galloylated sugars), and methyl gallate. This study examines the response of human colon cancer cells to treatment with fractions obtained from a witch hazel polyphenolic extract. The results are compared with those obtained previously with homologous fractions from grape (less galloylated) and pine (nongalloylated). Witch hazel fractions were the most efficient in inhibiting cell proliferation in HT29 and HCT116 human colon cancer cell lines, which clearly shows that the more galloylated the fractions, the more effective they were at inhibiting proliferation of colon cancer cells. Witch hazel fractions were, in addition, more potent in arresting the cell cycle at the S phase and inducing apoptosis; they also induced a significant percentage of necrosis. Interestingly, the apoptosis and cell cycle arrest effects induced were proportional to their galloylation. Moreover, witch hazel fractions with a high degree of galloylation were also the most effective as scavengers of both hydroxyl and superoxide radicals and in protecting against DNA damage triggered by the hydroxyl radical system. These findings provide a better understanding of the structure−bioactivity relationships of polyphenolics, which should be of assistance in choosing an appropriate source and preparing a rational design for formulations of plant polyphenols in nutritional supplements.

KEYWORDS: Antitumoral; DNA protection; gallate; scavenger; colon cancer; apoptosis; cell cycle

INTRODUCTION

Several phytochemicals (e.g., polyphenols) found in plants exert antioxidant and anticancer activities, including cell cycle arrest and induction of apoptosis in cancer cells (*1, 2*). Interest in plants as a source of bioactive compounds such as (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin (EC), and (+)-catechin (C) has recently increased. Some of these polyphenols can be found in green tea (*Camellia sinensis*), grape (*Vitis vinifera*), pine (*Pinus pinaster*), and witch hazel (*Hamamelis virginiana*) (*3*−*6*).

Plant polyphenols are natural antioxidants, and most of their pharmacological properties are understood to be based on their antioxidant capacity (*7*). This capacity is also generally considered to be linked to the scavenging of endogenously generated oxygen radicals or exogenous radicals produced by radiation or exposure to certain xenobiotics (*8*). A particularly valuable effect attributed to polyphenolics is their capacity to prevent oxidative DNA damage.

It has been suggested that EGCG, which is a galloylated compound and the major biologically active component of green tea, is associated with reduced risk of cancer through its prooxidant property (*9*). This anticancer effect has been linked to inhibition of cell growth, deregulation of cell cycle, and

* Author to whom correspondence should be addressed (telephone 0034934021593; fax 0034934021219; e-mail martacascante@ub.edu).
† University of Barcelona.
§ Maastricht University.
# Institute for Chemical and Environmental Research (IIQAB-CSIC).
⊥ University of Granada.

10.1021/jf802345x CCC: $40.75
Published on Web 11/26/2008

Table 1. Polyphenolic Composition of Witch Hazel, Grape, and Pine Extracts and Growth Inhibition Potency on HT29 and HCT116 Cells

plant source	fraction	% CT[a]	mDP[b]	% HT[c]	% G[b]	HT29 IC_{50}[d] (μg/mL)	HCT116 IC_{50}[d] (μg/mL)
witch hazel	VIIIH	4	1.1	96	97	35 ± 2	22 ± 2
	IVH	63	1.6	37	52	21 ± 2	36 ± 2
	VH	79	1	21	16	25 ± 2	27 ± 3
grape	VIIIG	>99	3.4	0	34	64 ± 4a	73 ± 4
	IVG	>99	2.7	0	25	59 ± 3a	82 ± 5
	VG	>99	1	0	0	119 ± 6a	122 ± 5
pine	VIIIP	>99	3	0	0	108 ± 5a	128 ± 6
	IVP	>99	2.9	0	0	106 ± 5a	128 ± 4
	VP	>99	1	0	0	422 ± 4a	447 ± 4

[a] CT, condensed tannins: monomeric catechins and proanthocyanidins; molar percentage. [b] mDP (mean degree of polymerization) refers only to condensed tannins. % G (percentage of galloylation) adds up the contributions of both condensed and hydrolyzable tannins. mDP and %G from refs *4*−*6*. [c] HT, hydrolyzable tannins (hamamelitannin, gallic acid, methyl gallate and pentagalloylglucose); molar percentage estimated by HPLC and standards. [d] IC_{50} of grape and pine in HT29 from ref *14*.

apoptosis induction (*10*−*12*). Moreover, other related compounds such as epicatechin, *trans*-resveratrol, and gallic acids have been described as antioxidant protectors in intestinal model systems (*13*). The influence of the polyphenolic structure on antioxidant activity, protective capacity, and, particularly, the mechanism of action remains open to debate, and further studies are required. It has been observed that polymerization and galloylation may render polyphenolics more or less reactive and bioavailable (*14, 15*).

It is usually argued that the extent of the potency of polyphenols in vivo is dependent on their metabolization and therefore their absorption (*16*). Some but not all polyphenols can be extensively degraded, metabolized, and absorbed through the gastrointestinal tract by several chemical reactions, but their final uptake is sometimes incomplete and their plasma and body levels are low (*16*). Indeed, there are several studies that demonstrate the presence of intact polyphenols in the gastrointestinal tract and suggest their importance as antioxidants, producing health-promoting activities in the colon (*17, 18*). The gallate esters are more stable than the simple catechins upon metabolization (*19*) and may be more bioavailable in the colon. Gallates have been reported to inhibit cell growth, trigger cell cycle arrest in tumor cell lines, and induce apoptosis (*20*). These chemical properties may be useful indicators for evaluating the potential of polyphenolic fractions for colon cancer prevention or treatment and the degree of polymerization related to the bioavailability in the colon.

For the present study we evaluated the inhibition of cell growth by witch hazel fractions on colorectal adenocarcinoma HT29 cells and the effect of those fractions on the cell cycle and induction of apoptosis through FACS analysis. Furthermore, we analyzed the properties of witch hazel fractions as scavengers of hydroxyl and superoxide radicals by ESR. We also compared the results obtained for the highly galloylated fractions from witch hazel with those from grape and pine. All of the fractions from the three sources (witch hazel, grape, and pine) were extracted using the same chemical procedures but differ in terms of gallate ester content (high, medium, and nil, respectively) and polymerization. All of the fractions were evaluated as protective agents against DNA damage through the measurement of 8-oxo-dG by HPLC. The results may help to clarify the role played by polyphenols from different sources and the relationship between structure and inhibition of cell proliferation, scavenger capacity, and DNA protection processes.

EXPERIMENTAL PROCEDURES

Materials. Dubelcco's Modified Eagle's Medium (DMEM) and Dubelcco's phosphate buffer saline (PBS) were obtained from Sigma Chemical Co (St. Louis, MO), antibiotics (10000 units/mL penicillin, 10000 μg/mL streptomycin) were from Gibco-BRL (Eggenstein, Germany), and fetal calf serum (FCS) was from Invitrogen (Carlsbad, CA). Trypsin EDTA solution C (0.05% trypsin−0.02% EDTA) was purchased from Biological Industries (Kibbutz Beit Haemet, Israel). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), propidium iodide (PI), and Igepal CA-630 were obtained from Sigma Chemical Co. NADH disodium salt (grade I) was supplied by Boehringer (Mannheim, Germany). RNase and agarose MP were obtained from Roche Diagnostics (Mannheim, Germany). Iron(II) sulfate heptahydrated was obtained from Merck (Darmstadt, Germany), α,α,α-Tris(hydroxymethyl)aminomethane was from Aldrich-Chemie (Steinheim, Germany) and moviol from Calbiochem (La Jolla, CA). Annexin V/FITC kit was obtained from Bender System (Vienna, Austria). The Realpure DNA extraction kit, including proteinase K, was obtained from Durviz S.L (Paterna, Spain). Blue/Orange loading dye and 1 kb DNA ladder were purchased from Promega (Madison, WI). 5,5-Dimethyl-1-pyrolline *N*-oxide (DMPO), hydrogen peroxide, phenazine methosulfate (PMS), and Hoescht were obtained from Sigma. DMPO was further purified by charcoal treatment. Salmon sperm DNA 2′-deoxyguanosine (dG) was obtained from Sigma.

Fractions. The polyphenolic fractions were obtained previously in our laboratories (*4*−*6*) and contain mainly monomeric catechins and procyanidins (OWG and OWP from grape and pine, respectively), which are soluble in both ethyl acetate and water. OWH from witch hazel contains mixtures of monomeric catechins, proanthocyanidins (procyanidins + prodelphinidins), and gallotannins (hydrolyzable tannins). The fractions derived from the extracts OWH, OWG, and OWP were generated by a combination of preparative reversed-phase high-performance liquid chromatography and semipreparative chromatography on Toyopearl TSK HW-40F (TosoHass, Tokyo, Japan), which separated the components by size and hydrophobicity. The phenolics were eluted from the latter column with MeOH (fractions VG and VP) and water/acetone 1:1 (fractions VIIIH, IVH, VH, VIIIG, IVG, VG, VIIIP, IVP, and V), evaporated almost to dryness, redissolved in Milli-Q water, and freeze-dried. The third to sixth columns in **Table 1** show the average chemical compositions of the fractions. Witch hazel fractions contain mainly small proanthocyanidins (monomers and dimers) so their mDPs are around 1 or 2. The composition of the fractions was estimated as previously described (*4*−*6*). Condensed tannins represented in **Figure 1** (monomeric catechins and proanthocyanidins) were estimated by thioacidolytic depolymerization and HPLC analysis of the cleaved units, and hydrolyzable tannins represented in **Figure 2** (HT, hamamelitannin, gallic acid, methyl gallate, and pentagalloylglucose) were determined directly from the fractions by HPLC and standards.

Cell Culture. Human colorectal adenocarcinoma HT29 cells (ATCC HTB-38) and HCT116 cells (ATCC CCL-247) were used in all experiments. HT29 and HCT116 cells were maintained in monolayer culture in an incubator with 95% humidity and 5% CO_2 at 37 °C. HT29

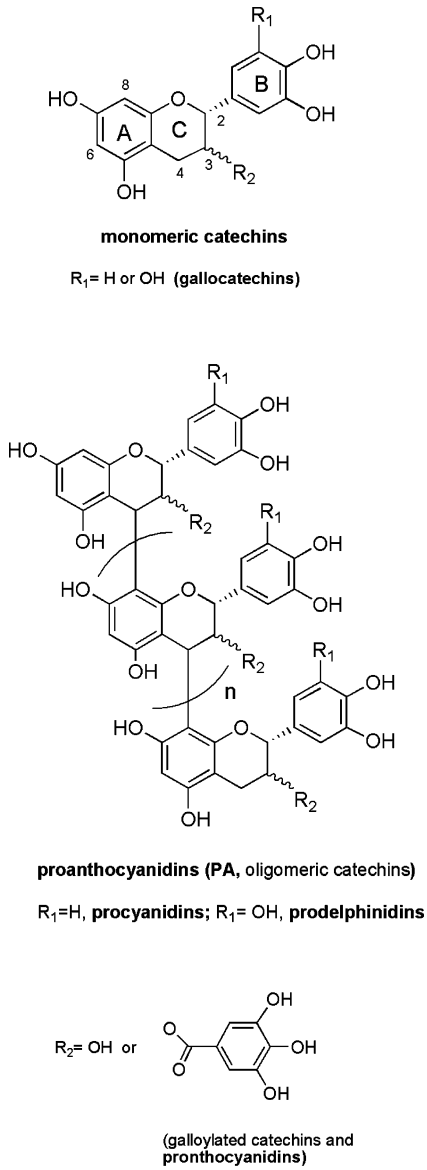


Figure 1. Structures of condensed tannins in *H. virginiana* bark extract.

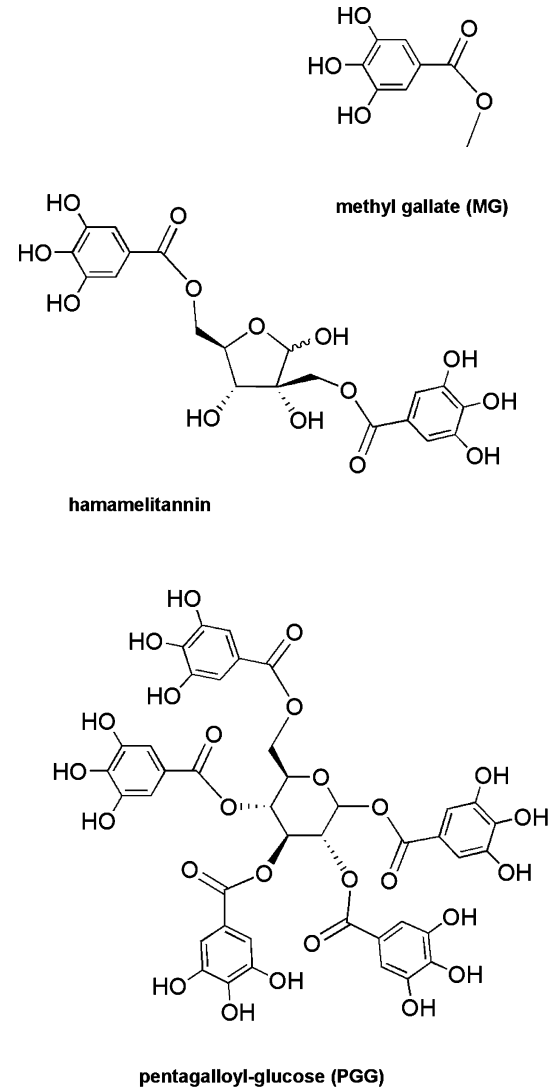


Figure 2. Structures of hydrolyzable tannins and methyl gallate in *H. virginiana* bark extract.

and HCT116 cells were passaged at preconfluent densities using trypsin−EDTA solution C. Cells were cultured and passaged in DMEM supplemented with 10% heat-inactivated fetal calf serum and 0.1% streptomycin/penicillin.

Cell Growth Inhibition. HT29 and HCT116 were seeded at densities of 3×10^3 and 1.7×10^3 cells/well, respectively, in 96-well flat-bottom plates. After 24 h of incubation at 37 °C, the polyphenolic fractions were added to the cells at different concentrations from 5 to 2300 μM in fresh medium. The culture was incubated for 72 h, after which the medium was removed and 50 μL of MTT (5 mg/mL in PBS) with 50 μL of fresh medium was added to each well and incubated for 1 h. The blue MTT formazan precipitated was dissolved in 100 μL of DMSO, and the absorbance values at 550 nm were measured on an ELISA plate reader (Tecan Sunrise MR20-301, TECAN, Salzburg, Austria). Absorbance was proportional to the number of living cells. The growth inhibition concentrations that caused 50% (IC_{50}) and 80% (IC_{80}) cell growth inhibition were calculated using Grafit 3.0 software. The assay was performed using a variation of the MTT assay described by Mosmann (*21*).

Cell Cycle Analysis. The assay was carried out using flow cytometry with a fluorescence-activated cell sorter (FACS). HT29 cells were plated in 6-well flat-bottom plates at a density of 87.3×10^3 cells/well. The number of cells was determined by cells/area well, as used in the cell growth inhibition assay. The culture was incubated for 72 h in the absence or presence of the polyphenolic fractions at their respective IC_{50} values. Cells were then trypsinized, pelleted by centrifugation, and stained in Tris-buffered saline (TBS) containing 50 μg/mL PI, 10 μg/mL RNase free of DNase, and 0.1% Igepal CA-630 in the dark for 1 h at 4 °C. Cell cycle analysis was performed by FACS (Epics XL flow cytometer, Coulter Corp., Hialeah, FL) at 488 nm (*22*).

Apoptosis Analysis by FAC. Annexin V-FITC and propidium iodide staining were measured by FACS. Cells were seeded, treated, and collected as described in the previous section. Following centrifugation, cells were washed in binding buffer (10 mM Hepes, pH 7.4, 140 mM sodium chloride, 2.5 mM calcium chloride) and resuspended in the same buffer. Annexin V-FITC was added using the Annexin V-FITC kit. Following 30 min of incubation at room temperature and in the dark, propidium iodide (PI) was added 1 min before the FACS analysis at 20 μg/mL.

HPLC-ECD for Measuring 8-Oxo-dg. HPLC-ECD of 8-oxo-dG was based on a method described previously (*23*). Salmon DNA (1 mg/mL) was incubated as blank with Milli-Q water. The positive control of the experiment was performed using salmon DNA (1 mg/mL) incubated with Milli-Q water, 1 μM $FeSO_4$, and 250 μM H_2O_2, generating the hydroxyl radical system that induced the DNA lesion (8-oxo-dG), as a marker of oxidative damage. To assay the protection

capacity of the fractions in the induction of 8-oxo-dG, the witch hazel, grape, and pine fractions (VIIIH, IVH, VH, VIIIG, IVG, VG, VIIIP, IVP, and VP) were preincubated at 10, 25, 50, and 100 μM. In all samples the incubation time was 10 min at room temperature, and then the hydroxyl radical systems were generated to simulate the oxidative stress exposure; samples were incubated for 30 min at 37 °C, and the reaction was stopped with a $^1/_{30}$ volume of 0.5 M NaAc. After that, DNA was digested into deoxyribonucleosides by treatment with nuclease P1 (0.02 unit/mL) and alkaline phosphatase (0.014 unit/mL) as was previously described (*23*). To minimize the possible induction of 8-oxo-dG during DNA handling, 10 mM 2,2,6,6-tetramethylpiperidine-1-oxide (TEMPO) was added to all solutions. The digest was then injected into a Gynkotek 480 isocratic pump (Gynkotek, Bremen, Germany) coupled with a Midas injector (Spark Holland, Hendrik Ido Ambacht, The Netherlands) and connected to a Supelcosil LC-18S column (250 × 4.6 mm) (Supelco Park, Bellefonte, PA) and a Decade electrochemical detector (Antec, Leiden, The Netherlands). The mobile phase consisted of 10% aqueous methanol containing 94 mM KH_2PO_4, 13 mM K_2HPO_4, 26 mM NaCl, and 0.5 mM EDTA. Elution was performed at a flow rate of 1.0 mL/min with a lower detection limit of 40 fmol absolute for 8-oxo-dG or 1.5 residues/106 2′-deoxyguanosine (dG). 8-Oxo-dG was detected at a potential of 850 mV, and dG was simultaneously monitored at 260 nm. Results are expressed as percentages of the ratios of 8-oxo-dG to dG, relative to control ratios.

Electron Spin Resonance Spectroscopy. ESR measurements were performed at concentrations that caused 50% cell growth inhibition (IC_{50}). Fifty micromolar witch hazel fractions (VIIIH, IVH, and VH) were then compared with grape and pine fractions (VIIIG, IVG, VG, VIIIP, IVP, and VP). The molar concentrations of the fractions were calculated using the mean molecular masses estimated from the fraction compositions in **Table 1**. The mean molecular masses of the condensed tannins were estimated by thiolysis with cysteamine, as described in ref *24*. $^{\bullet}OH$ and $O_2^{\bullet-}$ formation was detected by ESR spectroscopy using DMPO (100 mM) as a spin trap. ESR spectra were recorded at room temperature in glass capillaries (100 μL, Brand AG Wertheim, Germany) on a Bruker EMX 1273 spectrometer equipped with an ER 4119HS high-sensitivity cavity and 12 kW power supply operating at X-band frequencies. The modulation frequency of the spectrometer was 100 kHz. Instrumental conditions for the recorded spectra were as follows: magnetic field, 3490 G; scan range, 60 G; modulation amplitude, 1 G; receiver gain, 1×10^5; microwave frequency, 9.85 GHz; power, 50 mW; time constant, 40.96 ms; scan time, 20.97 s; number of scans, 25. Spectra were quantified by peak surface measurements using the WIN-EPR spectrum manipulation program (Bruker, Karlsruhe, Germany).

All incubations were done at room temperature; the hydroxyl radical generation system used 500 μM $FeSO_4$ and 550 μM H_2O_2, and hydroxyl radicals generated in this system were trapped by DMPO, forming a spin adduct detected by the ESR spectrometer. The typical 1:2:2:1 ESR signal of DMPO$^{\bullet}$−OH was observed. The superoxide radical generation system using 50 μM of the reduced form of β-nicotinamide adenine dinucleotide (NADH) and 3.3 μM phenazine methosulfate (PMS), and the superoxide radicals generated in this system were trapped by DMPO, forming a spin adduct detected by the ESR spectrometer. The typical ESR signal of DMPO$^{\bullet}$−OOH/ DMPO$^{\bullet}$−OH was observed. The $^{\bullet}OH$ and $O_2^{\bullet-}$ scavenging activity was calculated on the basis of decreases in the DMPO$^{\bullet}$−OH or DMPO$^{\bullet}$−OOH/ DMPO$^{\bullet}$−OH signals, respectively, in which the coupling constant for DMPO$^{\bullet}$−OH was 14.9 G.

Data Presentation and Statistical Analysis. Assays were analyzed using Student's *t* test and were considered to be statistically significant at $* = P < 0.05$ and $** = P < 0.001$. Data shown were representative of three independent experiments, with the exception of ESR experiments that were performed in duplicate. ESR experiments were analyzed separately by radicals, two-way ANOVA was applied (day was a block factor; due to the nonsignificant effect of the day factor, we have reanalyzed with a one-way ANOVA), and finally multicomparison was done between compounds with respect to the control. ANOVA with Bonferroni and Scheffe posthoc tests was performed in ESR experiments.

RESULTS

Growth Inhibition Capacity. Table 1 shows that witch hazel fractions containing highly galloylated tannins (VIIIH and IVH) dose-dependently reduced the proliferation of the carcinoma cell lines HT29 and HCT116 with IC_{50} average values of approximately 28 and 29 μg/mL, respectively, whereas the IC_{50} of fraction VH containing around 16% of galloylation was effective in almost the same order (25 and 27 μg/mL, respectively). It can also be observed that grape polyphenolic fractions VIIIG and IVG obtained by the same extraction procedure as witch hazel fractions VIIIH and IVH have a percentage of galloylation of around 30% (approximately half or one-third that of the witch hazel fractions). On HT29 and HCT116, VIIIG and IVG showed IC_{50} values of around 61 and 77 μg/mL, respectively. Those concentrations were approximately double those of the homologous VIIIH and IVH witch hazel fractions required to exert the same cell growth inhibition on these cells. Moreover, for both cell lines IC_{50} of grape fraction VG was higher (119 and 122 μg/mL, respectively). Pine fractions VIIIP and IVP required a concentration almost 4 times higher than their homologues from witch hazel to exert the same effect in HT29 and HCT116 cells. In addition, the IC_{50} for fraction VP was almost 16 times higher than its counterpart from witch hazel. As pine fractions are not galloylated and grape fractions show almost half the galloylation of witch hazel fractions, we conclude that the more galloylated fractions are much more efficient than the less galloylated fractions in inhibiting colon carcinoma cell proliferation. This effect might also be modulated by the degree of polymerization of some components of the fractions (e.g., proanthocyanidins). Among monomers, those from witch hazel (galloylated) were more efficient than the less galloylated grape and pine counterparts. The results show that the more galloylated the fractions, the more effective they are at inhibiting proliferation of colon cells. These results confirm that galloylation enhances the antiproliferative capacity of polyphenolic fractions and indicate that natural polyphenolic fractions with a high degree of galloylation are more suitable as potential antitumoral agents than those containing no galloylation. This might be due to an enhanced electron transfer capacity (each gallate moiety provides three electrons) or a more specific activity attributed to the gallate group and not to other phenolic moieties.

Cell Cycle Analysis. The growth inhibition capacity of the fractions in HT29 and HCT116 from colon cancer followed almost the same pattern. We examined the effects of witch hazel fractions on the cell cycle at concentrations equal to their IC_{50} values in HT29 colon cancer cells. Cells were treated with each fraction for 72 h and then analyzed by FACS cytometer (**Figure 3**). The cell cycle distribution pattern induced after witch hazel polyphenolic treatments showed that, at IC_{50}, fraction VIIIH induced a significant decrease in G1 phase with respect to the control. In addition, the three fractions induced significant increases in S phase; interestingly, this induction was proportional to their percentage of galloylation. In phase G2 a significant decrease was induced by VIIIH. The most galloylated fraction (VIIIH) was the one that induced more deregulation in all stages of cell cycle in HT29 cells.

Apoptosis Induction. HT29 cell incubations with witch hazel polyphenolic fractions were performed at concentrations described under Materials and Methods. As shown in **Figure 4**, at IC_{50} concentrations, the witch hazel polyphenolic fractions VIIIH and IVH induced significant percentages of early apoptosis in HT29 cells as measured by FACS analysis. In addition, the three fractions (VIIIH, IVH, and VH) induced significant

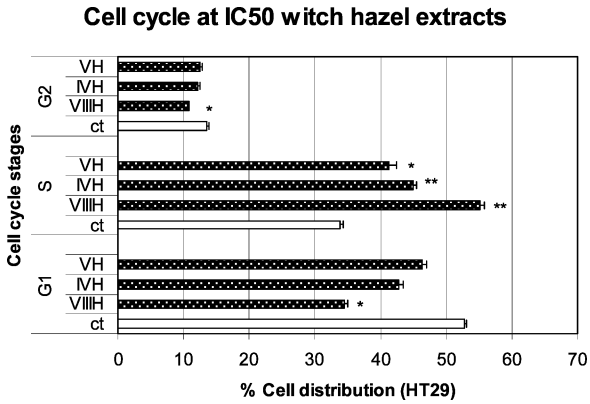


Figure 3. Cell cycle analysis of HT29 cells treated with witch hazel polyphenolic fractions at their respective grape IC_{50} concentrations. Percentages of cells are shown in the different cell stages. Cell phases analyzed were G1, S, and G2 (% cells ± SEM; ∗, $p < 0.05$; ∗∗, $p < 0.001$). Experiments were performed in triplicate.

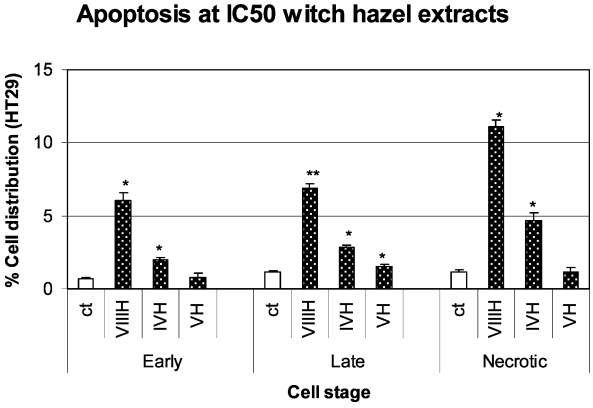


Figure 4. Apoptosis was induced in HT29 tumor cells. HT29 cells were treated with witch hazel polyphenolic fractions at their respective IC_{50} concentrations. Percentages of cells are shown in different cell stages (on the *x*-axis) (% cells ± SEM; ∗, $p < 0.05$; ∗∗, $p < 0.001$). Experiments were performed in triplicate.

percentages in early/late apoptosis. Interestingly, this effect is proportional to their galloylation percentage. Fractions VIIIH and IVH also induced a significant percentage of necrosis, which could be due to a prooxidant effect at that concentration (*25*). This effect has also been observed for different polyphenolic compounds such as EGCG in HT29 and other tumoral cells (*26, 27*).

Protection Exerted by the Fractions against DNA Oxidative Damage. To evaluate the protective capacity of phenolics of different origins and compositions against DNA oxidative damage, salmon DNA incubation with fractions VIIIH, IVH, VH, VIIIG, IVG, VG, VIIIP, IVP, and VP was performed at 10, 25, 50, and 100 μM as described under Materials and Methods. Cells were preincubated with the fractions and thereafter exposed to a hydroxyl radical system. As shown in **Figure 5**, VIIIH, IVH, VIIIG, IVG, VIIIP, and IVP induced a dose-dependent protection against the hydroxyl radical, as given by the amount of the oxidative marker 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxo-dg) (*28*) compared with the levels in 2′-deoxyguanosine (dG) of the cells. Low dose effects from fractions IVG, VIIIP, and IVP could be due to an interaction between polyphenols and traces of iron, which results in more radical-induced oxidative DNA damage in a hydroxyl radical generating system. A reasonable explanation for this phenomenon is that green tea polyphenols and flavonoids have metal-chelating properties combined with DNA-binding properties,

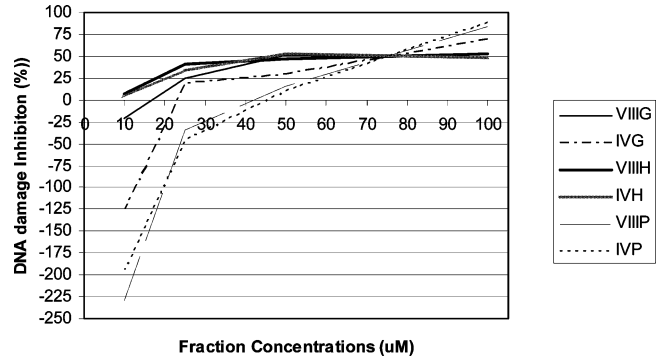


Figure 5. Witch hazel oligomeric fractions induced protection against DNA damage in the hydroxyl radical system. Percentages of protection produced at different fractions concentrations [concentrations (μM) shown on the *x*-axis] are shown (% protection ± SEM). Experiments were performed in triplicate.

thereby promoting hydroxyl radical production closely associated with the DNA (*29*). The dose-dependent decreases in the 8-oxo-dG to dG ratios indicate the efficiency of the fractions in protecting DNA from damage. The same DNA protection pattern has been observed for all of the fractions, with the witch hazel fractions providing the highest level of protection, followed by the grape fractions and finally by pine fractions (**Figure 5**). This property has recently been related to the antioxidant capacity exerted by several polyphenolic compounds. The monomeric fractions from witch hazel, grape, and pine (VH, VG, and VP) did not exert a protective effect (data not shown); this may be due to the concentration used in this assay. Moreover, to detect whether witch hazel fractions are also scavengers of the hydroxyl radical system, the scavenging capacity of the fractions was assayed by ESR.

Oxygen Radical Scavenging Activity As Detected by ESR Spectroscopy. The next set of experiments used ESR spectroscopy to test the radical scavenging capacity of the fractions against different biologically significant potentially harmful radicals. The results show that the VIIIH, IVH, VIIIG, IVG, VIIIP, and IVP, which were the most effective in antiproliferative assays on HT29 cells, were also the most efficient as hydroxyl radical and superoxide scavengers at 50 μM (**Figure 6A**). Fraction VIIIH was the most potent radical scavenger, followed by IVH, grape fractions VIIIG and IVG, and finally the pine fractions VIIIP and IVP. The same order of efficiency was also observed in the cell cycle arrest and induction of apoptosis observed in the present study and in our previous paper on grape and pine fractions (*14*). When fractions were tested at their respective IC_{50} (**Figure 6B**), witch hazel phenolics were less effective as hydroxyl scavengers than grape and pine fractions. The same radical scavenging was observed on the superoxide anion radical system. This may be due to the low concentration used. At 50 μM, VH, VG, and VP (**Figure 6A**) were efficient hydroxyl scavengers; the fraction VH was the most effective, followed by VG fraction and VP fraction. These monomers did not show significant activity as superoxide anion radical scavengers. Again, the different order of efficacies as hydroxyl scavenger observed at their IC_{50} (**Figure 6B**) for VH, VG, and VP may be attributed to the high concentrations used for monomers VG and VP (119 ± 6 and 422 ± 4 μg/mL, respectively) as opposed to the low concentration used for VH (25 μg/mL). In any case, VH from witch hazel was clearly more effective than VG and VP from grape and pine on the superoxide anion radical system.

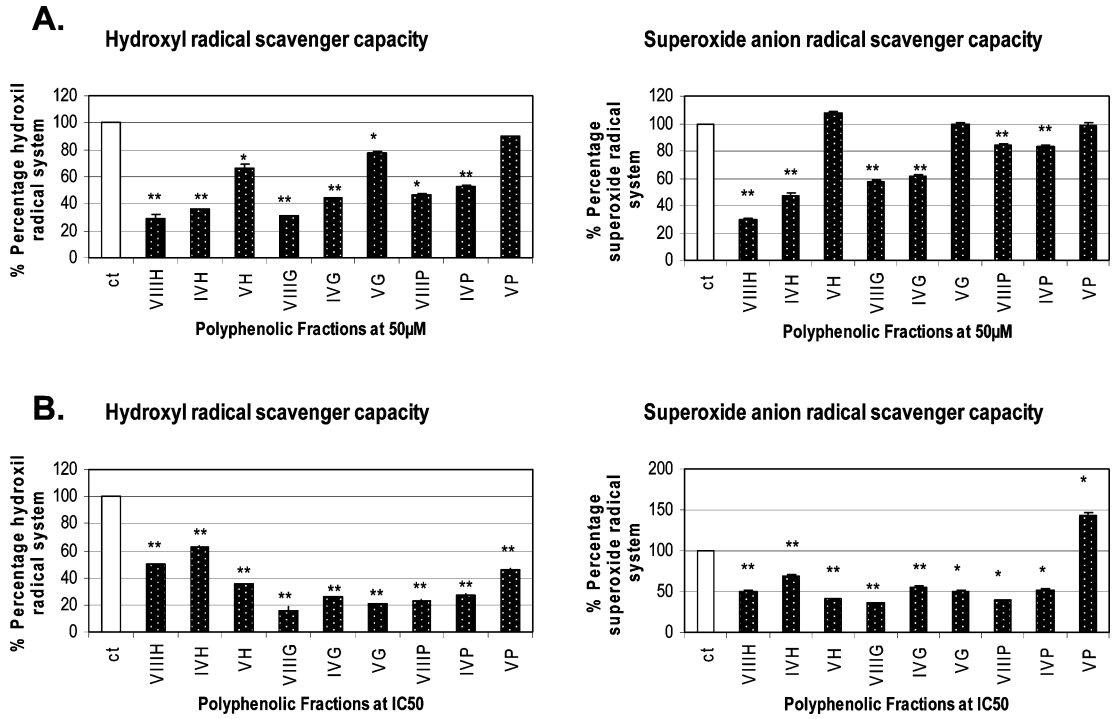


Figure 6. Scavenging activity of $OH^{\bullet}$ and $O_2^{\bullet -}$ analyzed by ESR. Witch hazel, grape, and pine fractions were evaluated at (**A**) 50 μM and (**B**) IC_{50} concentration in HT29 cells in hydroxyl radical and superoxide anion radical generating systems, described under Experimental Procedures. Experiments were performed in duplicate (∗, $p < 0.05$; ∗∗, $p < 0.001$). Data from grape and pine are from ref *18*.

DISCUSSION

The present study shows that significant differences in the inhibition of cell growth in HT29 and HCT116 cells brought about by phenolics of different origins (fractions VIIIH, IVH, VH, VIIIG, IVG, VG, VIIIP, IVP, and VP) may be related to the structural properties of their components, mainly the percentage of galloylation and perhaps the degree of polymerization. Witch hazel fractions VIIIH, IVH, and VH were more effective than VIIIG, IVG, VG, VIIIP, IVP, and VP from grape and pine (**Table 1**). The same differences were observed in cell cycle deregulation and apoptosis induction on HT29 cells, the protective activity against DNA damage by oxidative stress, and the free radical scavenging capacities at the same concentration (50 μM). Galloylation appears to play an important role in the inhibition of cell growth. Even with monomers, galloylated VH was more potent than nongalloylated VG and VP. Fraction VIIIH was the most potent cell cycle deregulator, inducing significant changes in cell percentages of all the phases of the cell cycle. This fraction was also the most effective scavenger among those tested. Because the increases of cell percentages on the S phase of the cell cycle were proportional to their galloylation, this structural feature appears to be clearly significant for the explanation of the activity of phenolics, in accordance with our previous studies (*14, 15*). This highly galloylated fraction was also the most active apoptotic inducer and best free radical scavenger. The cell cycle arrest in the S phase observed for the three witch hazel fractions tested might be triggered by a change in the intracellular redox balance. Perturbations of the intracellular redox signals may block proliferation of tumor cells. It has been observed that cell membrane potentials change in response to H_2O_2 depending on the cell cycle phase. Intracellular ROS are particularly high in the S phase (*30*). Therefore, the cell cycle arrest induced by witch hazel fractions may be explained by their ROS scavenger capacity. Witch hazel fractions may block the cell cycle of HT29 cells in the S phase by scavenging ROS, with subsequent inhibition of DNA synthesis.

Because each gallate moiety provides three hydroxyl groups, galloylation clearly enhances the scavenger capacity of the fractions. Interestingly, galloylation also enhanced the induction of apoptosis and necrosis on HT29 cells. Fractions VIIIH and IVH induced both effects, whereas VH induced only some apoptosis. In this assay VIIIH also was the most active fraction, inducing around 13% of apoptosis and 11% of necrosis. The latter may be due to a pro-oxidant effect. This pro-oxidant effect of some polyphenolic fractions has been extensively discussed in the literature, where it has been described as a protective mechanism by up-regulation of genes implicated in the intracellular defense and biotransformation of xenobiotics such as enzymes of phases I and II (*31*).

There is a clear relationship between high galloylation percentages with low IC_{50}, cell cycle deregulation, apoptosis−necrosis induction, and scavenging capacity. Witch hazel fractions, which are heavily galloylated (**Table 1**), were more potent scavengers than grape and pine fractions in the two radical generation systems (hydroxyl and superoxide) at 50 μM. This is due to the presence of pyrogallol moieties (three adjacent phenolic groups) in the form of gallates from both condensed and hydrolyzable tannins and gallocatechins (pyrogallol moieties in the condensed ring of proanthocyanidins). The main hydrolyzable tannins in the fractions are hamamelitannin (2′,5′-di-*O*-galloyl hamamelose) and pentagalloyl glucose (*6*), included in **Table 1** under HT. Moreover, witch hazel is a source of gallocatechins such as pyrogallol-containing epigallocatechin gallate (EGC), whereas grape and pine are mainly composed of epicatechin (EC) units. These structural features make witch hazel fractions effective electron donors (*6*). This could be behind the alleged pro-oxidant effect of some polyphenols. In fact, ROS may be formed by phenolics through one-electron reductions of molecular oxygen (O_2) to form the superoxide radical, which is primarily responsible for the cytotoxic effects

observed in aerobic organisms (*32, 33*). It has been observed that several pyrogallol-containing polyphenols, such as EGCG from green tea, exert their chemotherapeutical properties by modulating the cellular redox system on cells, including the production of reactive oxygen species, and influence glutathione metabolism and lipid peroxidation in different subcellular compartments (*9*). Moreover, it has been observed that antioxidant−prooxidant properties of ECGC are dose−response dependent (*34*). The redox states of polyphenolic flavonoids appear to influence their ability to induce oxidative molecular damage or antioxidant protective action (*35*). Nowadays, many natural polyphenolic extracts are commercialized, so it is crucial to increase knowledge of the polyphenolic structures within the extracts, the relationship with their putative antioxidant protective activity, and their mechanisms of action. The results presented here should be useful for a better understanding of structure−bioactivity relationships of polyphenolics, which should be of assistance in choosing an adequate source and a rational design for formulations of plant polyphenols in nutritional supplements and as chemotherapy in cancer.

LITERATURE CITED

(1) Seeram, N. P.; Adams, L. S.; Zhang, Y.; Lee, R.; Sand, D.; Scheuller, H. S.; Heber, D. Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. *J. Agric. Food Chem.* **2006**, *54*, 9329–9339.

(2) Lee, S. Y.; Munerol, B.; Pollard, S.; Youdim, K. A.; Pannala, A. S.; Kuhnle, G. G.; Debnam, E. S.; Rice-Evans, C.; Spencer, J. P. The reaction of flavanols with nitrous acid protects against *N*-nitrosamine formation and leads to the formation of nitroso derivatives which inhibit cancer cell growth. *Free Radical Biol. Med.* **2006**, *40*, 323–334.

(3) Reto, M.; Figueira, M. E.; Filipe, H. M.; Almeida, C. M. Chemical composition of green tea (*Camellia sinensis*) infusions commercialized in Portugal. *Plant Foods Hum. Nutr.* **2007**, *62*, 139–144.

(4) Torres, J. L.; Varela, B.; García, M. T.; Carilla, J.; Matito, C.; Centelles, J. J.; Cascante, M.; Sort, X.; Bobet, R. Valorization of grape (*Vitis vinifera*) byproducts. Antioxidant and biological properties of polyphenolic fractions differing in procyanidin composition and flavonol content. *J. Agric. Food Chem.* **2002**, *50*, 7548–7555.

(5) Touriño, S.; Selga, A.; Jiménez, A.; Juliá, L.; Lozano, C.; Lizárraga, D.; Cascante, M.; Torres, J. L. Procyanidin fractions from pine (*Pinus pinaster*) bark: radical scavenging power in solution, antioxidant activity in emulsion, and antiproliferative effect in melanoma cells. *J. Agric. Food Chem.* **2005**, *53*, 4728–4735.

(6) Touriño, S.; Lizárraga, D.; Carreras, A.; Lorenzo, S.; Ugartondo, V.; Mitjans, M.; Vinardell, M. P.; Juliá, Ll.; Cascante, M.; Torres, J. L. Highly galloylated tannin fractions from witch hazel (*Hamamelis virginiana*) bark: electron transfer capacity, in vitro antioxidant activity and effects on skin related cells. *Chem. Res. Toxicol.* **2008**, *21*, 696–704.

(7) Adhami, V. M.; Mukhtar, H. Anti-oxidants from green tea and pomegranate for chemoprevention of prostate cancer. *Mol. Biotechnol.* **2007**, *37*, 52–57.

(8) Hofmann, T.; Liegibel, U.; Winterhalter, P.; Bub, A.; Rechkemmer, G.; Pool-Zobel, B. L. Intervention with polyphenol-rich fruit juices results in an elevation of glutathione S-transferase P1 (hGSTP1) protein expression in human leucocytes of healthy volunteers. *Mol. Nutr. Food Res.* **2006**, *50*, 1191–1200.

(9) Raza, H.; John, A. Green tea polyphenol epigallocatechin-3-gallate differentially modulates oxidative stress in PC12 cell compartments. *Toxicol. Appl. Pharmacol.* **2005**, *207*, 212–220.

(10) Yang, G. Y.; Liao, J.; Kim, K.; Yurkow, E. J.; Yang, C. S. Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols. *Carcinogenesis* **1998**, *19*, 611–6.

(11) Jung, Y. D.; Kim, M. S.; Shin, B. A.; Chay, K. O.; Ahn, B. W.; Liu, W.; Bucana, C. D.; Gallick, G. E.; Ellis, L. M. EGCG, a major component of green tea, inhibits tumour growth by inhibiting VEGF induction in human colon carcinoma cells. *Br. J. Cancer* **2001**, *84*, 844–850.

(12) Shimizu, M.; Deguchi, A.; Lim, J. T.; Moriwaki, H.; Kopelovich, L.; Weinstein, I. B. (−)-Epigallocatechin gallate and polyphenon E inhibit growth and activation of the epidermal growth factor receptor and human epidermal growth factor receptor-2 signaling pathways in human colon cancer cells. *Clin. Cancer Res.* **2005**, *11*, 2735–2746.

(13) Kerem, Z.; Chetrit, D.; Shoseyov, O.; Regev-Shoshani, G. Protection of lipids from oxidation by epicatechin, *trans*-resveratrol, and gallic and caffeic acids in intestinal model systems. *J. Agric. Food Chem.* **2006**, *54*, 10288–10293.

(14) Lizárraga, D.; Lozano, C.; Briedé, J. J.; van Delft, J. H.; Touriño, S.; Centelles, J. J.; Torres, J. L.; Cascante, M. The importance of polymerization and galloylation for the antiproliferative properties of procyanidin-rich natural extracts. *FEBS J.* **2007**, *274*, 4802–4811.

(15) Lozano, C.; Juliá, L.; Jiménez, A.; Touriño, S.; Centelles, J. J.; Cascante, M.; Torres, J. L. Electron-transfer capacity of catechin derivatives and influence on the cell cycle and apoptosis in HT29 cells. *FEBS J.* **2006**, *273*, 2475–2486.

(16) Rice-Evans, C. Flavonoids and isoflavones: absorption, metabolism, and bioactivity. *Free Radical Biol. Med.* **2004**, *36*, 827–828.

(17) Jenner, A. M.; Rafter, J.; Halliwell, B. Human fecal water content of phenolics: the extent of colonic exposure to aromatic compounds. *Free Radical Biol. Med.* **2005**, *38*, 763–772.

(18) Halliwell, B.; Zhao, K.; Whiteman, M. The gastrointestinal tract: a major site of antioxidant action. *Free Radical Res.* **2000**, *33*, 819–830.

(19) Meselhy, M. R.; Nakamura, N.; Hattori, M. Biotransformation of (−)-epicatechin 3-*O*-gallate by human intestinal bacteria. *Chem. Pharm. Bull. (Tokyo)* **1997**, *45*, 888–893.

(20) Salucci, M.; Stivala, L. A.; Maiani, G.; Bugianesi, R.; Vannini, V. Flavonoids uptake and their effect on cell cycle of human colon adenocarcinoma cells (Caco2). *Br. J. Cancer* **2002**, *86*, 1645–1651.

(21) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55–63.

(22) Lozano, C.; Torres, J. L.; Julia, L.; Jiménez, A.; Centelles, J. J.; Cascante, M. Effect of new antioxidant cysteinyl-flavanol conjugates on skin cancer cells. *FEBS Lett.* **2005**, *579*, 4219–4225.

(23) de Kok, T. M.; ten Vaarwerk, F.; Zwingman, I.; van Maanen, J. M.; Kleinjans, J. C. Peroxidation of linoleic, arachidonic and oleic acid in relation to the induction of oxidative DNA damage and cytogenetic effects. *Carcinogenesis* **1994**, *15*, 1399–1404.

(24) Selga, A.; Torres, J. L. Efficient preparation of catechin thio conjugates by one step extraction/depolymerization of pine (*Pinus pinaster*) bark procyanidins. *J. Agric. Food Chem.* **2005**, *53*, 7760–7765.

(25) Azam, S.; Hadi, N.; Khan, N. U.; Hadi, S. M. Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: implications for anticancer properties. *Toxicol In Vitro* **2004**, *18*, 555–561.

(26) Hwang, J. T.; Ha, J.; Park, I. J.; Lee, S. K.; Baik, H. W.; Kim, Y. M.; Park, O. J. Apoptotic effect of EGCG in HT-29 colon cancer cells via AMPK signal pathway. *Cancer Lett.* **2007**, *247*, 115–121.

(27) Weisburg, J. H.; Weissman, D. B.; Sedaghat, T.; Babich, H. In vitro cytotoxicity of epigallocatechin gallate and tea extracts to cancerous and normal cells from the human oral cavity. *Basic Clin. Pharmacol. Toxicol.* **2004**, *95*, 191–200.

(28) Zinov'eva, V. N.; Ostrovskii, O. V. Free radical oxidation of DNA and its biomarker oxidized guanosine(8-oxodG). *Vopr. Med. Khim.* **2002**, *48*, 419–431.

(29) Leanderson, P.; Faresjo, A. O.; Tagesson, C. Green tea polyphenols inhibit oxidant-induced DNA strand breakage in cultured lung cells. *Free Radical Biol. Med.* **1997**, *23*, 235–242.

(30) Gamaley, I. A.; Klyubin, I. V. Roles of reactive oxygen species: signaling and regulation of cellular functions. *Int. Rev. Cytol.* **1999**, *188*, 203–255.

(31) Dhakshinamoorthy, S.; Long, D. J., 2nd; Jaiswal, A. K. Antioxidant regulation of genes encoding enzymes that detoxify xenobiotics and carcinogens. *Curr. Top. Cell Regul.* **2000**, *36*, 201–216.

(32) Halliwell, B.; Gutteridge, J. M. Biologically relevant metal ion-dependent hydroxyl radical generation. An update. *FEBS Lett.* **1992**, *307*, 108–112.

(33) Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M. T.; Mazur, M.; Telser, J. Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* **2007**, *39*, 44–84.

(34) Tian, B.; Sun, Z.; Xu, Z.; Hua, Y. Chemiluminescence analysis of the prooxidant and antioxidant effects of epigallocatechin-3-gallate. *Asia Pac. J. Clin. Nutr* **2007**, *16*, 153–157.

(35) Kim, B. H.; Cho, S. M.; Chang, Y. S.; Han, S. B.; Kim, Y. Effect of quercitrin gallate on zymosan A-induced peroxynitrite production in macrophages. *Arch. Pharm. Res.* **2007**, *30*, 733–738.

Received for review July 29, 2008. Revised manuscript received October 1, 2008. Accepted October 16, 2008. This work was supported by Grants PPQ2003-06602-C04-01 and -04, AGL2006-12210-C03-02/ALI, SAF2008-00164, doctoral fellowships to S.T. and D.L. from the Spanish Ministry of Education and Science, ISCIII-RTICC (RD06/0020/0046) from the Spanish government and the European Union FEDER funds and Generalitat de Catalunya (2005SGR00204; 2006ITT-10007).

JF802345X

VII. The maize ZmMYB42 represses the phenylpropanoid pathway and affects the cell wall structure, composition and degradability in *Arabidopsis thaliana*

Sonbol, F-M.; Capellades, M.; Fornalé, S.; Ruel, K.; ***Touriño, S.***; Torres, J.L.; Rovira, P.; Encina, A.; Puigdomènech, P.; Rigau, J. and Caparrós-Ruiz, D. ***Plant Mol. Biol.***; 70(3): 283-296, **2009**

Plant Mol Biol
DOI 10.1007/s11103-009-9473-2

The maize *Zm*MYB42 represses the phenylpropanoid pathway and affects the cell wall structure, composition and degradability in *Arabidopsis thaliana*

Fathi-Mohamed Sonbol · Silvia Fornalé · Montserrat Capellades · Antonio Encina · Sonia Touriño · Josep-Lluís Torres · Pere Rovira · Katia Ruel · Pere Puigdomènech · Joan Rigau · David Caparrós-Ruiz

Received: 4 December 2008 / Accepted: 12 February 2009

Abstract The involvement of the maize *Zm*MYB42 R2R3-MYB factor in the phenylpropanoid pathway and cell wall structure and composition was investigated by overexpression in *Arabidopsis thaliana*. *Zm*MYB42 down-regulates several genes of the lignin pathway and this effect reduces the lignin content in all lignified tissues. In addition, *Zm*MYB42 plants generate a lignin polymer with a decreased S to G ratio through the enrichment in H and G subunits and depletion in S subunits. This transcription factor also regulates other genes involved in the synthesis of sinapate esters and flavonoids. Furthermore, *Zm*MYB42 affects the cell wall structure and degradability, and its polysaccharide composition. Together, these results suggest that *Zm*MYB42 may be part of the regulatory network controlling the phenylpropanoid biosynthetic pathway.

Keywords *Arabidopsis thaliana* · Maize · Phenylpropanoids · Lignin regulation · R2R3-MYB factors

Introduction

Lignin is, after cellulose the most abundant component of biomass (Boerjan et al. 2003). This polymer is synthesised through the phenylpropanoid pathway, a metabolic grid that synthesises other secondary metabolites such as flavonoids and sinapate esters (Fig. 1). Lignin is deposited in the secondary cell wall of vascular plants and its presence increases the efficiency of water transport, the stiffness of mechanical tissues and constitutes by itself a physical barrier against microbial attacks. However, the covalent interaction of lignin with cell wall polysaccharides makes

Fathi-Mohamed Sonbol and Silvia Fornalé contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s11103-009-9473-2) contains supplementary material, which is available to authorized users.

F.-M. Sonbol · S. Fornalé · M. Capellades · P. Puigdomènech · J. Rigau (✉) · D. Caparrós-Ruiz
Consorci CSIC-IRTA-UAB, Centre de Recerca en AgriGenomica (CRAG), Jordi Girona 18-26, 08034 Barcelona, Spain
e-mail: rigau@ibmb.csic.es

A. Encina
Departamento de Ingeniería y Ciencias Agrarias, Universidad de León, 24071 León, Spain

S. Touriño · J.-L. Torres
Institute for Chemical and Environmental Research, IIQAB-CSIC, Barcelona, Spain

P. Rovira
Departament de Biologia Vegetal, Facultat de Biologia, Universitat de Barcelona, 08028 Barcelona, Spain

K. Ruel
Centre de Recherche sur les Macromolecules Vegetales, UPR-CNRS-5301, 38041 Grenoble Cedex 09, France

Present Address:
F.-M. Sonbol
Department of Biology, Faculty of Dentistry, Sinai University, P.O. Box 7, El Massaid-El Arish, North Sinai, Egypt

Present Address:
P. Rovira
Centre Tecnològic Forestal Catalunya, 25280 Solsona, Spain

Published online: 24 February 2009

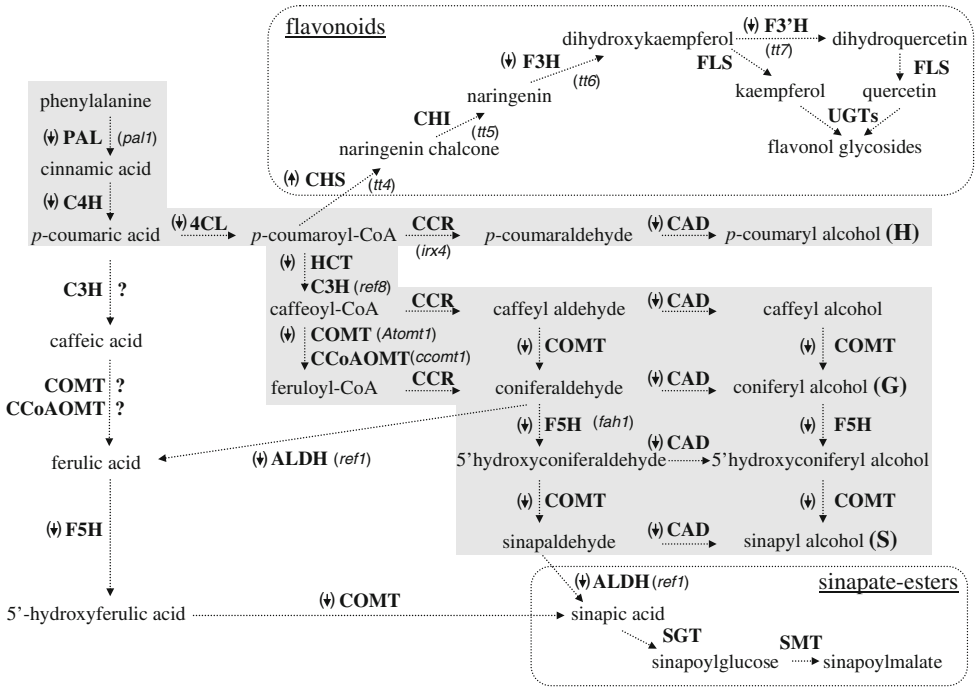


Fig. 1 The phenylpropanoid biosynthetic pathway. Monolignol biosynthesis: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase; HCT, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase; C3H, 4-coumarate 3-hydroxylase; COMT, caffeic acid *o*-methyltransferase; CCoAOMT, caffeoyl-CoA o-methyltransferase; F5H, ferulate-5-hydroxylase. Flavonoid biosynthesis: CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; FLS, flavonol synthase; UGTs, UDP sugar glycosyltransferases. Sinapate-ester biosynthesis: ALDH, aldehyde dehydrogenase; SGT, sinapate UDP-glucose sinapoyltransferase; SMT, sinapoylglucose malate sinapoyltransferase. Question mark refers to enzymatic steps not fully characterised. *Small arrows* in brackets indicate the genes whose expression is repressed by *Zm*MYB42 in *A. thaliana* (with the exception of *CHS* gene which is induced). *A. thaliana* mutants for genes involved in phenylpropanoid synthesis are indicated in brackets

this polymer undesired for biotechnological applications (Torney et al. 2007; Li et al. 2008; Sticklen 2008; Vanholme et al. 2008).

During the last years, a step forward to better understand how lignification takes place has been achieved through the identification of transcription factors regulating lignin biosynthesis. Several proteins belonging to families such as MYB, MADS-box, bHLH, KNOX, and LIM have been shown to be involved in the regulation of lignification (Tamagnone et al. 1998a; Jin et al. 2000; Kawaoka and Ebinuma 2001; Mele et al. 2003; Arnaud et al. 2007; Zhong and Ye 2007; Bomal et al. 2008).

Plant transcription factors containing the MYB domain have been involved in several physiological and biochemical processes (Martin and Paz-Ares 1997; Stracke et al. 2001; Bonke et al. 2003; Liang et al. 2005; Carlsbecker and Helariutta 2005) and belong to a large multigene family composed by 126 members in *A. thaliana* (Yanhui et al. 2006) distributed into 22 subgroups (Stracke et al. 2001). In the case of monocot plants, the maize genome is expected to encode more than 200 MYB proteins (Rabinowicz et al. 1999) while 183 MYB genes have been identified in the rice genome (Jia et al. 2004; Yanhui et al. 2006). The *Antirrhinum majus Am*MYB308 and *Am*MYB330 were the first R2R3-MYB factors associated with the down-regulation of lignification (Tamagnone et al. 1998a) and they were shown to down-regulate three structural genes of the lignin pathway (*4CL1*, *C4H* and *CAD*) when heterologously overexpressed in tobacco plants. Later on Jin et al. (2000) described a knock-out *Atmyb4* plant displaying an increase of the *C4H* gene expression and a decrease in *CCoAOMT* gene expression. In both cases, the overexpression of *AmMYB308*, *AmMYB330*, and *AtMYB4* in tobacco and *A. thaliana* respectively, affected plant growth and development (Tamagnone et al. 1998a, b; Jin et al. 2000). More recently, the *At*MYB32 factor has been proposed to repress the *A. thaliana COMT* gene as the *Atmyb32* mutant slightly increases the expression of *AtCOMT* (Preston et al. 2004). Similarly, the *Eucalyptus gunnii Eg*MYB1 factor has been also proposed as a repressor of lignification (Legay et al. 2007). Interestingly, all these repressors belong to the subgroup 4 of the R2R3-MYB transcription factors.

Many R2R3-MYB proteins belonging to other subgroups have been described as regulators of lignification, such as *Pinus taeda Pt*MYB1 and *Pt*MYB4 (Patzlaff et al. 2003a, b), poplar *Ptt*MYB21a (Karpinska et al. 2004),

*At*MYB61 (Newman et al. 2004), *Eg*MYB2 (Goicoechea et al. 2005), *Vitis vinifera Vv*MYB5a (Deluc et al. 2006) and tobacco *Nt*MYBJS1 (Gális et al. 2006). These factors have been associated with the regulation of lignin biosynthesis through their *in vitro* interaction with ACI, ACII, and ACIII *cis*-elements typically recognised by these transcription factors (Romero et al. 1998).

In maize, few members of the R2R3-MYB family have been characterised so far. This is the case for C1 and PL (Paz-Ares et al. 1987; Marocco et al. 1989; Cone et al. 1993; Pilu et al. 2003), P (Grotewold et al. 1991, 1994), *Zm*1 and *Zm*38 (Franken et al. 1989; Marocco et al. 1989), all of which are involved in the regulation of flavonoid biosynthesis. Another R2R3-MYB factor has been cloned and proposed to participate in maize heterosis (Ju et al. 2006) while other maize factors, such as *Zm*MYB-IF35, have been associated with secondary metabolic pathways (Heine et al. 2007). Recently five new maize R2R3-MYB factors, *Zm*MYB2, *Zm*MYB8, *Zm*MYB31, *Zm*MYB39, and *Zm*MYB42, have been identified and we have shown that two of them, *Zm*MYB31 and *Zm*MYB42, act as repressors of lignin biosynthesis (Fornalé et al. 2006).

In this work, we have investigated the role of *Zm*MYB42 in the biosynthesis of the lignin polymer and studied its role in the regulation of other branches of the phenylpropanoid pathway, such as the biosynthesis of sinapate esters and flavonoids. In addition, we have also investigated the effect of *Zm*MYB42 on cell wall structure, polysaccharides content and composition, and cell wall degradability.

Material and methods

Plant material

Arabidopsis thaliana (ecotype WS) plants were grown under standard greenhouse conditions (25°C day and 22°C night with 60% humidity) and a 16/8 hr photoperiod. For the *in vitro* culture, plants were grown in solid MS medium (Murashige and Skoog 1962) supplemented with 1% sucrose in Petri dishes and kept in growth chamber at 22 ± 2°C with a 16 h light period. The transgenic plants used in this work have been already published (Fornalé et al. 2006) and several independent transgenic lines were studied.

Isolation of total RNA, RT-PCR and PCR

Total RNA was extracted with Trizol Reagent (Invitrogen) and 4 µg of total RNA were reverse-transcribed using M-MLV Reverse Transcriptase (Invitrogen). First-strand cDNA was generated using an oligo(dT)$_{15}$ primer, and 2 µl of the first-strand cDNA used as a template in subsequent PCR reactions. Gene-specific primers were used to amplify *ZmMYB42*, and "no-RT" PCR assays were performed to confirm the absence of genomic DNA contamination. For each assay, several numbers of cycles were tested to ensure that the amplification was in the exponential range. The gene-specific primers used in this work are reported in the Supplementary Table S1 online.

Histology

Cross-sections (150 µm thick) of the basal part of inflorescence stems were obtained using a vibratome (Vibratome Series 1000, TPI Inc., St. Louis, MO, USA) and stained as follow. For the Wiesner staining, sections were incubated for 1 min in 2% Phloroglucinol (w/v) in 95% ethanol, then in 50% HCl previous to the observation under light microscope. For the Maüle staining, sections were stained for 10 min in 0.5% $KMnO_4$ (w/v) solution. After a brief wash in distilled water, samples were incubated for 5 min in 10% HCl, washed with distilled water and mounted onto microscope slides using a concentrated NH_4OH solution for observation under light microscopy.

Lignin auto-florescence was detected using UV-excitation under DAPI-filter and the thickness measurement of lignified tissues was performed using the ImageJ 1.38x program (Rasband 2007).

Assay of Klason lignin

Lignin content from *Arabidopsis thaliana* mature stems was quantitatively measured using the Klason method (Kirk and Obst 1988). Briefly, plant material was extracted four times in methanol and vacuum dried. 100 mg of the samples were hydrolysed in 2 ml of 72% (v/v) H_2SO_4 at 30°C for 1 h. The hydrolysate was diluted with 56 ml of water and autoclaved for 1 h. The sample solution was filtered through a fritted glass-crucible and lignin was measured and expressed as mg of Klason lignin per gram of cell wall residue.

Analysis of lignin monomer composition

The analysis was carried out by the CuO oxidation method (Kögel and Bochter 1985; Heddges and Mann 1979). Briefly, mature stems from 30 wt and *Zm*MYB42 plants respectively were collected and immediately frozen and grounded in liquid nitrogen. After extraction in 20 volumes of methanol, the extract-free samples were dried and aliquots of 50 mg of dried plant material were placed in a sealable Pyrex tube, together with 100 mg of CuO, 200 mg of Fe_2SO_4 and 10 ml of 2 M NaOH were added and a stream of N_2 was bubbled through the solution. The tube was sealed under a N_2 stream and placed at 170°C for 2 h, with occasional shaking. Tubes were cooled to room temperature and their content was transferred to polypropylene

tubes and centrifuged at 3,000 rpm. The pellet was washed with 20 ml of distilled water, centrifuged, and the new supernatant pooled with the previous one. The extract was then acidified with HCl to pH 1, centrifuged again and the pellet washed with 0.1 M HCl. The acidified extract was then filtered through a Waters sep-pack cartridge (Waters) and dried under a N_2 stream, and eluted, first with 2 ml ethyl acetate, then with 1 ml acetonitrile. Eluates were collected in Pyrex vials, dried under N_2 stream and re-dissolved with 1 ml methanol for the HPLC analysis.

The separation and quantitation of the phenolic units was performed with a chromatograph under the following conditions. Injected volume: 20 µl. Column: Teknokroma Tracer Extrasil ODS2 column of 5 mm, 25 × 0.46 cm. Eluents: (a) 0.05% H_3PO_4, (b) acetonitrile. Flux: 1 ml min^{-1}. Temperature: 40°C. Gradient: 10% A at the start, increasing slowly up to 17% in 40 min. The column was washed with 100% acetonitrile between two samples, following the sequence: 17% to 100% in 10 min, 100% during 5 min, dropping to 10% in 10 min, 5 minutes in 10% A to recover a total equilibrium. Phenolic compounds were detected at 280 nm.

Organic extraction of soluble phenolics and high-performance liquid chromatograph with diode array detection (HPLC/DAD)

Leaves or stems (ca. 500 mg) from wt and *Zm*MYB42 plants were frozen in liquid N_2 and extracted as previously reported (Pérez-Jiménez and Saura-Calixto 2008). Samples were analysed by HPLC/DAD on a Hitachi (San Jose, CA, USA) Lachrom Elite HPLC system equipped with a quaternary pump, temperature control unit and photo-diode array UV detector (DAD) fitted with a Kromasil C-18 (Tecknokroma, Madrid, Spain) column (25 × 0.4 cm i.d., 100 Å, 5 μ particle size). Acquisition was made using EZChrom Elite version 3.1.3 from Scientific Software Inc (Pleasanton, CA, USA). Loads: 50 µl. Elution with a binary system of solvents [A] 0.1% (v/v) aqueous TFA, [B] 0.05% TFA in CH_3CN under gradient conditions percentage [B] from 0 to 8 over 5 min; 8 to 10 over 10 min and 10 to 50 over 30 min followed by washing 100% CH_3CN for 10 min and re-equilibration of the column to the initial gradient conditions.

High-performance liquid chromatography–electrospray mass spectrometry (HPLC/ESI/MS)

Analyses were performed on a HPLC/MS system, consisting of a LC200 pump, PE Nelson 1050S integrator (Perkin Elmer, USA) fitted with a reversed phase Phenomenex (Torrance, CA, USA) Luna C18 (2) column (150 × 2.0 mm i.d, 5 µm particle size) coupled to a API3000 triple quadrupole mass spectrometer PE Sciex (Concord, Canada) with a turbo ion spray source that was used to obtain the MS/MS data. HPLC elution conditions: [A] 0,1% aqueous formic acid, [B] CH_3CN gradient from 5 to 23 over 30 min followed washing and equilibration of the column to the initial conditions. The flow rate was 0.4 ml min^{-1}. Ionisation (negative mode) conditions: capillary voltage −3500 V, nebulizer gas (N_2) 10 (arbitrary units), curtain gas (N_2) 12 (arbitrary units), focusing potential −200 V, entrance potential 10 V, drying gas (N_2) heated to 400°C. Analysis of the ions was carried out using full scan (FS) data acquisition from m/z 100 to 800 μ in profile mode and using a cycle time of 2 s with a step size of 0.1 μ and a pause between each of 2 ms. To confirm the identity of some of the compounds, neutral loss (NL) experiments by tandem mass spectrometry (HPLC/ESI/MS/MS) were performed.

HPLC/DAD/MS analysis of sinapate esters

The HPLC/DAD profile after injection of the polyphenolic extract from leaves included a major peak corresponding to sinapoylmalate as ascertained by UV spectrophotometry and LC/ESI/MS. The UV spectrum (190–600 nm) of this peak on HPLC-DAD was compatible with sinapate. Two major MS signals recorded in the negative mode, namely the molecular ion (*m/z* 339.2, MW sinapoylmalate 340) and a fragment corresponding to the sinapate moiety (*m/z* 223.1) revealed the structure of this major phenolic.

Auxin transport assay

Auxin transport was measured according to Besseau et al. (2007). A 2.5-cm segment of flowering stems was excised 5 cm above the base of the stem. Segments were placed in a 1.5-ml microcentrifuge tube and the apical ends submerged in 30 µl of MES buffer (5 mM MES and 1% sucrose, pH 5.5) containing 1 µM indole-3-acetic acid and 66 nM of tritiated indole-3-acetic acid. After 4 h of incubation in the dark, segments were removed and the last 5 mm of the non-submerged ends were excised and placed in 2.5 ml of liquid scintillation cocktail (Optiphase-high-safe 2, Perkin-Elmer). Samples were slowly shaken overnight before measuring radioactivity in a scintillation counter (Beckman LS6000 SC).

Cell wall analysis

Dried plant material (1 g) was added to Poly-Prep tubes and extracted with 10 volumes of 70% EtOH for 5 days at room temperature with wheel-shaking, then washed 6 times with 70% EtOH, 6 times with acetone, and air-dried to obtain the alcohol insoluble residue (AIR). AIR

was then de-starched, treated with acidified phenol and washed with organic solvents to obtain the cell wall residue as previously described (Encina et al. 2002).

Neutral sugar analysis was performed according to Albersheim et al. (1967). Dried cell walls were hydrolysed with 2 M TFA (trifluoroacetic acid) for 1 h at 121°C and the resulting sugars were derivatised to alditol acetates and analysed by gas chromatography (GC) on a Supelco SP-2330 column. Uronic acid contents were determined by the m-hydroxybiphenyl method (Blumenkrantz and Asboe-Hansen 1973), with galacturonic acid as a standard. Cellulose was quantified in crude cell walls by the Updegraff method (Updegraff 1969) with the hydrolytic conditions described by Saeman et al. (1963) and quantification of the glucose released by the anthrone method (Dische 1962) with glucose as a standard.

For the cell wall degradability assays, cell walls were hydrolysed (20 mg/1.5 ml) in a mixture of Cellulase R10 (1%); Macerozyme R-10 (0.5%) and purified Driselase (0.1%) dissolved in sodium acetate 20 mM (pH 4.8). Aliquots were taken at 6, 48 and 72 h, clarified by centrifugation and assayed for total sugars (Dubois et al. 1956).

UV treatment

UV-B treatment was performed according to Jin et al. (2000). Plants were grown for 10 days onto solid MS medium. The lids of Petri dishes were removed and plants were irradiated for 10 min with a short wave transilluminator. After exposure, plants were grown for one week more and then photographed.

Measurement of soluble phenolics

Total phenolic quantitation was performed according to Cliff et al. (2007). Stems were extracted with 10% EtOH (0.1 mg FW/µl). A 10 µl aliquot of each sample was added to 10 µl of 0.1% HCl in 95% ethanol and 182 µl of 2% HCl. Each sample was vortexed and allowed to stand for 15 min. Absorbance was measured at 280, 360, and 520 nm using a Shimadzu UV-1630 spectrophotometer. Phenolic content was determined from standard curves obtained using dilutions of gallic acid, rutin and cyanidin chloride at 280, 360, and 520 nm, respectively.

Electron microscopy assays

Hand-cut transverse sections of the basal region of stems of 5-week-old plants were processed for TEM as previously described (Day et al. 2005). Wall polysaccharides of ultrathin transverse sections (50 nm) were PATAg (Periodic acid-thio carbohydrazide silver proteinate) stained according to Ruel et al. (1977). Observations were performed at 80 kV with a Philips CM 200 Cryo-electron microscope.

Results

Phenotype of *A. thaliana* plants overexpressing *Zm*MYB42

Transgenic plants overexpressing *Zm*MYB42 show several phenotypic alterations (Fig. 2a) at the macroscopic level: they are dwarf with smaller leaves that present a severe adaxial curvature and an altered vascular network characterised by a reduced number of tertiary veins. Previous studies have shown that the overexpression of R2R3-MYB factors acting as repressors of lignification produces alterations of the leaf morphology, with the appearance of white lesions on the older leaves, and reduction of the growth rate when overexpressed in tobacco and *A. thaliana* (Tamagnone et al. 1998a; Jin et al. 2000). In the case of *Zm*MYB42, transgenic plants do not present these white lesions.

*Zm*MYB42 affects lignin biosynthesis

The lignification pattern in different tissues of wt and *Zm*MYB42 plants was analysed by Wiesner staining (Figs. 2b, 3). In stem cross-sections of wild type and transgenic plants, xylem vessels (X) and interfascicular fibres (IF) regions are stained with a red colour, indicative of lignin deposition. However, transgenic stems retain less colour intensity than wild type (Fig. 2b).

Transgenic stems are thinner than the wild type ones (about 65%) and while lignified tissues contain 5-6 cell layers in wild type, transgenic plants display a reduction of approximately 3 cell layers and do not develop secondary xylem at least in the interfascicular region (Fig. 2b). Despite the reduction of lignified layers, the thickness of vascular bundles is not affected in transgenic plants when compared to the stem size (Fig. 2c). In contrast, the thickness of the area of interfascicular fibres decreases by about 30% (Fig. 2c).

Mature siliques of plants expressing *Zm*MYB42 show reduced colour intensity both in the replum and pedicel, indicative of reduced lignin content (Fig. 3). Both the lignified valve marginal cell and endocarp b regions of the silique are also reduced in the transgenic lines (Fig. 3).

We determined the absolute lignin content by the Klason method. This result shows that *Zm*MYB42 plants have 60% reduction of total lignin content compared to wt plants (Fig. 4a). We also analysed the lignin monomer composition by staining stem cross sections with the Maüle reagent

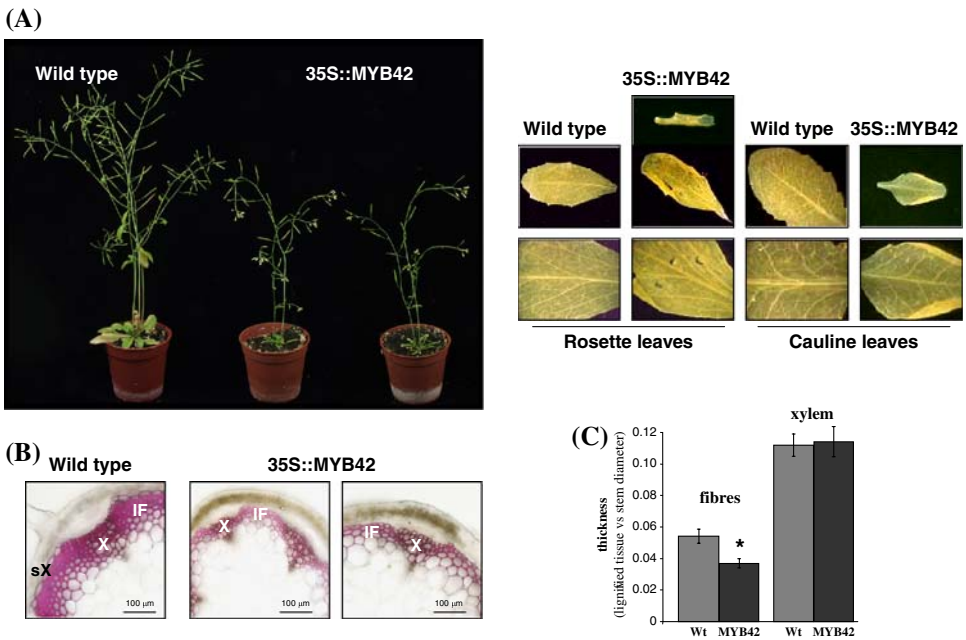


Fig. 2 *Zm*MYB42 affects *Arabidopsis* growth. **a** Phenotype of wt and two independent transgenic plants and leaves at the end of the inflorescence stage. **b** Wiesner staining of cross sections of lignified stalks. (X, xylem; sX, secondary xylem; IF, interfascicular fibres). **c** Quantification of thickness of xylem and fibres in wt and transgenic plants. Values are means of $n = 10$ independent measurements. (*) indicates significant differences ($P \leq 0.01$). The cell thickness was measured using the ImageJ 1.38x program (Rasband 2007)

that stains the S subunits of the lignin polymer specifically. Wild type plants exhibit two staining patterns (Fig. 3): dark red staining is detected in the IF region, indicative of S-enriched lignin, while the fascicular region stain slightly yellow-red, reflecting the deposition of G-enriched lignin. The transgenic *Zm*MYB42 stem show the same pattern,

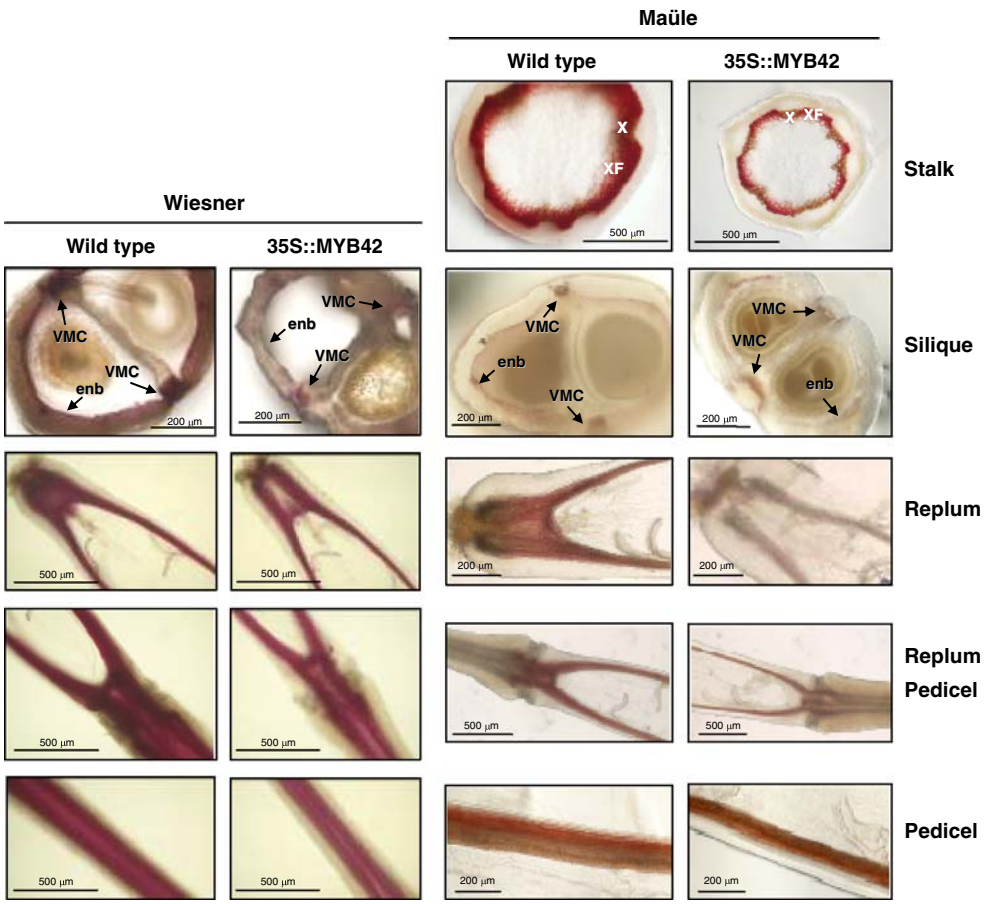


Fig. 3 Histochemical analyses of *Zm*MYB42 transgenic plants. Wiesner (*left*) and Maüle (*right*) staining of cross sections of lignified tissues (VMC, valve marginal cell; enb, endocarp-b)

even though the staining intensity is strongly reduced, in accordance with the reduced lignin content indicated by the Wiesner staining. However, the bright yellow staining of the fascicular region indicates a decrease of S-lignin in this tissue compared to the wild type plants.

A similar result was obtained with mature siliques: besides the general reduction of the stained tissues, the weaker red staining of transgenic siliques indicates a reduction of S-lignin in the lines expressing *Zm*MYB42.

To quantify the effect of *Zm*MYB42 on lignin monomer composition further, we performed HPLC analysis of the phenolic compounds obtained from cell walls of the control and transgenic plants (Fig. 4a). We observed a huge increase in *P*-hydroxybenzaldehyde accounting for a 4-fold increase in the H subunits in the lignin of *Zm*MYB42 expressing plants and a strong reduction of syringic acid, accounting for a 50% reduction of S subunits, even when this method overestimates the S subunits (Lewis and Yamamoto 1990; Heddges and Mann 1979). We observed a 70% increase in G subunits due to an increased accumulation of vanillin. All these changes lead to a 3.4-fold reduction of the S/G ratio of transgenic plants compared to control plants. Thus, the over-expression of *Zm*MYB42 leads to substantial differences in lignin composition compared to the control samples, in line with the histological staining obtained with the Maüle reagent. Interestingly, the expression of this maize transcription factor in *A. thaliana* leads to the production of a lignin polymer with a final composition more similar to that of maize.

In addition to the major lignin-derived compounds, levels of *P*-coumaric and ferulic acid were analysed. While the endogenous levels of *P*-coumaric acid are not affected in *Zm*MYB42 plants, ferulic acid increases 2-fold in transgenic plants compared to wt plants (Fig. 4b).

To further understand the function of *Zm*MYB42 we analysed the expression of all the genes involved in lignification (Fig. 4b). Our analyses showed that the accumulation of *PAL1* mRNA (in addition to *C4H*, and *4CL1;* Fornalé et al. 2006), is reduced in *Zm*MYB42 transgenic lines. This means that *Zm*MYB42 represses the expression of the three genes of the general phenylpropanoid metabolism which is in line with the reduction of the total lignin content in transgenic plants. In addition, the repression pattern of *HCT* and *F5H1* (in addition to *COMT1* and *CAD6;* Fornalé et al. 2006), is in line with the

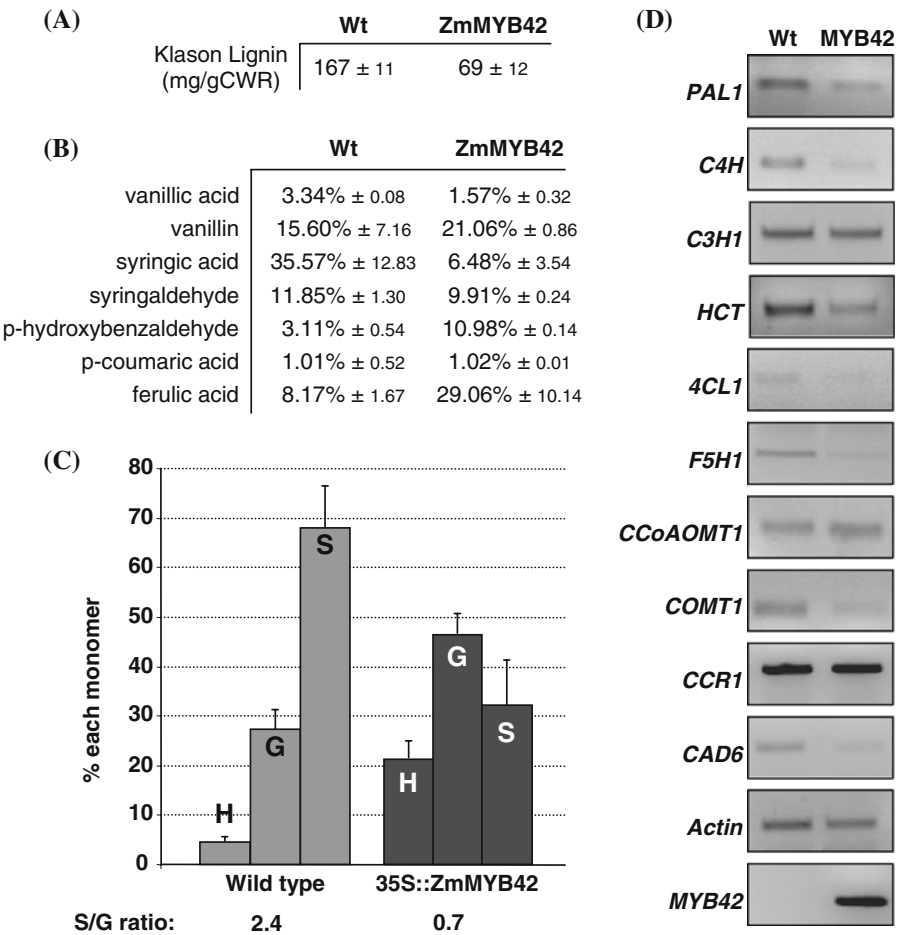


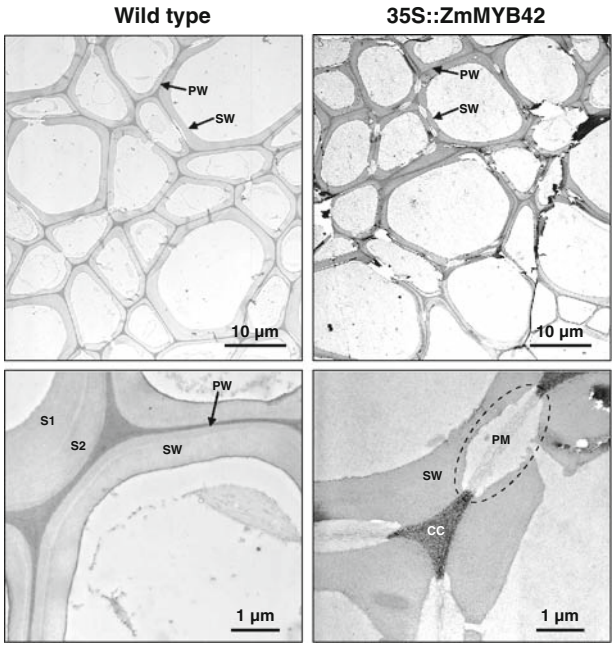
Fig. 4 *Zm*MYB42 alters lignin content and composition. **a** Quantitative lignin content determination from wt and *Zm*MYB42 mature stems by the Klason method. **b** HPLC analysis of lignin composition from wt and *Zm*MYB42 plants determined by the CuO oxidation method. Results are shown as relative amounts of each compound. **c** Histogram reporting the lignin composition of *Zm*MYB42 and wt plants. H refers to *P*-hydroxybenzaldehyde; G refers to the sum of vanillin and vanillic acid; S refers to the sum of syringaldehyde and syringic acid. Data correspond to the mean value ± SD of three independent assays. **d** Relative expression levels of all the monolignol biosynthetic genes

alteration in the final lignin composition of the transgenic plants. On the other hand, the expression of *C3H*, *CCoAOMT* and *CCR* genes is not affected by *Zm*MYB42.

*Zm*MYB42 affects cell wall structure, polysaccharide content, composition and degradability

The huge reduction of lignin and its altered S/G ratio led us to investigate whether these changes affected the structure of the cell wall of *Zm*MYB42 plants. Therefore, we analysed by TEM (transmission electron microscopy) the cell wall structure of wild type and transgenic plants (Fig. 5a). The modifications induced by *Zm*MYB42 essentially affect the interfascicular fibres. In *Zm*MYB42 plants, the secondary cell wall thickenings are interrupted by a greater number of wide "pits" in which the primary wall is clearly visible and sometimes extended between two cell corners. In addition, the interrupted secondary wall of the transgenic plants exhibits a weaker delimitation between S1 and S2 sub-layers. It is worth noting that the reactivity to PATAg of *Zm*MYB42 fibre walls appears enhanced. This may be explained by an enhanced accessibility of polysaccharides to the periodic acid treatment due to the reduced lignin content.

As lignin interacts with polysaccharides within the cell wall and putatively constitutes a competing carbon sink, we also investigated whether *Zm*MYB42 affected the cell wall polysaccharide content and composition by GC analysis. The cell wall yield does not vary between wild type (0.59 ± 0.02) and plants overexpressing *Zm*MYB42 (0.60 ± 0.02). As shown in Fig. 5b, while cellulose content is not significatively affected by the overexpression of *Zm*MYB42, a broad alteration in cell wall composition is detected. In particular, a general increase



(B)

µg mg^{-1} dry cell wall

		Wild type	35S::ZmMYB42	
	Cellulose	366.90 ± 38.2	354.21 ± 24.6	
Hemicelluloses	Fucose	0.32 ± 0.0	1.81 ± 0.3	*
	Arabinose	4.63 ± 0.1	25.82 ± 2.6	*
	Xylose	248.58 ± 0.5	219.27 ± 6.2	*
	Glucose	5.36 ± 0.2	6.33 ± 3.0	
	Mannose	2.59 ± 0.1	2.49 ± 0.6	
Pectins	Uronic acids	151.32 ± 9.7	233.73 ± 26.4	*
	Rhamnose	3.78 ± 0.1	9.50 ± 3.6	*
	Galactose	2.99 ± 0.2	13.05 ± 1.4	*
	Matrix poly.	419.50 ± 37.9	512.00 ± 83.7	
	Total sugars	786.40 ± 65.2	866.21 ± 103.4	

(C)

carbohydrates released (mg g^{-1} dry cell wall)

	Wild type	35S::ZmMYB42
6h	249.7 ± 11.5	406.2 ± 35.5
48h	270.9 ± 16.8	475.5 ± 27.9
72h	326.6 ± 14.9	545.2 ± 26.9
degradability ratio	1	1.68

Fig. 5 *Zm*MYB42 affects cell wall structure and composition. **a** Transmission electron microscopy after PATAg staining of interfascicular fibres of wt and transgenic plants (PM, Pit-Membrane; PW, primary wall; SW, secondary wall; S1 and S2, secondary cell wall sub-layers; CC, cell corner). Broken line circle indicates the appearance of wide "pits" in transgenic cell walls. **b** Sugar analyses of cell walls from stems of wt and transgenic plants. Data are expressed as mean value of six independent assays ± SD. (*) indicate significant differences by Student's t test ($P < 0.05$). **c** Resistance of cell walls to digestion with polysaccharide hydrolases. A time-course of cell wall degradability was performed using stems of wt and transgenic plants. Data correspond to the mean value ± SD of three independent assays

of polysaccharides habitually present in the primary cell wall (arabinose, galactose, uronic acids, rhamnose and fucose) is observed while a decrease of xylose, a secondary cell wall polysaccharide, is also observed. However, these changes do not lead to significant changes in the amounts of total sugars in transgenic cell walls with respect to wild type plants.

The observed changes in lignin, cell wall structure and polysaccharides in the cell wall prompted us to determine whether the transgenic cell walls were more susceptible to enzymatic degradability. Our results indicate that *Zm*MYB42 plants have cell walls more degradable when treated with the cellulase-macerozyme-driselase enzymatic cocktail (Fig. 5c).

*Zm*MYB42 plants have reduced levels of sinapoylmalate

Similarly to what observed in the case of the *fah1-2* and the *ref8* mutants (Franke et al. 2002), *Zm*MYB42 plants show a red colour when visualised under UV-A, suggesting a reduction of sinapoylmalate (Fig. 6a). We therefore analysed by HPLC/DAD/MS extracts of 4-week-old rosette leaves and the results obtained confirmed that transgenic plants show a strong reduction of this compound (Fig. 6b).

As sinapoylmalate plays a central role as a UV-protectant in *A. thaliana*, we investigated whether *Zm*MYB42 plants were more sensitive to the UV-B radiation. Thus, we treated 10-days old wild type and transgenic plants with 10 min UV-B light and we analysed their response. After 7 days, transgenic plants show yellowing leaves, indicative of a severe and extensive damage compared to the wild type plants (Fig. 6c). The expression analysis of the main genes involved in sinapoylmalate biosynthesis indicated that *Zm*MYB42 represses the *ALDH* gene (Fig. 6d). As *At*MYB4 has been shown to affect the synthesis of sinapoylmalate (Jin et al. 2000; Hemm et al. 2001) we also investigated whether *Zm*MYB42 could affect the expression of this transcription factor. Our results show that *AtMYB4* gene expression is reduced in *Zm*MYB42 plants (Fig. 6d).

*Zm*MYB42 represses flavonol biosynthesis

The observation of stem cross-sections of *Zm*MYB42 plants under UV light indicates an altered fluorescence, indicative of possible changes in flavonoid accumulation compared to wild type plants (Fig. 7a).

As *Zm*MYB42 represses the expression of genes of the core phenylpropanoid pathway, we investigated whether this factor could regulate other branches of this complex network, such as the one leading to the synthesis of flavonoids (Fig. 1).

Thus, we performed a quantitative analysis of the main phenolics from 2-month old plants, which revealed that *Zm*MYB42 plants present more than a 66% reduction of the total phenolic content compared to the wild type plants. This decrease is mainly due to the huge reduction of flavonols (about 50% of the wild type), while the anthocyanins level remains practically unchanged (Fig. 7b). The HPLC/DAD/MS analysis indicated that the reduced flavonol content is mainly due to a decrease in the accumulation of flavonols belonging to the kaempferol family (Fig. 7c).

As previous studies demonstrated that flavonoids are negative regulators of auxin transport (Jacobs and Rubery 1988), we investigated whether this reduction of flavonols affected the transport of these hormones using a radiochemical method. The results obtained indicate that auxin transport in *Zm*MYB42 plants is not affected compared to wild type plants (Fig. 7d).

Finally, we performed RT-PCR assays to study the effect of *Zm*MYB42 on the main genes of the flavonoid pathway (Fig. 1). Our results indicated that *Zm*MYB42 down-regulates the *F3H* and *F3′H* gene expression. In contrast, the mRNA accumulation of the *CHS* gene is increased compared to wild type plants (Fig. 7e).

Discussion

In a previous studies, we reported the isolation of *Zm*MYB42, a new R2R3-MYB factor belonging to the subgroup 4 that down-regulates the maize and the *A. thaliana COMT* genes and we showed that transgenic plants overexpressing this factor contain only half of the total lignin content (Fornalé et al. 2006). Here we characterised the effects of *Zm*MYB42 in relation to lignin biosynthesis and also to other branches of the phenylpropanoid pathway. *Zm*MYB42 plants are dwarf and their leaves are severely curved with a reduction of their vascular network. The reduced growth may be caused by the difficulty of plants in producing vascular and mechanical tissues. Similar phenotypes appear in mutants with reduced lignin content (e.g. *ref8* mutant; Franke et al. 2002).

The strong reduction of lignin content was observed in all lignified tissues when analysed by phloroglucinol staining. In addition, we showed that in transgenic plants, there is a strong reduction of the S/G ratio of the lignin polymer, due to both a decrease of the S subunits and an increase of the G subunits. Indeed, the appearance of high levels of H subunits makes the final lignin polymer of the *Zm*MYB42 *A. thaliana* plants more similar to that typically produced by maize.

The impact on lignin content and composition caused by *Zm*MYB42 relies on the downregulation of the phenylpropanoid pathway (Fig. 1); the general phenylpropanoid

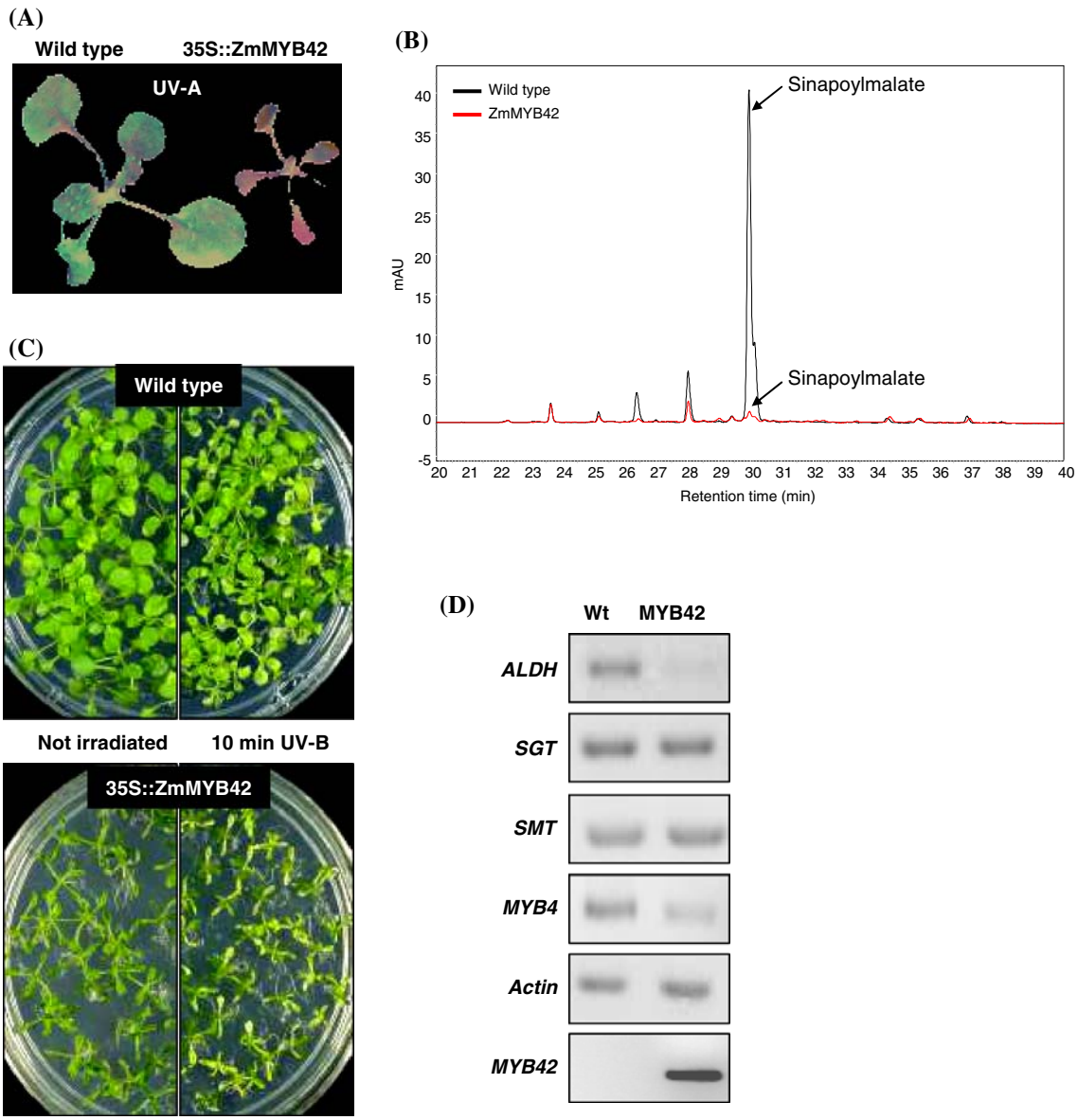


Fig. 6 *Zm*MYB42 represses sinapoylmalate biosynthesis. **a** Phenotype of wt and transgenic plantlets exposed to UV light. **b** HPLC-DAD analysis of sinapoylmalate content in leaves of wt and transgenic plants. **c** Phenotype of 20-days old wt and transgenic plantlets submitted to 10 min UV irradiation. **d** Relative expression of the genes involved in sinapoylmalate synthesis and of *AtMYB4* gene

genes (*PAL*, *C4H*, and *4CL*), several genes involved in the conversion from esters to aldehydes and alcohols (*HCT* and *CAD*) and genes belonging to the branches leading to the production of the three main monolignols (*F5H* and *COMT*). In contrast, *C3H*, *CCoAOMT* and *CCR* genes are not regulated by this transcription factor (Figs. 1, 4). Together, and in accordance with previous observations (Ralph et al. 2008) these results show once more the high level of flexibility of the phenylpropanoid pathway.

Several reports have shown that reduction of CAD (Sibout et al. 2003), F5H (Marita et al. 1999) and COMT (Goujon et al. 2003; Do et al. 2007) results in a decrease of the S units in the final lignin polymer. In agreement with these results, the repression of *F5H*, *COMT* and *CAD* genes produces a lignin polymer strongly depleted in S units. In addition, the increased levels of ferulic acid are not expected if this compound is produced by COMT, as this gene is repressed in *Zm*MYB42 plants. Thus, this finding is in line with other works indicating that COMT does not catalyse the *in vivo* methylation of caffeic acid to produce ferulic acid (Do et al. 2007). Together, the overall pattern of repression of the lignin genes accounts for the reduced capacity of transgenic plants to produce S units.

Cell walls of transgenic fibres show an altered structure characterised by a higher number of pit-membranes and a reduced thickness of secondary cell walls. The increase of

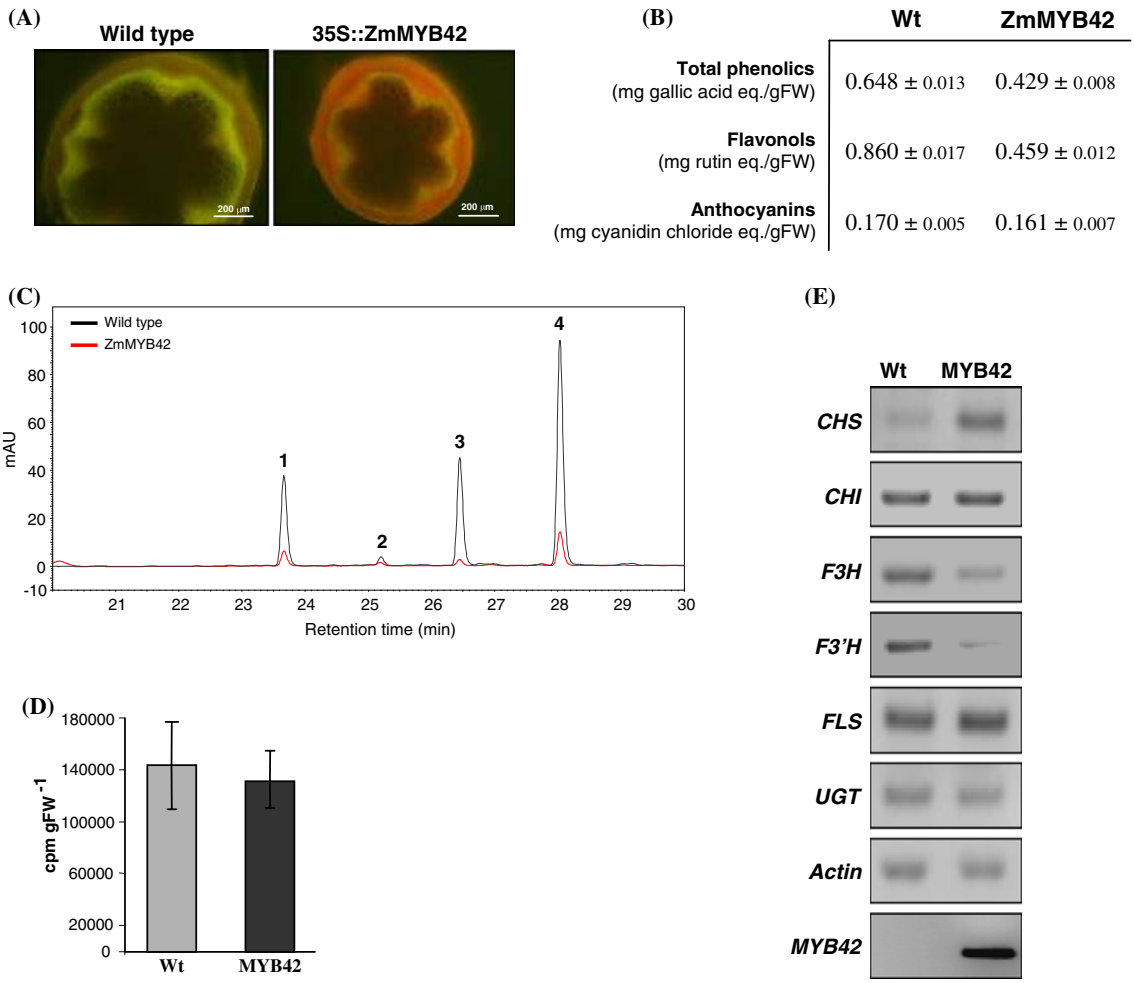


Fig. 7 *Zm*MYB42 represses flavonols biosynthesis. **a** Sections of wt and transgenic stalks visualised under UV microscope at the end of the inflorescence stage. **b** Quantification of phenolic compounds from wt and transgenic plants (eq means equivalents). **c** HPLC-DAD analysis of flavonols content in stalks of wt and transgenic plants. (a: Kaempferol 3-*O*-rhamnosyl-glucoside 7-*O*-rhamnoside (*m/z* 739.3, MW 740); b: Kaempferol 3-*O*-glucosyl-glucoside 7-*O*-rhamnoside (*m/z* 755.1, MW 756); c: Kaempferol 3-*O*-glucoside 7-*O*-rhamnoside (*m/z* 593.4, MW 594); d: Kaempferol 3-*O*-rhamnoside 7-*O*-rhamnoside (*m/z* 577.2, MW 578)). The structures were confirmed by neutral loss experiments (MS-MS). The losses detected were rhamnoside moieties (146 mass units) from each of the four flavonol glycosides and an additional glucoside moiety (162 mass units) from kaempferol 3-*O*-glucoside-7-*O*-rhamnoside. **d** Auxin transport determination in wt and transgenic plants. Data correspond to the mean value ± SD of three independent assays. **e** Relative expression of the main genes involved in flavonol synthesis

PATAg staining in transgenic plants also suggests that the reduction of the lignin content leads to a looser interaction between cellulose microfibrils which favours the enhancement of the size of silver grain deposits. Similar loosening of microfibrils association was previously observed in relation to a decrease in syringyl units, suggesting the important role of non-condensed substructures in the secondary wall cohesion (Ruel et al. 2001).

In addition to lignin content and composition, and cell wall structure, *Zm*MYB42 also affects the cell wall composition. Our results show that the majority of primary cell wall type monosaccharides are increased while xylose, present in the secondary cell wall, is decreased. Therefore, cell wall compositional analysis indicates an overall enrichment in matrix polysaccharides that are characteristics of the primary cell wall. Based on these results it seems that because of the reduction in lignin, *Zm*MYB42 stems retain primary cell wall enriched tissues.

In agreement with the reduction of total lignin content and the strong reduction of the S/G ratio, transgenic cell walls are more degradable. Histological studies, together with the reduction of the S subunits of the lignin polymer and the electron microscopy data all indicate that *Zm*MYB42 mainly affects the development of the mechanical tissues, which are normally enriched in S subunits (Mellerowicz et al. 2001).

Plants having a reduction of *C4H* (Jin et al. 2000), *F5H* (in the *fah1* mutant; Ruegger et al. 1999), *C3H* (in the *ref8*

mutant; Franke et al. 2002) and *COMT* gene expression (in the *Atomt1* mutant; Goujon et al. 2003; Do et al. 2007) show a reduction in the synthesis of sinapoylmalate and display red fluorescence when visualised under UV light. Accordingly, *Zm*MYB42 represses *C4H*, *F5H*, and *COMT* gene expression, and thus transgenic plants have a strong reduction in sinapoylmalate as demonstrated by HPLC/DAD/MS, the observation under UV light and the increased sensitivity to UV-B irradiation. In addition, *Zm*MYB42 also represses the expression of *ALDH* gene, which is the first specific enzyme for the synthesis of sinapoylmalate (Fig. 1). This result is in line with what observed in the Arabidopsis *ref1* mutant in which the mutation of the *ALDH* gene results in a strong reduction of the sinapate esters (Nair et al. 2004).

The involvement of *C4H* repression in the synthesis of sinapoylmalate was described through the study of *AtMYB4* gene (Jin et al. 2000), a repressor of sinapoylmalate synthesis. In *Zm*MYB42 plants, the expression of *AtMYB4* gene is downregulated. This behaviour could be the consequence of a homeostatic response by which the transgenic plants tend to counteract the effect produced by the overexpression of *Zm*MYB42.

As mentioned above, *Zm*MYB42 also represses the synthesis of *PAL1*, *C4H*, and *4CL* genes. These genes are involved in the core-phenylpropanoid pathway and catalyse the synthesis of hydroxycinnamic acid intermediates, which are precursors for the route-specific pathways leading to the synthesis of a wide range of secondary metabolites such as flavonoids (Fig. 1). Transgenic plants overexpressing *Zm*MYB42 have stems in which the cortex region appears orange under UV light, suggesting an alteration in the synthesis of some flavonoids. A deeper characterisation revealed that transgenic plants have decreased levels of total soluble phenolics and in particular, a strong reduction of flavonols belonging to the family of kaempferol. Although *Zm*MYB42 induces the expression of *CHS* gene, the entry point to flavonoid biosynthesis, no increased levels of naringenin chalcone were detected (data not shown). However, *Zm*MYB42 represses the expression of *F3H* and *F3′H* genes, which could explain the reduced levels of flavonols in transgenic plants. Therefore, in addition to the strong reduction of sinapoylmalate, the fact that transgenic plants are more sensitive to UV irradiation could be, at least partially, also caused by the reduction of the flavonol content as described in the case of the *transparent testa 4* (*tt4*) mutant (Li et al. 1993).

It has been reported that flavonoids are negative regulators of the auxin transport (Brown et al. 2001; Besseau et al. 2007). In our case, although *Zm*MYB42 have reduced levels of flavonoids, the auxin transport is not induced in transgenic plants, suggesting that a 50% reduction of flavonols is not sufficient to increase the levels of auxin transport.

Together, these results show that *Zm*MYB42 is a general repressor of the phenylpropanoid pathway affecting cell wall structure and composition when overexpressed in *A. thaliana*. The reduction of the total lignin content, the strong reduction of its S/G ratio, and the increased cell wall degradability, makes this factor a good candidate to control lignin metabolism in maize for biotechnological applications.

Acknowledgements This work received financial support from the CONSOLIDER-INGENIO programme (CSD2007-00036) from the Spanish Ministerio de Ciencia e Innovación. F.M. Sonbol was financed by the European Project INCO-II (ICA4-CT-2000-30017), by the CRAG laboratory, and by the "Instituto de Cooperación con el Mundo Árabe, Agencia Española de Cooperación Internacional". S. Fornale was financed by a post-doctoral grant from the "Generalitat de Catalunya" (2003PIV-A-00033) and by an I3P contract from the "Consejo Superior de Investigaciones Científicas". D. Caparrós-Ruiz was financed by the Spanish "Ministerio de Educación y Ciencia" ("Ramón y Cajal" Program). This work was carried out within the framework of the "Xarxa de Referència de Biotecnologia" (XarBa) from the Autonomous Government of Catalonia. We are indebted to Dr. Jean-Paul Joseleau (CERMAV-CNRS Grenoble) and Dr. Cathie R. Martin (John Innes Center) for critical reading of the manuscript. We also thank the sequencing and greenhouse teams of IBMB-CSIC as well as Dr. R.S. Joshi for the English revision of the manuscript.

References

Albersheim P, Nevins DS, English PD (1967) A method for the analysis of sugars in plant cell wall polysaccharides by gas liquid chromatography. Carbohydr Res 5:340–345. doi:10.1016/S0008-6215(00)80510-8

Arnaud D, Déjardin A, Leplé JC, Lesage-Descauses MC, Pilate G (2007) Genome-wide analysis of LIM gene family in *Populus trichocarpa*, *Arabidopsis thaliana*, and *Oryza sativa*. DNA Res 14:103–116. doi:10.1093/dnares/dsm013

Besseau S, Hoffmann L, Geoffroy P, Lapierre C, Pollet B, Legrand M (2007) Flavonoid accumulation in Arabidopsis repressed in lignin synthesis affects auxin transport and plant growth. Plant Cell 19:148–162. doi:10.1105/tpc.106.044495

Blumenkrantz N, Asboe-Hansen G (1973) New method for quantitative determination of uronic acids. Anal Biochem 54:484–489. doi:10.1016/0003-2697(73)90377-1

Boerjan W, Ralph J, Baucher M (2003) Lignin biosynthesis. Annu Rev Plant Biol 54:519–546. doi:10.1146/annurev.arplant.54.031902.134938

Bomal C, Bedon F, Caron S, Mansfield SD, Levasseur C, Cooke JE, Blais S, Tremblay L, Morency MJ, Pavy N, Grima-Pettenati J, Séguin A, Mackay J (2008) Involvement of *Pinus taeda* MYB1 and MYB8 in phenylpropanoid metabolism and secondary cell wall biogenesis: a comparative in planta analysis. J Exp Bot 59:3925–3939. doi:10.1093/jxb/ern234

Bonke M, Thitamadee S, Mähönen AP, Hauser MT, Helariutta Y (2003) APL regulates vascular tissue identity in Arabidopsis. Nature 426:181–186. doi:10.1038/nature02100

Brown DE, Rashotte AM, Murphy AS, Normanly J, Tague BW, Peer WA, Taiz L, Muday GK (2001) Flavonoids act as negative

regulators of auxin transport in vivo in Arabidopsis. Plant Physiol 126:524–535. doi:10.1104/pp.126.2.524
Carlsbecker A, Helariutta Y (2005) Phloem and xylem specification: pieces of the puzzle emerge. Curr Opin Plant Biol 8:512–517. doi:10.1016/j.pbi.2005.07.001
Cliff MA, King MC, Schlosser J (2007) Anthocyanin, phenolic composition, colour measurement and sensory analysis of BC commercial red wines. Food Res Int 40:92–100. doi:10.1016/j.foodres.2006.08.002
Cone KC, Cocciolone SM, Burr FA, Burr B (1993) Maize anthocyanin regulatory gene pI is a duplicate of c1 that functions in the plant. Plant Cell 5:1795–1805
Day A, Ruel K, Neutelings G, Crônier D, David H, Hawkins S, Chabbert B (2005) Lignification in the flax stem: evidence for an unusual lignin in bast fibers. Planta 222:234–245. doi:10.1007/s00425-005-1537-1
Deluc L, Barrieu F, Marchive C, Lauvergeat V, Decendit A, Richard T, Carde JP, Mérillon JM, Hamdi S (2006) Characterization of a grapevine R2R3-MYB transcription factor that regulates the phenylpropanoid pathway. Plant Physiol 140:499–511. doi:10.1104/pp.105.067231
Dische J (1962) Color reactions of carbohydrates. In: Whistler RL, Wolfrom ML (eds) Methods in carbohydrate chemistry, vol 1. Academic Press, New York, pp 475–514
Do CT, Pollet B, Thévenin J, Sibout R, Denoue D, Barrière Y, Lapierre C, Jouanin L (2007) Both caffeoyl Coenzyme A 3-O-methyltransferase 1 and caffeic acid O-methyltransferase 1 are involved in redundant functions for lignin, flavonoids and sinapoyl malate biosynthesis in *Arabidopsis*. Planta 226:1117–1129. doi:10.1007/s00425-007-0558-3
Dubois M, Giles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method of determination of sugars and related substances. Anal Chem 28:350–356. doi:10.1021/ac60111a017
Encina A, Sevillano JM, Acebes JL, Alvarez J (2002) Cell wall modifications of bean (*Phaseolus vulgaris*) cell suspensions during habituation and dehabituation to dichlobenil. Physiol Plant 114:182–191. doi:10.1034/j.1399-3054.2002.1140204.x
Fornalé S, Sonbol FM, Maes T, Capellades M, Puigdomenech P, Rigau J, Caparrós-Ruiz D (2006) Down-regulation of the maize and *Arabidopsis thaliana* caffeic acid O-methyl-transferase genes by two new maize R2R3-MYB transcription factors. Plant Mol Biol 62:809–823. doi:10.1007/s11103-006-9058-2
Franke R, Humphreys JM, Hemm MR, Denault JW, Ruegger MO, Cusumano JC, Chapple C (2002) The Arabidopsis REF8 gene encodes the 3-hydroxylase of phenylpropanoid metabolism. Plant J 30:33–45. doi:10.1046/j.1365-313X.2002.01266.x
Franken P, Schrell S, Peterson PA, Saedler H, Wienand U (1989) Molecular analysis of protein domain function encoded by the myb-homologous maize genes C1, Zm 1 and Zm 38. Plant J 6:21–30. doi:10.1046/j.1365-313X.1994.6010021.x
Gális I, Simek P, Narisawa T, Sasaki M, Horiguchi T, Fukuda H, Matsuoka K (2006) A novel R2R3-MYB transcription factor NtMYBJS1 is a methyl Jasmonate-dependent regulator of phenylpropanoid-conjugate biosynthesis in tobacco. Plant J 46:573–592. doi:10.1111/j.1365-313X.2006.02719.x
Goicoechea M, Lacombe E, Legay S, Mihaljevic S, Rech P, Jauneau A, Lapierre C, Pollet B, Verhaegen D, Chaubet-Gigot N, Grima-Pettenati G (2005) EgMYB2, a new transcriptional activator from Eucalyptus xylem, regulates secondary cell wall formation and lignin biosynthesis. Plant J 43:553–567. doi:10.1111/j.1365-313X.2005.02480.x
Goujon T, Sibout R, Pollet B, Maba B, Nussaume L, Bechtold N, Lu F, Ralph J, Mila I, Barrière Y, Lapierre C, Jouanin L (2003) A new Arabidopsis thaliana mutant deficient in the expression of O-methyltransferase impacts lignins and sinapoyl esters. Plant Mol Biol 51:973–989. doi:10.1023/A:1023022825098
Grotewold E, Athma P, Peterson T (1991) Alternatively spliced products of the maize P gene encode proteins with homology to the DNA-binding domain of myb-like transcription factors. Proc Natl Acad Sci USA 88:4587–4591. doi:10.1073/pnas.88.11.4587
Grotewold E, Drummond BJ, Bowen B, Peterson T (1994) The myb-homologous P gene controls phlobaphene pigmentation in maize floral organs by directly activating a flavonoid biosynthetic gene subset. Cell 76:543–553. doi:10.1016/0092-8674(94)90117-1
Heddges JI, Mann DC (1979) The characterization of plant tissues by their lignin oxidation products. Geochim Cosmochim Acta 43:1803–1807. doi:10.1016/0016-7037(79)90028-0
Heine GF, Malik V, Dias AP, Grotewold E (2007) Expression and molecular characterization of ZmMYB-IF35 and related R2R3-MYB transcription factors. Mol Biotechnol 37:155–164. doi:10.1007/s12033-007-0061-x
Hemm MR, Herrmann KM, Chapple C (2001) AtMYB4: a transcription factor general in the battle against UV. Trends Plant Sci 6:135–136. doi:10.1016/S1360-1385(01)01915-X
Jacobs M, Rubery PH (1988) Naturally occurring auxin transport regulators. Science 241:346–349. doi:10.1126/science.241.4863.346
Jia L, Clegg MT, Jiang T (2004) Evolutionary dynamics of the DNA-binding domains in putative R2R3-MYB genes identified from rice subspecies indica and japonica genomes. Plant Physiol 134:575–585. doi:10.1104/pp.103.027201
Jin H, Cominelli E, Bailey P, Parr A, Mehrtens F, Jones J, Tonelli C, Weisshaar B, Martin C (2000) Transcriptional repression by *AtMYB4* controls production of UV-protecting sunscreens in *Arabidopsis*. EMBO J 15:6150–6161. doi:10.1093/emboj/19.22.6150
Ju C, Zhang F, Gao Y, Zhang W, Yan J, Dai J, Li J (2006) Cloning, chromosome mapping and expression analysis of an *R2R3-MYB* gene under-expressed in maize hybrid. Mol Biol Rep 33:103–110. doi:10.1007/s11033-006-0016-5
Karpinska B, Karlsson M, Srivastava M, Stenberg A, Schrader J, Sterky F, Bhalerao R, Wingsle G (2004) MYB transcription factors are differentially expressed and regulated during secondary vascular tissue development in hybrid aspen. Plant Mol Biol 56:255–270. doi:10.1007/s11103-004-3354-5
Kawaoka A, Ebinuma H (2001) Transcriptional control of lignin biosynthesis by tobacco LIM protein. Phytochemistry 57:1149–1157. doi:10.1016/S0031-9422(01)00054-1
Kirk TK, Obst JR (1988) Lignin determination. Methods Enzymol 161:87–100. doi:10.1016/0076-6879(88)61014-7
Kögel I, Bochter R (1985) Characterization of lignin in forest humus layers by high-performance liquid chromatography of cupric oxide oxidation products. Soil Biol Biochem 17:637–640. doi:10.1016/0038-0717(85)90040-9
Legay S, Lacombe E, Goicoechea M, Briere C, Seguin A, Mackay J, Grima-Pettenati J (2007) Molecular characterization of EgMYB1, a putative transcriptional repressor of the lignin biosynthetic pathway. Plant Sci 173:542–549. doi:10.1016/j.plantsci.2007.08.007
Lewis NG, Yamamoto E (1990) Lignin: occurrence, biogenesis and biodegradation. Annu Rev Plant Physiol Plant Mol Biol 41:455–496. doi:10.1146/annurev.pp.41.060190.002323
Li J, Ou-Lee TM, Raba R, Amundson RG, Last RL (1993) Arabidopsis flavonoid mutants are hypersensitive to UV-B irradiation. Plant Cell 5:171–179
Li X, Weng JK, Chapple C (2008) Improvement of biomass through lignin modification. Plant J 54:569–581. doi:10.1111/j.1365-313X.2008.03457.x
Liang YK, Dubos C, Dodd IC, Holroyd GH, Hetherington AM, Campbell MM (2005) AtMYB61, an R2R3-MYB transcription factor controlling stomatal aperture in *Arabidopsis thaliana*. Curr Biol 15:1201–1206. doi:10.1016/j.cub.2005.06.041

Marita JM, Ralph J, Hatfield RD, Chapple C (1999) NMR characterization of lignins in Arabidopsis altered in the activity of ferulate 5-hydroxylase. Proc Natl Acad Sci USA 96:12328–12332. doi:10.1073/pnas.96.22.12328

Marocco A, Wissenbach M, Becker D, Paz-Ares J, Saedler H, Salamini F, Rohde W (1989) Multiple genes are transcribed in *Hordeum vulgare* and *Zea mays* that carry the DNA binding domain of the myb oncoproteins. Mol Gen Genet 216:183–187. doi:10.1007/BF00334354

Martin C, Paz-Ares J (1997) MYB transcription factors in plants. Trends Genet 13:67–73. doi:10.1016/S0168-9525(96)10049-4

Mele G, Ori N, Sato Y, Hake S (2003) The knotted1-like homeobox gene BREVIPEDICELLUS regulates cell differentiation by modulating metabolic pathways. Genes Dev 17:2088–2093. doi:10.1101/gad.1120003

Mellerowicz EJ, Baucher M, Sundberg B, Boerjan W (2001) Unravelling cell wall formation in the woody dicot stem. Plant Mol Biol 47:239–274. doi:10.1023/A:1010699919325

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497. doi:10.1111/j.1399-3054.1962.tb08052.x

Nair RB, Bastress KL, Ruegger MO, Denault JW, Chapple C (2004) The Arabidopsis thaliana REDUCED EPIDERMAL FLUORESCENCE1 gene encodes an aldehyde dehydrogenase involved in ferulic acid and sinapic acid biosynthesis. Plant Cell 16:544–554. doi:10.1105/tpc.017509

Newman LJ, Perazza DE, Juda L, Campbell MM (2004) Involvement of the R2R3-MYB, AtMYB61, in the ectopic lignification and dark-photomorphogenic components of the det3 mutant phenotype. Plant J 37:239–250

Patzlaff A, Newman LJ, Dubos C, Whetten RW, Smith C, McInnis S, Bevan MW, Sederoff RR, Campbell MM (2003a) Characterisation of Pt MYB1, an R2R3-MYB from pine xylem. Plant Mol Biol 53:597–608. doi:10.1023/B:PLAN.0000019066.07933.d6

Patzlaff A, McInnis S, Courtenay A, Surman C, Newman LJ, Smith C, Bevan MW, Mansfield S, Whetten RW, Sederoff RR, Campbell MM (2003b) Characterisation of a pine MYB that regulates lignification. Plant J 36:743–754. doi:10.1046/j.1365-313X.2003.01916.x

Paz-Ares J, Ghosal D, Wienand U, Peterson PA, Saedler H (1987) The regulatory c1 locus of *Zea mays* encodes a protein with homology to myb proto-oncogene products and with structural similarities to transcriptional activators. EMBO J 6:3553–3558

Pérez-Jiménez J, Saura-Calixto F (2008) Anti-oxidant capacity of dietary polyphenols determined by ABTS assay: a kinetic expression of the results. Int J Food Sci Technol 43:185–191

Pilu R, Piazza P, Petroni K, Ronchi A, Martin C, Tonelli C (2003) pl-bol3, a complex allele of the anthocyanin regulatory pl1 locus that arose in a naturally occurring maize population. Plant J 36:510–521. doi:10.1046/j.1365-313X.2003.01898.x

Preston J, Wheeler J, Heazlewood J, Li SF, Parish RW (2004) AtMYB32 is required for normal pollen development in *Arabidopsis thaliana*. Plant J 40:979–995. doi:10.1111/j.1365-313X.2004.02280.x

Rabinowicz PD, Braun EL, Wolfe AD, Bowen B, Grotewold E (1999) Maize R2R3 Myb genes: sequence analysis reveals amplification in the higher plants. Genetics 153:427–444

Ralph J, Kim H, Lu F, Grabber JH, Leplé JC, Berrio-Sierra J, Derikvand MM, Jouanin L, Boerjan W, Lapierre C (2008) Identification of the structure and origin of a thioacidolysis marker compound for ferulic acid incorporation into angiosperm lignins (and an indicator for cinnamoyl CoA reductase deficiency). Plant J 53:368–379. doi:10.1111/j.1365-313X.2007.03345.x

Rasband WS (2007) ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA. http://rsb.info.nih.gov/ij/, 1997–2004

Romero I, Fuertes A, Benito MJ, Malpica JM, Leyva A, Paz-Ares J (1998) More than 80 R2R3-MYB regulatory genes in the genome of *Arabidopsis thaliana*. Plant J 14:273–284. doi:10.1046/j.1365-313X.1998.00113.x

Ruegger M, Meyer K, Cusumano JC, Chapple C (1999) Regulation of ferulate-5-hydroxylase expression in Arabidopsis in the context of sinapate ester biosynthesis. Plant Physiol 119:101–110. doi:10.1104/pp.119.1.101

Ruel K, Comtat J, Barnoud F (1977) Localisation histologique et ultrastructurale des xylanes dans les parois primaires des tissus d' *Arundo donax*. C R Acad Sci Gen 284:1421–1424

Ruel K, Chabannes M, Boudet AM, Legrand M, Joseleau JP (2001) Reassessment of qualitative changes in lignification of transgenic tobacco plants and their impact on cell wall assembly. Phytochemistry 57:1–9. doi:10.1016/S0031-9422(01)00118-2

Saeman JF, Moore WE, Millet MA (1963) Sugar units present. In: Whistler RL (ed) Methods in carbohydrate chemistry, vol 3. Academic Press, New York, pp 54–69

Sibout R, Eudes A, Pollet B, Goujon T, Mila I, Granier F, Séguin A, Lapierre C, Jouanin L (2003) Expression pattern of two paralogs encoding cinnamyl alcohol dehydrogenases in Arabidopsis. Isolation and characterization of the corresponding mutants. Plant Physiol 132:848–860. doi:10.1104/pp.103.021048

Sticklen MB (2008) Plant genetic engineering for biofuel production: towards affordable cellulosic ethanol. Nat Rev Genet 9:433–443. doi:10.1038/nrg2336

Stracke R, Werber M, Weisshaar B (2001) The R2R3-MYB gene family in *Arabidopsis thaliana*. Curr Opin Plant Biol 4:447–456. doi:10.1016/S1369-5266(00)00199-0

Tamagnone L, Merida A, Parr A, Mackay S, Culianez-Macia FA, Roberts K, Martin C (1998a) The AmMYB308 and AmMYB330 transcription factors from antirrhinum regulate phenylpropanoid and lignin biosynthesis in transgenic tobacco. Plant Cell 10:135–154

Tamagnone L, Merida A, Stacey N, Plaskitt K, Parr A, Chang CF, Lynn D, Dow JM, Roberts K, Martin C (1998b) Inhibition of phenolic acid metabolism results in precocious cell death and altered cell morphology in leaves of transgenic tobacco plants. Plant Cell 10:1801–1816

Torney F, Moeller L, Scarpa A, Wang K (2007) Genetic engineering approaches to improve bioethanol production from maize. Curr Opin Biotechnol 18:193–199. doi:10.1016/j.copbio.2007.03.006

Updegraff DM (1969) Semi-micro determination of cellulose in biological materials. Anal Biochem 32:420–424. doi:10.1016/S0003-2697(69)80009-6

Vanholme R, Morreel K, Ralph J, Boerjan W (2008) Lignin engineering. Curr Opin Plant Biol 11:1–8. doi:10.1016/j.pbi.2008.03.005

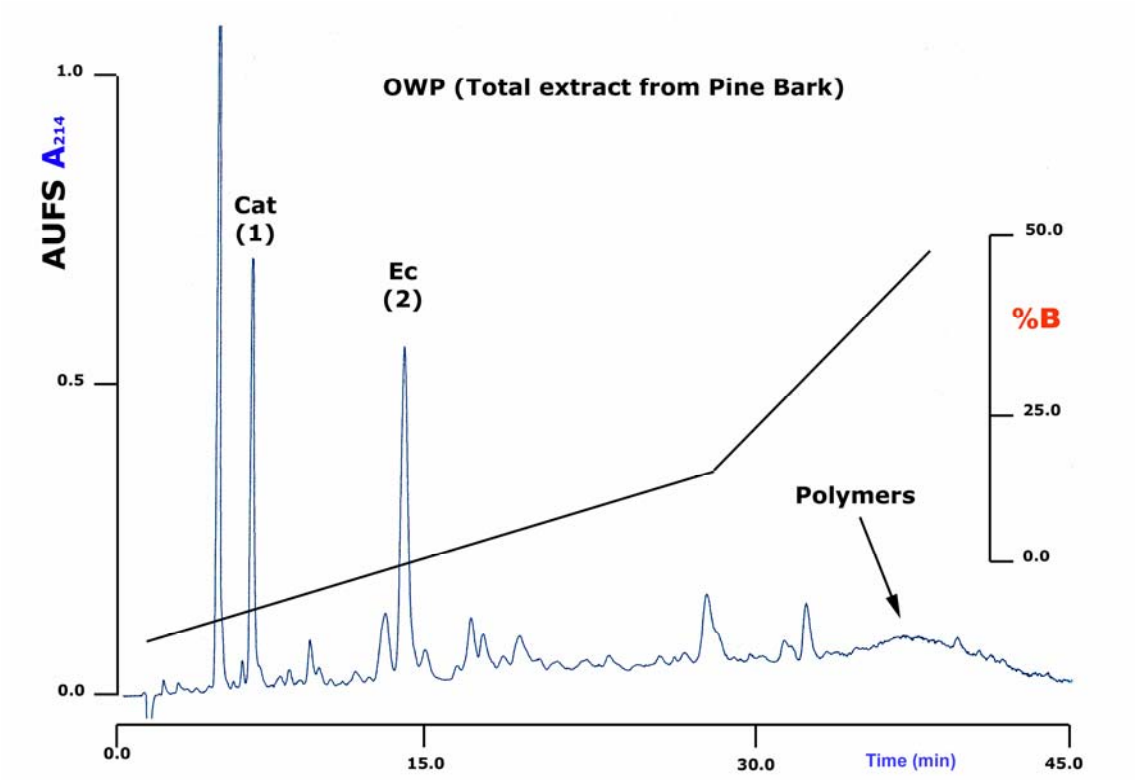
Yanhui C, Xiaoyuan Y, Kun H, Meihua L, Jigang L, Zhaofeng G, Zhiqiang L, Yunfei Z, Xiaoxiao W, Xiaoming Q, Yunping S, Li Z, Xiaohui D, Jingchu L, Xing-Wang D, Zhangliang C, Hongya G, Li-Jia Q (2006) The MYB transcription factor superfamily of Arabidopsis: expression analysis and phylogenetic comparison with the rice MYB family. Plant Mol Biol 60:107–124. doi:10.1007/s11103-005-2910-y

Zhong R, Ye ZH (2007) Regulation of cell wall biosynthesis. Curr Opin Plant Biol 10:564–572. doi:10.1016/j.pbi.2007.09.001

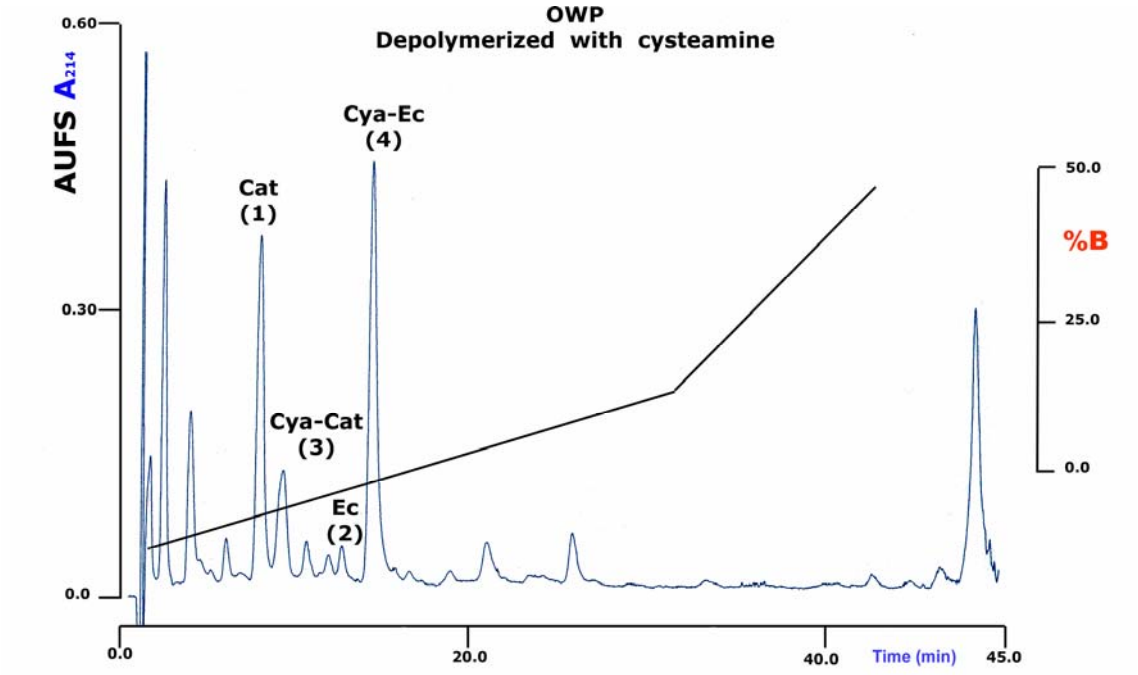
8.2 Material suplementario

8.2.1 Material suplementario de la publicación 1 (Publicación mostrada en el apartado 4.1.1)

a

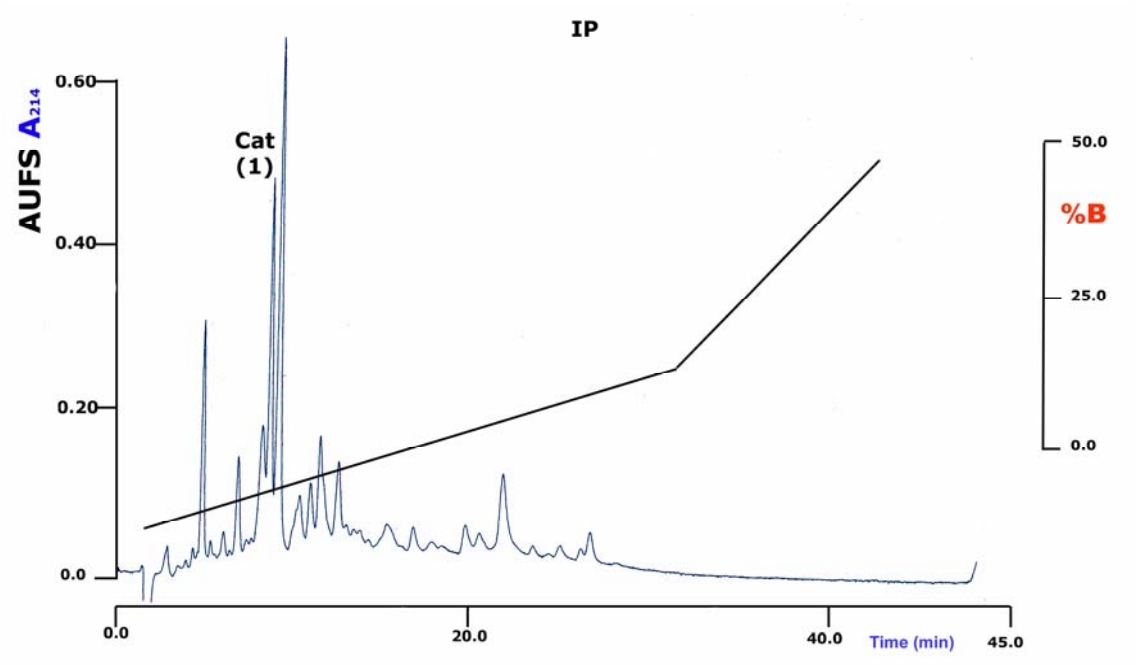


b

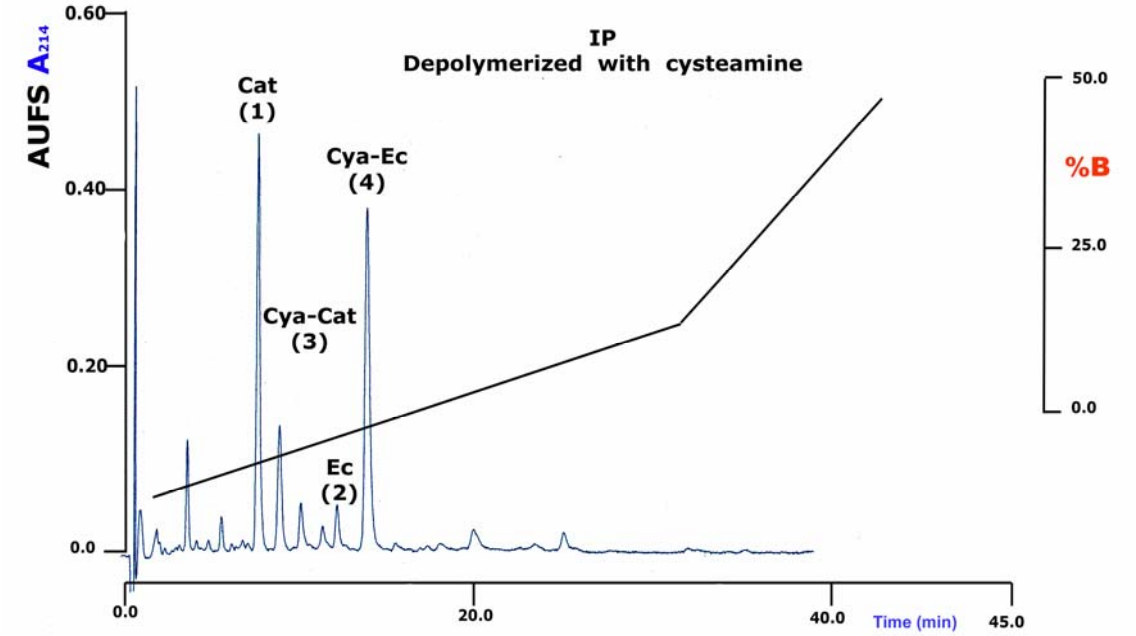


RP-HPLC chromatograms, column µRPC C2/C18 SC 2.1/10 (100 x 2.1mmi.d). Elution : [A] 0.10% (v/v) aqueous TFA, [B] 0.08 % (v/v) TFA in water /CH_3CN 1:4. Flow rate 200µL/min. Detection at 214nm. a) Load 5 µg crude fraction, gradient elution 8 to 23 % [B] over 38 min; b) Load 2.5 µg fraction after thioacidolysis, gradient elution 8 to 23 % [B] over 45 min.

a

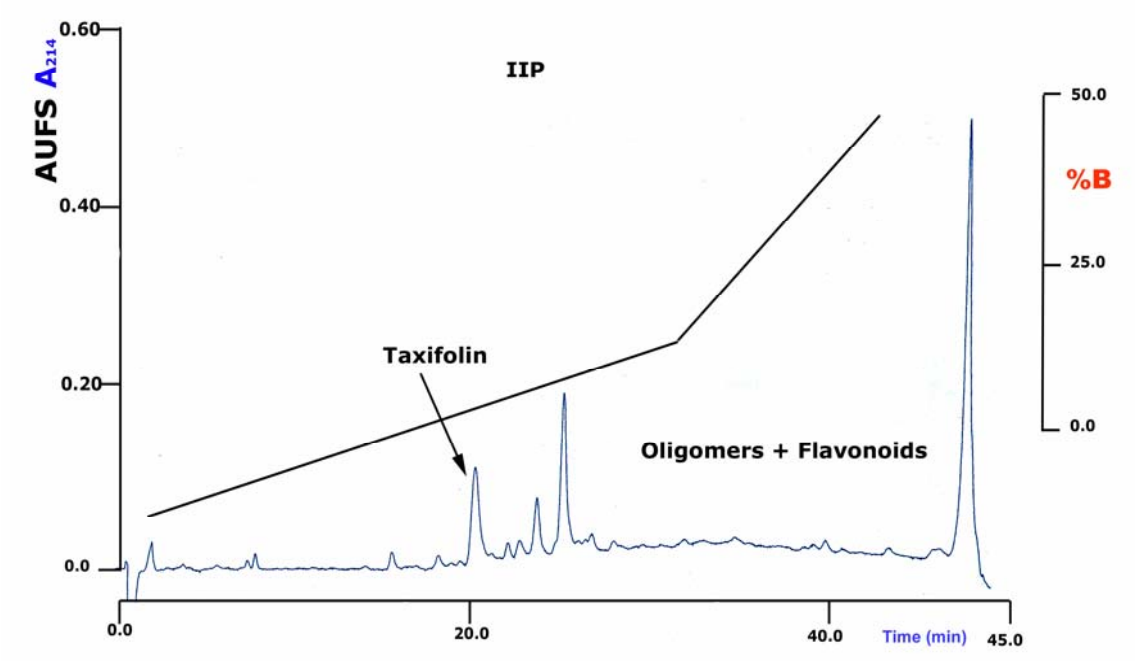


b

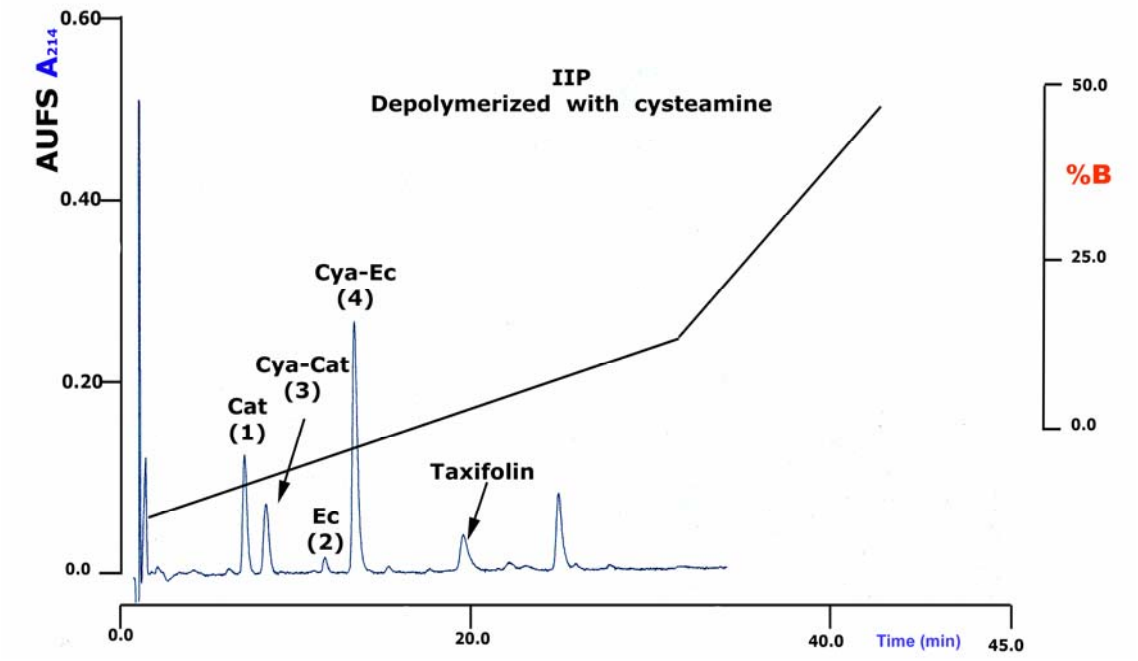


RP-HPLC chromatograms, column µRPC C2/C18 SC 2.1/10 (100 x 2.1mmi.d). Elution : [A] 0.10% (v/v) aqueous TFA, [B] 0.08 % (v/v) TFA in water /CH_3CN 1:4. Flow rate 200µL/min. Detection at 214nm. a) Load 5 µg crude fraction, gradient elution 8 to 23 % [B] over 38 min; b) Load 2.5 µg fraction after thioacidolysis, gradient elution 8 to 23 % [B] over 45 min.

a

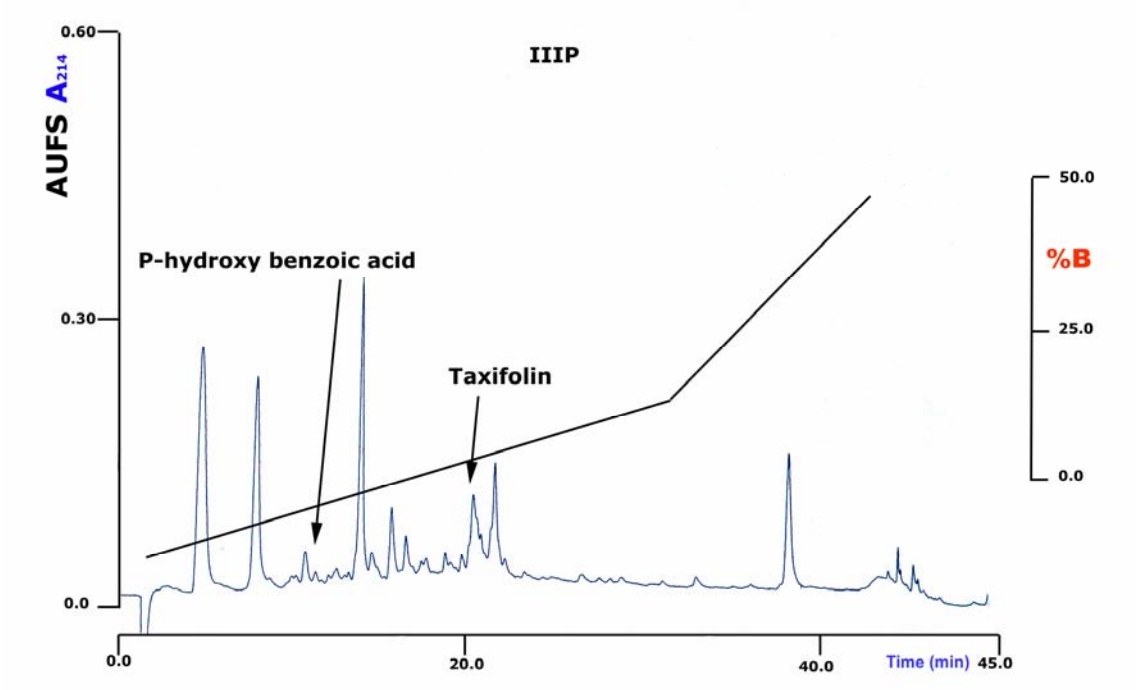


b

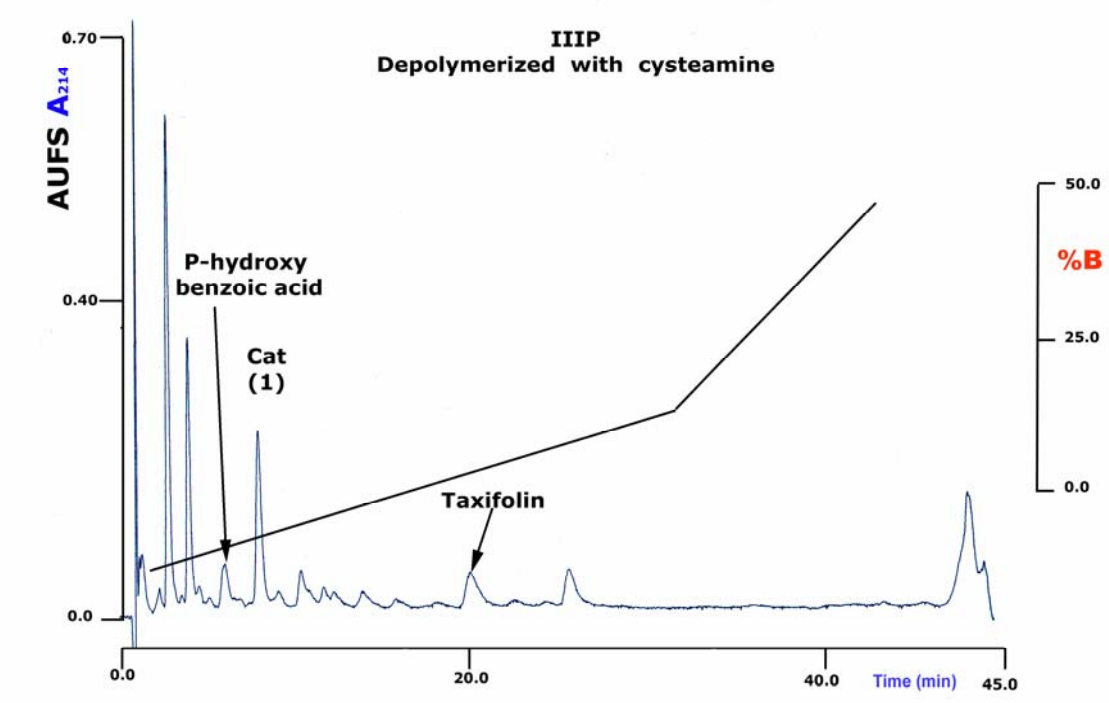


RP-HPLC chromatograms, column µRPC C2/C18 SC 2.1/10 (100 x 2.1mmi.d). Elution : [A] 0.10% (v/v) aqueous TFA, [B] 0.08 % (v/v) TFA in water /CH_3CN 1:4. Flow rate 200µL/min. Detection at 214nm. a) Load 5 µg crude fraction, gradient elution 8 to 23 % [B] over 38 min; b) Load 2.5 µg fraction after thioacidolysis, gradient elution 8 to 23 % [B] over 45 min.

a

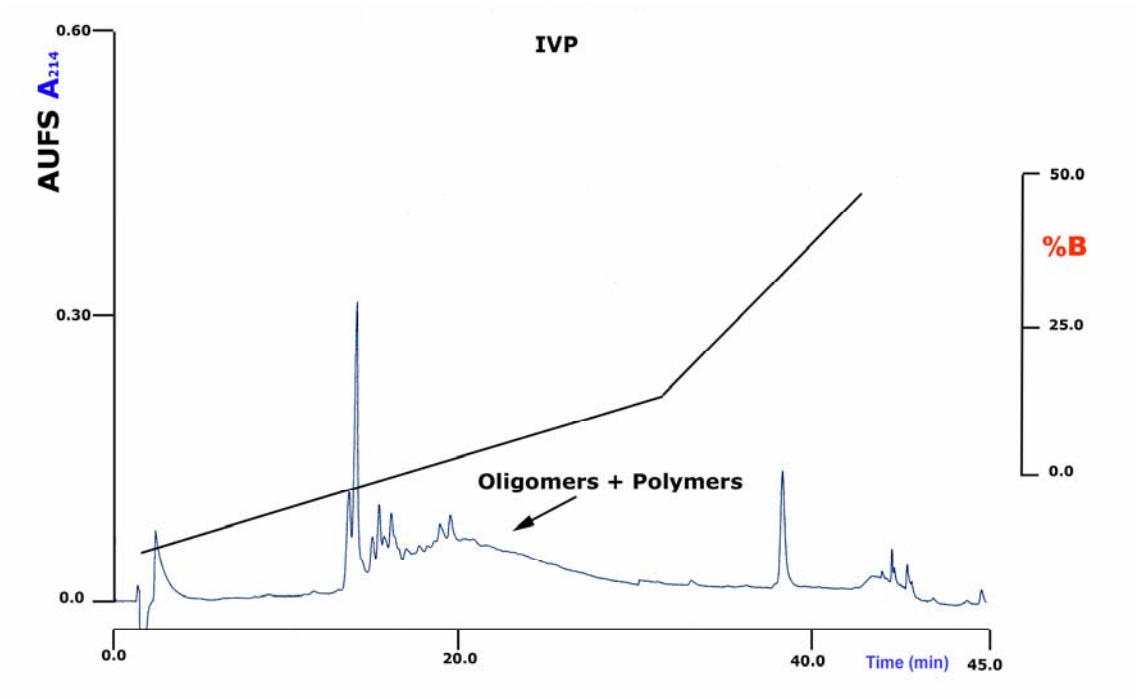


b

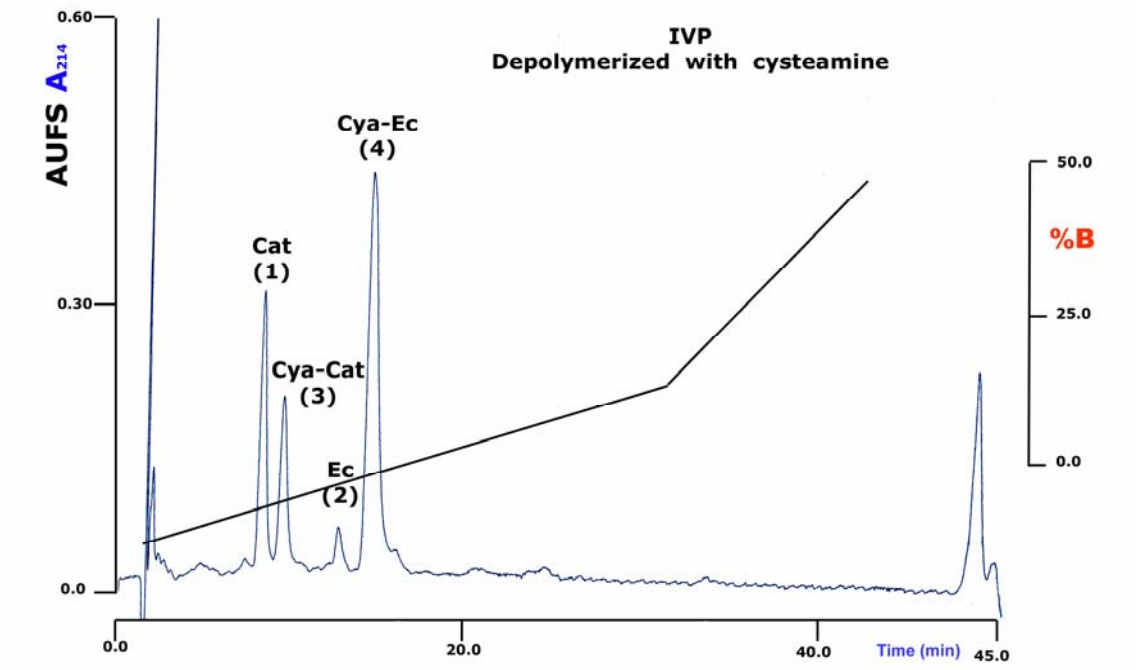


RP-HPLC chromatograms, column µRPC C2/C18 SC 2.1/10 (100 x 2.1mmi.d). Elution : [A] 0.10% (v/v) aqueous TFA, [B] 0.08 % (v/v) TFA in water /CH_3CN 1:4. Flow rate 200µL/min. Detection at 214nm. a) Load 5 µg crude fraction, gradient elution 8 to 23 % [B] over 38 min; b) Load 2.5 µg fraction after thioacidolysis, gradient elution 8 to 23 % [B] over 45 min.

a

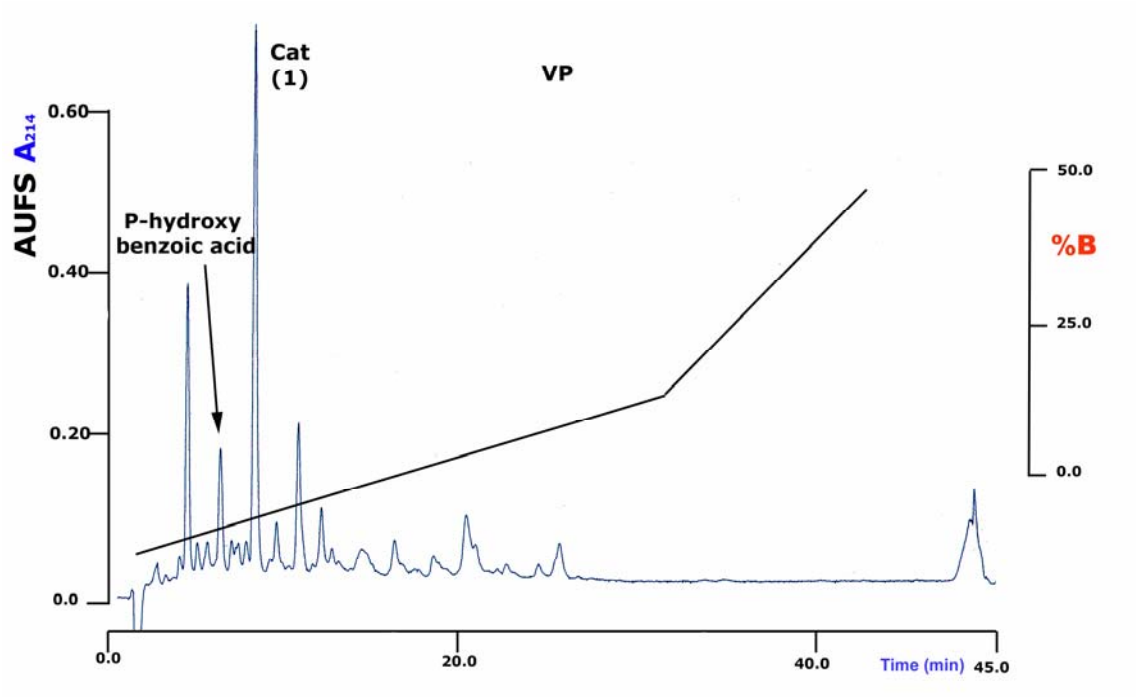


b

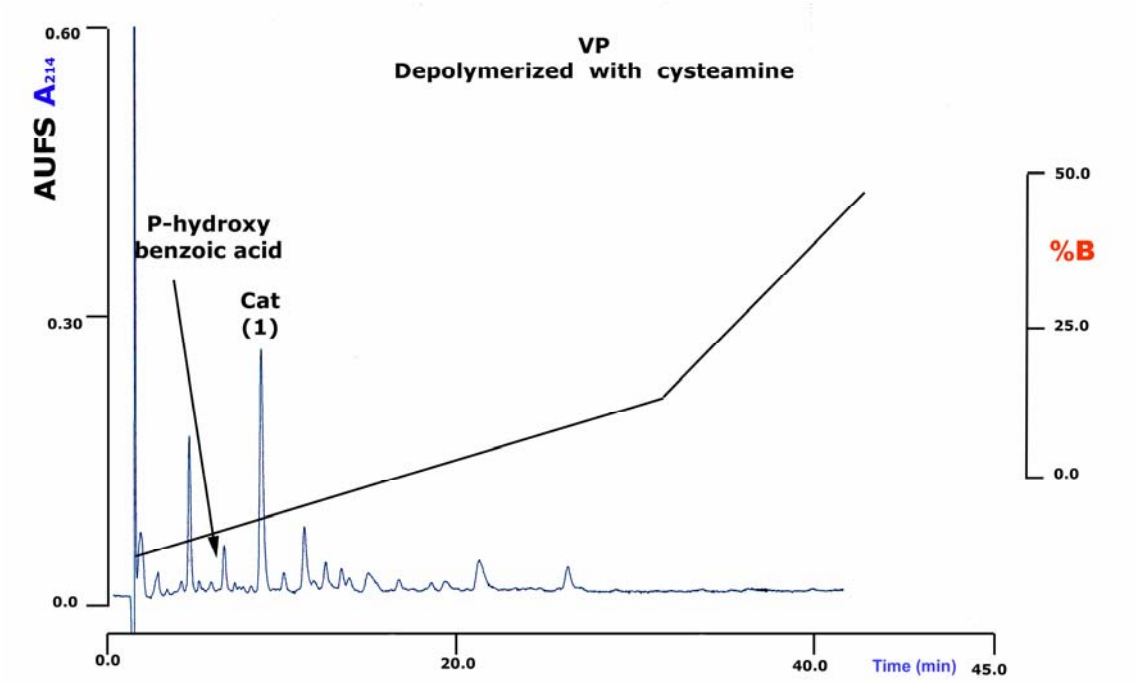


RP-HPLC chromatograms, column µRPC C2/C18 SC 2.1/10 (100 x 2.1mmi.d). Elution : [A] 0.10% (v/v) aqueous TFA, [B] 0.08 % (v/v) TFA in water /CH_3CN 1:4. Flow rate 200µL/min. Detection at 214nm. a) Load 5 µg crude fraction, gradient elution 8 to 23 % [B] over 38 min; b) Load 2.5 µg fraction after thioacidolysis, gradient elution 8 to 23 % [B] over 45 min.

a

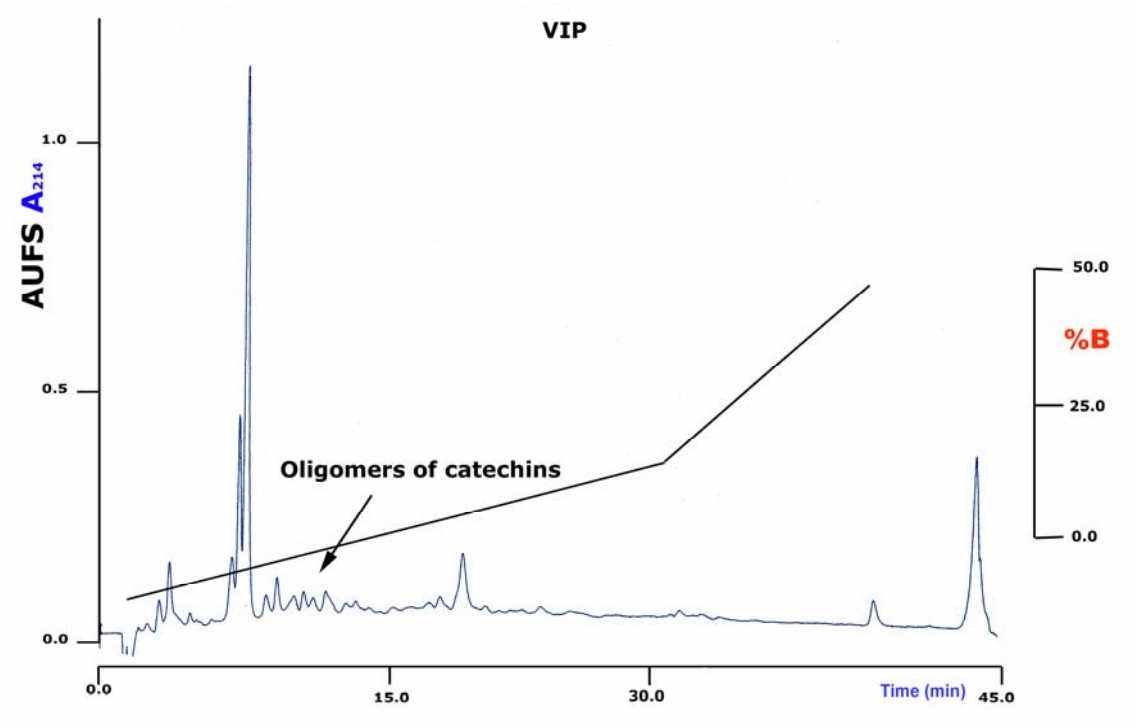


b

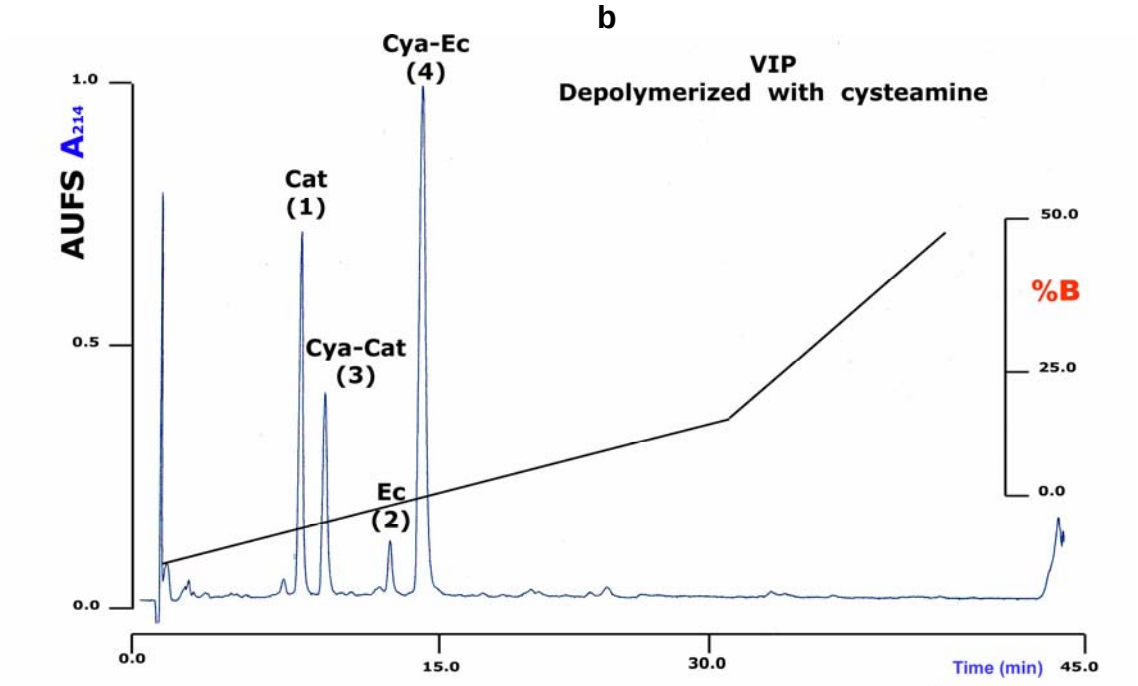


RP-HPLC chromatograms, column µRPC C2/C18 SC 2.1/10 (100 x 2.1mmi.d). Elution : [A] 0.10% (v/v) aqueous TFA, [B] 0.08 % (v/v) TFA in water /CH_3CN 1:4. Flow rate 200µL/min. Detection at 214nm. a) Load 5 µg crude fraction, gradient elution 8 to 23 % [B] over 38 min; b) Load 2.5 µg fraction after thioacidolysis, gradient elution 8 to 23 % [B] over 45 min.

a

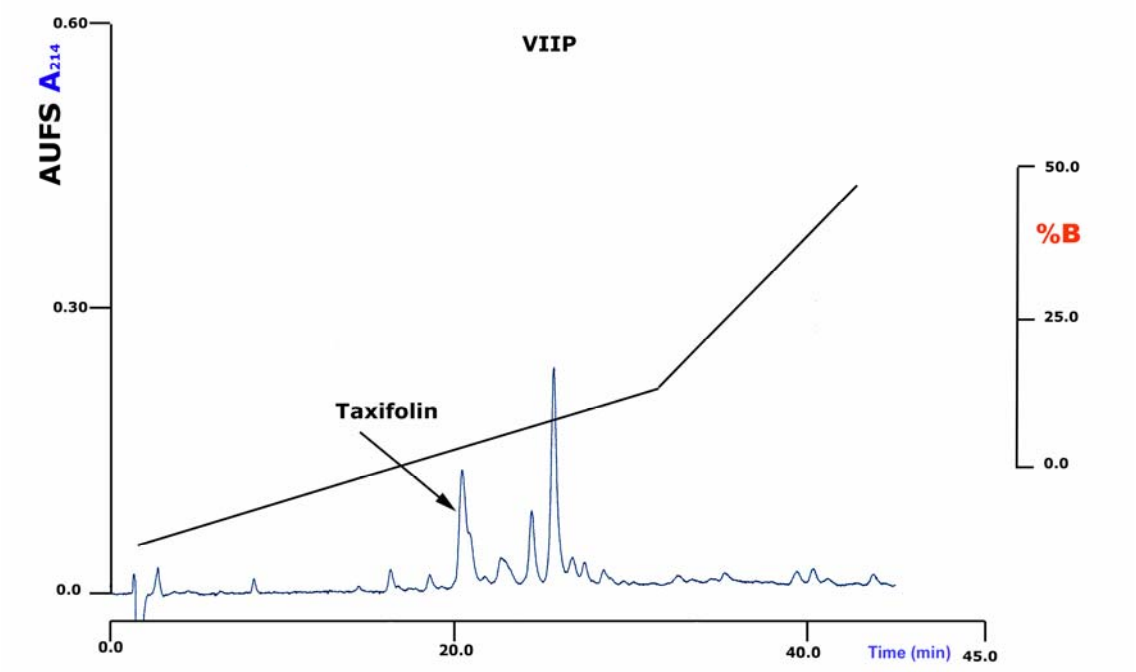


b

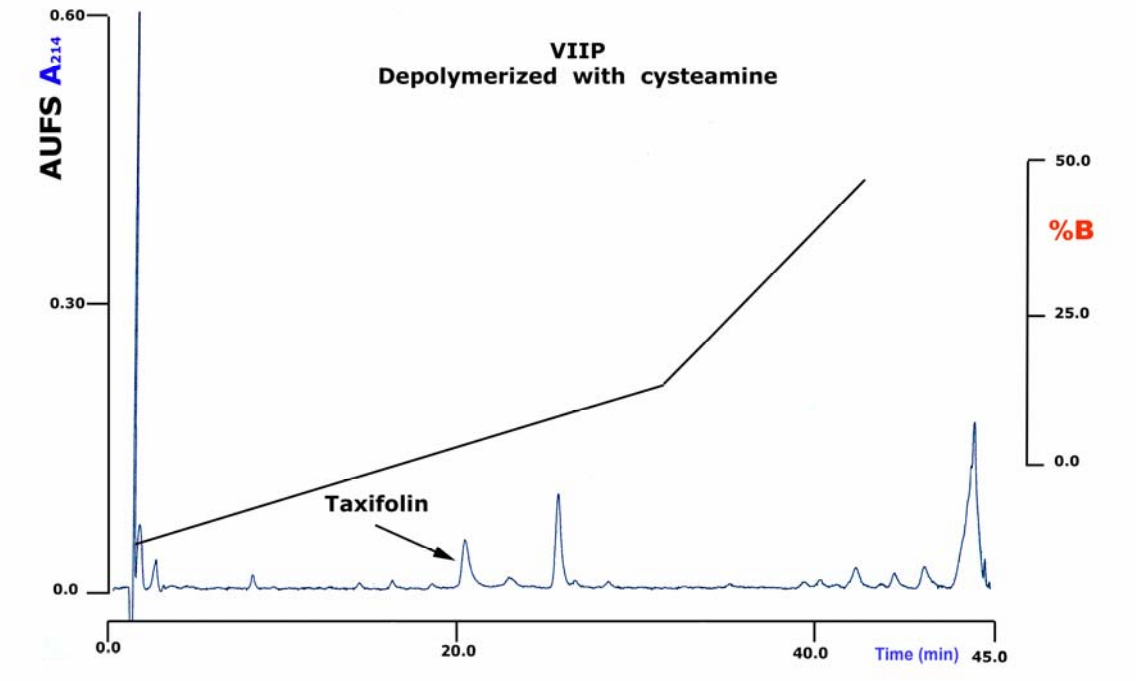


RP-HPLC chromatograms, column µRPC C2/C18 SC 2.1/10 (100 x 2.1mmi.d). Elution : [A] 0.10% (v/v) aqueous TFA, [B] 0.08 % (v/v) TFA in water /CH_3CN 1:4. Flow rate 200µL/min. Detection at 214nm. a) Load 5 µg crude fraction, gradient elution 8 to 23 % [B] over 38 min; b) Load 2.5 µg fraction after thioacidolysis, gradient elution 8 to 23 % [B] over 45 min.

a

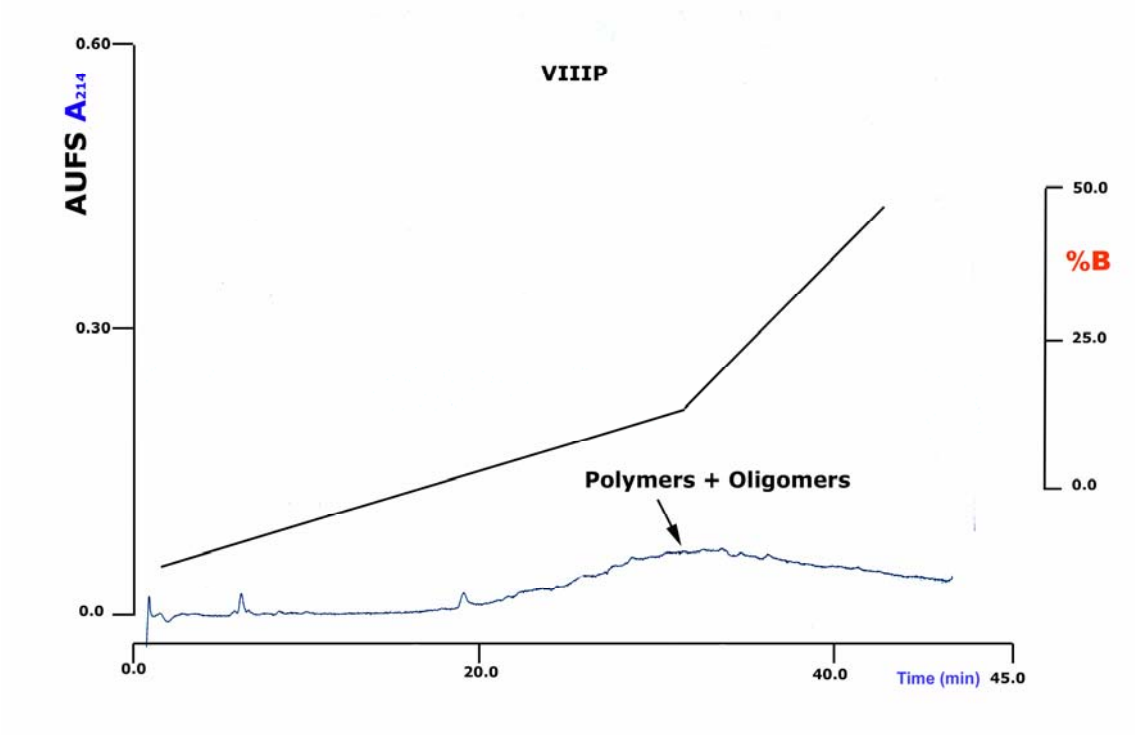


b

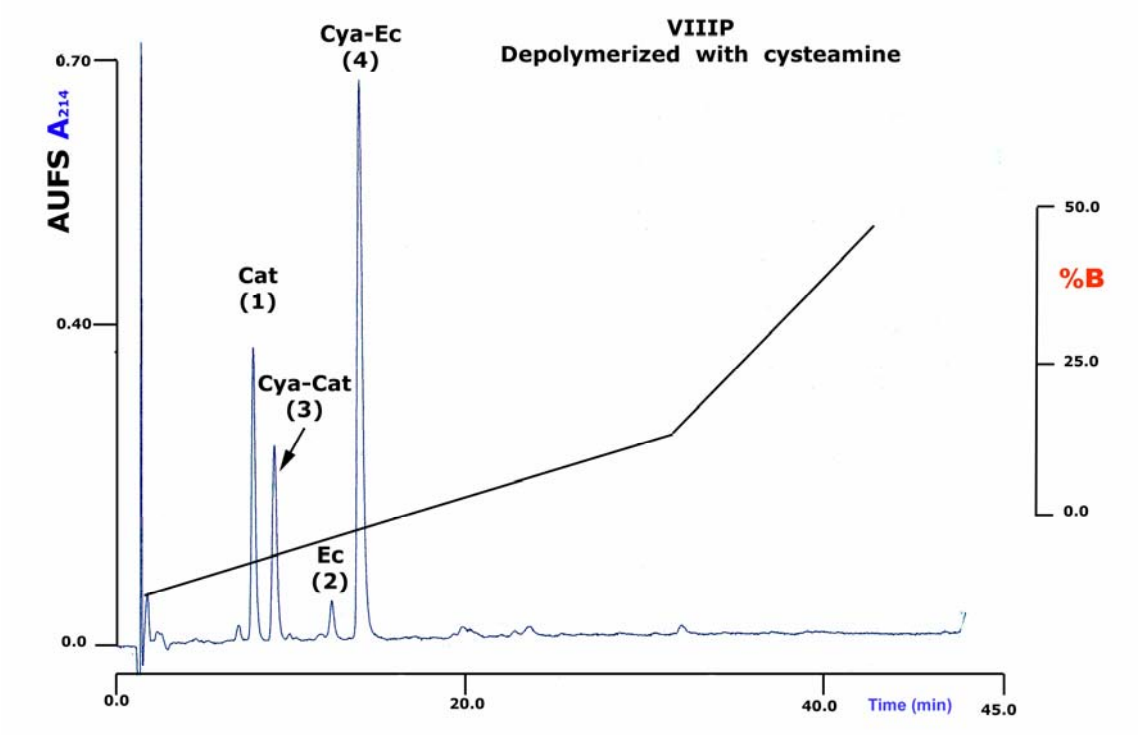


RP-HPLC chromatograms, column µRPC C2/C18 SC 2.1/10 (100 x 2.1mmi.d). Elution : [A] 0.10% (v/v) aqueous TFA, [B] 0.08 % (v/v) TFA in water /CH_3CN 1:4. Flow rate 200µL/min. Detection at 214nm. a) Load 5 µg crude fraction, gradient elution 8 to 23 % [B] over 38 min; b) Load 2.5 µg fraction after thioacidolysis, gradient elution 8 to 23 % [B] over 45 min..

a

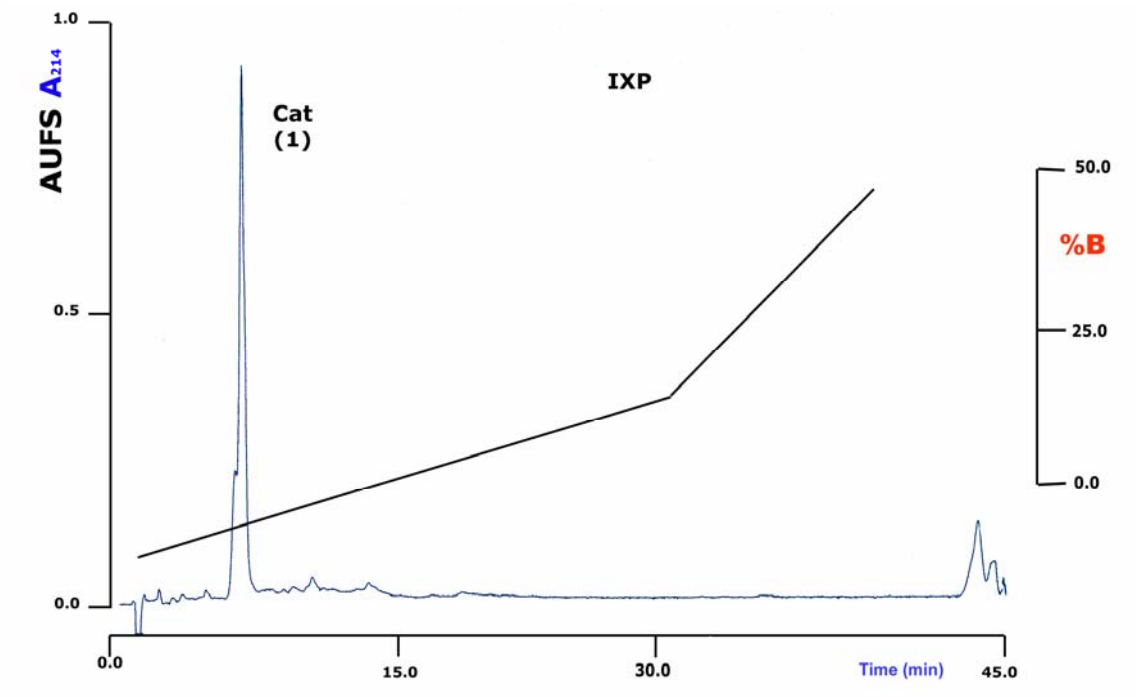


b

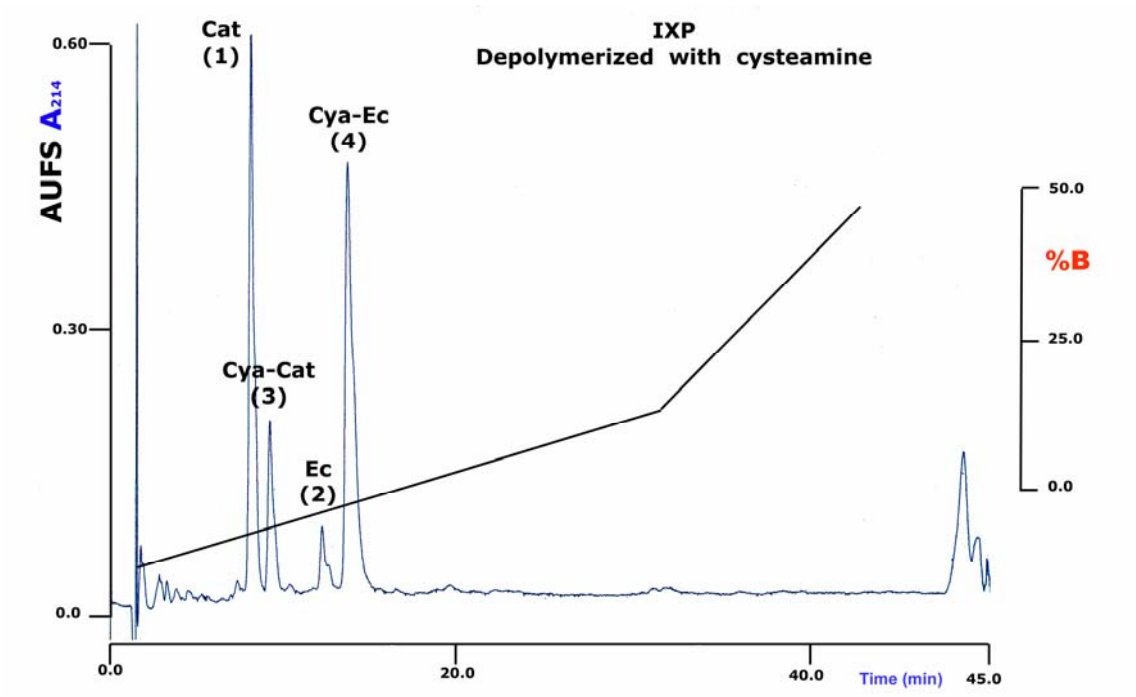


RP-HPLC chromatograms, column µRPC C2/C18 SC 2.1/10 (100 x 2.1mmi.d). Elution : [A] 0.10% (v/v) aqueous TFA, [B] 0.08 % (v/v) TFA in water /CH_3CN 1:4. Flow rate 200µL/min. Detection at 214nm. a) Load 5 µg crude fraction, gradient elution 8 to 23 % [B] over 38 min; b) Load 2.5 µg fraction after thioacidolysis, gradient elution 8 to 23 % [B] over 45 min..

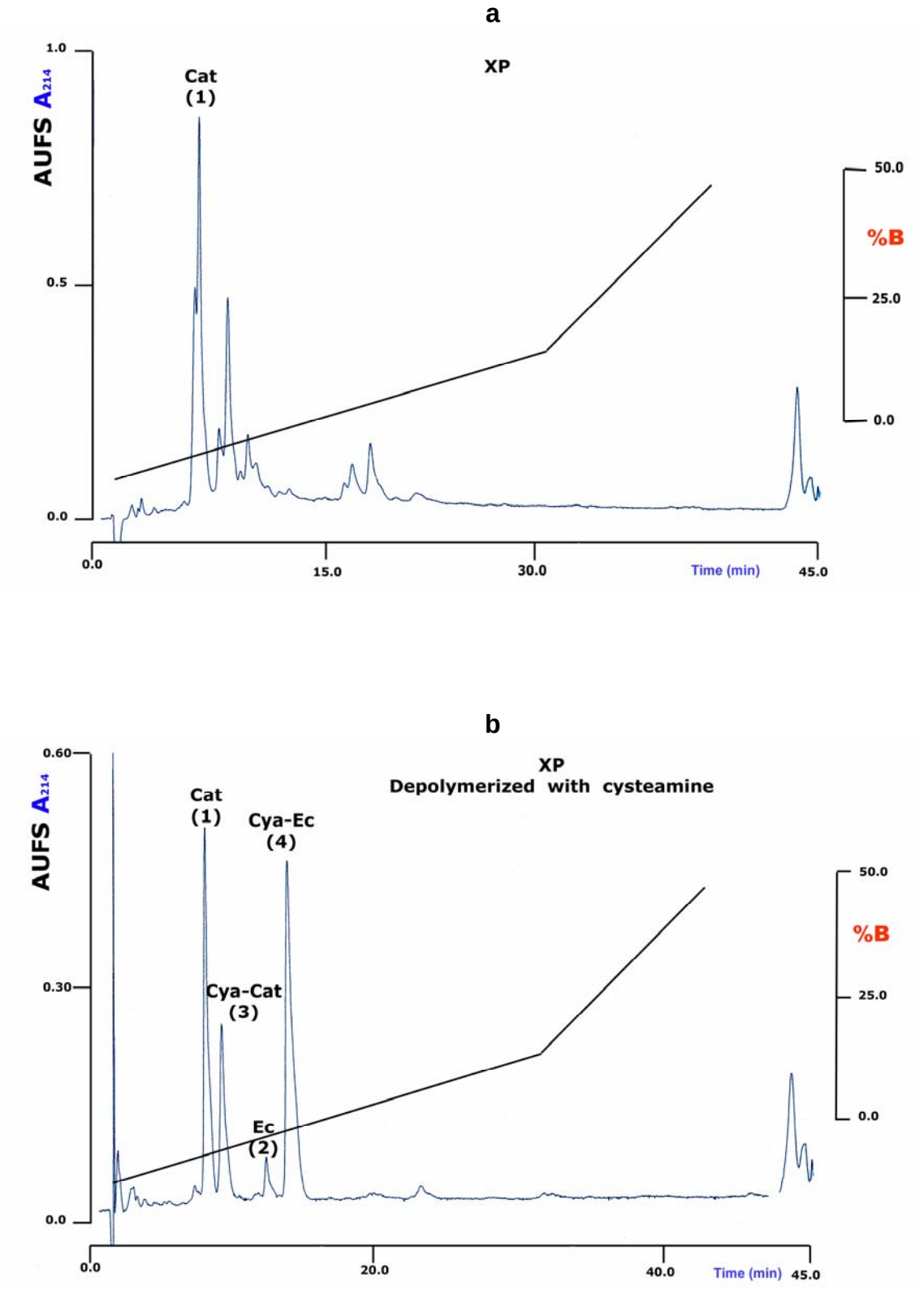
a



b

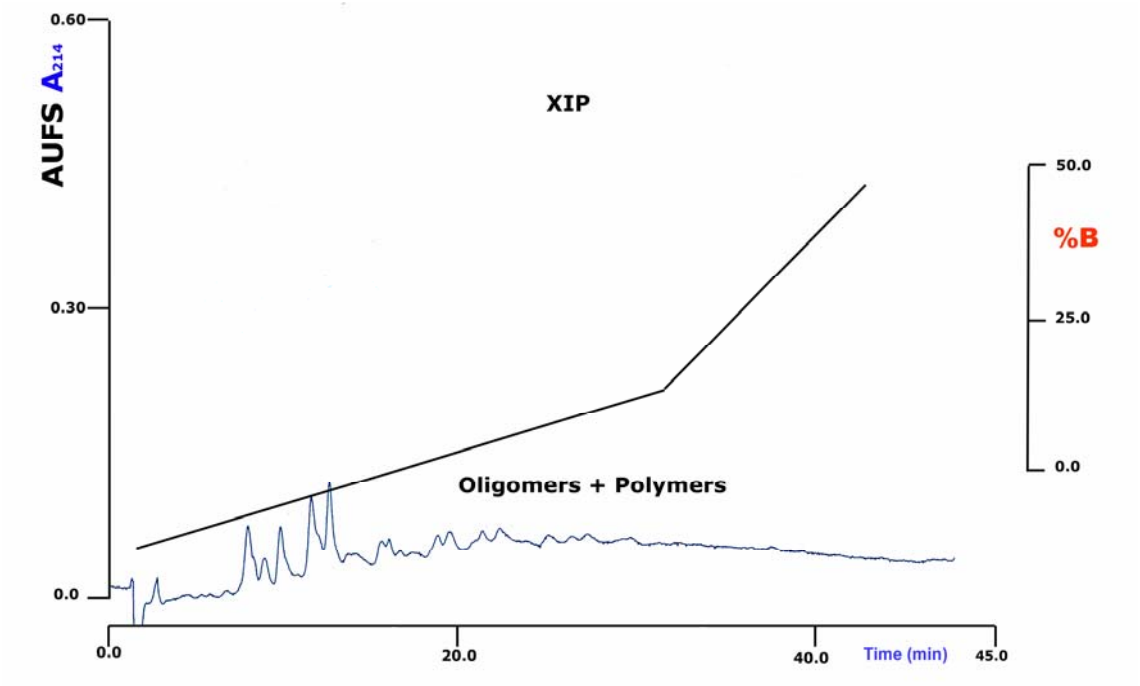


RP-HPLC chromatograms, column µRPC C2/C18 SC 2.1/10 (100 x 2.1mmi.d). Elution : [A] 0.10% (v/v) aqueous TFA, [B] 0.08 % (v/v) TFA in water /CH_3CN 1:4. Flow rate 200µL/min. Detection at 214nm. a) Load 5 µg crude fraction, gradient elution 8 to 23 % [B] over 38 min; b) Load 2.5 µg fraction after thioacidolysis, gradient elution 8 to 23 % [B] over 45 min.

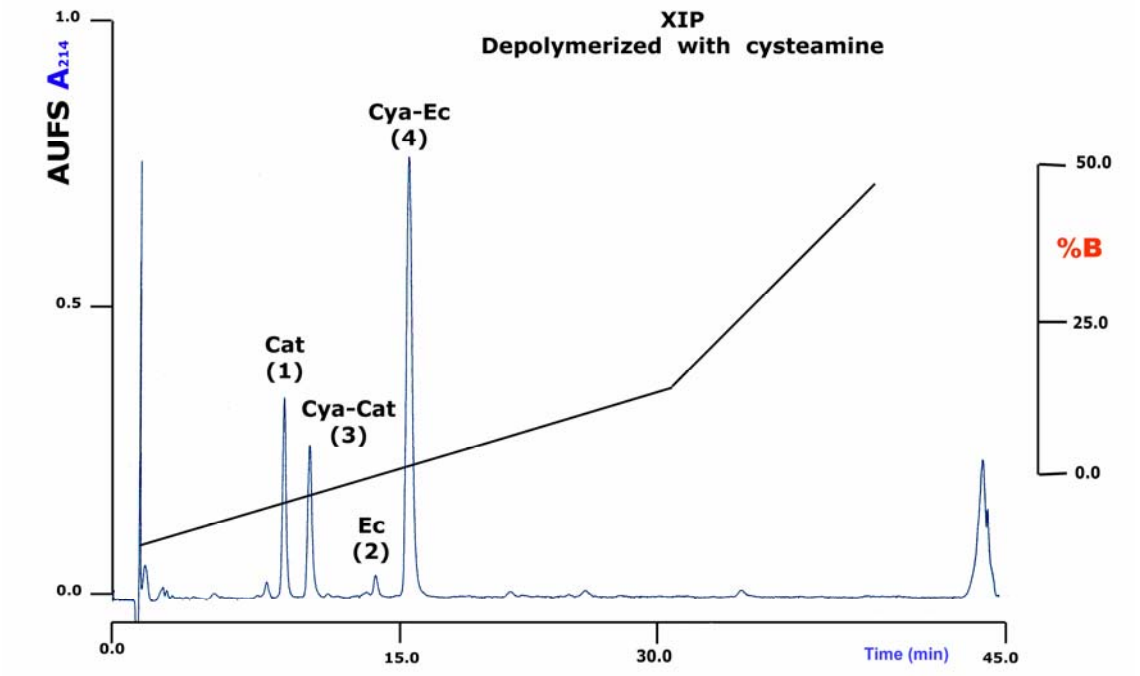


RP-HPLC chromatograms, column µRPC C2/C18 SC 2.1/10 (100 x 2.1mmi.d). Elution : [A] 0.10% (v/v) aqueous TFA, [B] 0.08 % (v/v) TFA in water /CH_3CN 1:4. Flow rate 200µL/min. Detection at 214nm. a) Load 5 µg crude fraction, gradient elution 8 to 23 % [B] over 38 min; b) Load 2.5 µg fraction after thioacidolysis, gradient elution 8 to 23 % [B] over 45 min.

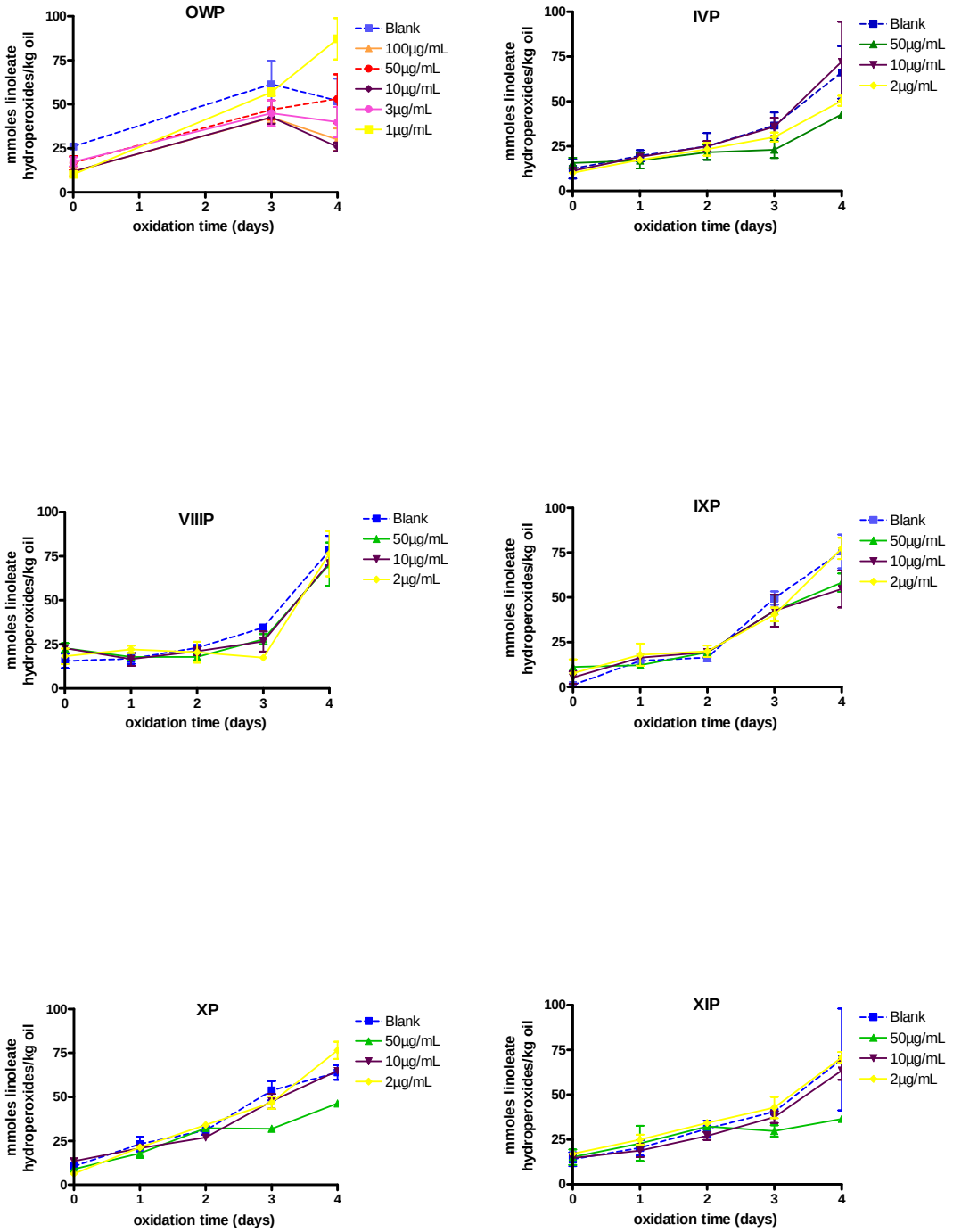
a



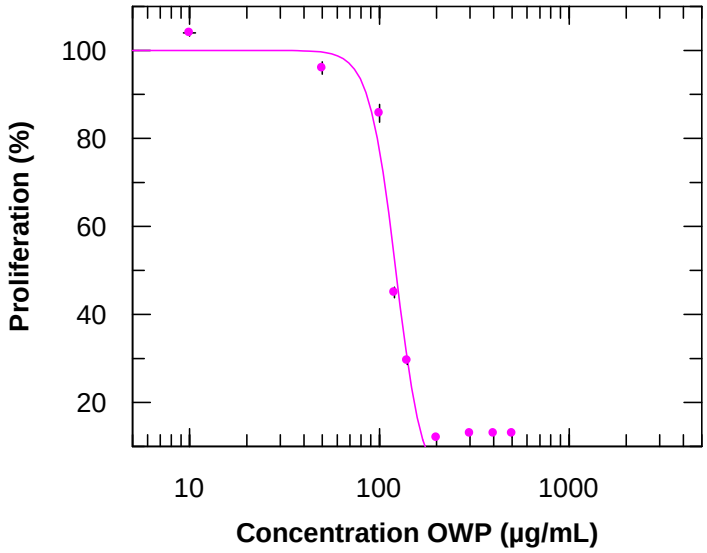
b



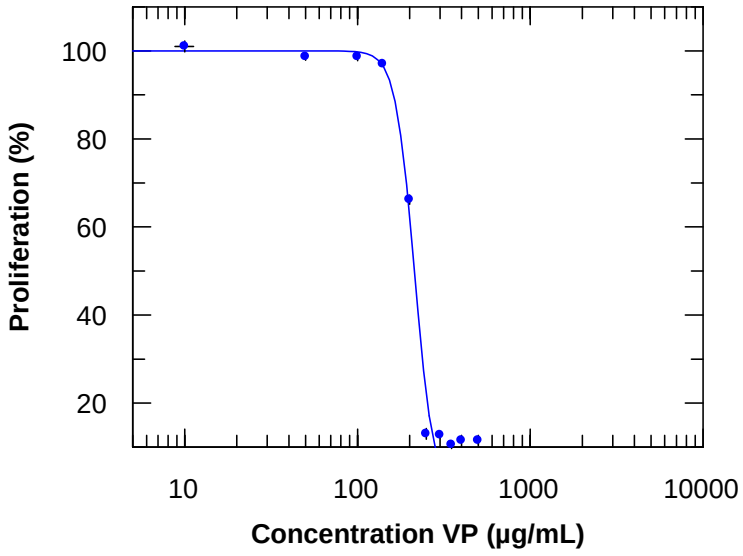
RP-HPLC chromatograms, column µRPC C2/C18 SC 2.1/10 (100 x 2.1mmi.d). Elution : [A] 0.10% (v/v) aqueous TFA, [B] 0.08 % (v/v) TFA in water /CH_3CN 1:4. Flow rate 200µL/min. Detection at 214nm. a) Load 5 µg crude fraction, gradient elution 8 to 23 % [B] over 38 min; b) Load 2.5 µg fraction after thioacidolysis, gradient elution 8 to 23 % [B] over 45 min.



Corn oil air oxidation in an oil-in-water emulsion in the presence of pine bark polyphenolic fractions.

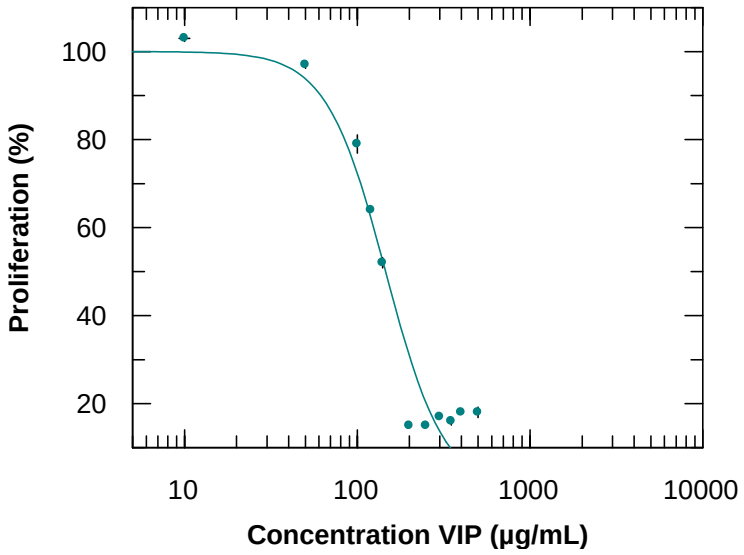


IC_{50}= 122 ± 5 µg/mL (mean ± SD, n=4)

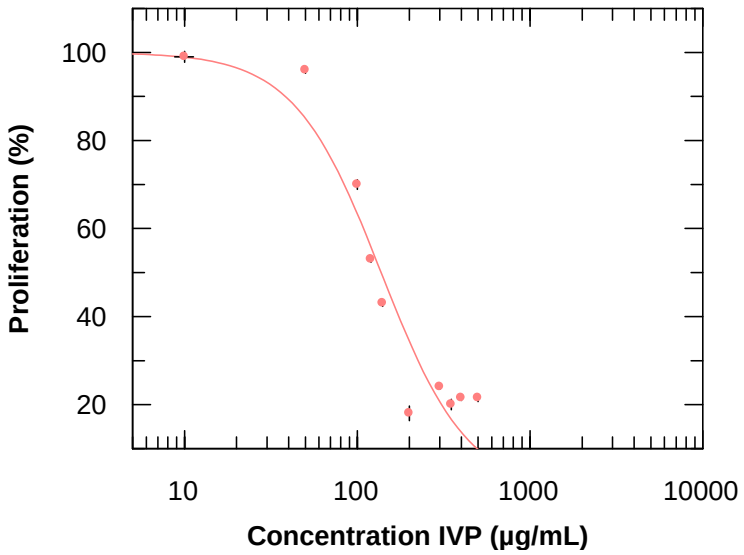


IC_{50}= 213 ± 7 µg/mL (mean ± SD, n=4)

Dose-response curves of SKMel28 melanoma cells proliferation in the presence of polyphenolic fractions from pine bark. Viability and proliferation expressed as per cent with respect to untreated control cells. IC_{50} in µg/mL ± SD, n=4 (four independent experiments).

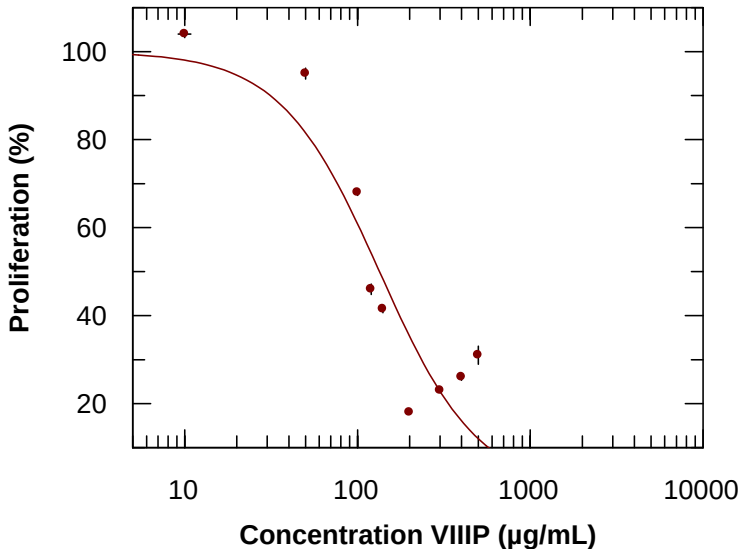


IC_{50}= 146 ± 10 µg/mL (mean ± SD, n=4)



IC_{50}= 137 ± 13 µg/mL (mean ± SD, n=4)

Dose-response curves of SKMel28 melanoma cells proliferation in the presence of polyphenolic fractions from pine bark. Viability and proliferation expressed as per cent with respect to untreated control cells. IC_{50} in µg/mL ± SD, n=4 (four independent experiments).

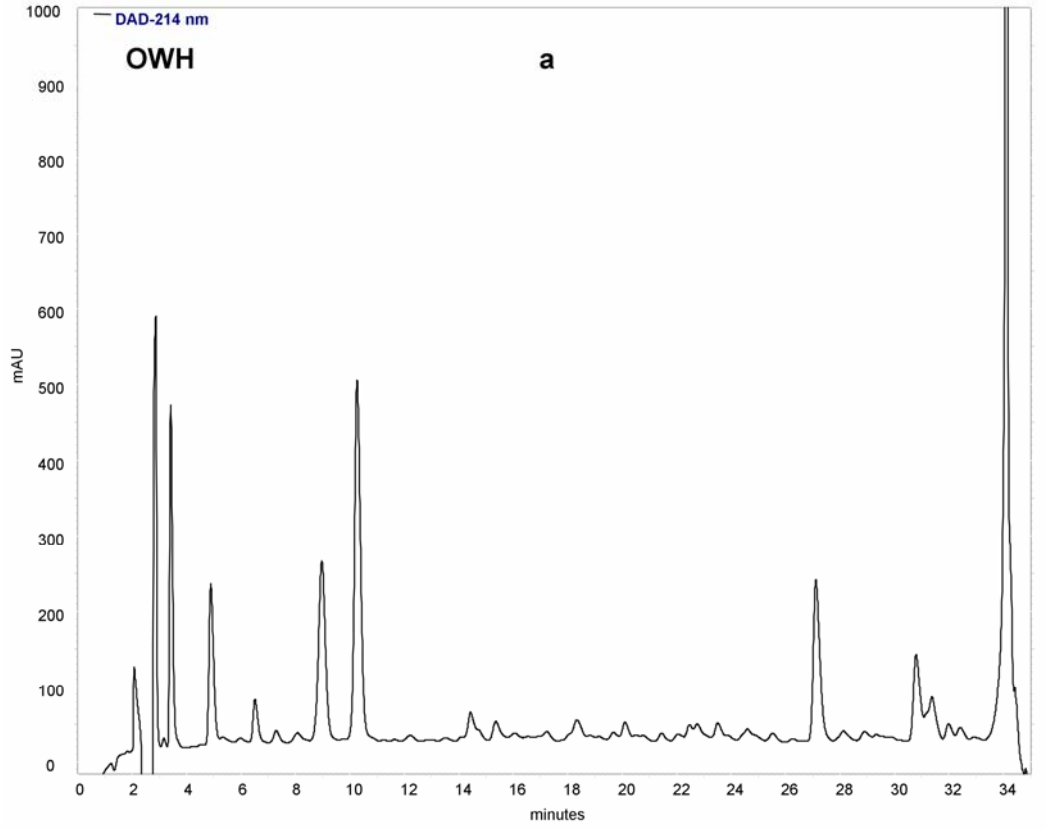


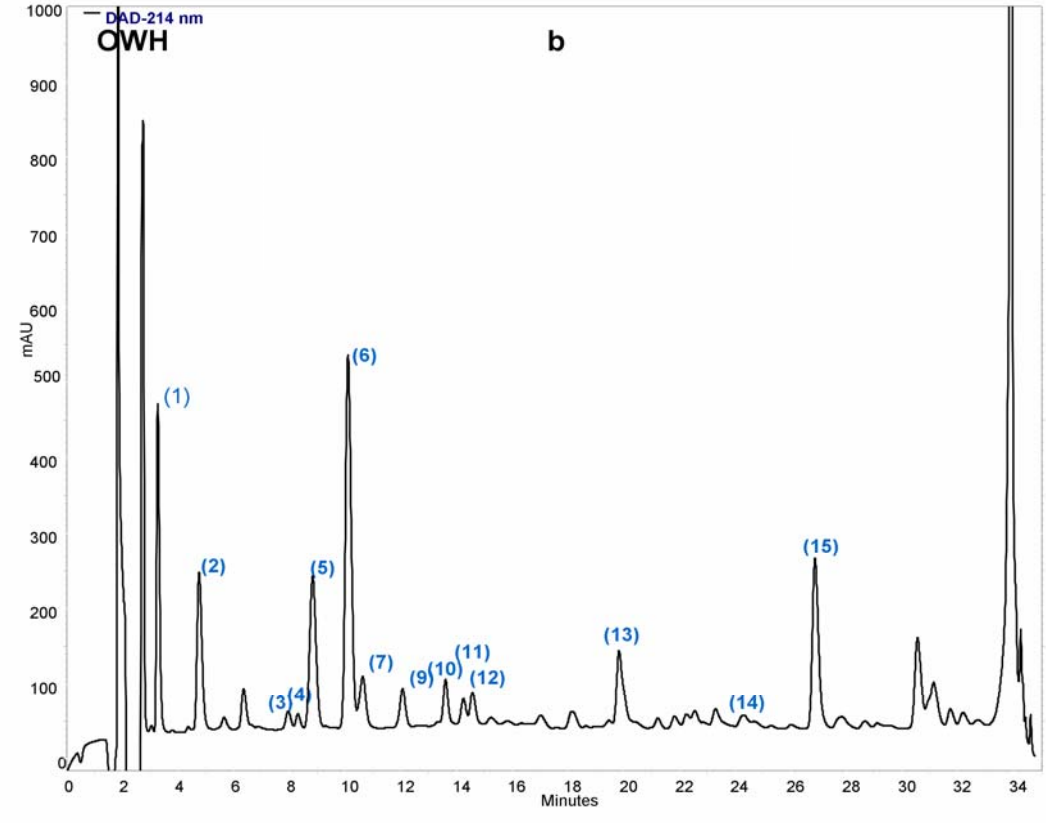
IC_{50}= 134 ± 21 µg/mL (mean ± SD, n=4)

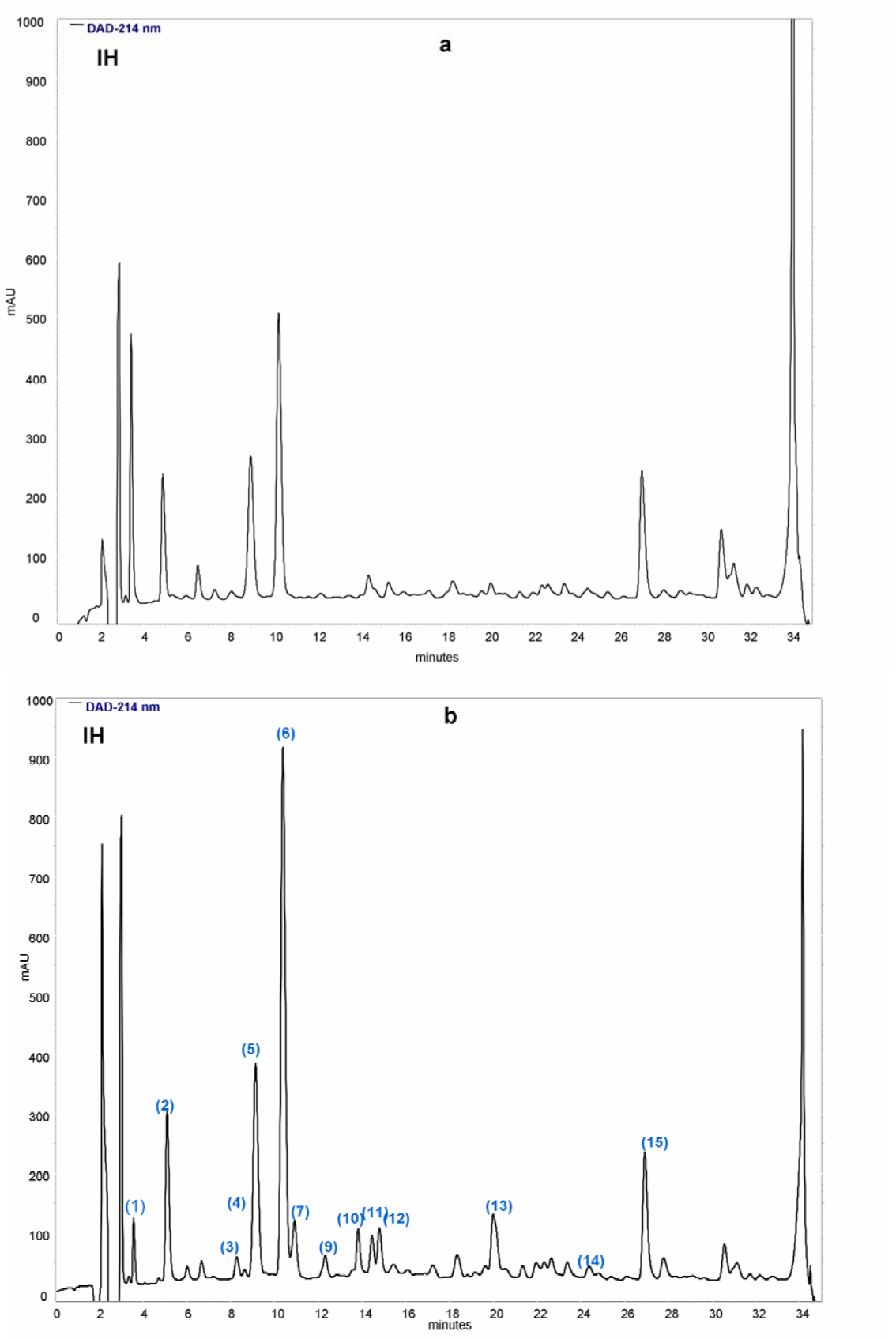
Dose-response curves of SKMel28 melanoma cells proliferation in the presence of polyphenolic fractions from pine bark. Viability and proliferation expressed as per cent with respect to untreated control cells. IC_{50} in µg/mL ± SD, n=4 (four independent experiments).

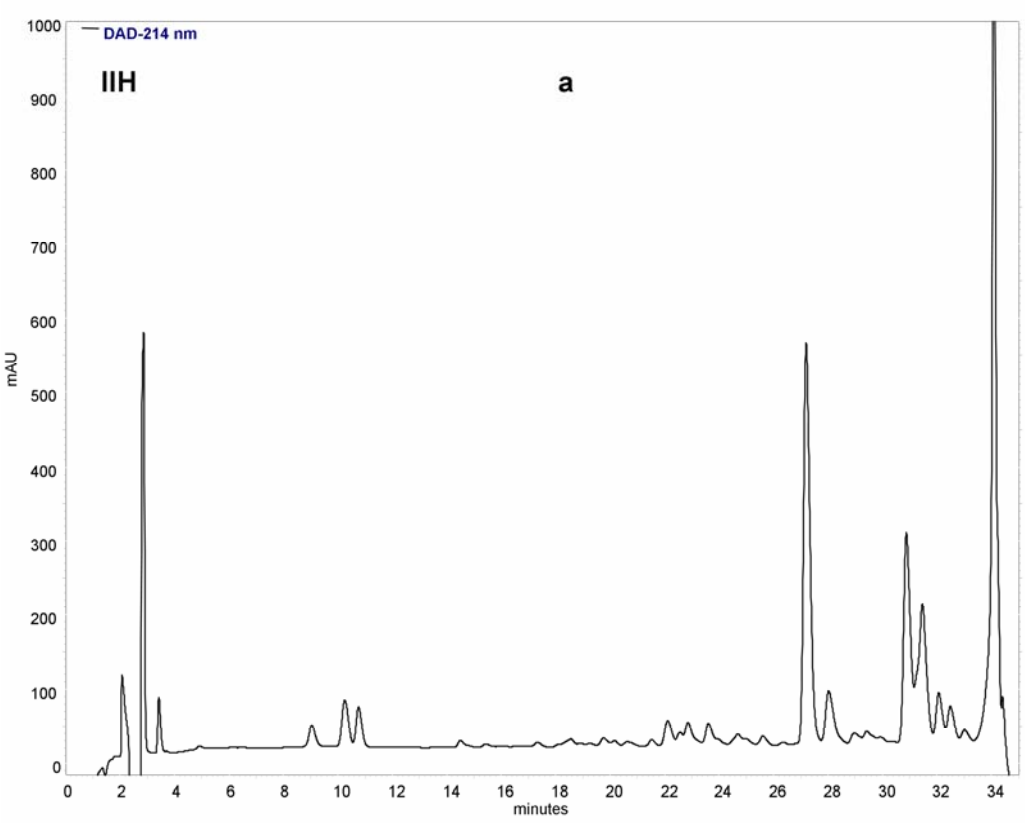
8.2.2 Material suplementario de la publicación 2 (Publicación mostrada en el apartado 4.1.2)

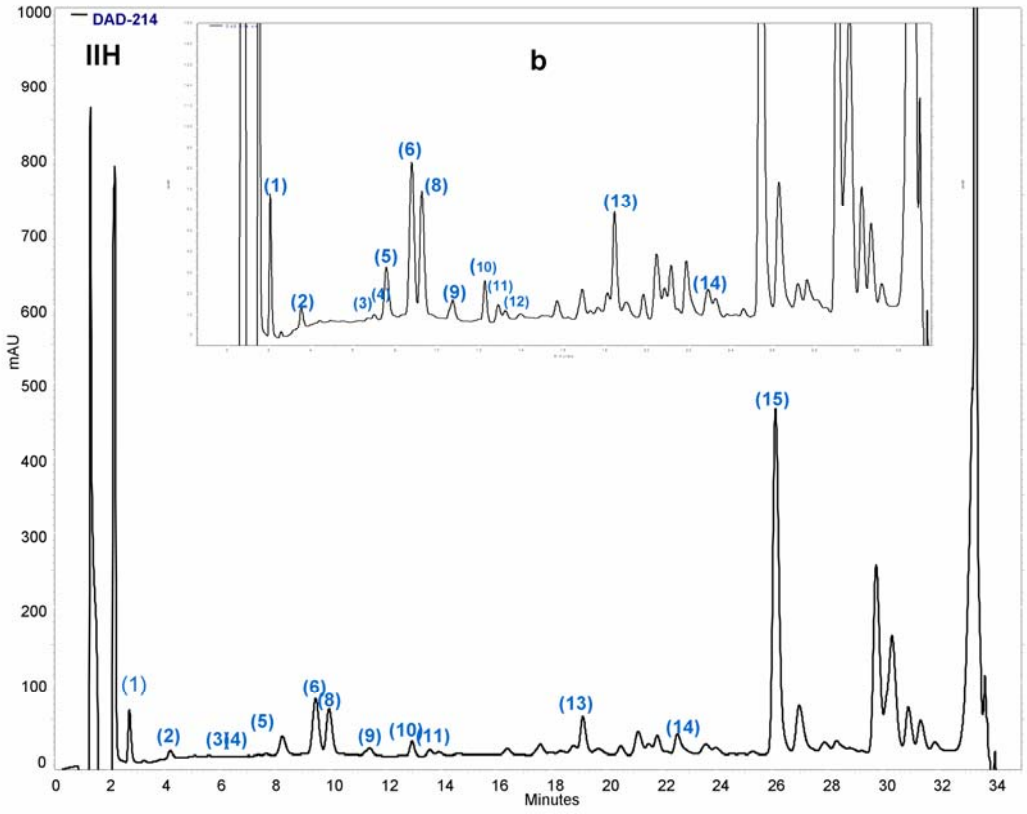
RP-HPLC chromatograms, column Kromasil 100 C18 (25 x 0,4cm i.d., 5 µm particle size) Elution: [A] 0.10% (v/v) aqueous TFA, [B] 0.08 % (v/v) TFA in water /CH3CN 1:4. Flow rate 1mL/min. DAD dectetion and intregation were perfomed to 214nm. a) Load 10 µg crude fraction, gradient elution 12 to 30 % [B] over 30 min; b) Load 10 µg fraction after thioacidolysis, gradient elution 12 to 30 % [B] over 30 min.

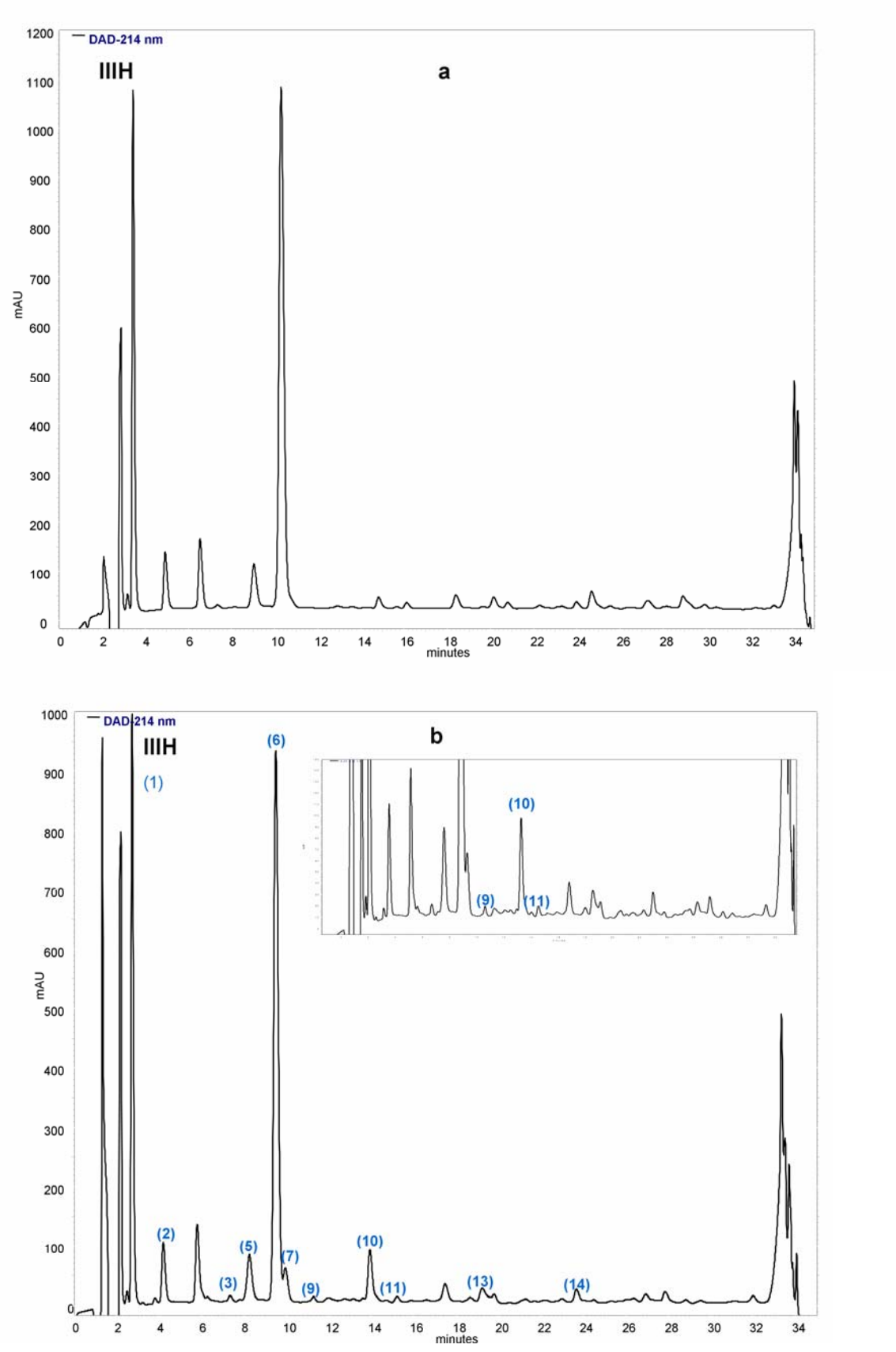


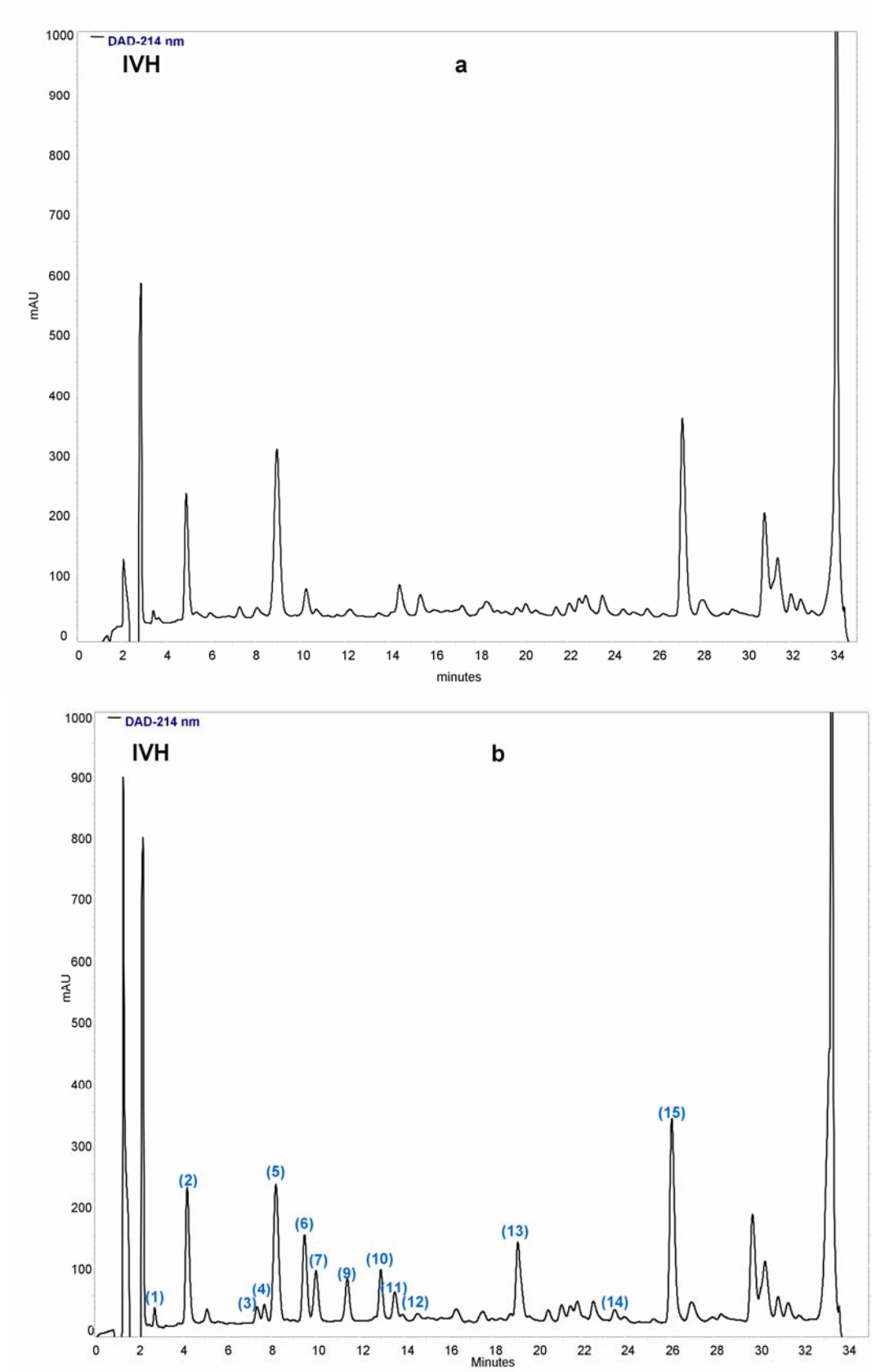


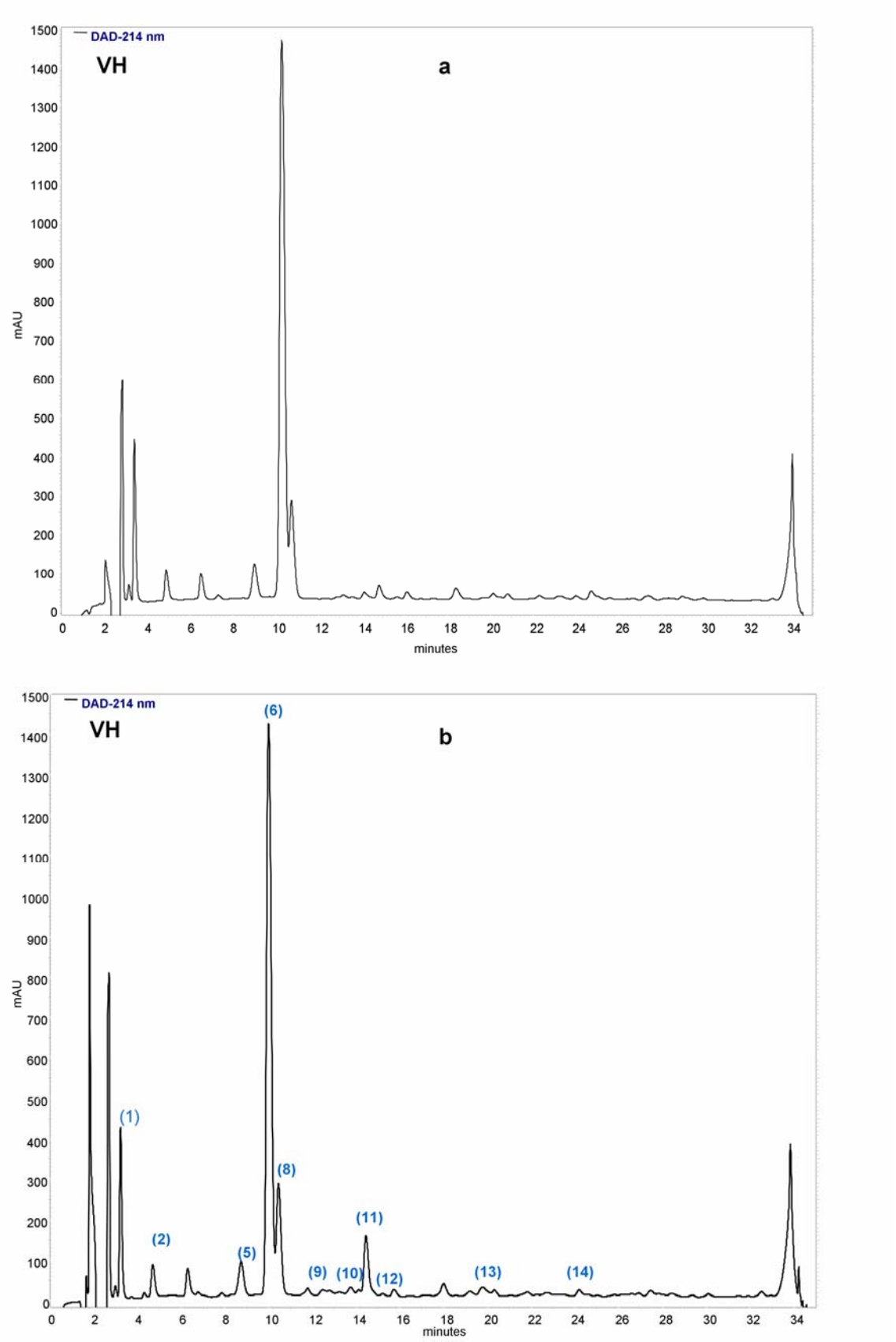

DAD-214 nm
IH
a
mAU
minutes
DAD-214 nm
IH
b
(1)
(2)
(3)
(4)
(5)
(6)
(7)
(9)
(10)
(11)
(12)
(13)
(14)
(15)
mAU
minutes

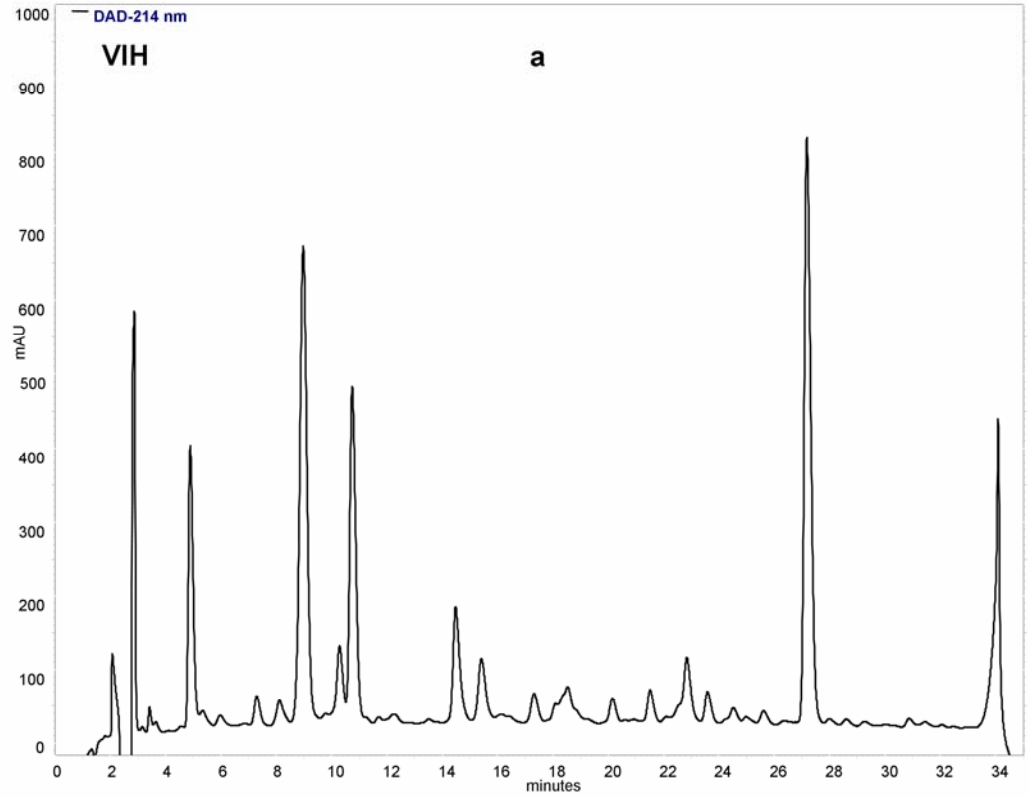

DAD-214 nm
IIH
a
mAU
minutes

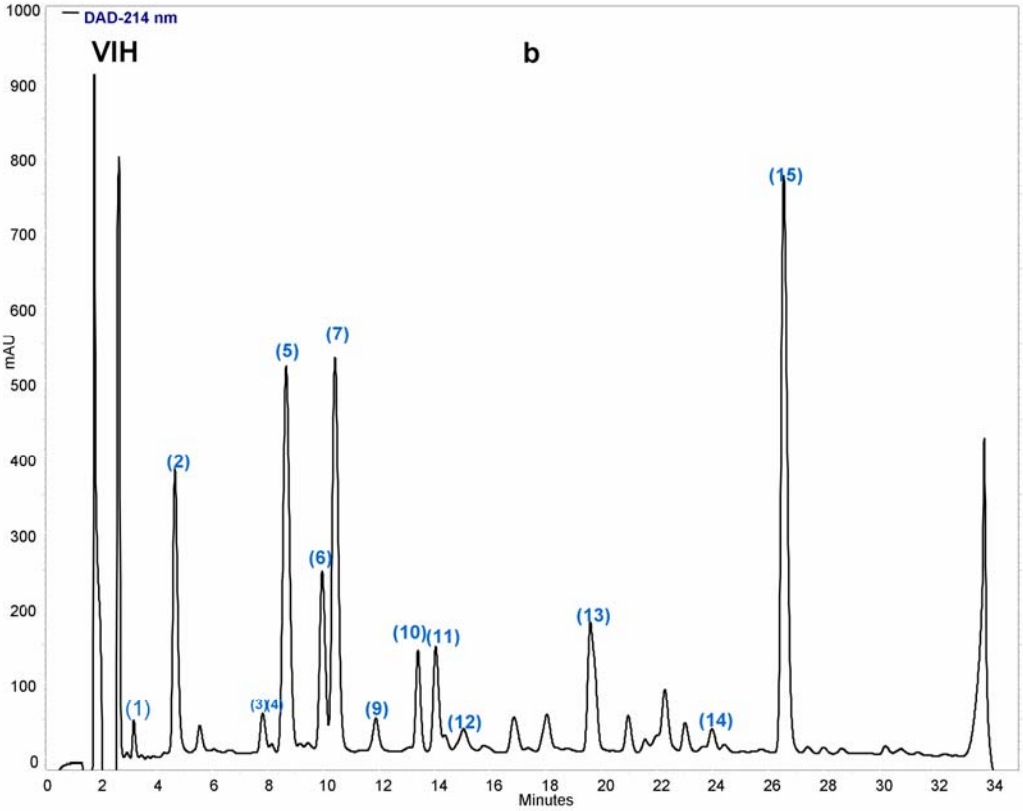

DAD-214
IIH
b
(1)
(2)
(3)
(4)
(5)
(6)
(8)
(9)
(10)
(11)
(12)
(13)
(14)
(15)
mAU
Minutes

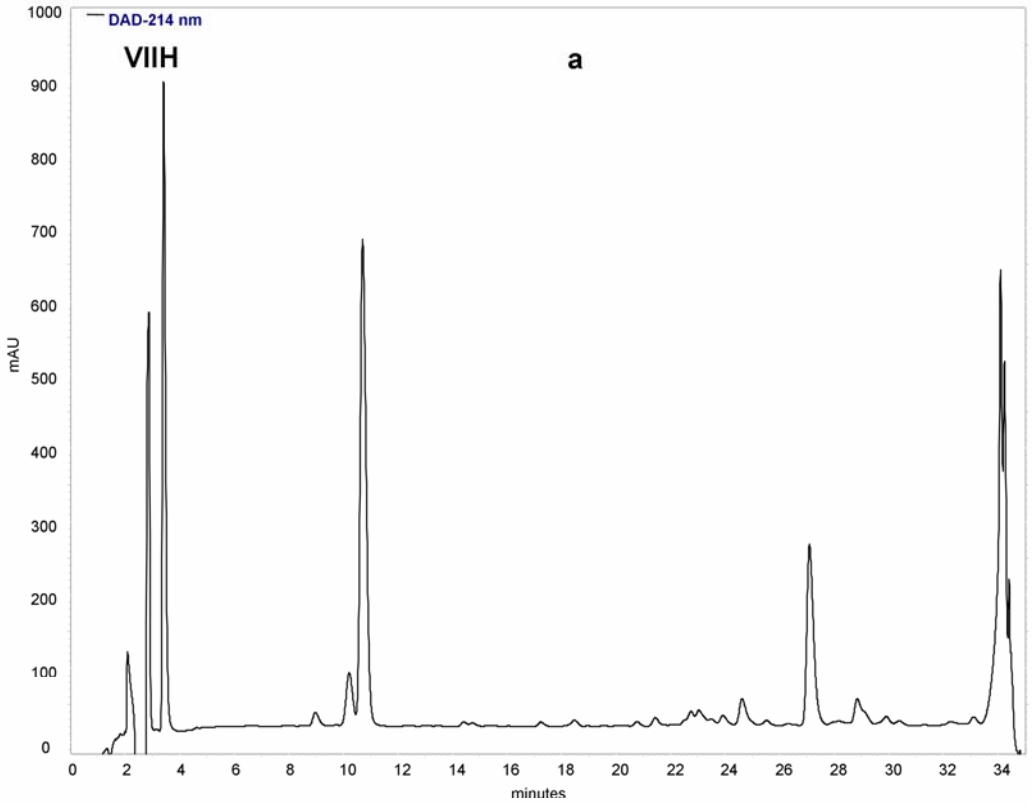

DAD-214 nm
IIIH
a
mAU
minutes
DAD-214 nm
IIIH
b
(1)
(2)
(3)
(5)
(6)
(7)
(9)
(10)
(11)
(13)
(14)
mAU
minutes

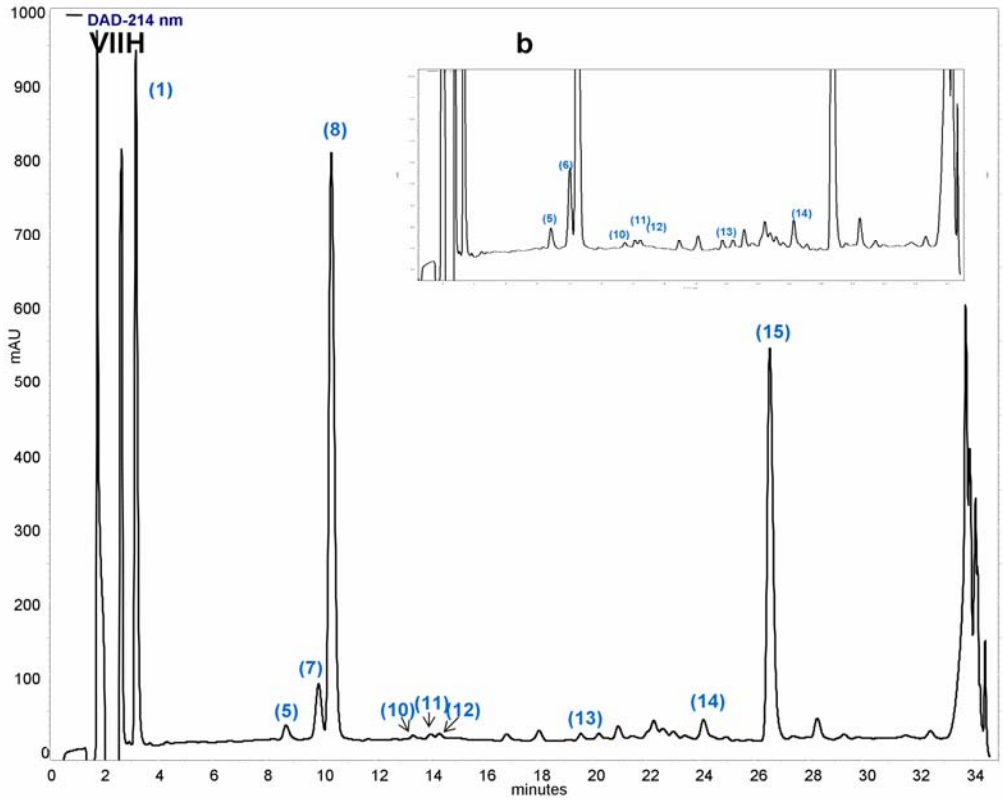

DAD-214 nm
IVH
a
mAU
minutes
DAD-214 nm
IVH
b
mAU
Minutes
(1)
(2)
(3)
(4)
(5)
(6)
(7)
(9)
(10)
(11)
(12)
(13)
(14)
(15)

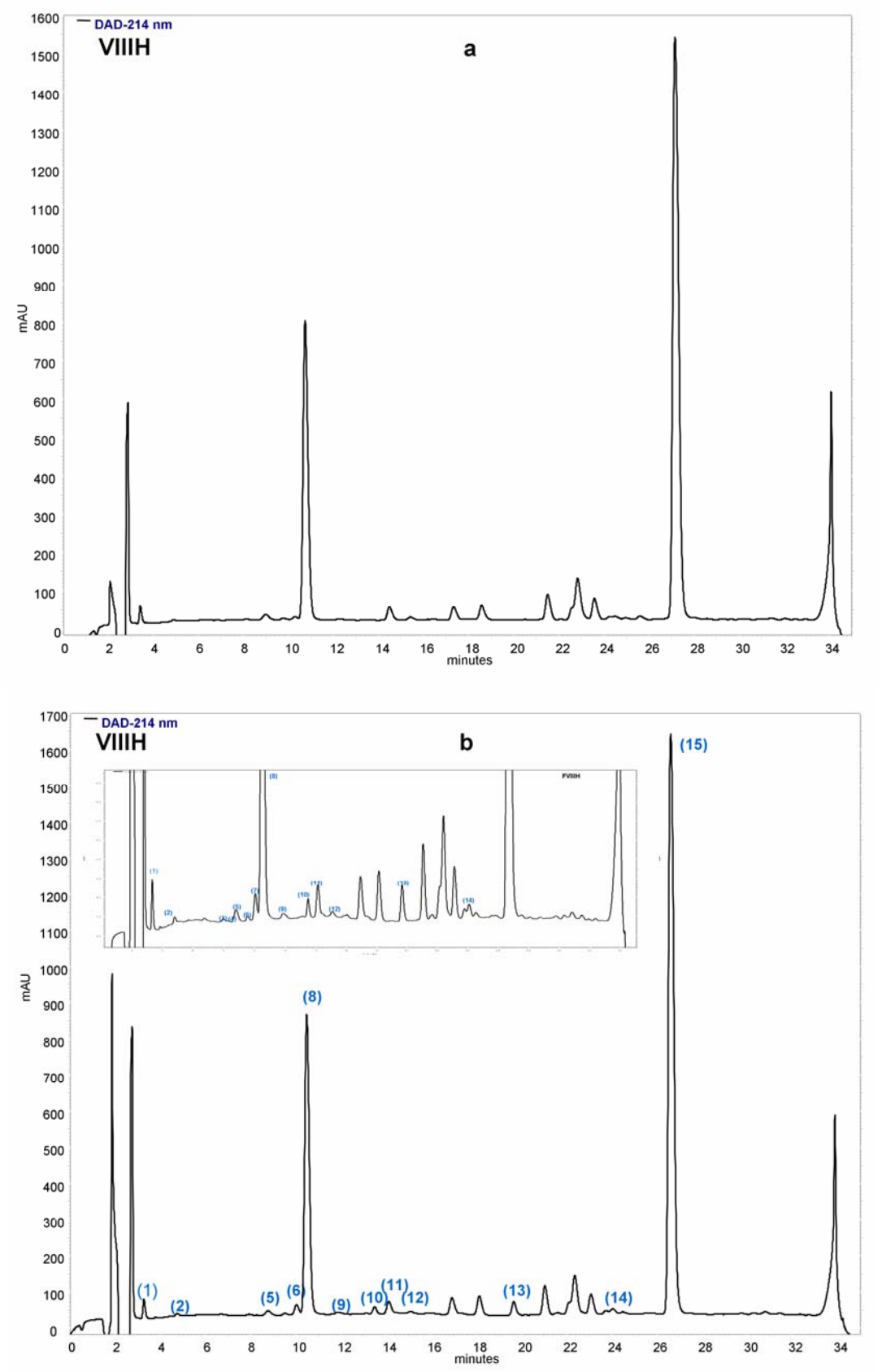

DAD-214 nm
VH
a
mAU
minutes
DAD-214 nm
VH
b
(1)
(2)
(5)
(6)
(8)
(9)
(10)
(11)
(12)
(13)
(14)
mAU
minutes


DAD-214 nm
VIH
a
mAU
minutes


DAD-214 nm
VIH
b
mAU
Minutes
(1)
(2)
(3)(4)
(5)
(6)
(7)
(9)
(10)
(11)
(12)
(13)
(14)
(15)

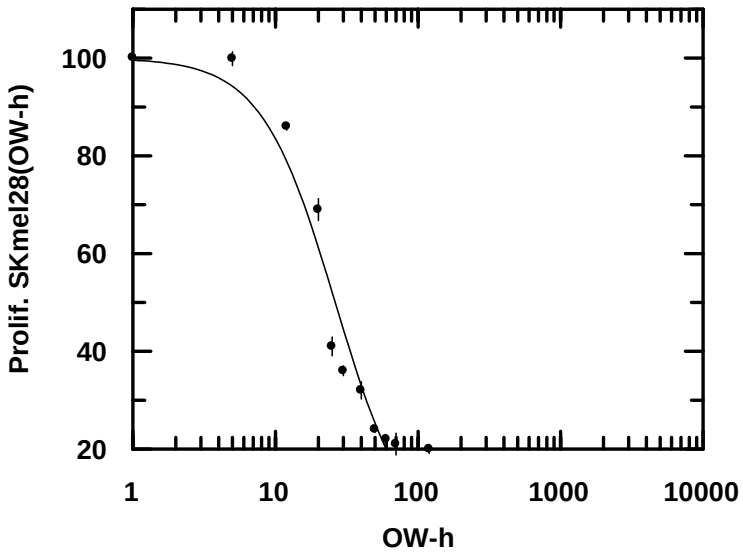

DAD-214 nm
VIIH
a
mAU
minutes


DAD-214 nm
VIIH
b
(1)
(5)
(6)
(7)
(8)
(10)
(11)
(12)
(13)
(14)
(15)
mAU
minutes

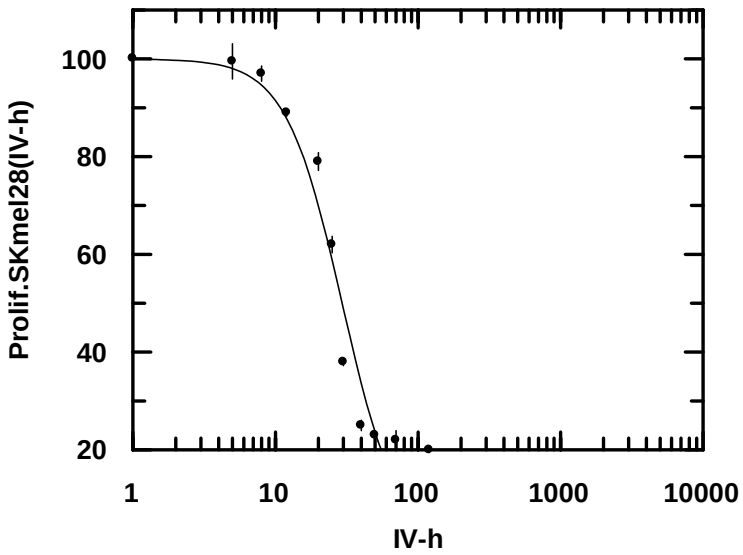


Gallic acid **(1)**; Gallocatechin **(2)**; Epigallocatechin **(3)**; Cysteamine-Epigallocatechin **(4)**; Hamamelitannin **(5)**; Catechin **(6)**; Cysteamine-Catechin **(7)**; Methyl Gallate **(8)**; Cysteamine-EpigallocatechinGallate **(9)**; Cysteamine-Epicatechin **(10)**; Epicatechin **(11)**; EpigallocatechinGallate **(12)**; Cysteamine-EpigallocatechinGallate **(13)**; EpicatechinGallate **(14)**; Pentagalloyl Glucose **(15)**.

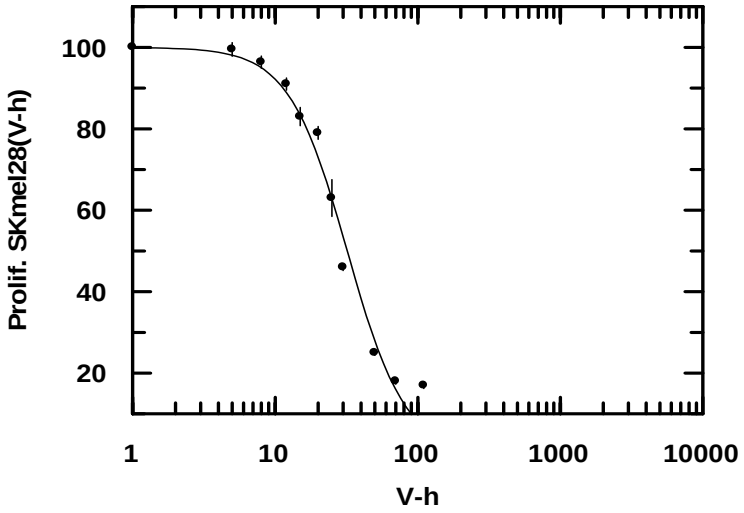
Dose-response curves of SKMel28 melanoma cells proliferation in the presence of polyphenolic fractions from Witch Hazel . Viability and proliferation expressed as per cent with respect to untreated control cells. IC_{50} in µg/mL ± SD, n=4 (four independent experiments).



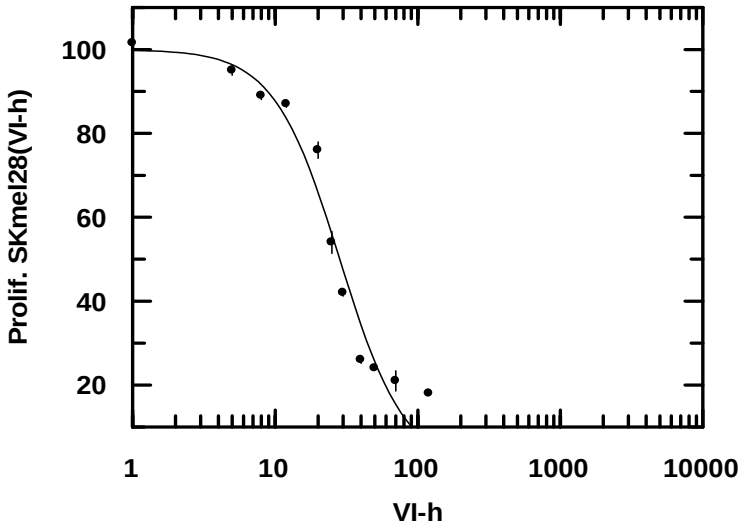
IC50 FOWH = 26±2 µg/mL



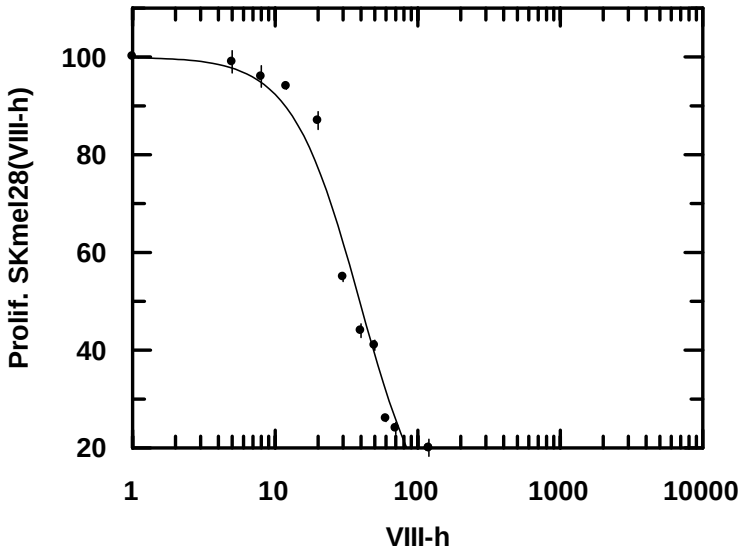
IC50 FIVH = 29±2 µg/mL



IC50 FVH = 32±2 µg/mL



IC50 FVIH = 28±2 µg/mL



IC50 VIIIH = 39±2 µg/mL

8.2.3 Material suplementario de la publicación 4 (Publicación mostrada en el apartado 4.2.2)

Table 3: Occurrence of conjugated metabolites of EC in urine from rats fed with GADF or EC at different sampling times.

Metabolite	Number	Occurrence					
		2 h	4 h	6 h	8 h	10 h	24 h
Gluc-EC-1	1	GADF, EC	GADF, EC	GADF, EC	GADF, EC	GADF, EC	GADF, EC
Gluc-EC-2	2	GADF, EC	GADF, EC	GADF, EC	GADF, EC	GADF, EC	GADF, EC
Gluc-EC-3	3	GADF, EC	GADF, EC	GADF, EC	GADF, EC	GADF, EC	GADF, EC
Sulf-EC-1	4	EC	EC	EC	EC	EC	EC
Sulf-EC-2	5	EC	EC	EC	EC	EC	EC
Me-Sulf-EC-1	6	GADF, EC	GADF, EC	GADF, EC	EC	EC	EC
Me-Sulf-EC-2	7	GADF, EC	GADF, EC	GADF, EC	GADF, EC	GADF, EC	GADF, EC
Me-Sulf-EC-3	8	GADF, EC	GADF, EC	GADF, EC	GADF, EC	GADF, EC	GADF, EC
Me-Sulf-EC-4	9	EC	EC	EC	EC	EC	EC
Gluc-Me-EC-1	10	GADF, EC	GADF, EC	GADF, EC	GADF, EC	GADF, EC	GADF, EC
Gluc-Me-EC-2	11	GADF, EC	GADF, EC	GADF, EC	GADF, EC	GADF, EC	GADF, EC
Gluc-Me-EC-3	12	GADF, EC	GADF, EC	GADF, EC	GADF, EC	GADF, EC	GADF, EC
Gluc-Me-EC-4	13	GADF, EC	GADF, EC	GADF, EC	GADF, EC	GADF, EC	GADF, EC
Gluc-Sulf-EC-1	14	EC	GADF, EC	EC	EC	EC	EC
di-Sulf-EC-1	15	GADF, EC	GADF, EC	GADF, EC	GADF, EC	GADF, EC	GADF, EC
di-Gluc-EC-1	16	EC	GADF, EC	EC	EC	EC	EC
di-Me-Gluc-EC-1	17	GADF, EC	GADF, EC	GADF, EC	EC	EC	EC
di-Me-Gluc-EC-2	18	GADF, EC	GADF, EC	GADF, EC	EC	EC	EC
di-Me-Gluc-EC-3	19	GADF, EC	GADF, EC	GADF, EC	EC	EC	EC
Me-di-Sulf-EC-1	20	EC	EC	EC	EC	EC	EC
Me-di-Sulf-EC-2	21	EC	EC	EC	EC	EC	GADF, EC
Gluc-Me-Sulf-EC-1	22	EC	EC	EC	EC	EC	EC
Gluc-Me-Sulf-EC-2	23	EC	EC	EC	EC	EC	EC
tri-Sulf-EC-1	24	EC	EC	EC	EC	EC	EC

Table 4. Occurrence of phenolic acids in urine from rats fed with GADF or EC.

Metabolite	Time (hours) GADF diet	Time (hours) EC diet
3-hydroxyphenylvaleric acid (3-HPhVA)	24	n.d.
3,4-di-hydroxyphenylpropionic acid (DHPhPA)	24	n.d.
4-hydroxyphenylpropionic acid (4-HPhPA)	4,6,8,10,24	8,10,24
Caffeic acid	n.d.	24
p-coumaric acid	2,4,6,8,10,24	2,4,6,8,10,24
m-coumaric acid	2,4,6,8,10,24	2,4,6,8,10
3,4-di-hydroxyphenylacetic acid (DHPhAcA)	24	n.d.
3-hydroxyphenylacetic acid (3-HPAcA)	2,4,6,8	10,24
4-hydroxybenzoic acid (4-HBA)	2,4,6,8,10,24	10,24
Sulf- di-hydroxyphenylpropionic acid-(Sulf-DHPhPA)	8,10,24	n.d.
Gluc-di-hydroxyphenylpropionic acid (Gluc-DHPhPA)	2,8	n.d.
Sulf-hydroxyphenylpropionic acid (Sulf-HPhPA)	8,24	n.d.
Sulf-coumaric acid	2,8,4,24	n.d.