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RELACIÓN ESTRUCTURA/ACTIVIDAD DE PROANTOCIANIDINAS PROCEDENTES DE FUENTES NATURALES DE ORIGEN VEGETAL

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RELACIÓN ESTRUCTURA/ACTIVIDAD DE PROANTOCIANIDINAS PROCEDENTES DE FUENTES NATURALES DE ORIGEN VEGETAL

Memoria presentada por Mª Luisa Mateos Martín para optar al título de Doctora por la Universidad de Barcelona.

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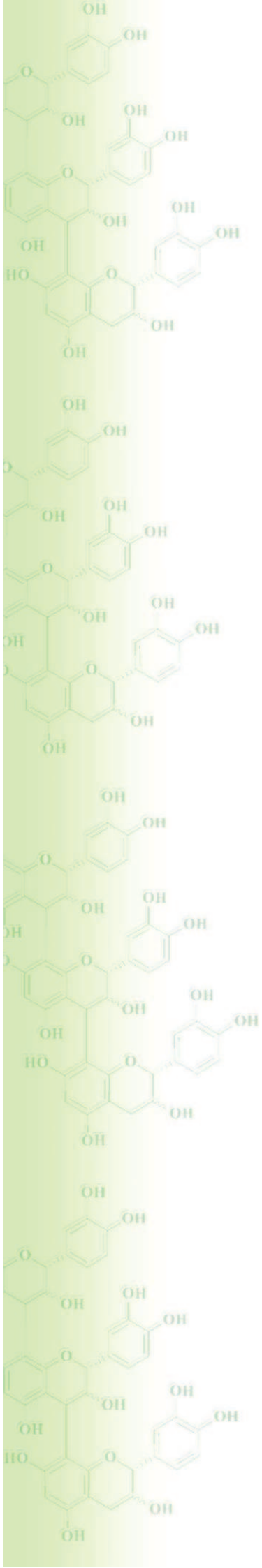

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ABREVIATURAS

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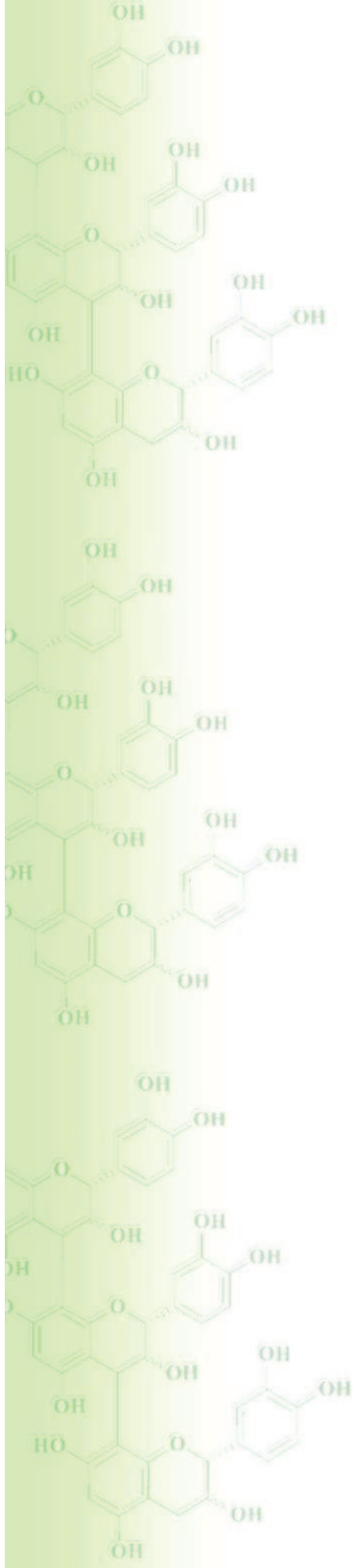
AAPH	2,2'-azo-bis (2-amidinopropano)dihidrocloruro
ABTS	2,2.azino-bis (3-etilbenzotiazolin-6-sulfonato)
$ABTS^{\cdot+}$	Radical catión ABTS
ADN	Ácido desoxirribonucleico
APCI	Ionización química a presión atmosférica
API	Ionización a presión atmosférica
ARN	Ácido ribonucleico
Aufs	Unidades de absorbancia
Cat	(+)-catequina
CID	Disociación inducida por colisión
Da	Dalton
DAD	Detector de diodos en línea
DP	Grado de polimerización
DPPH	Radical libre 2,2-difenil-1-picrilhidracil
EC	(Epi)catequina
Ec	(−)-epicatequina
EcG	(−)-epicatequina 3-*O*-galato
EGC	(−)-epigalocatequina
EGCG	(−)-epigalocatequin 3-*O*-galato
EPA	Proantocianidinas extraíbles
EPR	Resonancia paramagnética electrónica
ESI	Ionización por electrospray
FL	Fluoresceína
FRAP	Reducción de hierro/poder antioxidante
GADF	Fibra antioxidante dietética de uva
GHS	Glutatión
GlcA	Glucurónido
Gluc	Glucurónido
HAT	Transferencia de átomos de hidrogeno
HNTTM	Radical tris(2,4,6-tricloro-3,5-dinitrofenil)metilo
HPLC	Cromatografía líquida de alta resolución
HRF	Fisión del anillo heterocíclico

HSV	Virus de herpes simple
HSV-1	Virus de herpes simple de tipo 1
HSV-2	Virus de herpes simple de tipo 2
LC–MS/MS	Cromatografía líquida acoplada a espectrometría de masas en tándem
LC	Cromatografía líquida
LDL	Lipoproteínas de baja densidad
LID	Disociación inducida por laser
MALDI	Ionización por desorción mediante láser asistida por matriz
MALDI-TOF	Ionización por desorción mediante láser asistida por matriz acoplada a un detector de tiempo de vuelo
Me	Grupo metilo
MRM	Monitorización de reacciones múltiples
MS	Espectrometría de masas
NEPA	Proantocianidinas no extraíbles
NL	Pérdidas neutras
NP-HPLC	Cromatografía líquida de alta resolución de fase normal
ORAC	Capacitad de absorción de radicales libres
PA	Proantocianidinas
PC	Procianidinas
PTFE	Politetrafluoroetileno
Q	Detector de cuadrupolo de barras
QqQ	Triple cuadrupolo
Q-TOF	Cuadrupolo acoplado a un detector de tiempo de vuelo
RDA	Reacción Retro Diels Alder
ROS	Especies reactivas de oxígeno
RP-HPLC	Cromatografía líquida de alta resolución de fase reversa
SET	Transferencia de electrones
SIDA	Síndrome de inmunodeficiencia adquirida
SOD	Superóxido dismutasa
SPE	Extracción en fase sólida
Sulf	Sulfato
TEAC	Capacidad antioxidante equivalente de trólox
TFA	Ácido trifluoroacético

TIC	Cromatograma de iones totales
TNPTM	Radical tris(2,3,5,6-tetracloro-4-nitrofenil)metilo
TOF	Detector de tiempo de vuelo
Trólox	Ácido 6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxílico
uma	Unidad de masa atómica
UV	Luz ultravioleta (200-400 nm)
XIC	Cromatograma de iones extraídos



I. INTERÉS Y JUSTIFICACIÓN

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Parte de la importancia del consumo de alimentos de origen vegetal podría relacionarse con la presencia de compuestos antioxidantes. Dentro de este grupo de antioxidantes destacan los polifenoles, los cuales se pueden encontrar en una alta proporción en forma de polímeros en los extractos de plantas. Sin embargo, la mayoría de los estudios realizados sobre antioxidantes se han centrado en la composición y actividad de compuestos monoméricos.

Las proantocianidinas, a pesar de ser de los compuestos polifenólicos más abundantes en la dieta, se tienen poco en cuenta en estudios sobre la composición y propiedades funcionales de los alimentos. La utilización de métodos de espectrometría de masas para el análisis de alimentos puede proporcionar nuevos datos que relacionen la estructura con la función de las proantocianidinas y los posibles beneficios para la salud de éstas y de sus metabolitos. Es importante optimizar las técnicas de MS/MS para la mejor ionización y fragmentación de los compuestos para avanzar en la caracterización de proantocianidinas de alto grado de polimerización y galoización y así poder detectar y caracterizar nuevos compuestos bioactivos poliméricos en los alimentos y extractos naturales. Tanto el grado de polimerización como el de galoización son parámetros que se han relacionado con sus propiedades funcionales.

Se ha descrito que los alimentos ricos en proantocianidinas pueden presentar efectos beneficiosos para la salud al actuar como antioxidantes, anticancerígenos, cardiopreventivos, antimicrobianos, antivirales y como agentes neuroprotectores. Sus mecanismos de acción son objeto de estudio y hay poca información disponible sobre la metabolización de proantocianidinas poliméricas. Además, la mayoría de los estudios de investigación en el campo de compuestos polifenólicos se centran exclusivamente en polifenoles extraíbles de los alimentos vegetales con disolventes orgánicos. Sin embargo, gran parte de estos polifenoles no son extraídos y por lo tanto son ignorados en los estudios biológicos, nutricionales, y epidemiológicos. Los polifenoles no extraíbles son una parte

importante del total de polifenoles dietéticos y pueden exhibir una actividad biológica significativa.

Dentro de las proantocianidinas, las propiedades biológicas de las procianidinas (oligómeros de (epi)catequina) son las más estudiadas, sin embargo, existen otros tipos de proantocianidinas menos conocidas, que se encuentran en menor proporción en la naturaleza. Las proantocianidinas presentan una diversidad estructural que pueden explicar la actividad biológica más allá del efecto captador de radicales libres que normalmente se denomina antioxidante.

Esta tesis pretende ser una aportación al estudio de la relación estructura-actividad de las proantocianidinas mediante su caracterización estructural, el estudio de su metabolización y la evaluación de su actividad.

II. INTRODUCCIÓN

1. ANTIOXIDANTES DE ORIGEN VEGETAL Y NUTRICIÓN

Una dieta variada y rica en alimentos de origen vegetal, como puede ser la denominada dieta mediterránea, se caracteriza por tener múltiples efectos beneficiosos para la salud. Hay considerables evidencias que indican que un consumo adecuado de frutas y verduras ayudan a prevenir ciertas dolencias y nos hacen ser menos propensos a sufrir enfermedades degenerativas del sistema nervioso, cardiovasculares, diabetes, o ciertos tipos de cáncer (Lorgeril 1998; Trichopoulou *et al.* 2000; Esposito *et al.* 2004; Lorgeril *et al.* 2008).

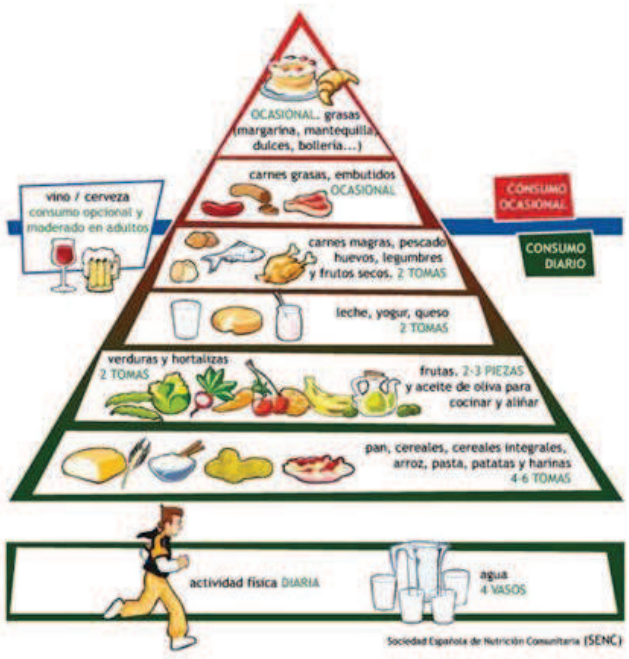
La influencia de los hábitos dietéticos sobre la salud es ampliamente conocida. Sin embargo, el abandono de estos buenos hábitos y costumbres alimentarias a otros menos saludables está haciendo que los índices de obesidad y diabetes en los denominados países desarrollados aumenten considerablemente, lo que conlleva un aumento de enfermedades cardiovasculares y lo convierte en el origen de uno de los mayores problemas de salud y unas de las principales causas de mortalidad de la sociedad actual en estos países (Chen *et al.* 2012).

Los hábitos dietéticos de la zona mediterránea han sido identificados en gran parte como responsables de los beneficios sobre la salud en estas poblaciones y su papel en la prevención de muchas enfermedades crónicas ha sido ampliamente estudiado. Se ha demostrado que la población en las regiones mediterráneas presentaba bajas tasas de enfermedad coronaria, así como menor incidencia de algunos tipos de cáncer, de enfermedades inflamatorias y enfermedades degenerativas, lo que se traduce en una mejor calidad de vida (Bonaccio *et al.* 2012). Algunos de los beneficios asignados a la dieta mediterránea pueden ser debidos a la presencia de diferentes compuestos bioactivos que actúan sinérgicamente. Esto ha provocado un creciente interés por conocer posibles factores que promuevan estos efectos beneficiosos para la salud.

La dieta mediterránea tradicional se caracteriza principalmente por una abundancia de alimentos vegetales (Willett et al. 1995), pero además de

estos patrones alimentarios la dieta mediterránea se caracteriza por el consumo de productos frescos y de temporada, dejando de ser un simple hábito alimentario para pasar a ser un estilo de vida, procedente de una herencia cultural y tradicional, asociado a la práctica de ejercicio físico diario moderado y a un disfrute de las comidas en compañía (**Figura 1.1**).

Figura 1.1. Pirámide dieta Mediterránea



La influencia de la dieta mediterránea en la salud no sólo se limita al hecho de que sea una dieta equilibrada y variada. A los beneficios de su riqueza en carbohidratos complejos y fibra, así como su bajo contenido en ácidos grasos saturados, hay que añadir los beneficios de su contenido en sustancias antioxidantes.

Los alimentos vegetales son la principal fuente de antioxidantes de la dieta, entre los que destacan distintos grupos de compuestos como las vitaminas, carotenoides o polifenoles (Moure et al. 2001). Las propiedades antioxidantes de estos compuestos retardan y/o previenen los procesos de oxidación y contribuyen así a la protección celular frente a los mismos. En la

naturaleza existe un elevado número de compuestos antioxidantes que pueden actuar sinérgicamente contra la oxidación del organismo. Este efecto sinérgico es difícil de ser valorado siendo la suma de los constituyentes de los alimentos la que produce los efectos saludables.

1.1. Fuentes de antioxidantes en la dieta

Como se ha mencionado anteriormente, algunos de los efectos saludables de los alimentos de origen vegetal se atribuyen a sus propiedades antioxidantes. De entre los posibles compuestos antioxidantes de la dieta humana destacan los polifenoles.

Los compuestos polifenólicos podrían participar en la protección celular contra el proceso oxidativo relacionado con enfermedades degenerativas (Rice-Evans *et al.* 1995; Halliwell 1996; Hollman *et al.* 1999; Aruoma 2003; Rossi *et al.* 2008), enfermedades relacionadas con el síndrome metabólico (Taubert *et al.* 2007), cáncer (Matito et al. 2003) y envejecimiento (Queen *et al.* 2010). Por esto, el consumo de polifenoles parece prevenir o disminuir el deterioro originado por un exceso de estrés oxidativo, dando como resultado un aumento de defensas contra la oxidación. Es por esto por lo que vamos a centrar nuestra atención en los antioxidantes más abundantes de la dieta humana que son los compuestos polifenólicos.

1.2. Compuestos polifenólicos

Los compuestos polifenólicos o polifenoles constituyen un amplio grupo de compuestos presentes en el reino vegetal que son producto del metabolismo secundario de las plantas y desempeñan en éstas importantes funciones ecológicas como regulación del crecimiento, defensa frente a agresiones externas de depredadores y microorganismos, resistencia a enfermedades y

estrés, pigmentación, polinización etc. (Scalbert *et al.* 1987; Haslam 1998). Además, son en gran medida responsables del sabor y color de la planta.

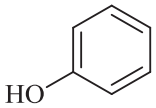
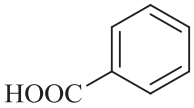
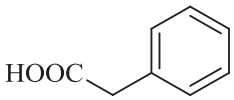
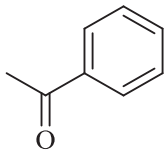
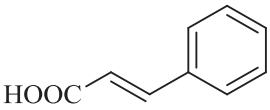
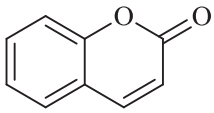
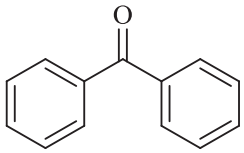
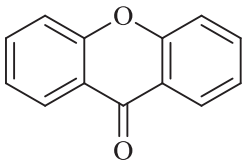
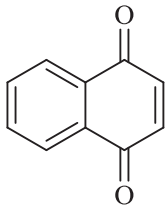
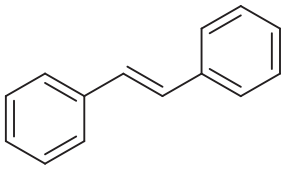
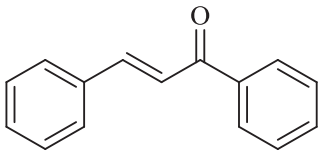
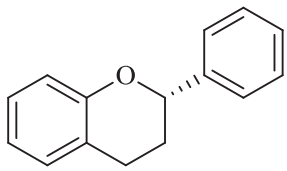
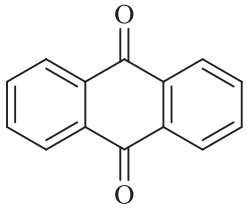
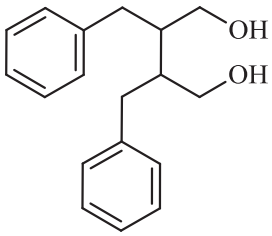
Los polifenoles son sintetizados por las plantas principalmente en respuesta a un estrés o agresión externa como pueden ser periodos prolongados de sequía, alta radiación ultravioleta o como defensa contra un herbívoro o infección por agentes patógenos. Por lo tanto, la presencia de estos compuestos en las plantas es muy variada dependiendo de múltiples factores como pueden ser aquellos intrínsecos de la planta como la especie, procedencia, variedad o parte del vegetal o extrínsecos como la intensidad lumínica, temperatura, maduración, zona de cultivo etc. Ya que la composición y concentración de polifenoles puede verse afectada por estos factores cabe esperar, por ejemplo, que las frutas y verduras que se cultivan de manera ecológica generen niveles mayores de compuestos polifenólicos para combatir con sus propios mecanismos de defensa posibles plagas (Vallverdú-Queralt et al. 2012).

La estructura de los polifenoles consiste en anillos aromáticos con uno o más grupos hidroxilo, incluyendo también derivados como ésteres, glicósidos y otros grupos con diferentes estructuras químicas y actividad.

1.2.1. Clasificación

Los compuestos fenólicos se clasifican en subcategorías dependiendo de su esqueleto estructural, variando su naturaleza desde moléculas simples como los ácidos fenólicos hasta compuestos de naturaleza polimérica como las proantocianidinas. Su clasificación se centra principalmente en el número de átomos de carbono que presenta su estructura (Spencer *et al.* 2008; Crozier *et al.* 2009) (**Tabla 1.1**).

Tabla 1.1. Principales clases de compuestos fenólicos en plantas, representados sin los grupos hidroxilo sustituyentes.

Fenoles simples C_6  	Ácidos fenólicos C_6-C_1  	Ácido fenilacético C_6-C_2 
Acetofenonas C_6-C_2  	Ácidos hidroxicinámicos C_6-C_3  	Cumarinas C_6-C_3 
Benzofenonas C_6-C_1-C_6  	Xantonas C_6-C_1-C_6  	Naftoquinonas C_6-C_4 
Estilbenos C_6-C_2-C_6 	Chalconas C_6-C_3-C_6  	Flavonoides C_6-C_3-C_6 
Androquinonas C_6-C_2-C_6  	Lignanos C_6-C_3-C_3-C_6  	Taninos hidrolizables $(C_6$-$C_1)_n$ Ligninas $(C_6$-$C_3)_n$

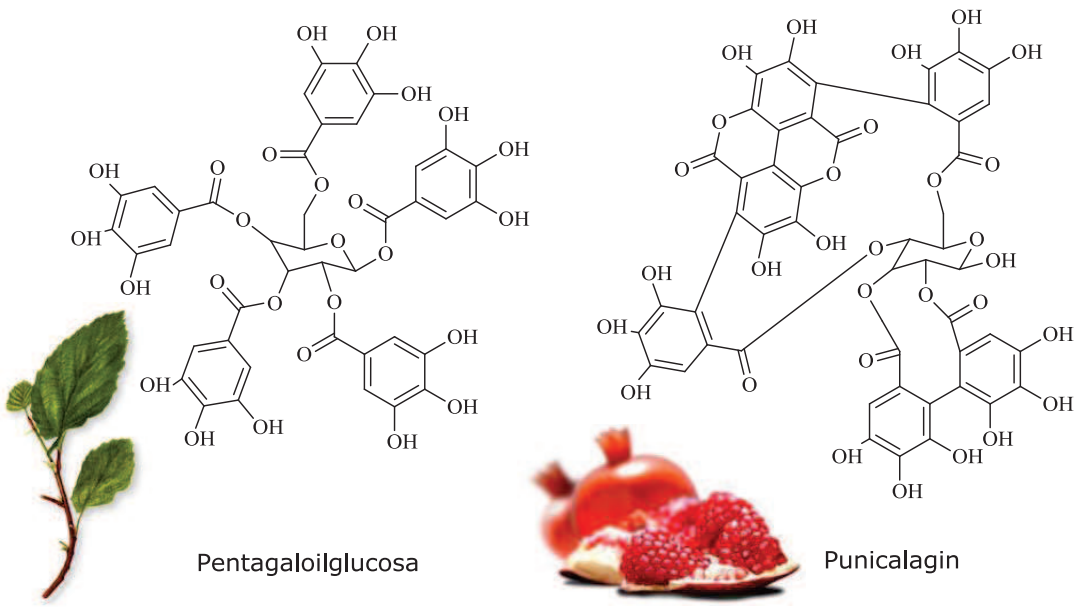
Los ácidos fenólicos son abundantes en extractos vegetales. Se diferencian dos grupos principales de ácidos fenólicos, los derivados de ácidos benzoicos y los derivados de ácidos cinámicos (Spencer *et al.* 2008). Los ácidos

fenólicos más frecuentes son el ácido cafeico, y el ácido ferúlico (Scalbert *et al.* 2000b).

Los **estilbenos** no son compuestos muy abundantes en los alimentos (Scalbert *et al.* 2000b). El estilbeno de mayor interés es el resveratrol (3,5,4'-trihidroxiestilbeno) presente en la uva y el vino. El resveratrol ha sido ampliamente estudiado mostrando actividad antivírica y antitumoral, además de poder prevenir trastornos relacionados con la obesidad y enfermedades del envejecimiento oponiéndose a los efectos de una dieta alta en calorías (Jang *et al.* 1997; Docherty *et al.* 1999; Baur *et al.* 2006).

Los **taninos hidrolizables** son polímeros constituidos por un carbohidrato, generalmente glucosa, cuyos grupos hidroxilos se encuentran esterificados por ácidos fenólicos, como el acido gálico para los galotaninos o el elágico para los elagitaninos (Arapitsas 2012) (**Figura 1.2**).

Figura 1.2. Estructura química de la pentagaloilglucosa (galotanino) presente en *Hamamelis virginiana* y punicalagin (elagitanino) presente en *Púnica granatum*



Los **flavonoides** son una de las familias de compuestos polifenólicos más conocidas. Tanto es así, que comúnmente los compuestos polifenólicos se dividen en flavonoides y no flavonoides.

1.2.2. Flavonoides

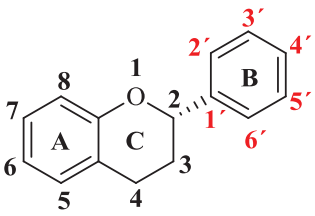


Figura 1.3. Estructura química de los flavonoides

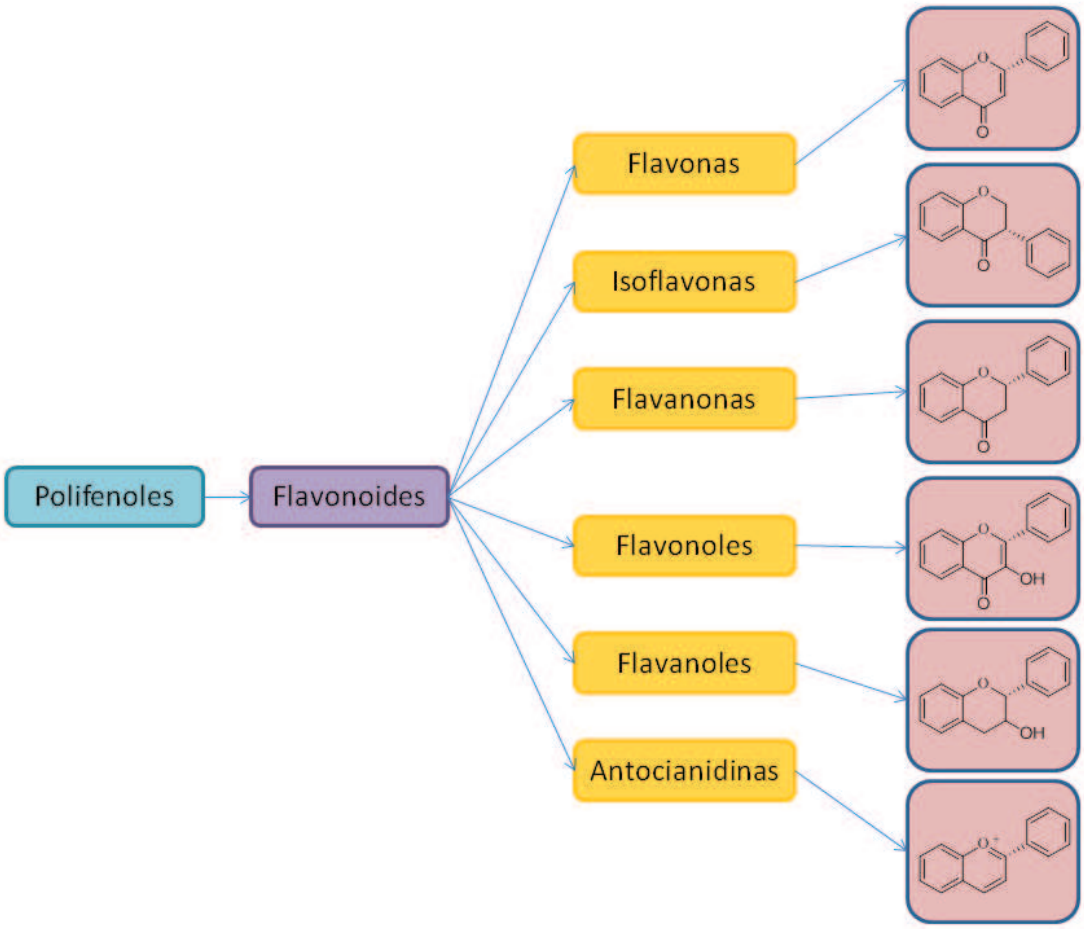
La estructura química de los flavonoides consta de un esqueleto difenilpropano (C_6-C_3-C_6) formado por dos anillos aromáticos (A y B) unidos a través de tres átomos de carbono que forman un heterociclo oxigenado (C) (Rice-Evans *et al.* 1996; Spencer *et al.* 2008) (**Figura 1.3**).

Los flavonoides a su vez se dividen en diferentes familias dependiendo del estado de oxidación del heterociclo. De este modo distinguimos entre flavonas, isoflavonas, flavanonas, flavonoles, flavanoles y antocianidinas (**Figura 1.4**).

Dentro de cada clase, los miembros difieren en el patrón de hidroxilación de sus dos anillos fenólicos, y por la naturaleza y la posición de los sustituyentes, que puede ser diferentes grupos como grupos metilo o azúcares.

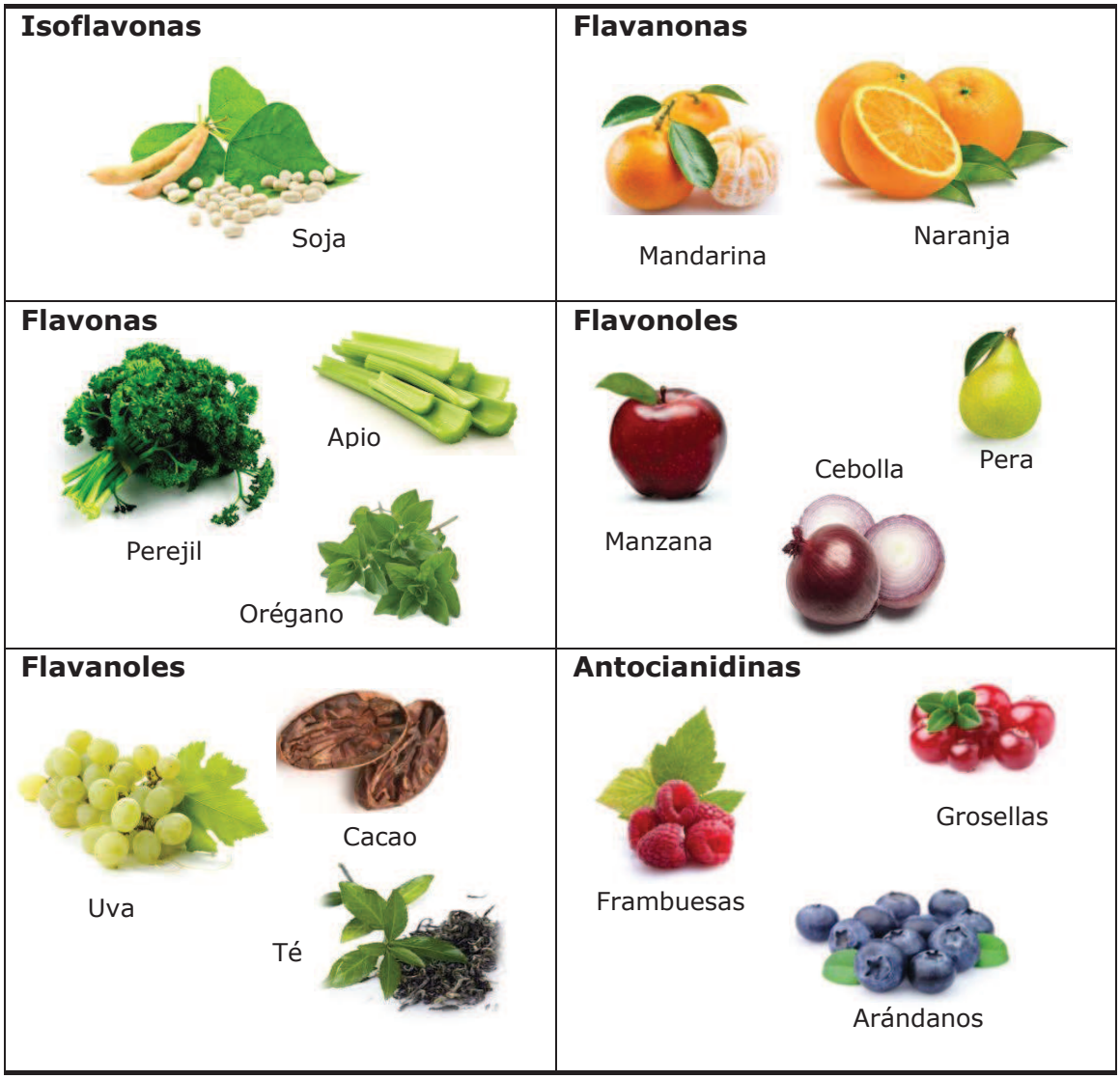
Los flavonoides que se encuentran libres, sin estar unidos a otros grupos, se denominan agliconas, mientras que los que se encuentran glucosilados se denominan glucósidos de flavonoides (Manach *et al.* 2004).

Figura 1.4. Estructuras básicas de los flavonoides.



Los flavonoides son el grupo de polifenoles más amplio y diverso en la naturaleza a la vez que los compuestos más abundantes en los alimentos de origen vegetal (**Figura 1.5**). En particular, los flavanoles están recibiendo una creciente atención. Estos se encuentran en la naturaleza principalmente como (epi)catequina o galatos de (epi)catequina, de forma monomérica o formando compuestos poliméricos. A estos polímeros de flavanoles se les llama proantocianidinas o taninos condensados (Cheynier 2005) y se encuentran en alimentos como el té, la uva y el cacao.

Figura 1.5. Especies vegetales ricas en flavonoides.



1.2.3. Proantocianidinas o taninos condensados

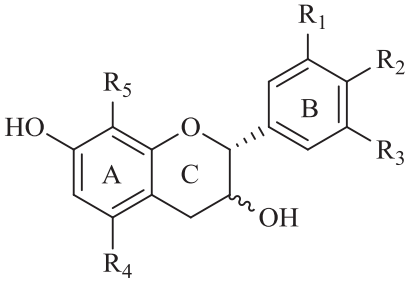
Los taninos condensados son estructuras poliméricas compuestas por la unión de flavanoles. Se encuentran en abundancia en la naturaleza formando mezclas complejas y son responsables en gran medida de la textura, color y sabor astringente y amargo de algunos alimentos como el té, café o el chocolate (Lea *et al.* 1978). Después de la lignina, representan la clase más abundante de compuestos fenólicos naturales y el principal compuesto

polifenólico en la dieta (Matthews *et al.* 1997; Scalbert *et al.* 2000a; Gu *et al.* 2004). Las proantocianidinas se han descrito como compuestos antimicrobianos, antioxidantes, anticancerígenos y antiinflamatorios, además de ser beneficiosos para la prevención de enfermedades relacionadas con síndrome metabólico (Cos *et al.* 2004; Serrano *et al.* 2009; Bladé *et al.* 2010; Castell-Auví *et al.* 2012). Hay gran variedad de clases de proantocianidinas dependiendo de la unidad monomérica de la que esté compuesta (**Figura 1.5**).

Las proantocianidinas se diferencian estructuralmente de acuerdo al número de grupos hidroxilos, su posición en los anillos aromáticos y su configuración espacial en las unidades de flavanoles constituyentes. Los flavan-3-oles poseen dos átomos de carbono quirales, es decir, existen cuatro diastereoisómeros para cada uno de ellos. Los enantiómeros con configuración cis llevan el prefijo "epi" mientras que las unidades que presentan una configuración trans no llevan este prefijo. En determinadas ocasiones el prefijo "epi" se incluye entre paréntesis para indicar indistintamente uno u otro compuesto.

Los polímeros de proantocianidinas pueden presentar una estructura homogénea constituida únicamente por unidades de un único flavanol o presentar una composición mixta con distintas unidades monoméricas, siendo las procianidinas (polímeros homogéneos de (epi)catequina) mayoritarias respecto a otras proantocianidinas en el reino vegetal (Prior *et al.* 2005; Aron *et al.* 2007).

Figura 1.5. Principales estructuras de los flavan-3-oles constituyentes de las proantocianidinas



Familia	Flavan-3-ol	R_1	R_2	R_3	R_4	R_5
Procianidinas[a]	(epi)catequina	H	OH	OH	OH	H
Propelargonidinas[b]	(epi)afzelequina	H	OH	H	OH	H
Profisetinidinas[b]	(epi)fisetinidol	H	OH	OH	H	H
Prodelfinidinas[b]	(epi)galocatequina	OH	OH	OH	OH	H
Proguibourtinidinas[b]	(epi)guibourtinidol	H	OH	H	H	H
Prorobinetinidinas[b]	(epi)robinetinidol	OH	OH	OH	H	H
Prodistenidina[b]	(epi)distenidina	H	H	H	OH	H
Proteracacinidina[b]	(epi)oritina	H	OH	H	H	OH
Promelacacinidina[b]	(epi)mesquitol	OH	OH	H	H	OH

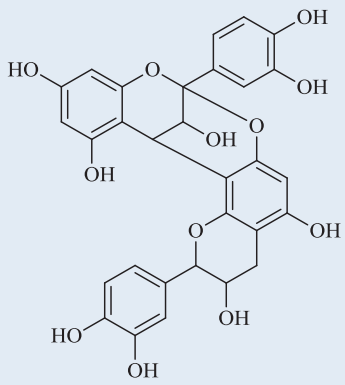
[a] contienen únicamente (epi)catequina

[b] contienen al menos una unidad diferente a (epi)catequina

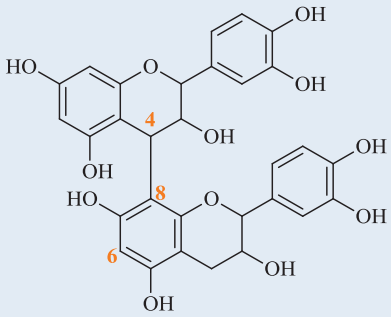
Otros muchos tipos de proantocianidinas han sido encontrados en extractos vegetales (Hümmer *et al.* 2008). Las profisetinidinas y prorobinetinidinas son las principales proantocianidinas en quebracho (Vivas *et al.* 2004), mientras que en habas pintas y canela se encuentran propelargonidinas (Gu *et al.* 2003b) y el té es estudiado por sus prodelfinidinas (Kiehne *et al.* 1997; El-Shahawi *et al.* 2012).

Según el tipo de unión las proantocianidinas pueden ser de tipo A y de tipo B (**Figura 1.6**). En los enlaces de tipo B, las unidades de flavan-3-ol se encuentran unidas por el enlace carbono-carbono entre el carbono C4-C6 o C4-C8, siendo los segundos más abundantes. Las proantocianidinas de tipo A, además de este enlace interflavánico, presentan una unión adicional entre el carbono C2 y el hidroxilo del carbono C7 principalmente (Hümmer *et al.* 2008). Además de este enlace C2-O7, se ha descrito en cacao enlaces de tipo A C2-O5 (Porter et al. 1991).

Figura 1.6. Unión de proantocianidinas de tipo A y B



Procianidina Tipo A



Procianidina Tipo B

Mientras que las proantocianidinas de tipo B, principalmente procianidinas, se encuentran en muchas especies de plantas como el cacao, manzana, té, uva etc., son escasas las fuentes naturales en las que se han identificado proantocianidinas de tipo A (ciruela, cacahuete, aguacate, canela y curry) (Lou *et al.* 1999; Gu *et al.* 2003a). Las proantocianidinas de tipo A son más resistentes a la despolimerización, por ejemplo, en condiciones de tiólisis.

La gran cantidad de grupos hidroxilo que poseen estos compuestos hace que presenten gran reactividad con otras moléculas, en particular presentan una alta afinidad por las proteínas (Hagerman *et al.* 1980; Santos-Buelga *et al.* 2000). Esta reactividad viene determinada por el grado de polimerización del compuesto, tipos de enlace interflavánicos y patrón de hidroxilación (**Figura 1.7**). Es necesario conocer estas diferencias para entender que efectos

biológicos pueden tener y cómo pueden afectar al metabolismo (Foo *et al.* 2000a; Prior *et al.* 2005).

Evaluar el contenido y la composición de estos compuestos poliméricos en diversos alimentos y fuentes vegetales implica una dificultad añadida debido a la complejidad de su extracción. Esto hace que, en muchos casos, estos compuestos no hayan sido debidamente caracterizados, y que en los datos encontrados en literatura sobre proantocianidinas en alimentos sólo se evalúen (epi)catequinas de bajo grado de polimerización por ser éstas más fácilmente extraíbles.

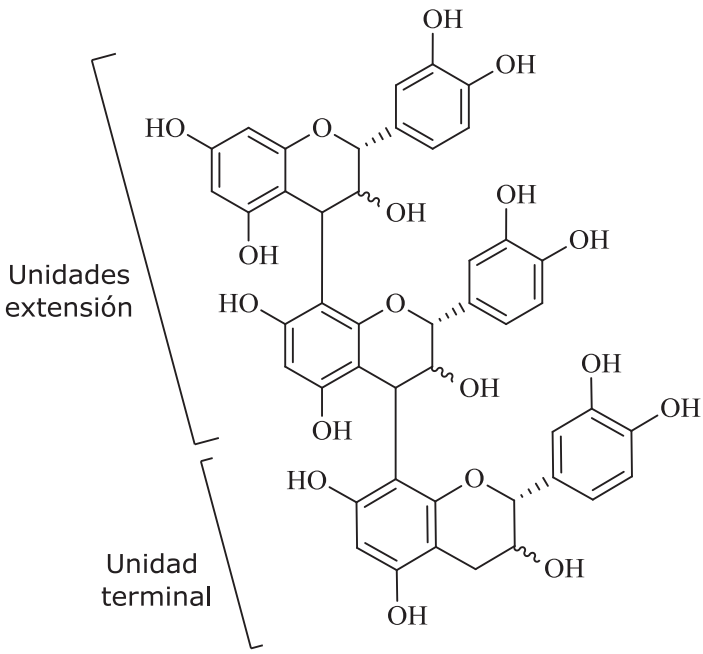


Figura 1.7. Ejemplo de tanino condensado

1.2.3.1. Proantocianidinas no extraíbles (NEPA)

Una parte de las proantocianidinas presentes en el tejido de la planta no son extraíbles. Un error común en la determinación del contenido de proantocianidinas en matrices vegetales es analizar únicamente las proantocianidinas extraíbles cuando se ha demostrado que los métodos más eficaces no logran extraer la mayor parte de las proantocianidinas, que quedan retenidas en la matriz y por eso se han definido como no-extraíbles (non-extractable proanthocyanidins, NEPA) (Arranz *et al.* 2009).

Varias técnicas han sido utilizadas y descritas para la extracción de compuestos polifenólicos de una matriz vegetal, como pueden ser la extracción por ultrasonidos, extractor soxhlet, microondas, extracción sólido-

líquido con distintos disolventes, etc. La elección del método está en función de los compuestos que se quieren extraer, pero hasta hoy no existe un método totalmente eficaz, siendo este paso de la extracción uno de los más críticos. El método más común para la obtención de proantocianidinas es la extracción con disolventes orgánicos. Concretamente, el método más efectivo se realiza con una mezcla de agua ácida y acetona (30:70, v/v) (Hussein *et al.* 1990), ya que la acetona está especialmente indicada para la extracción de proantocianidinas poliméricas (Prior *et al.* 2005).

El rendimiento de los métodos de extracción no depende solamente del disolvente, sino que la cantidad de proantocianidinas no extraídas también varía según la complejidad de la matriz y del grado de polimerización del compuesto. Además existen otros factores como la temperatura y el tiempo de extracción que pueden interferir en el proceso. La naturaleza de las proantocianidinas a extraer también es importante. Así, por ejemplo, las prodelfinidinas son mucho menos extraíbles que las procianidinas (Hümmer *et al.* 2008). De este modo la cantidad de compuestos polifenólicos no extraíbles puede llegar incluso hasta el 97% en algunos casos (Matthews *et al.* 1997).

Los procedimientos que se utilizan para la caracterización de proantocianidinas no extraíbles son exclusivamente espectrofotométricos, requieren la destrucción de la muestra y no son capaces de determinar el tipo de compuesto polifenólico presente en la planta. Debido a la diversidad química de los compuestos polifenólicos, es importante avanzar en la determinación de la composición de compuestos polifenólicos totales que se consumen en la dieta, teniendo en cuenta valores reales en los que se incluyen tanto extraíbles como no extraíbles. En las bases de datos de compuestos polifenólicos, se pueden encontrar la composición y concentración aproximada de polifenoles en algunos alimentos y fuentes naturales de origen vegetal, aunque generalmente las bases de datos no contemplan el contenido en polifenoles no extraíbles.

2. ACTIVIDAD BIOLÓGICA

Las plantas han sido desde siempre una fuente tradicional de remedios contra distintas enfermedades. Ya desde la antigüedad se pretendía conocer los secretos de la naturaleza y las estrellas. Hipócrates, considerado como el padre de la medicina y precursor de la dietética, se basaba en el poder curativo de la naturaleza. Hoy en día, el 80 % de la población mundial utiliza productos naturales para el cuidado de la salud, ya sea de una forma directa o indirecta (Atul Bhattaram *et al.* 2002).

Los extractos y otros productos procedentes de plantas contienen una multitud de componentes que podrían ser responsables de sus efectos terapéuticos. Los productos naturales son mezclas complejas de compuestos y sus actividades fisiológicas, preventivas o terapéuticas podrían ser el resultado de la acción combinada y/o sinérgica de varios componentes a veces desconocidos (Baur et al. 2006). El papel de los extractos naturales como activos alternativos o complementarios a los fármacos existentes es de particular importancia en el caso de desarrollo de resistencias de microorganismos. Por tanto, es necesario definir tantos componentes como sea posible con el fin de entender y explicar su actividad.

La actividad antioxidante es un foco de atención en el estudio de productos vegetales. Otra actividad relevante es la antiviral. El descubrimiento de nuevos agentes antivirales no tóxicos a partir de especies vegetales se considera un complemento o co-adyuvante para el tratamiento de infecciones víricas como el Herpes o el SIDA.

Las plantas medicinales pueden ser una fuente para el aislamiento de compuestos puros que actúan contra estas infecciones víricas. A pesar de la cantidad de información acerca de extractos de plantas contra HSV, no se han identificado, aislado y probado todos los compuestos bioactivos responsables de esta actividad (Khan *et al.* 2005).

Anteriormente han sido atribuidas propiedades antivirales a extractos polifenólicos de plantas (De Bruyne *et al.* 1999b). Por ejemplo, el

resveratrol, un estilbeno de origen natural que se encuentra en vinos, uvas y otras plantas, inhibe eficazmente la replicación del HSV-1 y HSV-2 (Docherty *et al.* 1999) aunque la relación entre su estructura y actividad no ha sido claramente establecida.

2.1. Bioactividad de proantocianidinas

Es importante destacar el gran número de posibles efectos funcionales, preventivos y terapéuticos que tienen las proantocianidinas. Entre los principales efectos biológicos estudiados están sus propiedades antivirales y antimicrobianas, además de las citadas anteriormente como antioxidantes, antitumorales, cardioprotectoras y antiarterioscleróticas (De Bruyne *et al.* 1999a; Shahat *et al.* 2002; Jerez *et al.* 2007). La estructura del esqueleto polifenólico y la posición de los grupos hidroxilo determinan las interacciones de los polifenoles con sistemas biológicos.

En los ensayos de actividad antibacteriana y actividad antiviral el grado de polimerización, el porcentaje de galoización, el tipo de enlace interflavánico y la estereoquímica influyen fuertemente en su capacidad inhibidora (Takechi *et al.* 1985; De Bruyne *et al.* 1999b; Gescher *et al.* 2011). Se cree que el principal mecanismo antiviral de las proantocianidinas es la unión a las proteínas de la envoltura del virus o de la membrana de la célula huésped impidiendo la unión del virus y la penetración de la membrana plasmática (Fukuchi *et al.* 1989).

Adicionalmente, se ha descrito que las proantocianidinas tienen efectos sobre la modulación de enzimas relacionadas con el estrés oxidativo (Middleton *et al.* 2000), la interacción con las membranas biológicas (Oteiza *et al.* 2005), la interacción con microbiota, y además son excelentes agentes quelatantes de metales (De Bruyne *et al.* 1999a; Scalbert *et al.* 2000a; Cos *et al.* 2004), aunque a generalmente se atribuyen sus múltiples efectos sobre la salud a su capacidad antioxidante.

3. ACTIVIDAD ANTIOXIDANTE

Un ***antioxidante*** es una molécula capaz de disminuir, retardar o inhibir la oxidación de otras moléculas o sustratos. Los antioxidantes actúan en reacciones redox cediendo un electrón o un átomo de hidrógeno. De esta manera pueden ejercer su acción reparando un daño oxidativo, captando directamente o neutralizando radicales libres o especies reactivas de oxígeno (ROS) o especies reactivas de nitrógeno (RNS) (Halliwell *et al.* 1995; Boots *et al.* 2008).

Un ***radical libre*** es una molécula que contiene un electrón desapareado en el orbital más externo de su estructura atómica. Estos electrones desapareados confieren al radical una gran reactividad química. Esta gran reactividad le concede una baja especificidad química de manera que pueden reaccionar con cualquier molécula adyacente para conseguir una configuración electrónica estable, es por esto que también son conocidas como especies reactivas (Aruoma 1998).

La importancia de las reacciones de oxidación-reducción en la naturaleza es manifiesta. En el organismo, debido al metabolismo aeróbico, se generan especies intermediarias altamente reactivas. La generación de ROS forma parte del metabolismo normal de una célula y está inevitablemente asociado a la vida, pero un desequilibrio puede conducir a un estado de estrés oxidativo (Gutteridge 1995).

Como ***estrés oxidativo*** nos referimos al desequilibrio entre las especies oxidantes y la capacidad de los sistemas de defensa del organismo para hacer frente a la agresión oxidativa. Este desequilibrio en favor de los oxidantes genera un daño oxidativo en las biomoléculas (Mayne 2003). Así, un exceso de oxidación y por lo tanto de radicales libres pueden dañar las macromoléculas y alterar procesos celulares. Se ha comprobado cómo el daño oxidativo causado por las ROS a las biomoléculas guarda relación con numerosas enfermedades crónicas y degenerativas, como puede ser enfermedades cardiovasculares, alzhéimer o ciertos tipos de cáncer entre otras (Halliwell 1997) ya que estas especies altamente reactivas pueden

alterar la estructura y función de membranas celulares, carbohidratos, proteínas, ARN y ADN. Esta es la causa principal de que cada vez haya más estudios focalizados en cómo los antioxidantes pueden prevenir el desarrollo de muchas de estas enfermedades (Bagchi et al. 2000).

Las fuentes de ROS pueden ser tanto endógenas como exógenas, destacando entre estas últimas el consumo de tabaco o la exposición a radiaciones. El propio organismo, para minimizar el daño que las ROS pueden producir en las biomoléculas, ha desarrollado un sistema de defensa enzimático para hacer frente a estas especies reactivas de oxigeno. Aun así, las células también utilizan una serie de compuestos antioxidantes de origen externo para su defensa que son obtenidos a través de la dieta.

Los antioxidantes naturales tienen un papel protector frente al estrés oxidativo y han sido usados como agentes quimiopreventivos (Moure *et al.* 2001). Estas propiedades beneficiosas podrían estar estrechamente vinculadas a la actividad antioxidante de estos compuestos gracias a la capacidad de captar radicales libres, por lo que se suele asociar la actividad biológica de los compuestos polifenólicos con su capacidad captadora de radicales libres que normalmente se asocia a la capacidad antioxidante (Rice-Evans *et al.* 1997; Aruoma 2003; Lee *et al.* 2006; Silva *et al.* 2007). Por otro lado, se ha demostrado que los organismos que viven más años son aquellos que tienen mejores defensas antioxidantes (Finkel *et al.* 2000).

3.1. Compuestos polifenólicos como antioxidantes

Concretamente los polifenoles, debido a su acción captadora de radicales libres, pueden prevenir del daño oxidativo causado por éstos, ya que son excelentes donadores de protones o electrones. Esta acción podría ser preventiva del envejecimiento celular y de ciertas enfermedades que cursan con estrés oxidativo.

La estructura química de estos compuestos, con un número variable de grupos hidroxilos, determina su capacidad antioxidante (Balasundram *et al.* 2006) ya que estos grupos hidroxilo ceden electrones o átomos de hidrógeno neutralizando los radicales. Se ha observado que los compuestos con mayor capacidad antioxidante los que presentan dos grupos hidroxilos en posición orto en el anillo B. Además, debido a la deslocalización por resonancia en el anillo aromático del electrón desapareado y a la ausencia de posiciones para el ataque por el oxígeno molecular, los compuestos polifenólicos tienen intermedios radicalarios relativamente estables (Siquet et al. 2006).

En el caso concreto de las proantocianidinas, son pocos los estudios publicados que relacionan su estructura y actividad debido a la complejidad de estas moléculas. Tanto el grado de galoización como el grado de polimerización son determinantes, ya que, como se ha mencionado, la actividad antioxidante depende directamente del número de grupos hidroxilos (Torres *et al.* 2002; Vissers *et al.* 2004; Touriño *et al.* 2005). Aún así, no hay resultados concluyentes sobre el efecto que puede tener el grado de polimerización sobre la capacidad antioxidante del compuesto y en muchos casos los resultados son contradictorios (Santos-Buelga *et al.* 2000; Heim *et al.* 2002). Existen estudios donde se indica una correlación positiva entre el contenido de proantocianidinas poliméricas y el porcentaje de inhibición de radicales mientras que en otros métodos de evaluación de actividad antioxidante la correlación es negativa (Zessner et al. 2008).

La eficacia de los antioxidantes en una matriz compleja y en sistemas biológicos se ve afectada por muchos factores, desde el mecanismo de acción hasta las condiciones de reacción, que pueden afectar tanto a la cinética de oxidación como a la propia matriz (Plumb et al. 1996). Por lo tanto, es necesario combinar más de un método de medida en la determinación de la capacidad antioxidante (Plumb *et al.* 1998; Frankel *et al.* 2000; Aruoma 2003; Pérez-Jiménez *et al.* 2008a). Además, los compuestos medidos individualmente no reflejan necesariamente la capacidad antioxidante total ya que estos pueden actuar de forma sinérgica variando sus reacciones redox.

También es necesario considerar si los compuestos responsables de esta capacidad antioxidante son biodisponibles en el tracto gastrointestinal y, por tanto, si podrán ejercer el efecto beneficioso sobre el organismo tras su ingesta.

Principalmente hay dos tipos de mecanismos químicos para la oxidación de los polifenoles (Rice-Evans *et al.* 1997; Cos *et al.* 2004):

- Mecanismos de transferencia de un átomo de hidrógeno (HAT)
- Mecanismos de transferencia electrónica (SET)

3.1.1. Actividad antioxidante basada en mecanismos HAT

Uno de los métodos más comunes para evaluar la actividad antioxidante de los polifenoles es estudiar los mecanismos de transferencia de átomos de hidrógeno. Se trata de la donación de un átomo de hidrógeno por parte de un antioxidante para neutralizar un radical en un único paso, siendo el nuevo compuesto formado mucho más estable que el inicial.

$$PhOH + R^{\bullet} \longrightarrow PhO^{\bullet} + HR$$

Para que estos métodos sean efectivos se deben realizar con un radical libre estable. Son reacciones muy rápidas, aunque en ciertos métodos basados en mecanismos HAT se ha comprobado que los disolventes pueden influir de forma significativa en los resultados (Fernández-Pachón et al. 2004; Villano et al. 2005).

3.1.1.1. Ensayo ORAC (Capacidad de absorción de radicales de oxígeno)

El ensayo ORAC es un método comúnmente utilizado para medir la actividad antioxidante en entornos tanto hidrofílicos como lipofílicos de vegetales (Wu et al. 2004). Los radicales peróxidos generados por la descomposición térmica del 2,2'-azo-bis(2-amidinopropano)dihidrocloruro (AAPH) reaccionan

con la fluoresceína (FL) inhibiendo su fluorescencia mientras que los antioxidantes presentes en la muestra neutralizan los radicales libres retrasando la pérdida de fluorescencia.

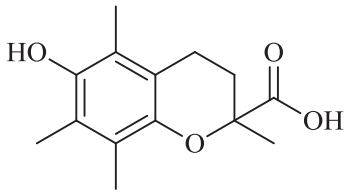


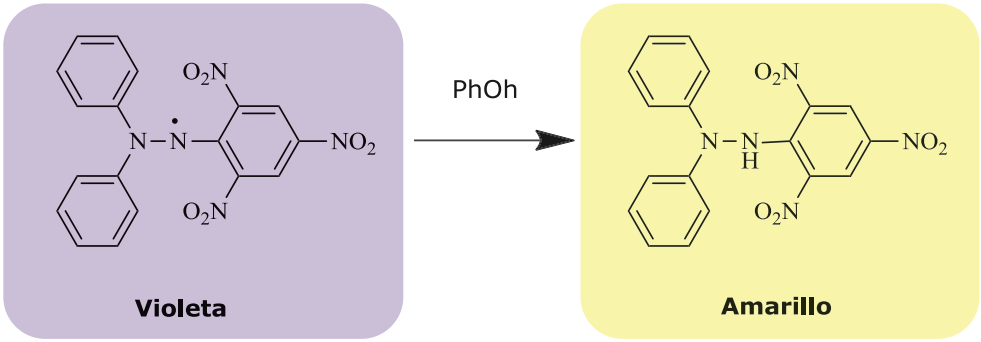
Figura 3.1. Trólox

La fluorescencia se mide durante 120 min hasta que se reduce a menos de un 5% de su valor inicial. Para cada concentración de antioxidante, se cuantifican las diferentes áreas bajo la curva en todas las cinéticas restando el valor del área del blanco. Se representan los valores del área neta frente a la cantidad de extracto siendo la pendiente de esta curva el valor ORAC. Usualmente se expresa como equivalentes de trólox (ácido 6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxílico), siendo este un análogo hidrosoluble de la vitamina E (**Figura 3.1**).

3.1.1.2. Ensayo del radical DPPH

El método del radical DPPH (2,2-difenil-1-picrilhidracil) es un método ampliamente utilizado para evaluación de la actividad antioxidante total en alimentos y extractos vegetales ya que es útil para el estudio de la actividad captadora de radicales libres, independientemente de cualquier actividad enzimática, además de su por su rapidez, sencillez y bajo coste. Este método colorimétrico de evaluación de actividad antioxidante se basa en la reducción del radical estable DPPH, el cual tiene una coloración violeta que se pierde progresivamente a medida que reacciona con los compuestos antioxidantes de la muestra, produciendo un descenso de la absorbancia a 515 nm (Brand-Williams *et al.* 1995) (**Figura 3.2**).

Figura 3.2. Reacción del radical DPPH



No obstante, este método presenta algunos inconvenientes. Uno de ellos es que existen otros compuestos que absorben a la misma longitud de onda que el radical, como pueden ser los carotenoides, por lo que interferirían en los resultados. Por otro lado, las moléculas pequeñas tienen menor impedimento estérico para reaccionar con el radical, por lo que darían mayor actividad antioxidante que moléculas con mayor peso molecular (Prior *et al.* 2005). El método no es adecuado para la determinación de la capacidad antioxidante del plasma, ya que las proteínas precipitan con el metanol del medio de reacción (Sánchez-Moreno 2002).

3.1.1.3. Ensayo TRAP (Parámetro antioxidante total de captura de radicales peroxilo)

Este ensayo se desarrolló principalmente para medir la capacidad antioxidante en plasma. Determina la eficacia con la que los compuestos antioxidantes captan los radicales peroxilos generados a partir del AAPH. Se mide por fluorescencia y como patrón de referencia se usa trólox (Wayner et al. 1985).

3.1.2. Actividad antioxidante basada en mecanismos SET

Los métodos basados en mecanismos de transferencia electrónica miden la capacidad que tiene un antioxidante para transferir un electrón y reducir un compuesto. Estas reacciones son lentas y dependen en gran medida del pH, por lo que el resultado de estos ensayos se basa en el porcentaje de disminución del radical. Esta transferencia electrónica depende directamente del potencial de ionización del compuesto antioxidante siendo más fácil la reacción a menores valores de estos (Wright *et al.* 2001).

3.1.2.1. Ensayo FRAP (Poder antioxidante de reducción del hierro)

Este método determina la capacidad del antioxidante para reducir el complejo de la tripiridiltriazina férrica a su forma ferrosa en medio ácido (Benzie *et al.* 1996). La reacción produce un cambio de color de intensidad proporcional a la actividad reductora, que es monitorizado midiendo la absorbancia a 595 nm durante 30 minutos para completar la reacción (Pulido et al. 2000).

Los inconvenientes de esta técnica son que el potencial de reducción del Fe (III) a Fe (II) es de 0,77 V, por lo que un compuesto con menor potencial redox podría reducir al Fe (III) (Ou et al. 2002). Además existen antioxidantes que no son capaces de llevar a cabo esta reacción dando resultados negativos. Asimismo, se han descrito interferencias de otros compuestos (Prior *et al.* 1999).

3.1.2.2. Ensayo TEAC (Capacidad antioxidante equivalente de trólox)

El ensayo TEAC o también conocido como ensayo ABTS (ácido 2,2-azinobis-(3-etilbenzotioazolín-6-sulfónico)), está basado en la captación del radical catión $ABTS^{\cdot+}$ por los antioxidantes. El radical catión del ABTS generado posee una coloración verde-azulada que disminuye debido a la reducción del radical por el antioxidante, es decir se produce una decoloración que es

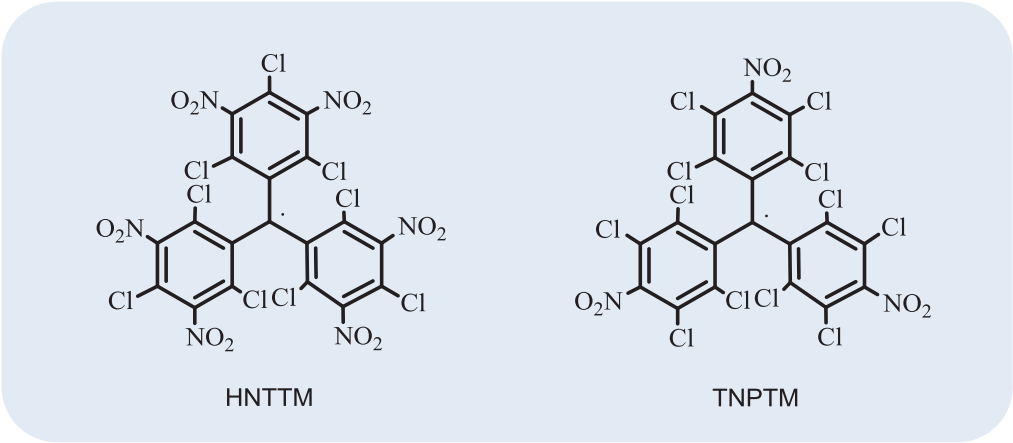
medida a una longitud de onda de 734 nm (Sánchez-Moreno 2002). Como patrón se emplea el trólox.

El $ABTS^{\cdot+}$ es soluble en disolventes orgánicos y acuosos, lo que hace que sea un método apto para determinar la capacidad antioxidante tanto lipofílica como hidrofílica de extractos y fluidos biológicos (Schlesier *et al.* 2002; Prior *et al.* 2005).

3.1.2.3. Ensayo de los radicales HNTTM y TNPTM

Los radicales HNTTM (tris(2,4,6-tricloro-3,5-dinitrofenil)metilo) y TNPTM (tris(2,3,5,6-tetracloro-4-nitrofenil)metilo) son radicales estables y solubles en solventes orgánicos de diferente polaridad (**Figura 3.3**). Presentan la propiedad de reaccionar exclusivamente por reacciones de transferencia electrónica por lo que se utilizan para monitorizar reacciones redox de compuestos polifenólicos con diferentes capacidades reductoras (Carreras *et al.* 2012).

Figura 3.3. Radicales HNTTM y TNPTM



Estos radicales han sido sintetizados expresamente para evaluar la capacidad de transferencia electrónica de los compuestos polifenólicos tanto naturales como sintéticos, pudiendo medir esta tanto por UV como por resonancia paramagnética electrónica (EPR) (Torres *et al.* 2007; Carreras *et al.* 2009).
La ventaja que presentan estos radicales con respecto al del ión férrico es que se evita la posibilidad de interferencias por la unión entre los compuestos polifenólicos y el hierro (Carreras *et al.* 2012).

El radical TNPTM reacciona solamente con los hidroxilos más reactivos, por lo tanto es una herramienta muy útil para la detección de otros polifenoles más potencialmente beneficiosos ya que las estructuras más reactivas son también las que tienen mayor efecto sobre la viabilidad celular in vitro (Carreras *et al.* 2012).

3.2. ¿Antioxidantes o prooxidantes?

A la actividad antioxidante constatada que tienen los polifenoles se le ha atribuido el efecto beneficioso y protector sobre la salud. Por otra parte, se han observado efectos adversos a dosis altas atribuidos a una acción pro-oxidante (Lambert *et al.* 2007).

Los compuestos prooxidantes son aquellos que promueven el daño oxidativo en sistemas modelo mientras que los antioxidantes lo inhiben. Los polifenoles pueden ser prooxidantes en función de su naturaleza y concentración. En general, en dosis bajas tienen un efecto beneficioso pero a altas dosis pueden causar daño celular (Gutteridge *et al.* 2010).

Cada vez hay más estudios sobre cómo actúan estos compuestos a nivel celular modulando actividades enzimáticas (Puiggròs *et al.* 2005; Del Bas *et al.* 2008; Montagut *et al.* 2010b). Un efecto pro-oxidante leve puede resultar en un efecto final protector o antioxidante porque estimulan los sistemas antioxidantes endógenos. Este fenómeno es conocido como hormesis y explica los efectos beneficiosos del ejercicio físico moderado o la restricción calórica (Schulz *et al.* 2007; Touriño *et al.* 2009).

Aunque existen numerosos resultados sobre actividad antioxidante *in vitro* de múltiples extractos vegetales, no está claro que los polifenoles tengan un efecto *in vivo.* Hay quien piensa que los resultados *in vitro* son en muchos casos biológicamente irrelevantes (Gutteridge *et al.* 2010). Esto es debido en gran parte a que los polifenoles sufren una metabolización extensiva en el organismo y muchos de sus metabolitos son inocuos como captadores de

radicales libres. Para esclarecer el papel de los polifenoles, en particular los flavonoides como antioxidantes o prooxidantes en el organismo, es crítico comprender la naturaleza química de las formas absorbidas, bioaccesibles y biodisponibles. Los efectos que puedan ejercer los flavonoides podrían ser debidos, en gran manera, a sus metabolitos. Por ello es importante tanto la caracterización de los compuestos polifenólicos en extractos vegetales como los metabolitos generados a partir de éstos.

4. CARACTERIZACIÓN DE COMPUESTOS POLIFENÓLICOS

La complejidad de las estructuras químicas de los polifenoles y su amplia diversidad hace complicada su caracterización y el estudio de la relación entre la estructura y la actividad.

El estudio de la composición de los compuestos polifenólicos en muestras de origen vegetal está limitado a algunas variedades de plantas. Existe gran variedad de las mismas, en concreto las tropicales, que no han sido identificadas y pueden representar una importante fuente de polifenoles. Además, aparecen nuevas técnicas más potentes para detectar y caracterizar nuevos compuestos por lo que la composición de una gran parte de alimentos, incluso los que están ampliamente caracterizados, puede quedar obsoleta.

A continuación se presentan algunas de las metodologías analíticas para la caracterización de compuestos polifenólicos en matrices vegetales.

4.1. Espectroscopía UV-VIS

La espectroscopía UV-VIS es uno de los métodos más comúnmente utilizado para la detección y cuantificación de los distintos tipos de polifenoles.

Gracias a los anillos fenólicos que presentan estos compuestos, los polifenoles son excelentes cromóforos, lo que facilita su detección con espectroscopía UV-vis. Las diferentes familias absorben a una longitud de onda característica, la cual depende del número, posición y tipo de sustituyentes del compuesto. Así, los flavanoles presentan su máximo de absorbancia a 280 nm, los flavonoles a 360 nm y las antocianidinas a 550 nm. La utilización de detectores de diodos en línea (DAD) permite analizar una muestra a diferentes longitudes de onda y por lo tanto determinar diferentes familias de compuestos fenólicos (Merken *et al.* 2000).

Existen varios métodos colorimétricos para la determinación de compuestos polifenólicos. Estos ensayos son generalmente utilizados para la detección y cuantificación de polifenoles en plantas debido a su simplicidad. Los más usuales son citados a continuación.

4.1.1. Ensayo de Folin-Ciocalteu

El ensayo Folin-Ciocalteu se utiliza como medida del contenido en compuestos fenólicos totales en productos naturales (Singleton *et al.* 1965). El mecanismo de este ensayo se rige por una reacción redox basado en la oxidación de los grupos hidroxilos, por lo que se emplea frecuentemente en el estudio de las propiedades antioxidantes de alimentos vegetales para la medida de la actividad antioxidante total (Prior *et al.* 2005). La oxidación de los fenoles presentes en la muestra por un reactivo de molibdeno y wolframio en medio básico causa la aparición de una coloración azul que presenta un máximo de absorción a 765 nm y que se cuantifica por espectrofotometría tomando como referencia una recta patrón de ácido gálico.

El inconveniente principal de este ensayo es su escasa robustez frente a variaciones de volúmenes de muestra, concentraciones de reactivos, tiempo y/o temperatura de incubación utilizados. Esto hace que haya diferencias significativas en las medidas obtenidas, lo que dificulta la comparación de

resultados. Además, es un método poco selectivo puesto que existen diversas sustancias de naturaleza no fenólica que interfieren dando lugar a resultados erróneos (Singleton *et al.* 1999).

4.1.2. Ensayo de la vainillina

Otro método usado comúnmente para la determinación flavanoles, tanto monoméricos como poliméricos, es el ensayo de la vainillina (Price et al. 1978; Sun et al. 1998). La vainillina (3-metoxi-4-hidroxibenzaldehído) en medio ácido reacciona con el flavanol formando un cromóforo de color rojo cereza que es medido a 500 nm. La principal desventaja de este método consiste en que se reduce la capacidad de reaccionar con la vainillina con el grado de polimerización de las proantocianidinas, porque la reacción se da preferentemente con las unidades terminales (Butler *et al.* 1982).

4.1.3. Ensayo de proantocianidinas

Existe un método analítico específico para la cuantificación de proantocianidinas. Este método está basado en la despolimerización de estos polifenoles transformando en carbocationes de sus unidades de extensión (antocianidinas) gracias a la acción de una mezcla HCl/BuOH en caliente. Estas antocianidinas presentan color rojo intenso que son detectadas a un máximo de absorbancia de 550 nm.

El principal inconveniente de este método es que la reacción no es completa ya que depende de la estructura y grado de polimerización de las proantocianidinas. Además, puede haber otras reacciones secundarias interferentes que absorben a la misma longitud de onda. Este hecho, puede dar lugar a un error de estimación y por lo tanto su aplicación para el análisis cuantitativo de proantocianidinas es limitado (Scalbert 1992).

4.2. Espectrometría de masas

La espectrometría de masas consiste en la separación de especies químicas iónicas en función de su relación masa/carga (*m/z*) mediante un campo electromagnético. Esta técnica analítica es excepcional para identificar, cuantificar y elucidar la estructura de compuestos polifenólicos debido a su robustez, alta sensibilidad, especificidad, reproducibilidad y buenos límites de detección. Además, combinada con técnicas cromatográficas, hace posible la separación, purificación e identificación de compuestos polifenólicos siendo la técnica más utilizada para caracterizar compuestos en extractos vegetales.

Los dos aspectos críticos de la técnica de espectrometría de masas son el sistema de ionización y el analizador o detector de masas. Tanto la elección de la fuente de ionización como del detector de masas es sumamente importante dependiendo de la naturaleza de la muestra a analizar y del objeto de estudio ya que cada instrumento presenta diferente resolución, sensibilidad, velocidad de barrido, rango dinámico lineal o precisión, intervalo y exactitud de masas entre otros.

Existen diferentes tipos de detectores, los más utilizados son el detector de tiempo de vuelo, cuadrupolo y de trampa de iones. Por otro lado, la técnica de ionización más utilizada para los compuestos polifenólicos es la ionización por electrospray, ya que es una ionización suave y sensible y por lo tanto es adecuada para un amplio rango de compuestos (Guyot *et al.* 1997; Wu *et al.* 2003).

La evolución de las técnicas de espectrometría de masas ha facilitado la caracterización de moléculas, puesto que la opción de realizar MS/MS con una alta resolución posibilita la confirmación de compuestos elucidando fragmentos de su estructura con una masa exacta.

4.2.1. Espectrometría de masas en tándem

La espectrometría de masas en tándem se aplica habitualmente para la caracterización y cuantificación de compuestos fenólicos, ya que el estudio de la fragmentación es una técnica excelente para la caracterización estructural.

En la espectrometría de masas en tándem el primer detector selecciona, de entre todos los iones producidos por el sistema de ionización, un ión con un valor de *m/z* concreto, a este ión se le denomina ión precursor. Este ión precursor es fragmentado en la cámara de colisión. Allí es excitado por un potencial eléctrico y colisiona con moléculas de un gas inerte (helio, nitrógeno o argón) dando los iones producto que serán analizados en el segundo detector de masas.

A continuación se describen los espectrómetros de masas en tándem más utilizados para el análisis de compuestos fenólicos.

4.2.1.1. Triple cuadrupolo (QqQ)

El detector triple cuadrupolo (QqQ) ha sido particularmente utilizado para la cuantificación e identificación de metabolitos procedentes de compuestos polifenólicos en diferentes muestras biológicas complejas. Consiste en el acoplamiento en serie de tres cuadrupolos sencillos, donde el primer y tercer cuadrupolo pueden escanear o seleccionar masas mientras el segundo actúa como celda de colisión. En cada cuadrupolo se aplica un potencial de corriente continua y un potencial de radiofrecuencia. Sólo los iones que incluyan ese campo de radiofrecuencia en relación a su *m/z* alcanzarán el detector (**Figura 4.1**).

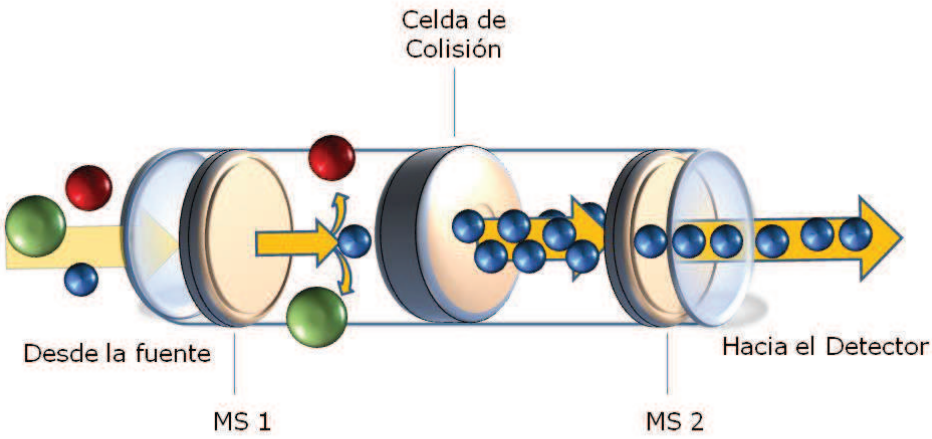


Figura 4.1. Analizador de Triple Cuadrupolo

El triple cuadrupolo es uno de los detectores de masas con mayor sensibilidad. Esta alta sensibilidad permite identificar analitos a bajas concentraciones en muestras complejas. Es por esto que esta técnica ha sido ampliamente utilizada para identificar y cuantificar metabolitos de compuestos fenólicos en muestras complejas de orina y plasma (Mata-Bilbao *et al.* 2007).

Otra ventaja del análisis con este detector es la posibilidad de realizar mediciones de masas con diferentes modos de MS/MS como son el barrido de iones producto, ión precursor, monitorización de reacción múltiple (MRM) y barrido de pérdidas neutras (NL), lo cual facilita la elucidación y cuantificación de compuestos.

En la monitorización de reacción múltiple (MRM) es seleccionado un ión precursor que contenga un ión producto específico y así se discrimina una molécula de todas las que no presenten esta combinación. Gracias además a su alta sensibilidad, este modo permite analizar cuantitativamente compuestos en mezclas complejas.

Otros modos que presentan una elevada selectividad son los de pérdidas neutras y de barrido de ión precursor. En el modo NL se monitorizan todos los iones producto que provienen de una pérdida de masa neutra concreta,

obteniendo así todos los iones precursores capaces tener esta pérdida. Por otro lado, el barrido del ión precursor selecciona todos los iones precursores que generan un ión producto característico.

El mayor inconveniente que presenta esta técnica es su baja resolución, el estrecho rango de masas y la dificultad para la determinar masas exactas. Además si no se conoce la estructura química y el perfil de fragmentación de los analitos a estudiar la elección de este equipo no es la más adecuada.

Entre las fuentes de ionización a presión atmosférica, para los compuestos polifenólicos la ionización por electrospray presenta mejor eficiencia (Rauha et al. 2001), empleada en el estudio de compuestos naturales tanto en modo de ionización positivo (ganancia de protones) como negativo (pérdida de protones).

4.2.1.2. Cuadrupolo- tiempo de vuelo (Q-TOF)

El detector Q-TOF junto con el Orbitrap son de los mejores detectores de espectrometría de masas para el descubrimiento de sustancias no conocidas y análisis de compuestos en muestras complejas. En concreto, el Q-TOF permite obtener el perfil de fragmentación de un ión precursor a la vez que tenemos una alta precisión en masa (masa exacta) tanto del precursor como de los fragmentos originados. La separación y detección de cada uno de los iones según su relación *m/z* se realiza mediante la aplicación de un campo magnético y aprovechando el diferente tiempo de vuelo de cada uno de ellos. La obtención de la masa exacta es imprescindible para elucidar la estructura molecular de los compuestos polifenólicos desconocidos en la muestra.

Por esto, el detector Q-TOF es una de las mejores herramientas para la identificación de compuestos en productos naturales que están formados por mezclas complejas que a menudo son difíciles de discernir si no se conocen aproximadamente las estructuras químicas que se buscan.

4.2.1.3. Ionización por desorción mediante láser asistida por matriz acoplado a tiempo de vuelo-tiempo de vuelo (MALDI-TOF/TOF)

Esta técnica ha sido empleada habitualmente en proteómica para el estudio de biomoléculas y recientemente está siendo utilizada para caracterizar polímeros naturales como las proantocianidinas (Dalluge 2002). La ionización por MALDI es una potente herramienta para el análisis de proantocianidinas gracias a la suave ionización que ejerce. La ionización del analito se realiza mediante un pulso corto de láser irradiado en una matriz que es cocristalizada con la muestra a estudiar. Como producto de esta irradiación, se produce la desorción de los iones de fase sólida a fase gaseosa dando lugar a una nube electrónica que es dirigida hacia el detector. La matriz que se incorpora a la muestra absorbe la mayor parte de la energía, de forma que protege los analitos de la energía directa del láser. Así, las moléculas se ionizan sin que se produzca apenas fragmentación y son finalmente analizadas en el detector (Pasch et al. 2001). Un aspecto importante de esta técnica es la preparación de la muestra que debe ser lo más homogénea posible entre las moléculas del analito y la matriz durante el proceso de cocristalización.

Actualmente es la única técnica capaz de detectar estructuras de proantocianidinas de alto grado de polimerización (Monagas *et al.* 2010a). Esta técnica presenta otras ventajas como la rapidez de análisis y su alta sensibilidad comparada con otros métodos, lo que nos permite una completa identificación de los compuestos. Además, se puede utilizar con muestras sólidas y líquidas independientemente de su volatilidad y polaridad.

Esta técnica de ionización suele ir acoplada principalmente a un detector de masas de tiempo de vuelo (TOF). Estas dos técnicas combinadas presentan la ventaja de proporcionar un espectro de masas completo con un alto rango de masas (Prasain *et al.* 2004) (**Figura 4.2**).

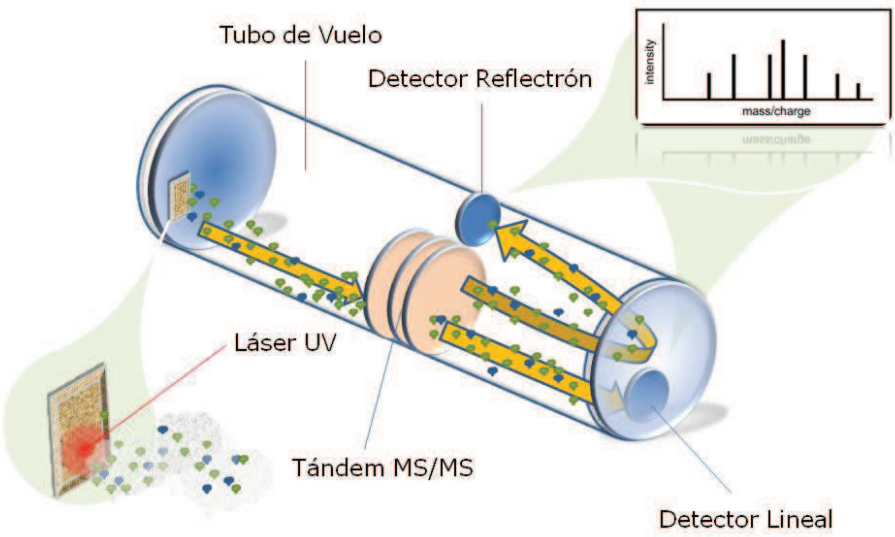


Figura 4.2. MALDI-TOF/TOF

En caso de tener dos analizadores de TOF acoplados al MALDI, además de obtener el valor exacto de la masa del compuesto, es posible obtener también información sobre la fragmentación de los analitos. En este caso el primer detector de TOF selecciona un ión precursor específico que se fragmenta en la cámara de colisión siendo los iones resultantes analizados por el segundo detector TOF. La fragmentación de los iones en MALDI se puede realizar por disociación inducida por laser (LID) o por disociación inducida por colisión (CID).

En resumen, el tiempo de vuelo (TOF) es una excelente herramienta para estudios donde se necesitan una elevada resolución y mediciones exactas de masa.

5. BIOACCESIBILIDAD Y BIODISPONIBILIDAD

Los efectos beneficiosos para el organismo derivados del consumo de compuestos polifenólicos dependen principalmente de la cantidad consumida y de su biodisponibilidad, por lo que determinar estos parámetros en la dieta es un factor indispensable para evaluar el papel de éstos en la prevención de enfermedades. Es difícil estimar la ingesta diaria de polifenoles en la dieta (Duthie et al. 2003) ya que, como mencionamos anteriormente, su concentración depende de diferentes factores: genéticos, geográficos, agronómicos, etc. Por otro lado, la metabolización de los compuestos polifenólicos depende de su estructura química, su interacción con la matriz alimentaria y la combinación de alimentos en la dieta (Aruoma 2003).

Nutracéutico o **Alimento Funcional** es un alimento o componente que proporciona efectos beneficiosos sobre la salud siendo capaz de mejorar la salud porque ayuda a mantener el correcto funcionamiento fisiológico del organismo.

La **bioaccesibilidad** se define como la capacidad de una sustancia de interaccionar y ser absorbida por un organismo. Se refiere principalmente a la interacción con el sistema digestivo. La **biodisponibilidad** se define como la cantidad de sustancia que se absorbe y que llega a la circulación sistémica (Aggett 2010).

El estudio del perfil metabólico a diferentes niveles (intestino, sangre, orina) proporciona información sobre qué compuestos podrían ser los responsables de la posible actividad beneficiosa para el organismo. Dentro de la bioaccesibilidad y biodisponibilidad se integran los procesos de liberación, absorción, distribución, metabolismo y excreción que se definen a continuación:

Liberación: separación de un compuesto de su matriz después de la administración.

Absorción: transporte o difusión de un compuesto desde la administración hasta el torrente sanguíneo.

Distribución: difusión de un compuesto a los diferentes tejidos del organismo.

Metabolismo: conversión o transformación química que sufre un compuesto en el organismo. Fase I, degradación de la molécula en entidades más pequeñas. Fase II, conjugación con otras moléculas para facilitar el transporte y la excreción.

Excreción: eliminación de un compuesto y/o sus metabolitos del organismo.

5.1. Absorción y metabolismo

Tal como se ha comentado anteriormente, la biodisponibilidad de los polifenoles depende de su estructura química, la cual determina su absorción intestinal y metabolización (Manach et al. 2004). Otro de los factores a tener en cuenta es la complejidad y variabilidad de la matriz. Los polifenoles interactúan con otros compuestos de la matriz como por ejemplo las proteínas, que pueden modificar el proceso de absorción (D'Archivio *et al.* 2010). Se han realizado pocos estudios sobre cómo influyen los componentes de los alimentos en la biodisponibilidad de los polifenoles (Donovan *et al.* 1999).

Una vez ingeridos, la mayoría de los compuestos polifenólicos de bajo peso molecular, como pueden ser algunos ácidos fenólicos y polifenoles monoméricos y diméricos, se mantienen estables en condiciones ácidas resistiendo la hidrólisis ácida del estómago. Así llegan intactos al intestino delgado donde pueden ser absorbidos o parcialmente absorbidos, metabolizados y excretados rápidamente (Okushio *et al.* 1999; Kuhnle *et al.* 2000; Donovan *et al.* 2001). Además una vez absorbidos y metabolizados en

el hígado pueden regresar al intestino vía bilis y alcanzar el colon (Monagas *et al.* 2010b) (**Figura 5.1**).

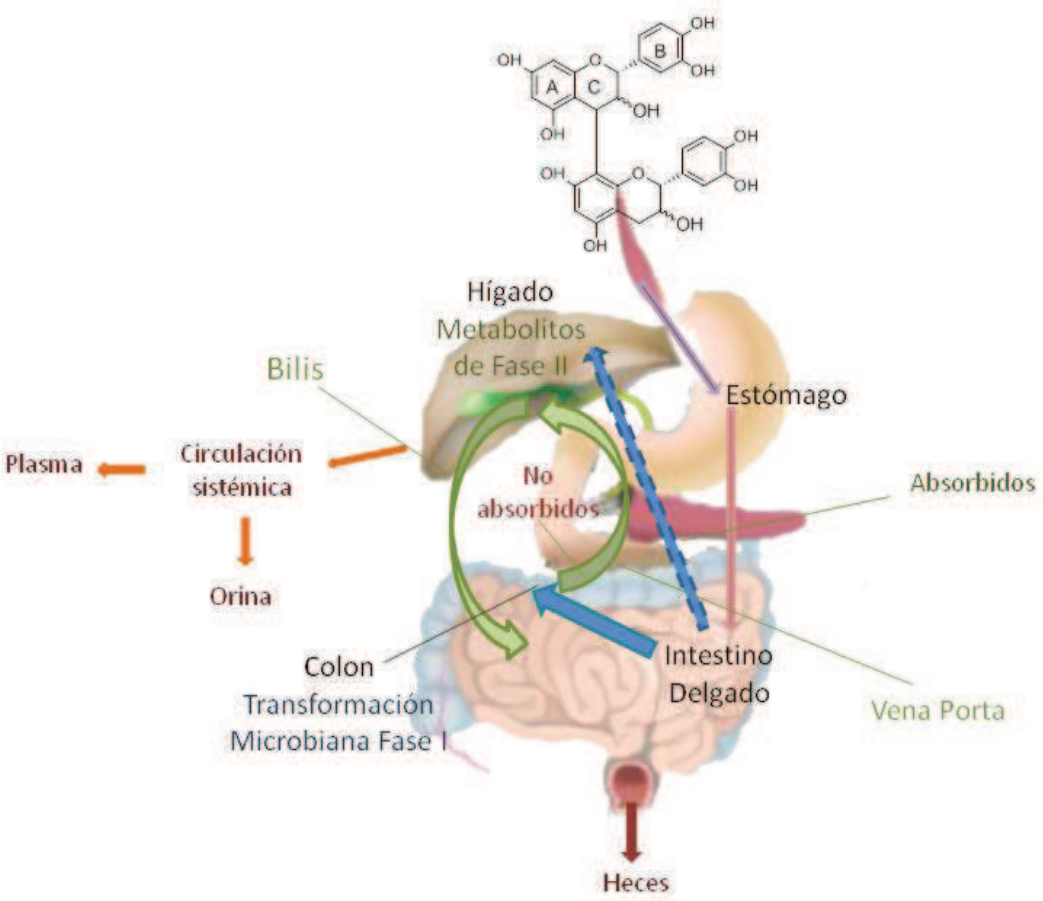


Figura 5.1. Esquema de metabolización

Una vez que los compuestos polifenólicos pasan por el estómago donde comienza el proceso de absorción, alcanzan el intestino delgado donde empiezan los procesos de transformación enzimática. Estos se suelen dividir en procesos de metabolismo de Fase I y de Fase II.

En el proceso de ***metabolismo de Fase I***, los compuestos son sometidos a reacciones de reducción, oxidación e hidrólisis y pueden ser desconjugados y degradados a unidades más pequeñas. Los compuestos polifenólicos que no puedan ser absorbidos directamente en el intestino deben ser previamente

hidrolizados por enzimas intestinales o por la microbiota del colon. En el colon se produce la hidrólisis por acción de las bacterias presentes dando lugar a ácidos aromáticos. Estos compuestos pueden ser reabsorbidos prolongando la presencia de los compuestos fenólicos en el organismo (Bosscher *et al.* 2009).

Las transformaciones de ***metabolismo de Fase II*** se dan principalmente en el hígado ya que su finalidad es modificar la polaridad de los compuestos para favorecer su excreción. Durante la absorción, los compuestos fenólicos son conjugados en el intestino e hígado mediante reacciones de metilación, glucuronidación, sulfatación, glutationación o sus combinaciones. Estos conjugados son transportados en plasma a los distintos tejidos y órganos, siendo excretados principalmente en orina y bilis.

5.2. Metabolización de las proantocianidinas

La absorción y metabolización de los compuestos polifenólicos está influenciada por factores tales como la solubilidad, la unión a azúcares o ácidos. El grado de polimerización de los compuestos polifenólicos también determina su absorción en el intestino. Los compuestos poliméricos, como las proantocianidinas, no son bioaccesibles como tales (Manach *et al.* 2004). Las proantocianidinas no se absorben en el intestino y llegan al colon donde son metabolizadas por la microbiota bacteriana (Déprez *et al.* 2000). Sin embargo, el establecimiento de la zona del sistema digestivo donde se realiza la absorción de las proantocianidinas poliméricas presenta cierta controversia (Donovan *et al.* 2002; Touriño *et al.* 2009).

Pocos son los estudios de biodisponibilidad que se centran en las proantocianidinas. Estudios realizados *in vitro* sugieren que la absorción de las proantocianidinas es baja ya que no pueden ser absorbidas por los enterocitos que forman la pared intestinal. Durante el curso de la absorción, los compuestos polifenólicos monoméricos son conjugados en el intestino e hígado mediante reacciones de metilación, glucuronidación, sulfatación, o

sus combinaciones. A pesar de su baja absorción, las proantocianidinas podrían ejercer efectos fisiológicos en el tracto gastrointestinal a nivel local antes y después de su degradación a ácidos fenólicos por la acción bacteriana (Manach *et al.* 2004).

Por otro lado, el estudio de estos compuestos se ve dificultado por la escasez de patrones para evaluar su respuesta directa. Se han llevado a cabo diversos experimentos con proantocianidinas marcadas con isótopos radiactivos para demostrar la presencia de metabolitos procedentes de estos compuestos en orina, heces, plasma y diversos órganos (Perez-Maldonado *et al.* 1996).

5.2.1. Despolimerización de proantocianidinas

Estudios *in vitro* confirman que las procianidinas poliméricas incubadas en condiciones anóxicas con microbiota procedente de colon humano son completamente degradadas después de 48 h (Déprez *et al.* 2000). Parte de los polifenoles que llegan intactos al intestino grueso son fermentados por la microbiota intestinal y despolimerizados a metabolitos fenólicos de bajo peso molecular, proporcionando un medio antioxidante en el colon y pasando después los productos obtenidos a través de la vena porta hasta el hígado, donde son metabolizados (Santos-Buelga *et al.* 2000; Heim *et al.* 2002). Especialmente, los compuestos polifenólicos que están fuertemente unidos al material vegetal que no son absorbidos en el intestino pueden contribuir a la captación de mutágenos fecales y genotoxinas (Stone *et al.* 1997). Las proantocianidinas, resistentes a la degradación por enzimas intestinales, podrían proteger de un posible daño oxidativo durante la digestión (Hagerman *et al.* 1998; Bagchi *et al.* 2000).

5.2.2. Metabolización de proantocianidinas no extraíbles

Además de los pocos estudios de biodisponibilidad realizados con proantocianidinas, los estudios que se han llevado a cabo de consumo de bebidas y alimentos de origen vegetal se han realizado haciendo una extracción previa de compuestos polifenólicos, donde se encuentran las proantocianidinas. Esto hace que los estudios estén incompletos ya que ignoran un elevado porcentaje de proantocianidinas que no son extraídas y no se han tenido en cuenta en los estudios de biodisponibilidad. Realmente no se sabe si estos compuestos están biodisponibles en el organismo ya que están fuertemente unidos a su matriz, por lo que es importante realizar estos estudios.

III. OBJETIVOS

OBJETIVOS

1. Objetivo general

El objetivo general de esta tesis es contribuir al estudio de la composición polifenólica de productos naturales de origen vegetal con alto contenido en proantocianidinas, de su metabolización en el organismo y de su actividad antioxidante y antiviral. La novedad del estudio radica en:

- La elección de productos con composición proantocianidínica heterogénea (canela) poco conocida (proantocianidinas no extraíbles de uva) o desconocida (extracto de *Chamaecrista nictitans*).
- La aplicación y puesta a punto de técnicas de espectrometría de masas para la caracterización de proantocianidinas del alto peso molecular en los tres productos estudiados.
- Relación entre la estructura de proantocianidinas y la actividad antivírica y antioxidante de éstas.

Los productos a estudiar fueron:

- Canela (*Cinnamomum zeylanicum*) en polvo como producto rico en proantocianidinas con actividad funcional sobre el metabolismo de la glucosa.

- Fracción de proantocianidinas no extraíbles (non-extractable proanthocyanidins, NEPA) de fibra antioxidante dietética de uva (Grape antioxidant dietary fiber, GADF), subproducto de la industria vitivinícola, como producto parcialmente responsable de la actividad preventiva de GADF sobre cáncer de colon.

- Extracto de *Chamaecrista nictitans* como producto seleccionado de la biodiversidad de Costa Rica por su actividad antiviral.

2. Objetivos específicos

1.- Caracterización de la composición polifenólica de productos naturales de origen vegetal mediante espectrometría de masas.

- Desarrollo y puesta a punto de un método analítico para la determinación de proantocianidinas de alto grado de polimerización procedente de canela común (*Cinnamomum zeylanicum*) por MALDI-TOF/TOF.

2.- Estudio de la metabolización de proantocianidinas procedentes de fuentes naturales con distinta matriz.

- Metabolización de proantocianidinas de alto peso molecular en canela (*Cinnamomum zeylanicum*).

- Metabolización de proantocianidinas no extraíbles de fibra antioxidante dietética de uva (*Vitis vinífera*).

3.- Establecimiento de la relación entre la estructura de proantocianidinas y compuestos relacionados y la actividad antivírica y antioxidante de un extracto vegetal de origen tropical.

- Elucidación de la composición polifenólica de *Chamaecrista nictitans* por LC-MS/MS (Q-TOF).

IV. RESULTADOS

RESULTADOS

Los resultados obtenidos en esta tesis doctoral se engloban en cuatro publicaciones firmadas como primera autora en revistas del primer cuartil del Science Citation Index.

Los resultados se presentan en tres bloques.

1. **Caracterización de la composición polifenólica de proantocianidinas de alto grado de polimerización procedente de canela por MALDI-TOF/TOF.**

   Publicación 1. New identification of proanthocyanidins in cinnamon (*Cinnamomum zeylanicum* L.) using MALDI-TOF/TOF mass spectrometry.

2. **Estudio de la absorción/metabolización de proantocianidinas.**

   2.1. Estudio de bioaccesibilidad y biodisponibilidad de proantocianidinas de canela.

   Publicación 2. Profile of urinary and fecal proanthocyanidin metabolites from common cinnamon (*Cinnamomum zeylanicum* L.) in rats.

   2.2. Estudio de bioaccesibilidad y biodisponibilidad de proantocianidinas no extraíbles de uva.

   Publicación 3. Non-extractable proanthocyanidins from grapes are a source of bioavailable (epi)catechin and derived metabolites in rats.

3. Estudio de la relación entre la estructura de proantocianidinas y su actividad antioxidante y antiviral.

Publicación 4. Identification of polyphenols from the antiviral *Chamaecrista nictitans* (Fabaceae) extract using high resolution LC-ESI-MS/MS.

1. Caracterización de la composición polifenólica de proantocianidinas de alto grado de polimerización procedente de canela por MALDI-TOF/TOF.

Publicación 1. Identificación de proantocianidinas en canela (*Cinnamomum zeylanicum* L.) usando MALDI-TOF/TOF.

New identification of proanthocyanidins in cinnamon (*Cinnamomum zeylanicum* L.) using MALDI-TOF/TOF mass spectrometry

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2012

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RESUMEN

La canela (*Cinnamomum zeylanicum* L.) es una de las especies más empleada en todo el mundo y ha sido utilizada en diversas culturas durante siglos. Tradicionalmente, la canela se ha empleado para uso culinario por su agradable olor y sabor y se ha aplicado para el tratamiento de distintas enfermedades en medicina tradicional (Gruenwald *et al.* 2010). El consumo de canela se ha asociado a propiedades antiinflamatorias, antibacterianas, a la reducción de la formación de coágulos sanguíneos y a la disminución de colesterol. Así mismo, ayuda al tratamiento de la diabetes (tipo 2) mediante la reducción de los niveles de azúcar en sangre y otros parámetros relacionados con el síndrome metabólico (Jean-Jacques Dugoua 2007; Kirkham *et al.* 2009) asociándose, en muchos casos, estos efectos beneficiosos a las proantocianidinas (Jiao *et al.* 2013).

A pesar de las múltiples propiedades que se citan y los estudios realizados en la caracterización de esta especie, hay pocos datos disponibles sobre el contenido de oligómeros y polímeros de flavanoles en alimentos debido a la falta de una adecuada metodología analítica y de patrones comerciales.

El objetivo principal de este trabajo es el desarrollo de un método analítico para la identificación de proantocianidinas con un alto grado de polimerización. En concreto, se identifican las proantocianidinas presentes en el extracto de canela con la técnica de ionización por desorción mediante láser asistida por matriz (MALDI) acoplada a detección por tiempo de vuelo (TOF). Esta es la primera vez que se realizan estudios con MALDI-TOF/TOF para este tipo de compuestos. Se utilizó como patrón un extracto de uva ampliamente caracterizado y conocido por su alto contenido en proantocianidinas.

Los resultados indican que la canela posee una alta heterogeneidad de polímeros de proantocianidinas de alto peso molecular con enlaces de tipo A, por lo que presentan una resistencia a la despolimerización mayor que las proantocianidinas de tipo B comunes en otras fuentes como la uva y la corteza de pino. Además, se han identificado ésteres de galatos en prodelfinidinas y procianidinas que no se habían descrito antes en canela. Este resultado es importante por cuanto los ésteres de galato se han relacionado con la actividad biológica en modelos de cáncer de colon (Lizárraga *et al.* 2007; Sánchez-Tena *et al.* 2013).

MALDI-TOF/TOF permite una detallada elucidación de los compuestos y la capacidad de obtener información estructural a través de los patrones de fragmentación obtenidos en los experimentos de MS/MS. La técnica destaca por tener una alta sensibilidad por compuestos con alto grado de polimerización y galoización.

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ORIGINAL PAPER

New identification of proanthocyanidins in cinnamon (*Cinnamomum zeylanicum* L.) using MALDI-TOF/TOF mass spectrometry

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Abstract The inner bark of Ceylon cinnamon (*Cinnamomum zeylanicum* L.) is commonly used as a spice and has also been widely employed in the treatment and prevention of disease. The positive health effects associated with the consumption of cinnamon could in part be due to its phenolic composition; proanthocyanidins (PA) are the major polyphenolic component in commercial cinnamon. We present a thorough study of the PA profile of cinnamon obtained using matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI-TOF/TOF) mass spectrometry. In addition to the advantages of MALDI-TOF as a sensitive technique for the analysis of high-molecular-weight compounds, the tandem arrangement allows the identification of the compounds through their fragmentation patterns from MS/MS experiments. This is the first time that this technique has been used to analyze polymeric PA. The results show that cinnamon PA are more complex than was previously thought. We show here for the first time that they contain (epi)gallocatechin and (epi)catechingallate units. As gallates (galloyl moieties) and the pyrogallol group in gallocatechins have been related to the biological activity of grape and tea polyphenols, the presence of these substructures may explain some of the properties of cinnamon extracts. MALDI-TOF/TOF reveals that cinnamon bark PA include combinations of (epi)catechin, (epi)catechingallate, (epi)gallocatechin, and (epi)afzelechin, which results in a highly heterogeneous mixture of procyanidins, prodelphinidins, and propelargonidins.

Keywords MALDI-TOF/TOF · Cinnamon · Polyphenols · Proanthocyanidins · Mass spectrometry

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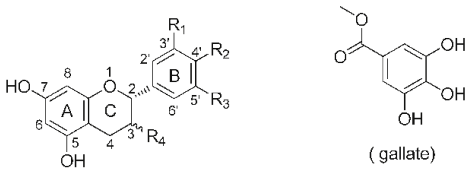
Introduction

Ceylon cinnamon (*Cinnamomum zeylanicum* L.) has been used as a spice in several cultures for centuries. In addition to its culinary uses, cinnamon has been employed in traditional herbal medicine to treat a variety of health conditions [1]. Generally, the part used as a spice or for medical purposes is the inner bark (cinnamomi cortex). Recently, the efficacy of cinnamon in the maintenance of physiologically balanced parameters has been studied, and new properties of interest for clinical use have been discovered. In particular, the most well-documented health benefit provided by this spice is related to the prevention and treatment of type 2 diabetes [2–5]. In addition, other evidence suggests that cinnamon may be effective in the treatment of cancer [6, 7] and infectious diseases [8, 9], and that it also shows anti-inflammatory [10, 11], antimicrobial [12–14], antioxidant [15–17], hypotensive [18], and cholesterol-lowering effects [5, 19].

The positive health effects associated with the consumption of cinnamon could in part be attributed to its phenolic composition [20–22]. Proanthocyanidins (PA), also known as condensed tannins, constitute the major type of polyphenols in commercial cinnamon [23]. PA are mixtures of oligomers and polymers composed of flavan-3-ol units (Fig. 1). PA consisting exclusively of (epi)catechin are



Flavan-3-ol	R_1	R_2	R_3	R_4
(Epi)afzelechin	H	OH	H	OH
(Epi)catechin	H	OH	OH	OH
(Epi)gallocatechin	OH	OH	OH	OH
(Epi)catechingallate	H	OH	OH	gallate
(Epi)gallocatechingallate	OH	OH	OH	gallate

Fig. 1 Most important structures of the flavan-3-ol units in proanthocyanidins

named procyanidins (PC), while those containing (epi) gallocatechin and (epi)afzelechin are called prodelphinidins and propelargonidins, respectively. Additionally, PC can be esterified with gallic acid, as happens in the case of grape tannins [24, 25]. The flavan-3-ol units are linked by C4 → C8 or C4 → C6 bonds (B type) and occasionally by an additional C2 → O7 ether bond (A-type) (Fig. 2). A particular characteristic of cinnamon is that most of its PA are A type [26], in contrast to most common sources of PA, such as grape, in which most are B type [24, 25].

The size of the proanthocyanidin molecules is an important feature that may influence their physiological role. The presence of polymeric PA with a high degree of polymerization is particularly interesting since in vitro and in vivo studies have suggested that large PA progressively release (epi) catechin units during their transit along the intestinal tract [27]. Most of these units, together with intact PA, reach the colon [28, 29], where they are processed by the colonic microbiota into phenolic acids and other absorbable metabolites [30, 31]. The array of compounds released and

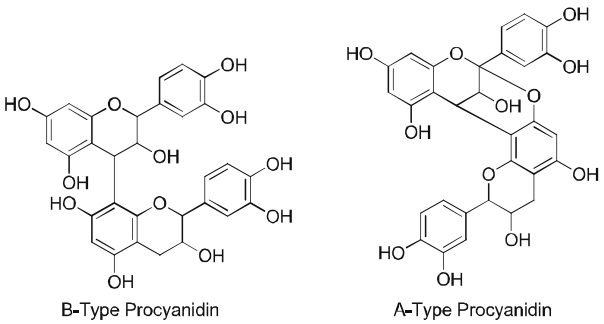


Fig. 2 Dimeric structures of procyanidins with B-type and A-type linkages

absorbed in the intestines by different physiological processes may contribute to the preventive effects of polyphenols.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) is a soft ionization MS technique which provides mass spectra that are mainly composed of signals corresponding to ions of intact molecules. It has the capacity to detect high-molecular-weight compounds and also to detect patterns of oligomers with small differences in mass. One of the main advantages of MALDI-TOF over other MS systems is its high sensitivity over a large mass range. However, the elucidation of complex polymeric species requires more sophisticated fragmentation analysis. MALDI-TOF/TOF provides high resolution, and in addition, MS/MS experiments can be performed on particular *m/z* values to obtain important information on the molecular structures of the polymeric compounds. MALDI-TOF has been widely used in the characterization of polymeric condensed tannins [32], but rarely in the analysis of cinnamon PA. Indeed, very few studies have looked at cinnamon PA at all [26, 33, 34]. MALDI-TOF/TOF, a technique widely used in proteomics, has not yet been applied to the analysis of PA. The aim of this work was to advance in the characterization of cinnamon PA and to show that MALDI-TOF/TOF is a sensitive and rapid technique suitable for detecting and characterizing new bioactive polymeric compounds in foods and natural extracts.

Experimental

Reagents and materials

Common cinnamon was purchased at a local market in Barcelona (Spain), and grape seed extract was from JF-NATURAL (Tianjin, China). The MALDI matrix, 2,5-dihydroxybenzoic acid (99%), and cesium trifluoroacetate (6 M aqueous solution) were from Sigma Chemical (Saint Louis, MO, USA); sodium chloride (99%) was from Carlo Erba Reagents (Rodano, Italy). For the extraction and fractionation of polyphenols, analytical grade hexane, acetone, and acetic acid (Merck, Darmstadt, Germany) were used. Trifluoroacetic acid (TFA; biotech grade distilled in-house) was from Fluorochem (Hadfield, UK). Water was purified using a Milli-Q plus system from Millipore (Bedford, MA, USA) to a resistivity of 18.2 MΩ cm.

Procedures

Sample extraction

Commercial cinnamon (5 g) was defatted with hexane (3× 40 mL) and air-dried overnight. The dried extract was extracted with 4 mL of a mixture of acetone/water/acetic acid

(7:2.5:0.5, *v*:*v*:*v*). The supernatant was decanted, filtered, and freeze-dried.

Sample preparation

Freeze-dried cinnamon extract or commercial grape extract (approximately 1 mg) was dissolved in aqueous 1% aqueous TFA (1 mL). The matrix solution was prepared by dissolving 2,5-dihydroxybenzoic acid (10 mg) and either sodium chloride or cesium trifluoroacetate (cationization reagent, 1 mg) in aqueous 1% aqueous TFA (1 mL). Aliquots of sample and matrix solutions were mixed (1:1, *v*:*v*), vortexed, and then deposited (2 μL) on the target plate. Once the solvent was dried (at room temperature), the crystals were analyzed by MALDI-TOF/TOF as detailed below.

MALDI-TOF/TOF mass spectrometry

A MALDI-TOF/TOF mass spectrometer (AutoFLEX III, Bruker Daltonics, Bremen, Germany) equipped with a pulsed N_2 laser (337 nm) controlled by the Flexcontrol 1.1 software package was used to obtain MS and MS/MS data. The accelerating voltage was 20 kV and the reflectron voltage 21 kV. Spectra are the sum of 500 shots with a frequency of 200 Hz. Both positive and negative reflectron modes were tried and the positive mode finally chosen, in agreement with the literature for this type of compounds [32]. The MS/MS spectra were obtained in the collision-induced dissociation (CID) mode using argon as the collision gas.

Results and discussion

Procyanidins in cinnamon

As this was the first time that cinnamon PA were analyzed by MALDI-TOF/TOF, we first examined the behavior of grape PA, which has been widely studied using several MS techniques, including MALDI-TOF MS [24, 25, 35–38]. The mass range analyzed was the same for both the cinnamon and grape extracts; to confirm the identity of the compounds, MS/MS was performed on the fragments that gave the peaks with the highest intensities.

It is known that PC are the main constituents of cinnamon extracts [26, 33]. To date, analyses performed using LC-ESI-MS have reported oligomeric structures mainly consisting of (epi)catechin units with a high proportion of A-type structures. Larger PA were only detected via doubly charged ions. Here, we report a series of oligomeric and polymeric cinnamon PA molecular ions detected by MALDI-TOF/TOF (Table 1). The masses recorded correspond to sodium ion adducts (+23 Da) in the positive ion mode. All the masses were confirmed, using cesium trifluoroacetate as the cationizing agent, to be the corresponding cesium ion adducts (+133 Da, not shown). Using this technique, we have clearly detected PA polymers with a degree of polymerization (DP) of up to 11. Figure 3 shows the spectrum of the cinnamon extract from *m*/*z* 500 to 2,000 (Fig. 3a) and from *m*/*z* 2,000 to 3,500 (Fig. 3b). Different series can be clearly identified. The first series (procyanidins) starts at $[M + Na]^+$ 599.5, which corresponds to an A-type dimer of (epi)catechin and shows mass increments of 288 Da, which correspond to one (epi)catechin moiety. This first series corresponds, therefore, to structures composed exclusively of (epi)catechin units, where all the compounds have at least one A-type substructure.

The presence of A-type linkages can easily be observed in the mass spectrum because the signals are shifted two mass units from the signals of B-type PC, corresponding to

Table 1 Calculated and observed masses of cinnamon PC by MALDI-TOF/TOF MS (all structures contain one A-type linkage)

Polymer	Galloyl units	Calculated $[M + Na^+]$	Observed $[M + Na^+]$
Dimer	0	599.5	599.5
	1	751.7	753.7
	2	903.8[a]	903.9
Trimer	0	887.8	887.9
	1	1,039.9	1,040.1
	2	1,192.0[a]	1,192.1
Tetramer	0	1,176.0	1,176.1
	1	1,328.1	1,328.3
	2	1,480.2[a]	1,480.4
Pentamer	0	1,464.3	1,464.4
	1	1,616.4	1,616.5
	2	1,768.5[a]	1,768.6
Hexamer	0	1,752.5	1,752.6
	1	1,904.7	1,904.7
	2	2,056.8[a]	2,056.9
Heptamer	0	2,040.8	2,040.9
	1	2,192.9	2,193.0
	2	2,345.0[a]	2,345.1
Octamer	0	2,329.1	2,329.1
	1	2,481.2	2,481.2
	2	2,633.3[a]	2,633.4
Nonamer	0	2,617.3	2,617.4
	1	2,769.4	2,769.4
	2	2,921.5[a]	2,921.5
Decamer	0	2,905.6	2,905.6
	1	3,057.7	3,057.7
	2	3,209.8[a]	3,210.0
Undecamer	0	3,193.8	3,193.8
	1	3,345.9	3,341.8

[a] *m*/*z* also compatible with a prodelphinidin one unit larger with one (epi)gallocatechin moiety and without (epi)catechingallate

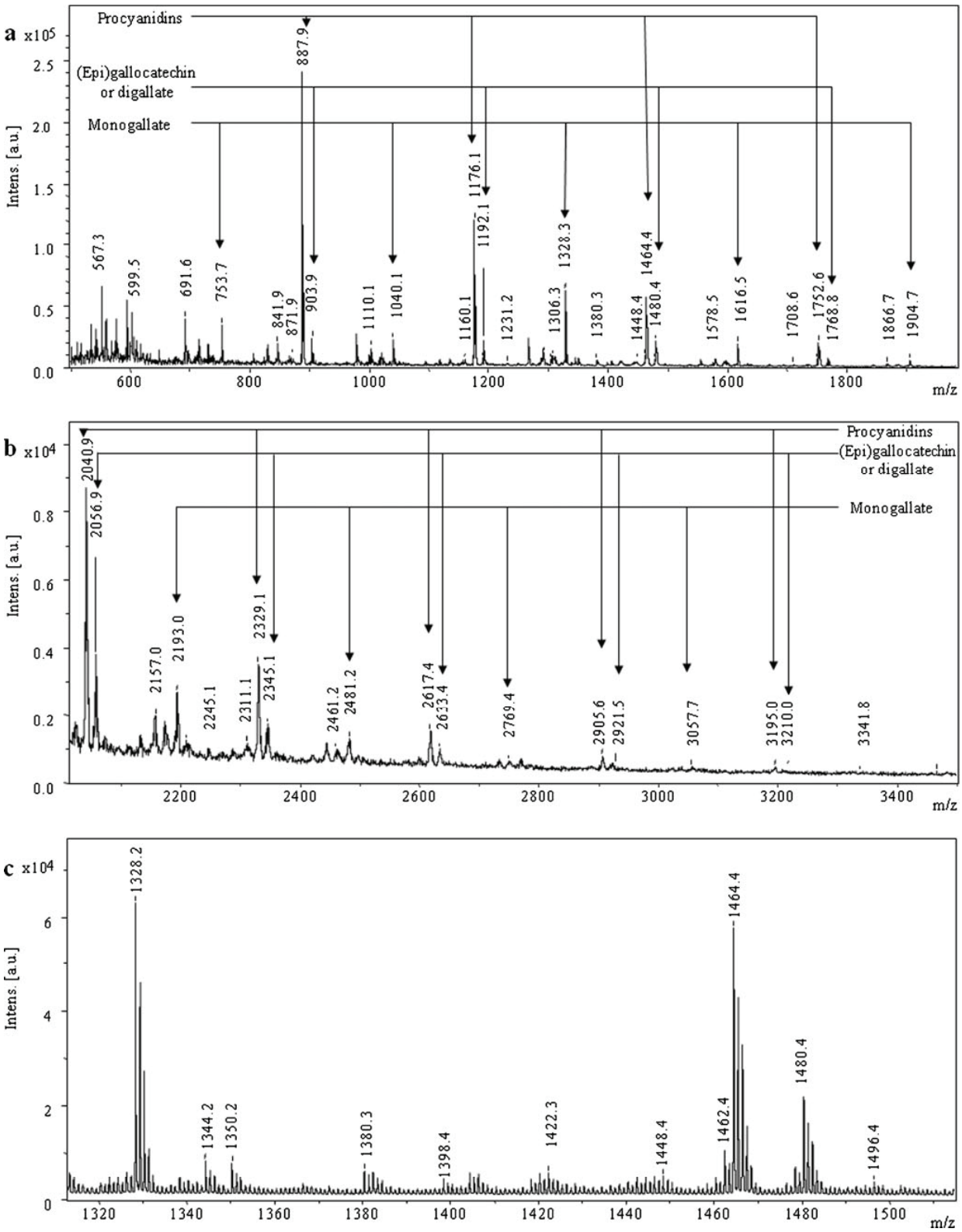


Fig. 3 Mass spectrum of cinnamon extract in the *m/z* range 500–2,000 (**a**) and in the *m/z* range 2,000–3,500 (**b**). **c** Amplification of the cinnamon extract spectrum in the *m/z* range 1,310—1,515

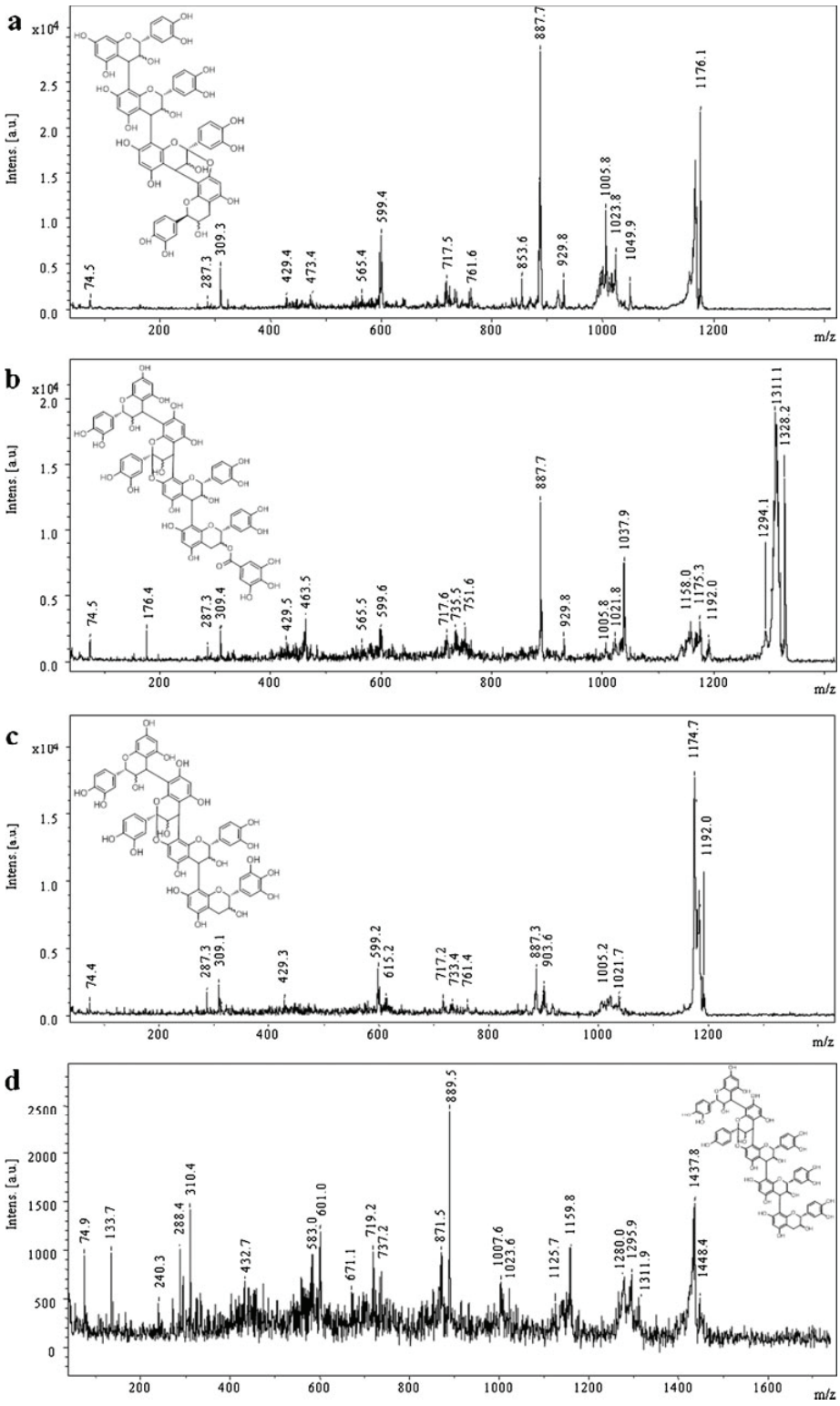


Fig. 4 Product ion mass spectra of: an (epi)catechin tetramer (*m/z* 1,176.1) (**a**); an (epi)catechin tetramer with one (epi)catechin-*O*-gallate unit (*m/z* 1,328.2) (**b**); an (epi)catechin tetramer with one (epi)gallocatechin unit and/or a trimer with two (epi) catechingallate units (*m/z* 1,192.1) (**c**); and an (epi)catechin pentamer with one (epi)afzelechin unit (*m/z* 1,448.3) (**d**). All of them have one A-type linkage

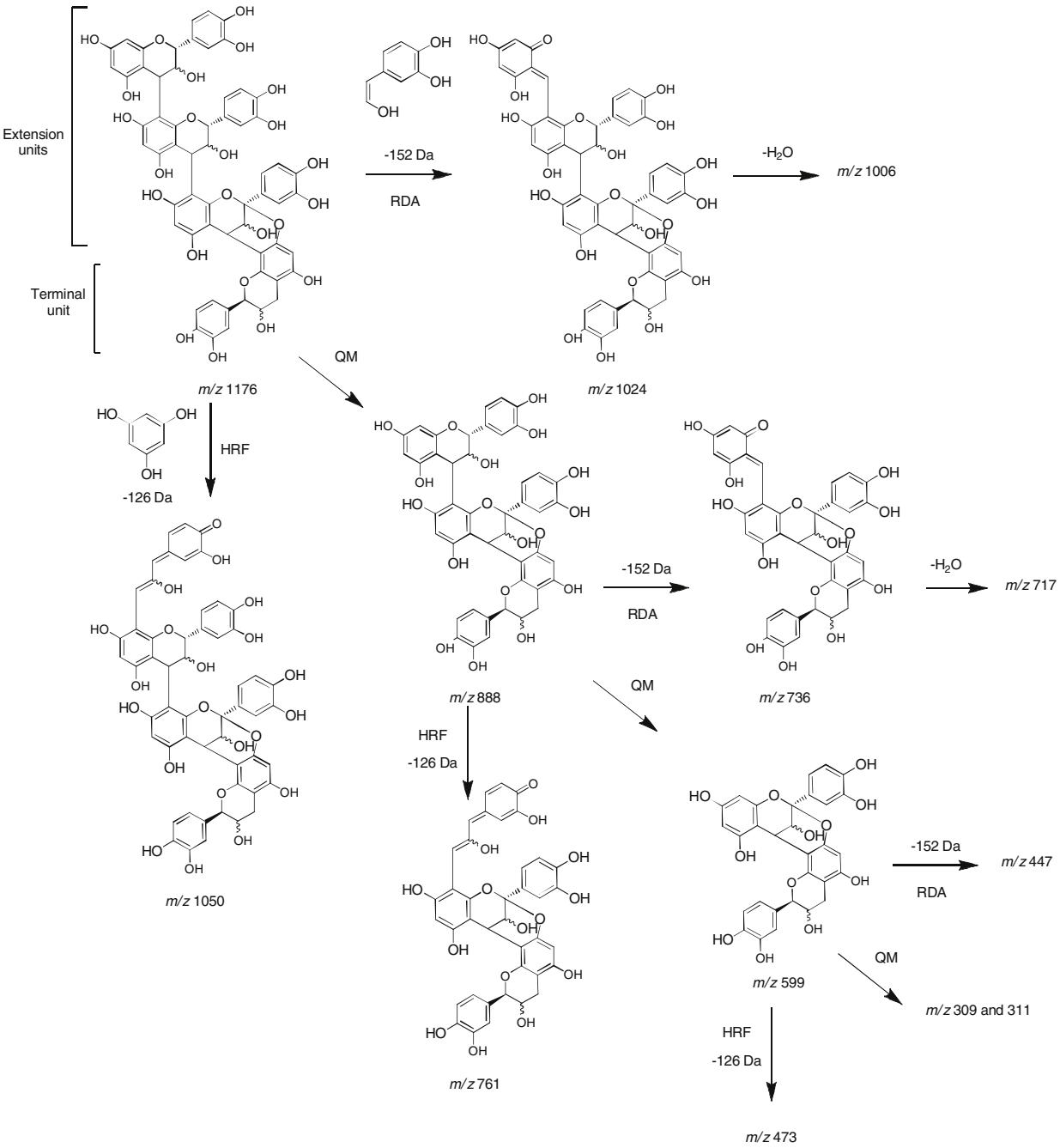


Fig. 5 Fragmentation pattern of an (epi)catechin tetramer with one A-type linkage (*m/z* 1,176)

the two hydrogen atoms that are lost in the formation of the extra interflavanic linkage. While the dimeric structures present are either B type or A type, the trimers and all the species with higher DP contain at least one A-type substructure. The full mass range spectrum obtained for the grape extract was very similar to that presented in Fig. 3, with the main difference being that B-type linkages were more abundant, as described in the literature [35, 36].

MALDI-TOF/TOF allows an accurate and highly sensitive elucidation of compounds through their MS/MS spectra. The spectrum of a tetrameric procyanidin composed only of (epi) catechin units (*m/z* 1,176.1, $[M + Na]^+$) is shown in Fig. 4a.

Figure 5 shows the fragmentation pattern of this compound. The observed fragments agree with the typical fragmentation patterns already described in the literature for polyphenolic compounds [26, 38–41]. The fragment at *m/z* 1,023.8 is the result of retro-Diels–Alder (RDA) cleavage of an (epi) catechin unit (−152 Da), and the fragment at *m/z* 1,005.8 is the corresponding loss of water (−18 Da). The fragment at *m/z* 1,049.9 is caused by heterocyclic ring fission (HRF) of the upper (epi)catechin unit (loss of 126 Da). The fragments at *m/z* 887.7 and 599.4 correspond to the trimer and the dimer PC, respectively, and are originated by quinone methide (QM) cleavages. Again, the fragmentation of the trimer (*m/z* 887.7) and the dimer (*m/z* 599.4) was observed through an RDA cleavage (*m/z* 735.5 and 447.4 for the trimer and the dimer, respectively), that then yields ions at *m/z* 717.5 and 429.4 due to the consecutive loss of a water molecule. HRF is also observed for the trimer and the dimer, generating ions at *m/z* 761.6 and 473.4, respectively. Finally, a QM reaction with the dimer results in the fragments at *m/z* 311.3 and 309.3. The two consecutive losses of 288 Da from the molecular mass, together with the presence of the ion at *m/z* 599.4 (A-type dimer), locate the A-type linkage in the terminal unit of the tetramer.

Some of the *m/z* recorded and listed in Table 1 are compatible with structures composed of (epi)catechin mixed with (epi)catechingallate (one or even two galloyl units). To our knowledge, this is the first time that gallates, which are common among grape PC [42], are detected in cinnamon extracts. Galloylated PA are particularly interesting because gallates appear to be important for the biological activity of these polyphenols [43]. A second series of signals neatly identified in Fig. 3 (monogallate) corresponds to structures with one (epi)catechingallate unit. The highest degree of polymerization for this series was also 11. Mass increments of 288 Da due to the (epi)catechin moieties are observed throughout the series that is shifted 152 mass units (gallate moiety) with respect the signals of the first series (PC). The patterns of fragmentation and MS/MS experiments confirmed the presence of galloylated moieties for all the compounds listed in Table 1. An example is given in Fig. 4b which shows the spectrum corresponding to an A-type tetramer with one (epi)catechingallate unit (*m/z* 1,328.2). A direct comparison with Fig. 4a (same compound without the gallate moiety) reveals that, in addition to the peaks coincident with Fig. 4a (*m/z* 1,175.3, 887.7, 717.6, 599.6, 309.4), some new signals characteristic of the gallate moiety appear. The first peak is at *m/z* 463.5 and matches the sodium adduct of an (epi)catechingallate loss. In fact, this peak, together with the peak at *m/z* 887.7 (A-type (epi) catechin trimer), is the result of a QM reaction of the tetramer. The second characteristic peak is the one at *m/z* 751.6, also a QM product, now between the second and the third monomeric units of the tetramer. This peak matches a dimer composed by one (epi)catechin and one (epi)catechin-gallate. The presence of these peaks suggests that the gallate moiety may be located in the terminal unit of the tetramer.

A third series ((epi)gallocatechin or digallate) can also be observed in Fig. 3. The increment of 152 mass units with respect to the monogallate series suggests the presence of a digallate series. Other peaks that would match trigallate or even tetragallate oligomers have been observed, but are not included in Table 1 because the low intensity of these peaks did not allow confirmation by MS/MS experiments.

Prodelphinidins in cinnamon

By carefully examining the MS spectra of the cinnamon extract, a series of signals that are shifted 16 mass units with respect to the (epi)catechin series can be observed ((epi) gallocatechin or digallate series). This series is compatible with digallates, as suggested above, but also with species containing (epi)gallocatechin units (Fig. 1). Because MALDI spectrometers are not coupled to a separation system, experiments are performed by direct insertion of the whole sample without any previous separation, so compounds with equal mass appear together in the spectrum. MS/MS may then be used to identify each of the different species with equal mass, provided the signals are strong enough. We performed MS/MS experiments on those molecular ions that gave high-intensity signals. Figure 4c is the MS/MS spectrum corresponding to the molecular ion with *m/z* 1,192.1. This mass matches either a tetramer that contains one (epi)gallocatechin unit and one A-type linkage or a trimer with two (epi)catechingallate units and one A-type linkage. When examining the MS/MS spectrum, there are several pieces of evidence that support the hypothesis that we are dealing with the tetramer with one (epi)gallocatechin unit. First, the characteristic (epi)catechingallate peak at *m/z* 463 does not appear in the spectrum; second, a peak at *m/z* 887.4 corresponding to the loss of an (epi)gallocatechin unit (304 mass units) directly from the molecular ion can be clearly observed; in addition, the peaks at *m/z* 1,049.5 and *m/z* 1,005.2 could be the result of HRF and an RDA cleavage plus the loss of a water molecule, respectively, from the (epi) gallocatechin unit. Although the peaks at *m/z* 1,039.7 (loss of 152 mass units) and *m/z* 1,021.8 (consecutive loss of water) may support the hypothesis that this is a digallate series, they could also correspond to an RDA cleavage of an (epi) catechin unit in a tetramer that contains one (epi)galloca-techin. In fact, the large number of fragments observed indicates that the spectrum may come from a mixture of compounds that may include a digallate trimer and a mixture of tetramers of (epi)gallocatechin with the (epi)gallocatechin unit in different positions. For instance, the fragment at *m/z* 887.4 ((epi)catechin trimer) would come from a tetramer with a terminal (epi)gallocatechin moiety, and the fragments

at *m/z* 903.6 and 615.4 (consecutive losses of 288 mass units: (epi)catechin monomers) would support the hypothesis of the (epi)gallocatechin moieties as extension units of the oligomer. The presence of prodelphinidins in the cinnamon extract is evident, which, as also noted for the galloyl moieties, has never been reported before. This may also be of biological significance since the pyrogallol moieties of (epi)gallocatechins (e.g., in tea) are more reactive than the catechol moieties of (epi)catechins (e.g., in grape). They can even promote the formation of free radicals, so-called pro-oxidant activity, which may end up stimulating endogenous antioxidant responses that result in more effective overall antioxidant activity in vivo than by direct free radical scavenging [44].

Many other low-intensity peaks attributable to prodelphinidins can be observed in the spectrum in Fig. 3. These peaks, included in Table 2, would match oligomers of (epi)catechin with two (epi)gallocatechin units or PA that contain both (epi)catechin-3-*O*-gallate and (epi)gallocatechin units. Amplification of a section of the cinnamon extract spectrum (Fig. 3c) reveals the presence of peaks corresponding to an (epi)catechin tetramer with one gallate unit (*m/z* 1,328.2) and an (epi)catechin tetramer with one gallate and one (epi)gallocatechin moieties (*m/z* 1,344.2) as well as pentameric PA. Other peaks that would match prodelphinidins with one (epi)gallocatehin unit and one or even two (epi)catechingallates have been observed, but are not included in Table 2 because the low intensity of these peaks did not allow a confirmation by MS/MS experiments.

Table 2 Calculated and observed masses of cinnamon prodelphinidins by MALDI-TOF/TOF MS (all structures contain one A-type linkage)

Polymer	(Epi)gallocatechin units	Calculated $[M + Na^+]$	Observed $[M + Na^+]$
Dimer	1	615.5	617.5
	2	631.5	633.5
Trimer	1	903.7[a]	903.9
	2	919.7	919.9
Tetramer	1	1,192.0[a]	1,192.1
	2	1,208.0	1,208.2
Pentamer	1	1,480.4[a]	1,480.4
	2	1,496.4	1,496.4
Hexamer	1	1,768.7[a]	1,768.6
	2	1,784.7	1,784.6
Heptamer	1	2,056.9[a]	2,056.8
	2	2,072.9	2,072.9
Octamer	1	2,345.2[a]	2,345.1
	2	2,361.2	2,361.2
Nonamer	1	2,633.4[a]	2,633.4
	2	2,649.4	2,649.4
Decamer	1	2,921.7[a]	2,921.5
	2	2,937.7	2,937.8

[a] *m/z* also compatible with a proanthocyanidin one unit shorter with two (epi)catechingallate moieties and without (epi)gallocatechin

Table 3 Calculated and observed masses of the main propelargonidins in cinnamon by MALDI-TOF/TOF MS

Polymer	(Epi)afzelechin units	A-type linkages	Calculated $[M + Na^+]$	Observed $[M + Na^+]$
Dimer	1	1	583.5	583.5
	2	1	567.5	567.3
Trimer	1	1	871.8	871.9
	1	2	869.8	869.9
	3	1	841.9	841.9
Tetramer	1	1	1,160.0	1,160.1
	2	3	1,140.0	1,140.1
	4	3	1,110.0	1,110.1
Pentamer	1	1	1,448.3	1,448.4
	4	2	1,398.3	1,398.4
	5	3	1,380.3	1,380.3
Hexamer	1	1	1,736.5	1,736.6
	1	2	1,734.5	1,734.6
	4	2	1,686.5	1,686.6
	5	2	1,670.5	1,670.6
	6	2	1,654.5	1,654.6
Heptamer	1	1	2,024.8	2,024.8
	5	2	1,958.8	1,958.8
	6	2	1,942.8	1,942.8
	7	2	1,927.8	1,927.8
Octamer	1	1	2,313.1	2,313.1
	1	2	2,311.1	2,311.1
	5	3	2,245.1	2,245.1
	7	5	2,209.1	2,208.9
	8	5	2,193.1	2,193.0
Nonamer	1	1	2,601.3	2,601.3
	7	5	2,497.3	2,497.2
	8	5	2,481.3	2,481.2
	9	7	2,461.3	2,461.2
Decamer	1	1	2,889.6	2,889.6
	1	2	2,887.6	2,887.6
	8	5	2,769.6	2,769.5
Undecamer	1	1	3,177.8	3,176.7

Propelargonidins in cinnamon

In addition to PC and prodelphinidins with and without gallate moieties, MALDI-TOF/TOF enabled us to detect heteropolymers with (epi)afzelechin moieties (propelargonidins) with a DP of up to 11. Propelargonidins of lower DP have been reported before in cinnamon [33, 34]. The propelargonidins we identified in cinnamon were composed

of (epi)catechin and (epi)afzelechin units, with at least one A-type linkage. The most intense peaks corresponded to oligomeric species with a single (epi)afzelechin unit. Table 3 shows the masses of the propelargonidins that yield intense MS signals. The product ion spectrum of a pentameric propelargonidin composed of four (epi)catechin units and one (epi)afzelechin unit (*m/z* 1,448.4, $[M + Na]^+$) is shown in Fig. 4d. The fragment at *m/z* 1,159.8 corresponds to a tetramer with one (epi)afzelechin unit and one A-type linkage. The fragment at *m/z* 889.5 (B-type (epi)catechin trimer) implies the loss of the (epi)afzelechin unit, and the signals at *m/z* 601.0 and 310.4 (B-type dimer and monomer, respectively) are the result of the consecutive loss of (epi) catechin units. Several RDA reactions are also observed (*m/z* 1,295.9, 1,007.6, and 737.2). These fragments indicate the following structure for the compound: (epi)cat-(epi)cat-(epi) cat-A-(epi)afz-(epi)cat, although other combinations may also be present.

Conclusions

We present here the first report of cinnamon PA determined by MALDI-TOF/TOF. MALDI-TOF/TOF in CID mode is a powerful technique for the structural analysis of polyphenolic polymers. The technique combines high sensitivity for high-molecular-weight compounds with the possibility of extracting structural information via the fragmentation patterns obtained from MS/MS experiments. We show that cinnamon PA contain substructures never described before for this source. Apart from the most common flavanol (epi)catechin and (epi)afzelechin, cinnamon PA contain (epi)catechingallate and (epi)gallocatechin units. As gallates (galloyl moities) and the pyrogallol group in (epi)gallocatechins have been related to the biological activity of grape and tea polyphenols, the presence of these substructures may explain some of the properties of cinnamon extracts. MALDI-TOF/TOF reveals that cinnamon bark PA include combinations of (epi)catechin, (epi)catechingallate, (epi)gallocatechin, and (epi)afzelechin, resulting in a highly heterogeneous mixture of procyanidins, prodelphinidins, and propelargonidins.

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2. Estudio de la absorción/metabolización de proantocianidinas

2.1. Estudio de bioaccesibilidad y biodisponibilidad de proantocianidinas de canela

Publicación 2. Estudio del perfil de metabolitos de proantocianidinas procedentes de canela (*Cinnamomum zeylanicum* L.) en ratas.

Profile of urinary and fecal proanthocyanidin metabolites from common cinnamon (*Cinnamomum zeylanicum* L.) in rats

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RESUMEN:

Una vez identificados los compuestos principales de la canela (*Cinnamomum zeylanicum*) en el estudio anterior, se realizó un estudio de metabolización *in vivo*. Las propiedades biológicas que se atribuyen a la canela podría estar relacionada con sus compuestos fenólicos, mayoritariamente proantocianidinas poliméricas con enlaces de tipo A. En concreto, este tipo de proantocianidinas doblemente enlazadas podrían potenciar la acción de la insulina, siendo beneficiosas en el control de la intolerancia a la glucosa y en el tratamiento de la diabetes (Anderson *et al.* 2004).

Existen pocos estudios sobre bioaccesibilidad y biodisponibilidad de proantocianidinas y éstos nos indican que estos compuestos poliméricos no se absorben o se absorben después de ser despolimerizados y degradados. Cabe esperar en consecuencia que las proantocianidinas con enlaces de tipo

A, al tener una unión más estable, sean menos biodisponibles en el organismo.

El presente estudio analiza los metabolitos de proantocianidinas detectados en orina y heces después de administrar una dosis aguda de canela en polvo a un grupo de ratas. Se han identificado en total 36 metabolitos, incluyendo 12 metabolitos de fase II derivados de (epi)catequina y 24 ácidos fenólicos con y sin conjugar, analizados por HPLC-ESI-MS/MS en modo de monitorización de reacciones múltiples (MRM) y barridos de pérdidas neutras (NL) e iones precursor (PI). Las heces contienen monómeros y dímeros de (epi)catequina intactos (sin conjugar). Esto indica que especies captadoras de radicales libres, potencialmente antioxidantes, están en contacto con las paredes del intestino durante horas después de la ingesta de canela en polvo.

Este estudio muestra, por primera vez, de qué forma las proantocianidinas de canela son biodisponibles después de una transformación extensiva por la microbiota intestinal.

FOOD & FUNCTION

Profile of urinary and fecal proanthocyanidin metabolites from common cinnamon (*Cinnamomum zeylanicum* L.) in rats

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Cinnamon (*Cinnamomum zeylanicum* L.) bark is widely used as a spice and in traditional medicine. Its oligomeric and polymeric proanthocyanidins are believed to be partly responsible for the beneficial properties of the plant. We describe here the metabolic fate of cinnamon proanthocyanidins in the urine and feces of rats fed a suspension of the whole bark. The metabolites include ten mono-, di-, and tri- conjugated (epi)catechin phase II metabolites and more than 20 small phenolic acids from intestinal microbial fermentation. Some of these are sulfated conjugates. Feces contain intact (epi)catechin and dimers. This suggests that free radical scavenging species are in contact with the intestinal walls for hours after ingestion of cinnamon. The phenolic metabolite profile of cinnamon bark in urine is consistent with a mixture of proanthocyanidins that are depolymerized into their constitutive (epi)catechin units as well as cleaved into smaller phenolic acids during their transit along the intestinal tract, with subsequent absorption and conjugation into bioavailable metabolites.

Keywords:
Bioavailability / Cinnamon / Mass spectrometry / Polyphenols / Proanthocyanidins

Common cinnamon or Ceylon cinnamon (*Cinnamomum zeylanicum)*, an aromatic plant from the family of the Lauraceae, is one of the most commonly consumed spices around the world. The bark, either whole or powdered, as well as being a highly prized spice, has been used in traditional medicine for centuries [1]. During recent years, interest has increased in how cinnamon modulates parameters related with the metabolic syndrome (e.g. blood glucose level, lipid profile), as observed in several animal studies and human clinical trials [1–3].

The biological properties of cinnamon are attributed in part to the phenolic compounds it contains, which mostly belong to the class of proanthocyanidins (PA: oligomers/polymers of flavan-3-ols). In particular, it has been suggested that A-type doubly linked procyanidins (PA constituted of (epi)catechin, EC, units) may have insulin-like biological activity in vitro [4]. Recent studies conducted by our group have revealed that cinnamon bark includes oligomers and polymers that contain (epi)gallocatechin and (epi)catechin gallate units [5], which may contribute to its biological activity [6]. Nevertheless, plant PA are neither directly bioaccessible nor bioavailable since they suffer transformations during transit along the intestinal tract [7]. Therefore, to study the potential biological effects of the PA, it is necessary to define their metabolic fate, i.e. the different transformations that they undergo once ingested and thereby the species that will be in contact with the intestinal tract as well as those that will be absorbed and will circulate in different biological fluids for hours after intake. Once ingested, monomeric and dimeric flavan-3-ols are absorbed in the small intestine and converted into several phase II conjugated metabolites in the liver; these then pass into the bloodstream and are later excreted in urine, or return via the bile to the small intestine. PA that are not absorbed in the small intestine reach the colon intact where, after depolymerization and fermentation by microbiota, they may release small metabolites that are absorbed, transformed in the liver, and have the same fate as the metabolites derived from absorption in the small intestine [7, 8]. The metabolites found in urine indicate the fraction of PA that is bioavailable and that may have an effect on target tissues, while the metabolites excreted in

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Abbreviations: EC, (epi)catechin; **GSH**, glutathione; **Gluc**, glucuronide; **Me**, methyl group; **MRM**, multiple reaction monitoring; **PA**, proanthocyanidins; **Sulf**, sulfate

Table 1. (Epi)catechin and conjugated metabolites in urine and feces from rats fed whole cinnamon bark[a)]

Metabolite	MRM parent	Identification	Urine	Feces
EC	289→245	Standard retention time	X	X
Procyanidin dimer	577→289	MS/MS: 577; 451; 425; 405; 289; 245		X
Mono-conjugated metabolites				
Gluc-EC-1	465→289	MS/MS: 465; 289; 245; 163; 113	X	
Gluc-EC-2	465→289	MS/MS: 465; 289; 245; 113	X	
Gluc-EC-3	465→289	MS/MS: 465; 289; 245; 217; 113	X	
Sulf-EC-1	369→289	MS/MS: 369; 289; 245; 113	X	
Sulf-EC-2	369→289	MRM daughter 289→245	X	
GSH-EC-1	594→289	MRM daughter 289→245	X	
Di-conjugated metabolites				
Me-Gluc-EC-1	479→303	MS/MS: 479; 303; 289; 245; 175; 137; 113	X	
Me-Gluc-EC-2	479→303	MS/MS: 479; 303; 285; 259; 175; 137; 113	X	
di-Gluc-EC-1	641→289	MS/MS: 641; 465; 289	X	
Tri-conjugated metabolites				
di-Me-Sulf-EC-1	397→289	MRM daughter 289→245		X

MRM: multiple reaction monitoring; Gluc: glucuronide; EC: (epi)catechin; Sulf: sulfate; GSH: glutathione; Me: methyl group.
a) Metabolites not detected in the control group or detected from signals at least tenfold stronger.

feces are in contact with the colonic tissue and may influence gut health [8, 9].

The study we report here analyzes the urinary and fecal PA metabolites derived from cinnamon. Female Sprague–Dawley rats ($n = 5$), fed a polyphenol-free diet (TD 94048, Harlan Interfauna Ibérica SL, Barcelona, Spain) were administered an acute dose of powdered common cinnamon (1 g/kg body weight via a solution of 0.6 g/10 mL) by oral gavage after 12 h fasting. PA metabolites, including EC derivatives and microbial-derived phenolic metabolites, were analyzed in urine and feces samples collected over the 24 h after intake, and compared to those of a control group that was only administered tap water. The experimental design, included in the Spanish National Research Project AGL-2009–12374-C03–03/ was approved by the Bioethics-Committee-CSIC.

Urine samples were concentrated by nitrogen stream and then resuspended in 2 mL of acid water (addition of phosphoric acid to pH 3). An Oasis HLB (60 mg) cartridge from Waters Corp. (Milford, MA, USA) was used for the solid phase extraction. The cartridge was activated with 1 mL of methanol and 2 mL of acid water 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 [9]. Feces samples (0.5 g) were defatted with 10 mL of hexane and the remnant was extracted at room temperature with 10 mL methanol:water:acetic acid (8.0:1.9:0.1, v/v/v) and concentrated by nitrogen stream. Taxifolin (50 mg/L solution) was added to each sample as the internal standard, to obtain a final concentration of 5 mg/L. The temperature of evaporation was kept under 30°C to avoid deterioration of the phenolic compounds. All the samples were filtered through a polytetrafluoroethylene (PTFE) 0.45-μm membrane into amber vials for liquid chromatography (LC)–mass spectrometry (MS)/MS analysis.

A Quattro LC triple quadrupole mass spectrometer with an electrospray source (Waters Corp.) was used in the negative mode to obtain MS and MS/MS data. LC separations were performed on an Alliance 2695 system from Waters Corp. equipped with a Phenomenex (Torrance, CA, USA) Luna C18 (50 × 2.1 mm id) 3.5-μm particle size column and a Phenomenex Security guard C18 (4 × 3 mm id) 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 were identified by multiple reaction monitoring (MRM) transitions of the putative metabolites using a dwell time of 100 ms and additionally by product ion scan experiments. The cycle time used was 2 s. Cone energy and collision energy in MRM mode were optimized for each group of metabolites.

Table 1 lists all the EC conjugates detected by high-performance liquid chromatography coupled to mass spectrometry (HPLC–MS) and HPLC–MS/MS in urine and feces from the rats fed cinnamon. The metabolites were first identified by MRM transitions previously reported in the literature [9] that correspond to the molecular ion and the main fragment, and the final elucidation was confirmed by a second MRM transition and/or by product ion scan experiments.

Free EC (MRM transition 289 → 245) was detected in both urine and feces from the rats fed cinnamon and signals corresponding to EC dimers (577 → 289) were detected in feces.

Nine conjugated EC metabolites were detected in urine: three glucuronidated forms (465 → 289), two sulfated forms (369 → 289), a monoconjugated metabolite with glutathione (GSH) (594 → 289), two methylated and glucuronidated forms (479 → 303), and a di-glucuronidated form (641 → 289). Figure 1A shows the HPLC–ESI–MS/MS (where ESI is

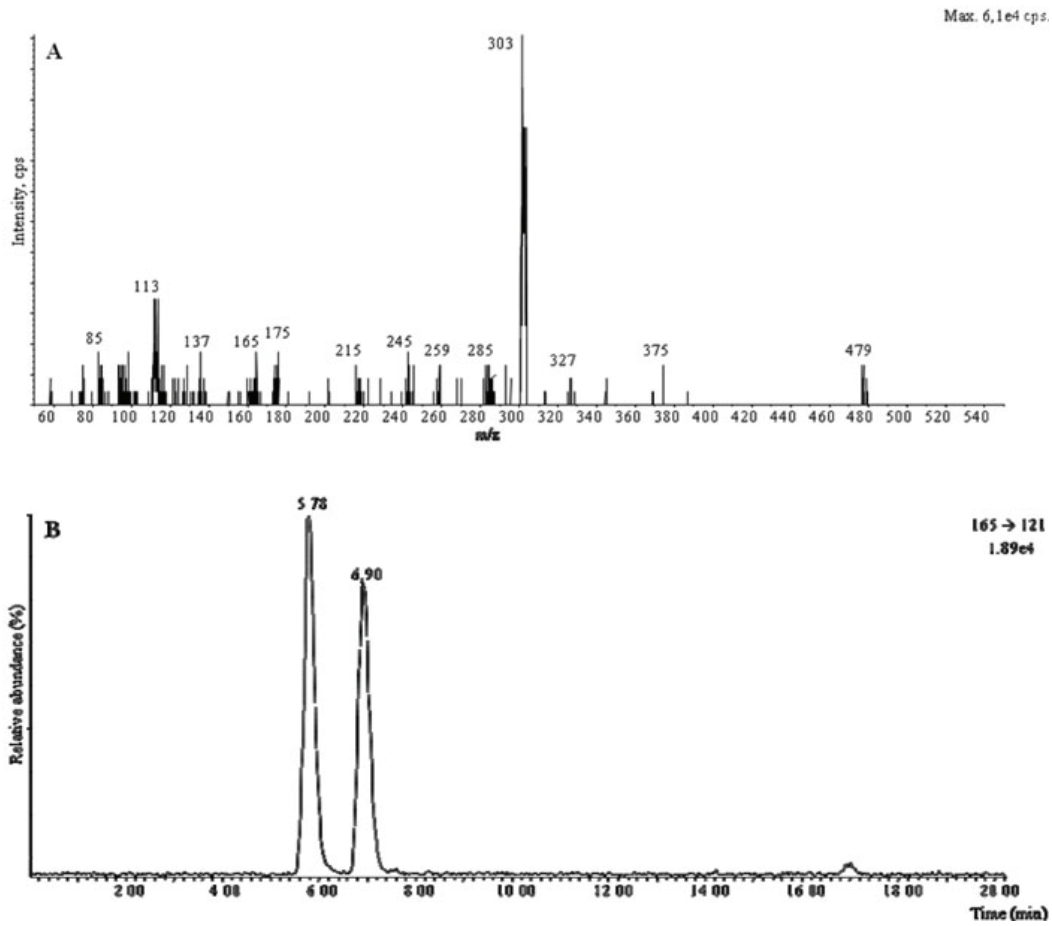

Figure 1. (A) HPLC–ESI–MS/MS product ion scan spectrum of methyl-glucuronide-(epi)catechin (*m/z* 479); and (B) HPLC–ESI–MS profile corresponding to 3- and 4-hydroxyphenylpropionic acid (*m/z* 165) in urine from rats fed whole cinnamon bark.

electrospray ionization) product ion scan spectrum of methyl-glucuronide-EC in urine. The only EC derivative detected in feces from the rats fed cinnamon was dimethylated-sulfated-EC (397 → 289), whose identity was confirmed by a second transition (289 → 245).

The microbial-derived PA metabolites detected in urine and/or feces from the rats fed whole cinnamon bark are listed in Table 2. There are 24 metabolites detected in urine and 6 detected in feces. These metabolites included some direct products of microbial fermentation of PA, such as 3- and 4-hydroxyphenylpropionic acid (165 → 121) as well as phenolic acids derived from further transformations in the liver, such as ferulic acid (193 → 134). Other metabolites derived from PA fermentation, such as 3-hydroxyphenylacetic acid (151 → 107) or 3,4-dihydroxyphenylacetic acid (167 → 123) were only detected in urine samples. The HPLC–ESI–MS profile corresponding to the detection of 3- and 4-hydroxyphenylpropionic acid in urine is shown in Fig. 1B.

Some conjugated microbial-derived PA metabolites were found in urine, indicating that microbial metabolites formed in the colon are absorbed, conjugated in the liver, transferred into the bloodstream, and finally excreted in urine. These metabolites were sulfate (sulf)-dihydroxyphenylvaleric acid (289 → 209), sulf-3,4-dihydroxyphenylpropionic acid (261 → 181), sulf-3- or 4-hydroxyphenylpropionic acid (245 → 165) and gluc-3- or 4-hydroxyphenylacetic acid (327 → 151). Finally, hippuric acid (178 → 134), from the conjugation of benzoic acid with glycine, as well as its methylated form (193 → 178) was also detected in urine from the rats fed whole cinnamon bark. It should be noticed that a fraction of the detected hippuric acid may be derived from cinnamaldehyde, also present in common cinnamon [10].

It was commonly thought that only PA dimers and trimers were metabolized in the intestine, while the modification of PA polymers would be negligible [11, 12]. More recent studies have suggested that the metabolism of PA is more significant than previously thought since a large number of metabolites

Table 2. Detection of microbial-derived proanthocyanidin metabolites in urine and feces from rats fed with whole cinnamon bark[a)]

Metabolite	MRM parent	Identification	Urine	Feces
Valerolactones				
Dihydroxyphenylvalerolactone	207→163	MRM daughter 163→119	X	X
Phenylvaleric acids				
Sulf-dihydroxyphenylvaleric acid	289→209	MRM daughter 209→165	X	
Phenylpropionic acids				
3-Hydroxyphenylpropionic acid	165→121	MS/MS: 165; 121; 93	X	X
4-Hydroxyphenylpropionic acid	165→121	MS/MS: 165; 121; 93	X	X
3,4-Dihydroxyphenylpropionic acid	181→137	Standard retention time	X	
Sulf-3,4-dihydroxyphenylpropionic acid	261→181	MRM daughter 181→137	X	
Suf-3 or 4-hydroxyphenylpropionic acid	245→165	MS/MS: 245; 165; 121	X	
Phenylacetic acids				
3-Hydroxyphenylacetic acid	151→107	Standard retention time	X	
4-Hydroxyphenylacetic acid	151→107	Standard retention time	X	
Gluc-3- or 4-hydroxyphenylacetic acid	327→151	MS/MS: 327; 151; 107	X	
3,4-Dihydroxyphenylacetic acid	167→123	MS/MS: 167; 123; 105; 95	X	
Benzoic acids				
Benzoic acid	121→77	Standard retention time	X	
4-Hydroxybenzoic acid	137→93	Standard retention time	X	X
Sulf-3 or 4-hydroxybenzoic acid	217→137	MS/MS: 217; 137; 121	X	
3,4-Dihidroxybenzoic acid	153→109	Standard retention time	X	
Hippuric acid	178→134	Standard retention time	X	
Me-hippuric acid	193→178	MS/MS: 193; 178; 134	X	X
Cinnamic acids				
Caffeic acid	179→135	Standard retention time	X	
m-coumaric acid	163→119	Standard retention time	X	
p-Coumaric acid	163→119	Standard retention time	X	
Sulf-coumaric acid	243→163	MRM daughter 163→119	X	
Ferulic acid	193→134	Standard retention time	X	X
Lignans				
Enterodiol	301→107	Standard retention time	X	
Sulf-enterolactone	377→297	MRM daughter 297→253	X	

MRM: multiple reaction monitoring; Sulf: sulfate; Gluc: glucuronide.
a) Metabolites not detected in the control group or detected from signals at least tenfold stronger.

have been detected in plasma and/or urine after ingestion of foodstuffs rich in polymers [8, 9, 13]. Many of these metabolites are conjugates of monomeric EC that cannot come exclusively from the small amounts of this monomer contained in the food. Moreover, the observation that some small phenolics are detected in plasma several hours after ingestion confirms the hypothesis that polymers are transformed by the intestinal microbiota [9, 14]. The results obtained in the present study with whole cinnamon are in agreement with those obtained for other polymer-rich sources such as grape pomace and almond skin [8, 9, 13], and are in keeping with the observation that cinnamon is a rich source of PA [5, 15]. Common cinnamon contains more than 3000 mg/100 g of PA (from dimers to polymers; more than 80% of which have a degree of polymerization equal to or higher than 10), while its monomeric EC content is only 24 mg/100 g [15]. We report here the detection of a large number of EC metabolites in the urine of rats fed whole cinnamon bark. The strong MS signals cannot come only from the transformation of monomeric EC, which is a very minor component of the bark. Moreover, we detected 3,4-dihydroxyphenylacetic acid, the major phenolic acid from procyanidin dimer fermentation, which has not been identified in studies performed with pure monomeric flavan-3-ols [16].

In the study reported here, we fed the animals a water suspension of whole cinnamon bark instead of the extracts commonly used in other studies. Therefore, both the extractable and the nonextractable PA fractions were administered. This is important since nonextractable PA are mostly polymeric and are present in higher amounts than extractable PA in many foodstuffs [17]. It may be expected that the PA metabolites detected in this study derive not only from extractable PA, but also from nonextractable PA which have been shown to be bioavailable as phase I and II metabolites [18]. The present results also confirm that polymeric PA are depolymerized into EC units in the intestine and not only directly cleaved into smaller phenolic acids [8, 9]. Furthermore, it should be noted that intact EC and an EC dimer were present in feces. This means that intact free radical scavenging catechol groups are in contact with the intestinal walls for hours after ingestion.

In summary, the bioavailability of cinnamon PA after ingestion of whole bark powder has been evaluated for the first time. A wide variety of PA metabolites, including phase II

EC metabolites and microbial-derived metabolites, were detected in the urine and feces of rats fed cinnamon. They would appear to be derived from the degradation of PA polymers. These circulating metabolites may contribute to the reported beneficial health effects of cinnamon.

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2.2. Estudio de bioaccesibilidad y biodisponibilidad de proantocianidinas no extraíbles de uva.

Publicación 3. Estudio de biodisponibilidad de proantocianidinas no extraíbles de uva.

Non-extractable proanthocyanidins from grapes are a source of bioavailable (epi)catechin and derived metabolites in rats

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RESUMEN:

La fibra dietética antioxidante de uva (GADF) es un subproducto del proceso industrial del prensado de la uva que se caracteriza por combinar las ventajas de la fibra con una alta concentración de proantocianidinas (Goñi *et al.* 2009). GADF puede tener un impacto importante sobre la salud al reducir significativamente la presión arterial y el colesterol en sangre (Pérez-Jiménez *et al.* 2008b) además de ejercer un efecto protector de la mucosa colónica (López-Oliva *et al.* 2010) y protegerlo del desarrollo de tumores (Lizarraga *et al.* 2011). En estudios anteriores en nuestro laboratorio, se identificaron los compuestos mayoritarios en orina y heces de rata tras la ingesta de GADF. Estos estudios demuestran que las proantocianidinas se van liberando durante el tránsito intestinal y que la GADF termina siendo extensamente metabolizada (Touriño et al. 2009; Touriño et al. 2011).

La fracción no extraíble de muchas frutas y verduras contiene compuestos polifenólicos bioactivos que, en la mayoría de los casos, no han sido bien caracterizadas estructuralmente. Las proantocianidinas no extraíbles (NEPA) de naturaleza polimérica son parte significativa de la fracción de fibra dietética de los alimentos y son ignoradas en los estudios de biodisponibilidad de compuestos polifenólicos ya que en la mayoría de estudios y bases de datos sólo se refieren a las proantocianidinas extraíbles. Al no existir información sobre la contribución de la fracción de proantocianidinas no extraíbles a la bioaccesibilidad de polifenoles de GADF, se planteó la purificación de NEPA y un estudio de su metabolización en ratas.

La GADF ha sido ampliamente caracterizada tanto por su contenido de proantocianidinas extraíbles como no extraíbles por lo que la hace muy interesante para este estudio, ya que comparado con los anteriores sobre GADF total, muestra que las NEPA contribuye al conjunto de metabolitos de GADF biodisponibles. Además, el estudio proporciona una primera prueba, por espectrometría de masas, de la naturaleza estructural de las NEPA (polímeros de (epi)catequina). Hasta el momento solamente se había demostrado que las NEPA producía una coloración en el ensayo de degradación ácida.

En este estudio de las proantocianidinas no extraíbles de uva se distingue entre el enfoque químico, corroborando la alta concentración de NEPA y el enfoque fisiológico, señalando que son una fuente de polifenoles de liberación gradual durante el tránsito intestinal.

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Non-extractable proanthocyanidins from grapes are a source of bioavailable (epi)catechin and derived metabolites in rats

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Abstract

The non-extractable fraction of many fruit and vegetables contains putatively bioactive polyphenolic compounds that, in most cases, have not been well characterised structurally. Non-extractable proanthocyanidins (NEPA) of a polymeric nature are part of the dietary fibre fraction of food. Using liquid chromatography coupled to a mass spectrometer equipped with an electrospray ionisation chamber and a triple quadrupole mass analyser for tandem analysis (HPLC–ESI–QqQ–MS/MS) techniques, we examine the phenolic metabolites present in urine and faeces from rats 24 h after ingestion of an NEPA-rich fraction. We show that NEPA are partially depolymerised during their transit along the intestinal tract, as evidenced by the presence of (epi)catechin (EC) monomers and dimers in faeces and phase II conjugates of EC in urine. Moreover, NEPA are further metabolised by the intestinal microbiota into smaller metabolites including phenolic acids that are present in urine as both free phenolics and conjugates with glucuronate or sulphate moieties. For the first time, we report evidence that NEPA behave *in vivo* as a source of phenolics that are released progressively and deliver phenolic species that come into contact with the intestinal walls and are bioavailable for at least 24 h after ingestion.

Key words: Bioavailability: MS: Non-extractable proanthocyanidins: Polyphenols

Proanthocyanidins (PA) are a class of dietary polyphenols. They are polymers of flavan-3-ols present in a wide variety of plant-based foodstuffs, such as berries, cocoa or certain nuts[1]. Several supplementation studies both in animals and in human subjects using PA-rich products have shown that PA play a preventive role against several conditions including CVD[2–4] or diabetes[1,5,6]. Similarly, a recent epidemiological study showed an inverse association between the intake of polymeric PA and the risk of colorectal cancer[7]. To advance knowledge of the possible effects of PA on human health, it is important to characterise their metabolic fate in detail, that is to say, the degradation they may suffer once ingested and the distribution of their metabolites through the different organs and fluids.

Most studies of PA, including those that address their metabolism, assume that the PA in foodstuffs correspond exclusively to the supernatants obtained after extracting the food with acetone; the most common procedure for their analysis[8]. However, these PA would actually only correspond to the extractable proanthocyanidins (EPA), which represent only a fraction of dietary PA. Recent work has emphasised that a considerable proportion of PA, the non-extractable proanthocyanidins (NEPA), remains in the residue from such extractions[9] and may very well play a more significant functional role than EPA. NEPA are associated with other components of the food matrix, mainly dietary fibre, and in fact constitute a part of it, according to current definitions of dietary fibre[10,11]. To date, there is no common method for determining NEPA. They are usually determined by destructive spectrophotometric methods and only a few recent papers have attempted structurally meaningful analysis[12,13]; therefore, structural evidence regarding the composition of NEPA as essential constituents of many foodstuffs is still scarce. The currently available evidence suggests that NEPA may be more abundant than EPA in much food[12–14] and, therefore, that significant amounts of NEPA are ingested daily.

Over the last decade, several studies have addressed the metabolism of PA. Although initial studies emphasised fairly poor intestinal absorption that was limited to dimers[15–17], later observations indicate that once intact PA reach the colon they are widely transformed by the colonic microbiota into small phenolic acids[18–21]. These metabolites are absorbed, and then transformed in the liver, and the resulting conjugates are transferred to the bloodstream. A recent study

Abbreviations: EC, (epi)catechin; EPA, extractable proanthocyanidins; GADF, grape antioxidant dietary fibre; GlcA, glucuronide; MRM, multiple reaction monitoring; NEPA, non-extractable proanthocyanidins; PA, proanthocyanidins; ppm, parts per million.

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in which [^{14}C]procyanidin B2, a labelled PA dimer, was administered to rats, reported bioavailability of around 80 %, based on total urinary ^{14}C[20].

Nevertheless, these papers have mostly addressed the bioavailability of dimers or trimers, while the most abundant PA in food are polymers[22]. Indeed, some recent studies have suggested that PA are also depolymerised into (epi)catechin (EC) units before cleavage into smaller species and further metabolism[21,23–25]. These studies suggest that phenolics that are bioavailable after the ingestion of PA-rich foodstuffs must have come from NEPA[24–26], but this has not been proved as the specific metabolism of NEPA has never been reported.

Grape antioxidant dietary fibre (GADF) is a food product obtained from red grapes that is rich in dietary fibre and polyphenols[27]. Besides extractable polyphenols, including EPA[28], GADF contains a significant amount (14·8 %) of NEPA[27] and was used in the studies which suggest that non-extractable polyphenols are an important source of metabolites that are bioavailable in rats[24–26]. To study the contribution of NEPA to the pool of phenolic metabolites from fruit and vegetables, we considered using a NEPA-rich fraction from GADF.

Our objective was to evaluate the fate of NEPA in rats 24 h after ingestion of a preparation free from any extractable polyphenols. NEPA metabolites, including hepatic and microbially derived metabolites, were analysed in urine and faeces using liquid chromatography coupled to a mass spectrometer equipped with an electrospray ionisation (ESI) chamber and a triple quadrupole mass analyser for tandem analysis (HPLC–ESI–QqQ–MS/MS).

Experimental methods

Reagents and samples

GADF was obtained from red grapes (*Cencibel variety*, harvested in 2005 in *La Mancha* region of Spain) by a patented procedure[29]. The NEPA content of GADF has previously been reported to be 14·8 g/100 g of dry weight[27]. To obtain an EPA-free (and therefore NEPA-rich) fraction, GADF (4 g) was defatted with hexane (3 × 40 ml), air-dried overnight and the residue was extracted with methanol–water (50:50, v/v, 200 ml) and then with acetone–water–acetic acid (70:29·5:0·5, by vol., 200 ml) once each at room temperature. The supernatant was decanted and the residue, including the NEPA-rich fraction, was vacuum filtered and lyophilised. The actual NEPA content of this residue is around 25 % according to published information[26].

Standards of EC (≥97 %), 3- and 4-hydroxyphenylacetic acid (≥98 %), 3,4-dihydroxyphenylacetic acid (≥98 %), 3- and 4-hydroxybenzoic acid (≥97 %), vanillic acid (≥97 %), caffeic acid (≥95 %), 3,4-di-hydroxyphenylpropionic acid (≥98 %), 4-hydroxyphenylpropionic acid (>98 %), protocatechuic acid (≥97 %), ferulic acid (≥99 %), isoferulic acid (≥97 %), *p*-coumaric acid (≥98 %), *m*-coumaric acid (≥97 %) and taxifolin (≥85 %) were obtained from Sigma Chemical (St Louis, MO, USA). Methanol (analytical grade), phosphoric acid (≥85 %) and acetic acid were purchased from Panreac (Castellar del Vallès, 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 mΩ/cm.

Animal experiments

The study was carried out on female Sprague–Dawley rats (*n* 10, body weight 233 (SD 9·3) g, 12 weeks of age) provided by Harlan Interfauna Ibérica SL (Barcelona, Spain). The animals were fed with a polyphenol-free diet (TD94048), also purchased from Harlan Interfauna Ibérica SL, and they were maintained in plastic cages at room temperature (22 ± 2°C) and 55 (SD 10) % relative humidity, with a 12 h light–12 h dark cycle for 1 week, in accordance with European Union regulations. After food deprivation for 12 h with free access to water, a group of animals (*n* 5) was administered a suspension of NEPA from GADF in tap water (1 g NEPA-rich fraction/10 ml, 1·6 g NEPA-rich fraction/kg body weight) by oral gavage, while a control group (*n* 5) was administered tap water (16 ml/kg body weight). The animals were then placed in metabolism cages and urine and faeces were collected over 24 h and stored at −80°C until extraction and analysis. These experimental protocols were approved by the Experimental Animal Ethical Research Committee of the CSIC in accordance with the current regulations for the use and handling of experimental animals.

Sample preparation

The biological samples were prepared according to previously described procedures for the extraction of phenolic metabolites[21,24,25]. Briefly, urine samples were concentrated via a nitrogen stream at room temperature and then resuspended in 1 ml of acid water (addition of phosphoric acid to reach pH 3). Taxifolin (100 μl of a 50 parts per million (ppm) solution) was added as an internal standard, to obtain a final concentration of 5 ppm. Then the samples were subjected to solid phase extraction in Oasis HLB (60 mg) cartridges from Waters Corporation (Mildford, MA, USA). The cartridges were activated with methanol (1 ml) and acid water (2 ml) and the samples loaded. To remove interfering components, the samples were washed with acid water (9 ml) and then the phenolic compounds were eluted with methanol (1 ml).

Faeces (0·5 g) were defatted with hexane (10 ml) and the residue was extracted with methanol–water–phosphoric acid (8:1·9:0·1, by vol., 10 ml) and concentrated down to 1 ml by nitrogen stream at room temperature. Taxifolin (100 μl of a 50 ppm solution, final concentration 5 ppm) was added to each sample as an internal standard.

Extracts from both urine and faeces were filtered through a polytetrafluoroethylene 0·45-μm membrane from Waters Corporation into amber vials for HPLC–MS/MS analysis.

HPLC–electrospray ionisation–MS/MS analysis

A Quatro LC from Waters Corporation triple quadrupole mass spectrometer with an electrospray source was used in negative mode to obtain MS and MS/MS data. Liquid chromatography separations were performed using an Alliance 2695 system from Waters Corporation equipped with a Phenomenex (Torrance, CA, USA) Luna C18 (50 × 2·1 mm internal diameter) 3·5 μm particle size column and a Phenomenex Securityguard C18 (4 × 3 mm internal diameter) 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) was used (*t* (min), %B): 0, 8; 10, 23; 15, 50; 20, 50; 21, 100, followed by a re-equilibration step.

Metabolites were detected in multiple reaction monitoring (MRM) experiments, and their identity was confirmed by product ion scan experiments. The dwell time for the MRM experiments was 100 ms and the cycle time for all the experiments was 2 s. Cone energy and collision energy in MRM mode were optimised for each group of metabolites: 30 V and 10 eV for taxifolin, 30 V and 15 eV for microbially derived phenolic metabolite derivatives, 30 V and 20 eV for EC, and 40 V and 20 eV for EC derivatives.

Results

(Epi)catechin and its phase II metabolites

Free EC (MRM transition 289 → 245) and a signal corresponding to a dimer (577 → 289) were detected in faeces by liquid chromatography–ESI–MS/MS. The product ion spectrum of this dimer provided characteristic fragments at *m/z* 425 and *m/z* 405 originated by cleavage of the C-ring of one of the EC units through a retro Diels–Alder reaction and consecutive loss of water. A fragment at *m/z* 451, caused by heterolytic ring fission, was also observed.

A total of ten EC conjugates derived from the activity of phase-II enzymes in both the intestinal tract and liver were detected in urine samples (Table 1). All these compounds were either not detected in the control group or detected at concentrations that were at least 10-fold lower. The metabolites were initially identified by previously reported MRM transitions corresponding to the main fragments[24,30] and their identity was confirmed by a second MRM transition and/or by product ion scan experiments.

The EC metabolites included three glucuronidated forms (465 → 289), two sulphated forms (369 → 289), a monoconjugated metabolite with glutathione (594 → 289), two methylated and glucuronidated forms (479 → 303), a di-glucuronidated form (641 → 289) and a tri-conjugated metabolite (397 → 289) corroborated by MS/MS experiments. All these derivatives were detected in urine samples and the di-glucuronidated conjugate was also detected in faeces.

Fig. 1 shows the product ion spectrum of glucuronide (GlcA)-EC-3 (*m/z* 465). Fragments at *m/z* 289 and *m/z* 245, corresponding to the loss of the conjugate moiety and the respective cleavage of CO_2 from the free EC unit, were observed; as were two fragments at *m/z* 175 and 113, from the fragmentation of the GlcA moiety. The MS/MS spectra of other conjugates showed characteristic fragments corresponding to a B-ring retro Diels-Alder fission of the EC, such as the fragment at *m/z* 137 for Me-GlcA-EC, which corresponds to a B-ring fragment with attached methyl group and GlcA moieties, indicating that the conjugation was located on the B-ring.

Microbially derived proanthocyanidin metabolites

A total of twenty microbially derived PA metabolites were identified in urine from rats fed the NEPA-rich fraction; two of them were also detected in faeces (Table 2). All these metabolites were either not detected in the control group or detected at concentrations that were at least 10-fold lower. Microbially derived PA metabolites detected in urine included valerolactones, phenylvaleric acids, phenylpropionic acids, phenylacetic acids, benzoic acid, cinnamic acids and lignans. 3,4-Dihydroxyphenylacetic acid, detected by MRM transition (167 → 123), has been reported to be as a specific metabolite of polymeric PA, since it has never been identified in studies performed with pure monomeric flavan-3-ols[31]. 3,4-Dihydroxyphenylacetic acid may be metabolised to

Table 1. (−)-(Epi)catechin (EC) and conjugated metabolites in urine and faeces from rats fed a non-extractable proanthocyanidin-rich fraction from grape antioxidant dietary fibre

Metabolite	MRM parent	Identification	Urine	Faeces
(−)-EC	289 → 245	Standard retention time		×
PC dimer	577 → 289	MS/MS: 575; 451; 424; 405; 327; 289; 123		×
Mono-conjugated metabolites				
GlcA-EC-1	465 → 289	MS/MS: 465; 289; 245; 137; 113	×	
GlcA-EC-2	465 → 289	MS/MS: 465; 289; 257; 175;113	×	
GlcA-EC-3	465 → 289	MS/MS: 465; 289; 245; 175; 113	×	
Sulf-EC-1	369 → 289	MS/MS: 369; 289; 245; 228; 184; 113	×	
Sulf-EC-2	369 → 289	MRM daughter 289 → 245	×	
GSH-EC-1	594 → 289	MRM daughter 289 → 245	×	
Di-conjugated metabolites				
Me-GlcA-EC-1	479 → 303	MS/MS: 479; 303; 289; 245; 173; 137; 113	×	
Me-GlcA-EC-2	479 → 303	MS/MS: 479; 303; 285; 259; 173; 137; 113	×	
Di-GlcA-EC-1	641 → 289	MS/MS: 641; 465; 289	×	×
Tri-conjugated metabolites				
Di-Me-Sulf-EC-1	397 → 289	MRM daughter 289 → 245	×	

MRM, multiple reaction monitoring; PC, procyanidins; GlcA, glucuronide; Sulf, sulphate; GSH, glutathione; Me: methyl group.

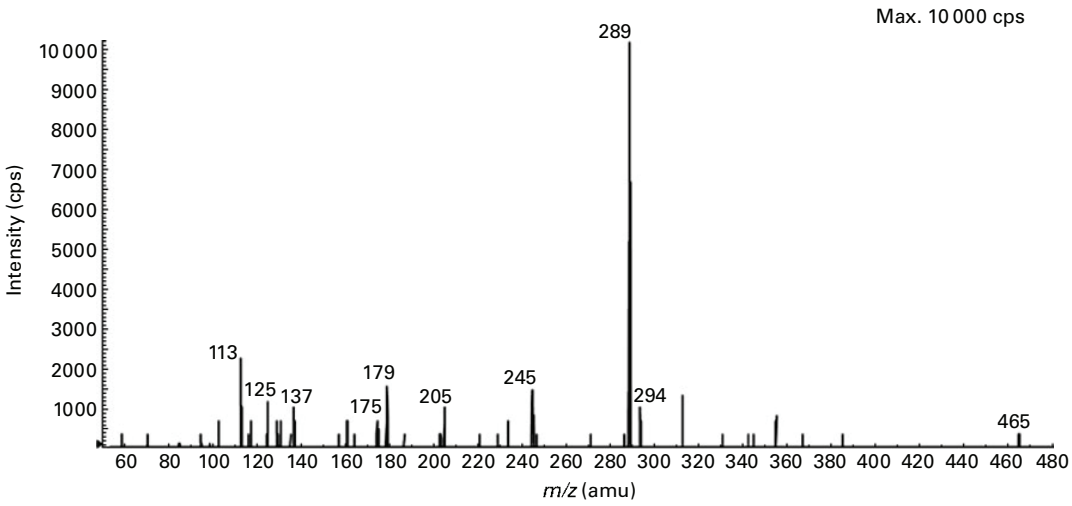

Fig. 1. HPLC–electrospray ionization-MS/MS product ion scan spectrum of glucuronide-(epi)catechin (*m/z* 465). cps, Counts per second; amu, atomic mass unit; max., maximum.

3-hydroxyphenylacetic acid and 4-hydroxyphenylacetic acid (151 → 107), which were also detected. Similarly, 3,4-dihydroxyphenylpropionic acid, 3-hydroxyphenylpropionic acid and 4-hydroxyphenylpropionic acid were detected in urine. Both 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxyphenylpropionic acid were absorbed and later conjugated in the liver as shown by the detection of the derivatives GlcA-3 or 4-hydroxyphenylacetic acid (327 → 151), Sulf-3, 4-dihydroxyphenylpropionic acid (261 → 181) and Sulf-3 or 4-hydroxyphenylpropionic acid (245 → 165). Conjugated forms of phenylvaleric and hippuric acid were detected in urine.

Two microbially derived phenolic metabolites were identified in the faeces from rats fed the NEPA-rich fraction: 4-hydroxyphenylpropionic acid (165 → 121) and 3,4-dihydroxyphenylpropionic acid (181 → 137).

Phenolic acids generated fragments corresponding to the successive loss of two CO_2 molecules. Similarly, MS/MS spectra of sulphated forms showed signals corresponding to the loss of sulphate and CO_2. These fragments confirm the identity

Table 2. Detection of microbially derived proanthocyanidin metabolites in urine and faeces from rats fed a non-extractable proanthocyanidin-rich fraction from grape antioxidant dietary fibre

Metabolite	MRM parent	Identification	Urine	Faeces
Valerolactones				
Sulf-dihydroxyphenylvalerolactone	287 → 207	MRM daughter 207 → 163	×	
Phenylvaleric acids				
Sulf-dihydroxyphenylvaleric acid	289 → 209	MRM daughter 209 → 165	×	
Phenylpropionic acids				
4-Hydroxyphenylpropionic acid	165 → 121	MS/MS: 165; 121; 93	×	×
3,4-Dihydroxyphenylpropionic acid	181 → 137	Standard retention time	×	×
Sulf-3,4-dihydroxyphenylpropionic acid	261 → 181	MRM daughter 181 → 137	×	
Suf-3 or 4-hydroxyphenylpropionic acid	245 → 165	MS/MS: 245; 165; 121	×	
Phenylacetic acids				
3-Hydroxyphenylacetic acid	151 → 107	Standard retention time	×	
4-Hydroxyphenylacetic acid	151 → 107	Standard retention time	×	
GlcA- 3- or 4-hydroxyphenylacetic acid	327 → 151	MS/MS: 327; 151; 107	×	
3,4-Dihydroxyphenylacetic acid	167 → 123	MS/MS: 167; 123; 105; 95	×	
Benzoic acids				
Benzoic acid	121 → 77	Standard retention time	×	
4-Hydroxybenzoic acid	137 → 93	Standard retention time	×	
3,4-Dihidroxybenzoic acid	153 → 109	Standard retention time	×	
Hippuric acid	178 → 134	Standard retention time	×	
Me-hippuric acid	193 → 178	MS/MS: 193; 178; 134	×	
Cinnamic acids				
m-Coumaric acid	163 → 119	Standard retention time	×	
p-Coumaric acid	163 → 119	Standard retention time	×	
Ferulic acid	193 → 134	Standard retention time	×	
Lignans				
Enterodiol	301 → 107	Standard retention time	×	
Sulf-enterolactone	377 → 297	MRM daughter 297 → 253	×	

MRM, multiple reaction monitoring; Sulf, sulphate; GlcA, glucuronide; Me, methyl group.

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of some of the microbially derived metabolites; others were confirmed by the use of standards, i.e. 4-hydroxybenzoic acid (Fig. 2).

Discussion

Several studies have addressed the metabolism of dietary oligomeric PA, mostly dimers. That work provides quite a clear picture of the different steps in the metabolism of PA dimers in laboratory animals and human subjects[18–21]. This process comprises the absorption of monomers and small oligomers (dimers) of PA in the small intestine and the absorption of microbially derived metabolites in the large intestine, after direct fermentation of the oligomers by microbiota without prior depolymerisation into EC. The absorbed metabolites may be conjugated in the liver, mostly resulting in GlcA, sulphates and methyl derivatives which pass to the bloodstream and eventually reach other tissues. Finally, the metabolites are excreted in urine and the fraction of PA that is not absorbed is excreted in faeces.

The metabolic fate of larger PA polymers is believed to follow the same pattern as that of dimers and trimers: essentially, direct cleavage of the EC units into smaller phenolic acids by the intestinal microbiota. By examining the metabolic fate of GADF, we have recently suggested that polymeric PA undergo depolymerisation into EC units during their transit along the intestinal tract[24,25]. This is important because it implies that the polymers may gradually release EC moieties during the postprandial period. Using a NEPA-rich fraction, devoid of EC monomers and extractable oligomers, we show here that this is indeed occurring. The faeces of rats fed with NEPA contained monomeric and dimeric EC and

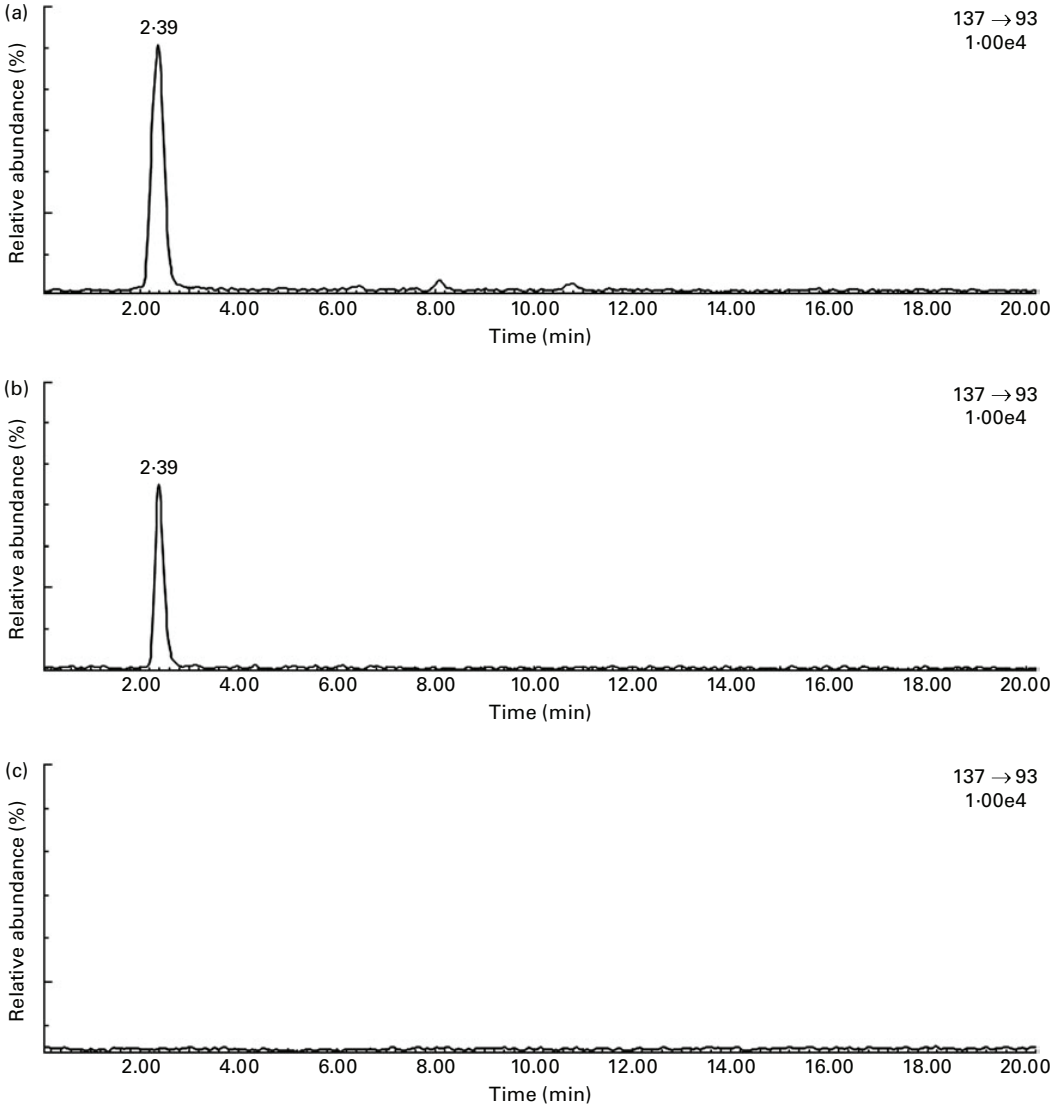

Fig. 2. HPLC–electrospray ionisation-MS profile corresponding to the detection by multiple reaction monitoring of 4-hydroxybenzoic acid (transition 137 → 93): (a) urine from rats fed non-extractable proanthocyanidins from grape antioxidant dietary fibre, (b) 4-hydroxybenzoic acid standard, (c) urine from rats fed water.

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their urine contained 10 phase II EC metabolites. These results clearly show that this fraction of dietary fibre generates bioavailable derivatives of EC. The di-glucuronidated EC derivative detected in faeces further demonstrates that those monomers that are released from NEPA efficiently enough to reach the liver suffer conjugation and are transported back to the intestine via bile. Our evidence for intestinal depolymerisation is consistent with a recent observation by Jové *et al.*[32] who report a 600 % recovery of free EC in the caecal content of rats after providing them with a single dose of PA-rich

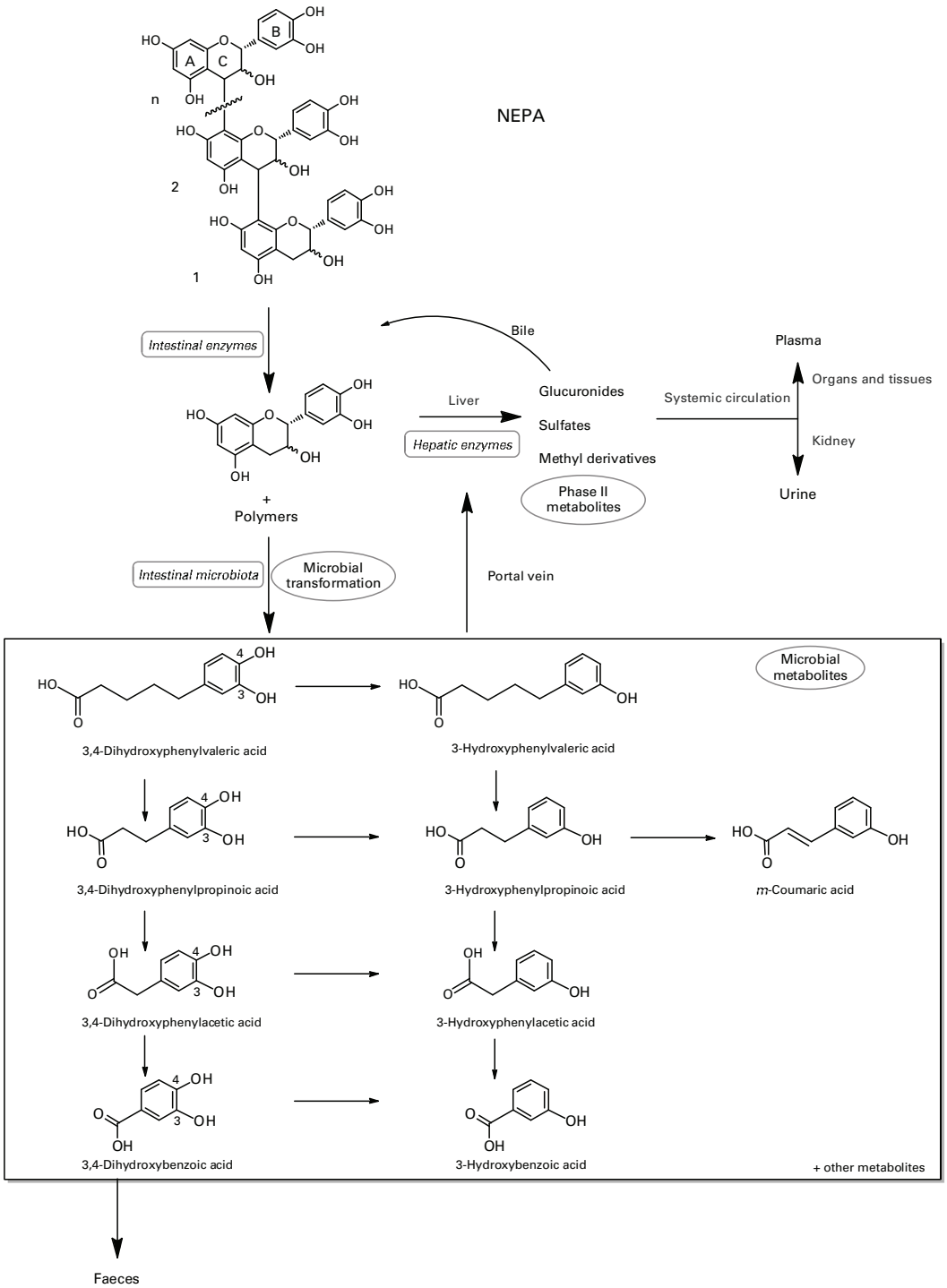

Fig. 3. Metabolism of non-extractable proanthocyanidins (NEPA).

almond extract. Moreover, the more than twenty EC-derived smaller metabolites detected in urine are consistent with the previous description of microbial fermentation and absorption. Our results also corroborate NEPA as PA, since direct evidence of the structure of these insoluble polymers is scarce. In fact, the residue after extraction with 70 % acetone is commonly not considered to be a source of polyphenols. Our results agree with those which report that the residues of the common extraction with 70 % acetone contain significant amounts of PA[12–14].

The transformation of NEPA (Fig. 3) differs in part from the process suggested for the transformation of small EPA, previously described. In the case of NEPA, a proportion of the larger PA polymers appears to be depolymerised during their transit along the intestinal tract, resulting in delivery of EC monomers and possibly oligomers. The posterior degradation by the intestinal microbiota into small units may also differ between NEPA and EPA. As NEPA are associated with the food matrix in foodstuffs, particularly with other insoluble polymers constitutive of dietary fibre, their conversion may be slow compared to that of EPA. This deferred release would make NEPA metabolites bioavailable for particularly long times after intake and may result in them having health effects for a long time. This may explain the previously reported delay in the increase of plasma antioxidant capacity after the intake of GADF by human subjects compared to that observed after the intake of food rich in EPA, such as red wine[33]. In addition, the metabolites detected in faeces prove that putatively active species remain in contact with the colonic epithelium for at least 24 h after ingestion. Indeed, the intake of PA, and particularly PA with a high degree of polymerisation, has been associated with a reduced risk of colorectal cancer[7] and our results suggest that food sources of NEPA could provide such putative cancer-preventative PA.

Previous nutritional studies have considered the extractable fraction of PA as the only source of dietary polyphenols. We show here that NEPA should be taken into account as most of the food in these studies contains significant amounts of NEPA. Further work is needed, both on the systematic analysis of NEPA in foodstuffs and on the metabolism of NEPA from different food sources, in order to unravel the contribution of this fraction of dietary PA to the health-promoting effects of fruit and vegetables.

In conclusion, we show here that NEPA are a source of polymeric PA that are progressively depolymerised during their transit along the intestinal tract into EC monomers and dimers, and later metabolised by the intestinal microbiota into smaller units. As a result, EC, phenolic acids and their phase II metabolites are in contact with the intestinal tract and bioavailable for at least 24 h after ingestion.

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3. Estudio de la relación entre la estructura de proantocianidinas y su actividad antioxidante y antiviral.

Publicación 4. Identificación de polifenoles procedentes de un extracto antiviral de *Chamaecrista nictitans* (Fabaceae) usando LC-ESI-MS/MS.

Identification of polyphenols from the antiviral *Chamaecrista nictitans* (Fabaceae) extract using high resolution LC-ESI-MS/MS

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RESUMEN:

El extracto de *Chamaecrista nictitans* ha sido seleccionado dentro de un programa de estudio sobre la biodiversidad en Costa Rica por su actividad contra el virus del herpes (HSV) (Herrero-Uribe *et al.* 2004). Anteriormente han sido atribuidas propiedades antivirales a extractos polifenólicos de plantas pero su relación estructura/actividad no ha sido claramente establecida (De Bruyne *et al.* 1999b).

El objeto de este estudio es el análisis del extracto y las fracciones por cromatografía líquida acoplada con espectrometría de masas (Q-TOF) para la identificar los polifenoles presentes en esta planta, posibles causantes de su actividad biológica. El descubrimiento de nuevos agentes antivirales no tóxicos es de suma importancia para el tratamiento de infecciones víricas.

Los compuestos oligoméricos y poliméricos fueron los principales componentes encontrados. Todas las estructuras oligoméricas y poliméricas se identificaron como mezclas de flavan-3-ol (proantocianidinas). A diferencia de otras mezclas de proantocianidinas homogéneas con respecto a las unidades constituyentes (p.e. epicatequina en uva y corteza de pino), el extracto de *Chamaecrista nictitans* contiene estructuras heterogéneas con fenoles monohidroxilados ((epi)catequina con (epi)afzelequina o (epi)guibourtinidol).

En un principio se consideró que el mecanismo biológico de acción de los compuestos polifenólicos de *Chamaecrista nictitans* estaba relacionado con su capacidad para actuar como antioxidantes. Sin embargo, los resultados obtenidos sugieren que la actividad biológica que presentan puede ser también debida a su configuración estructural (posición de los grupos hidroxilos) y no sólo a la capacidad de estos compuestos de ceder electrones, ya que los compuestos mayoritarios del extracto son menos activos como captadores de radicales libres que otras proantocianidinas (p.e. de uva) que no presentan actividad antiviral.

El presente trabajo sugiere una relación entre la estructura de proantocianidinas oligoméricas y su actividad antiviral. Las estructuras monofenólicas y los enlaces del tipo A podrían ser responsables de la actividad antiviral por inhibición de la transcripción tardía del ADN vírico. Una fracción rica en (epi)fisetinidol y (epi)afzelequina presenta potencial como agente preventivo contra el virus herpes simple.

Identification of polyphenols from the antiviral *Chamaecrista nictitans* extract using high resolution LC-ESI-MS/MS

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Chamaecrista nictitans (L) extract posesses antiviral properties against herpes-simplex virus that may be attributed to its constituent phenolics. We have used high resolution LC-ESI-MS/MS to identify the phenolic components of the most potent fraction of the extract (FII). The fraction is a complex mixture of oligomeric proanthocyanidins with a high content of monophenol moieties ((epi)fisetinidol, (epi)afzelechin and (epi)guibourtinidol) and A-type linkages, uncommon in other proanthocyanidin rich phenolic extracts such as those from grape seeds or pine bark. The major component in the fraction is the trimer (epi)fisetinidol-(epi)fisetinidol-(epi)fisetinidol. As monophenolic structures and A-type linkages have been related to antiviral effects, particularly through the inhibition of late transcription, we suggest that FII from the *C. nictitans* extract exerts its action by a particularly effective combination of proanthocyanidins that include these two structural features.

Keywords: LC-ESI-MS/MS, *Chamaecrista nictitans*, Polyphenols, Proanthocyanidins, Free radical scavenging activity, Antiviral activity

INTRODUCTION

Plants have always been the tradicional source of remedies for human disease. Tropical regions with their combination of sun and moisture are the planet's reservoir of plant biodiversity and an immense source of possible new drugs or coadjuvants for the treatment of many conditions. The role of natural extracts as alternative actives complementary to existing drugs is of particular importance in the case of development of microorganism resistances. The discovery of new non-toxic antiviral agents is of the utmost importance for the treatment of viral infections with high mutation rate. Costa Rican biodiversity is a particularly interesting working area as 4% of the planet species are concentrated in a small area (0.03% of the total dry surface) and about 25% of its area is protected by conservation policies.

In a study going back more than a decade an array of plant extracts (mostly *Euphorbiaceae*, *Fabaceae/Caesalpinaceae* and *Rubiaceae*) were tested as inhibitors of herpes-simplex virus (HSV) replication. Among the chosen extracts the one from *Chamaecrista nictitans* (L) (*Fabaceae/ Caesalpinaceae*) was one of the most promising. The extract was tested against HSV-1 and HSV-2 viral strains and compared with the antiviral drug acyclovir (*1, 2*). *C. nictans* (L) extract (CNE) and acyclovir inhibited the typical HSV's cytopatic effect (CPE) and it was determined that while acyclovir inhibits the secondary transcription of the virus, CNE inhibition was effective in two steps of HSV replication, namely adsorption and secondary transcription (*2*).

CNE appears to be a complex mixture of phenolic compounds of oligomeric nature (*2*). Antiviral properties have been attributed before to plant polyphenolic extracts but no clear structure/activity

relationships have been established. The antiviral effect has been related to the free radical scavenging of polyhydroxylated species (*3*), to unkown actions of saccharide moieties (*4*) or to unspecific inhibition of the adsorption phase or of some protein-protein interactions by polymeric tannins (*5*). Because we have demonstrated that CNE not only inhibits the adsorption step but also the virus replication, the structural characterization of the extract appears to be of great importance for the design of efficient antiviral natural preparations.

MATERIALS AND METHODS

Reagents and materials. *C. nictitans* (L) was collected in different locations of Costa Rica. Plant material consisted of aerial parts, mostly separated from roots. The original specimen was identified by Prof. Luis Poveda, at the Herbarium of the National University of Costa Rica. Plant Vouchers are deposited at the National Institute of Biodiversity Herbarium INB0002844961-67, INB0003393900-03, INB0003385491-4 and INB0003833609-12.

For the extraction and fractionation of polyphenols, the following reagents were used: methanol (ACS, 99,8%), hexane (ACS, 99,8%) methylene chloride (ACS, 99,8%,) and ethyl acetate (ACS, 99,8%) from ARVI (Cartago, Costa Rica). For the free radical scavenging activity assays 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH) (85%) from Sigma-Aldrich Chemical (Steinheim, Germany) was used. The positive control for the antiviral assay was Acyclovir (Sigma-Aldrich, Saint Louis, MO, USA)

For the characterization of polyphenols by HPLC-MS/MS the following reagents were used: methanol (analytical grade) was purchased from Panreac (Castellar del Vallès, Spain), and acetonitrile (HPLC grade) and formic acid (analytical grade) from Merck (Darmstad, Germany). Standards of ellagic acid (>96%), fisetin (>98%), and apigenin (>95%) were from Sigma Aldrich. Luteolin (>99%) was from Extrasynthese (Genay, France).

Water was purified by a Milli-Q plus system from Millipore (Bedford, MA, USA) to a resistivity of 18.2 MΩcm.

Apparatus and experimental conditions. **A** QSTAR Elite hybrid quadrupole-TOF system (Applied Biosystem Sciex, Foster City, CA, USA) equipped with a turbo spray source and coupled on line to an Agilent series 1200 HPLC instrument (Agilent, Waldbronn, Germany) fitted with a binary pump, an UV detector, an autosampler and a column oven was used for HPLC-UV-ESI-MS/MS experiments. The separations were conducted on a Phenomenex Luna C18 (Torrance, CA, USA) 3.5 µm particle size column (50 x 2.1 mm i.d.) equipped with Phenomenex Securityguard C18 column (4x 3 mm i.d.). The injection volume was 10 µL, the flow rate was 400 µL min^{-1}, and the temperature 25 ºC. Gradient elution was carried out with a binary system consisting of [A] 0,1% aqueous formic acid and [B] 0,1% formic acid in acetonitrile. An increasing linear gradient (v/v) of [B] was used, [*t*(min), %B]: 0, 8; 10, 23; 15, 50; 20, 50; 30, 70; 31, 100 followed by a re-equilibration step. Detection was at 280 nm. The QSTAR system was calibrated in the negative ion mode with taurocholic acid ($[M-H]^-$= 514.2844) for a *m/z* range from 100 to 900, and with PPG for a *m/z* range from 900 to 3000. Analyst QS 2.0 software from AB Sciex was used for data acquisition and processing.

Standards were solubilized in methanol at a concentration of 1 mg mL^{-1}, filtered through a 0.45 µm syringe filter (Millex-LH Millipore, Bedford, MA, USA), and diluted to 1 mg L^{-1} prior to injection. For free radical scavenging activity assays a Cary 50 UV-Vis Spectrophotometer (Varian, USA) was used.

Extraction and fractionation. An assay-guided fractionation strategy was used to concentrate the activity on active components (*6*). Crude extract (16.83 g) was dissolved in 90% aqueous methanol. The resulting hydroalcoholic solution was extracted with hexane (3 x 150 mL); the hexane fractions were combined and evaporated to dryness (6.14 g). The remaining methanolic aqueous solution was evaporated, diluted with water to 200 mL and extracted with methylene chloride (3 x 125 mL). The methylene chloride fractions were evaporated to dryness (3.36 g). In the same maner the remaining aqueous fractions were combined and evaporated

(6.41 g). A portion of the methylene chloride fraction (2.89 g) was submitted to vacuum liquid chromatography on a column (5 x 9 cm) filled with silica gel 60F (Merck). The column was washed with hexane and the extract fractionated by eluting stepwise with increasing amounts (10% each) of ethyl acetate in hexane up to 100% ethyl acetate. The final elution was done with an ethyl acetate/methanol 1:1 mixture, followed by a washing step with 100% methanol. With this procedure, 35 fractions were obtained, which were pooled into 8 final fractions after TLC evaluation. Fraction number number 7, labeled as 3-31D (a positive fraction against HSV-1 activity) was further sub-fractionated by reversed phase chromatography using the protocol described by Sun et al. (*6*) with minor modifications from 100 mg of 3-31D. Briefly, a glass SPE cartridge filled with 2 grams of LiChroprep RP-18 (Merck) was preconditionioned with deionizated water, methanol and water at pH 7. Phenolic acids were eluted with 10 mL of water. Then, oligomers were separated and concentrated by elution with with 25 mL of ethyl acetate (fractions F1 and F2) and finally with 10 mL of methanol (FIII). The combined oligomers fractions (F1 and F2) were re-eluted with 25 mL of methylene chloride to obtain FI (rich in monomeric species) and with 10 mL of methanol to obtain FII (rich in oligomeric species). These final fractions where tested for antiradical and antiviral activity.

Antiradical activity. The capacity of the fractions to scavenge free radicals was measured by the DPPH method (*7*). A 120 μM DPPH fresh methanolic solution was prepared and stored in the dark. Solutions of the fractions were prepared in methanol at several concentrations (from 0.5 to 18 mg L^{-1}). Then, 1 mL of the DPPH solution was added to 1 mL of each fraction solutions. DPPH is reduced in presence of antioxidant compounds, so after 30 minutes the absorbance values (A) were measured at 517 nm. DPPH blanks were also measured (A_0).

Results were plotted as degree of absorbance disappearance at 517 nm ($(1-A/A_0)x100$) against the concentration of sample divided by the initial DPPH concentracion. From these plots, the ED_{50} value, which corresponds to the concentration of antioxidant required to decrease the initial free radical activity by 50%, and the antiradical power (ARP), defined as the inverse of ED_{50} multiplied by 1000 were calculated. These assays were carried out in triplicate.

Antiviral activity. Toxicity: the cytotoxicity was determined using the protocol described in Herrero *et al.* (*2*). The solid sample dissolved in 20% DMSO phosphate buffer solution (PBS) (1 mg mL^{-1}). Two fold dilutions were performed in minimal essential medium (MEM) suplemented with 2% fetal calf serum, sodium bicarbonate and penicillin/streptomycin. Confluent Vero cell monolayers (ATCC-CCL-81) were overlayed with the different dilutions of the extract and were incubated at 37 °C with 5% CO_2 for three days. Toxicity was determined by observation of the morphology of the cells in comparison with the cell control without the extract. After three days of incubation, neutral red was added for confirmation of cell viability. The maximum concentration at which no cytotoxicity was observed, was used as the starting concentration for the antiviral activity determination.

HSV-inhibition test: the antiviral determination was conducted using the protocol described in Herrero *et al.* (*2*). The F strain of herpes simplex virus (ATCC-VR-733) was used at 100 $ID_{50\%}$ and viral dilutions were performed using PBS as diluent. Vero cell monolayers in 96 well microplates were inoculated with 100 mL of the virus and were adsorbed at 37 °C. After one hour, the inocula were removed and the monolayers were overlayed with the extract diluted to 1/50 in the maintenance medium and were incubated at 37 °C with 5% CO_2 for three days. Inhibition of the typical herpes simplex cytopathic effect (CPE) was recorded as the inhibitory effect of the extract, fraction or pure compound at the assayed concentrations. The inhibitory potency was tested performing two fold dilutions starting at the maximum non-toxic concentration. Samples were tested in triplicate.

RESULTS AND DISCUSSION

As the antiviral activity of *Chamaecrista nictitans* crude extract and fractions is believed to be related to its polyphenols (*2*) we monitored the free radical

scavenging activity of the fractions as well as the antiviral activity.

Free radical scavenging activity . The fractionation of *C. nictitans* (L) crude extract provided four fractions, in which polyphenols were separated according to their size and polarity, i.e., small phenolic acids in the aqueous fraction (FAQ), monomeric (FI), oligomeric species (FII), and polymeric species (FIII). The free radical scavenging activity of the different polyphenolic fractions was tested with DPPH (Table 1). Fraction II was clearly the most active, whereas FIII showed an antiradical scavenging activity similar to one of the crude extract.

Table 1: Free radical scavenging activity of the different fractions expressed as ED_{50} and ARP.

Fractions	ED_{50}*	ARP
Crude phenolic extract	112.1	8.9
FII	78.60	12.7
FIII	115.6	8.7

* ED_{50} in microgram fraction or control/ micromoles DPPH

Antiviral activity. FII exerted 72 h protection to the cells at a concentration of 68.7 µg/mL. Acyclovir was used as the positive control and the lowest cell protecting concentration was 21,9 µg/mL.

As fraction FII was the most active in both assays we analyzed the composition of this fraction by LC-ESI-MS/MS in an attempt to establish structure-activity relationships.

Identification of polyphenolic species. Structural elucidation of the main compounds in FII was performed through high resolution LC-UV-ESI-MS/MS analysis. The use of a high resolution tandem mass spectrometer provided not only the fragmentation patterns of the different compounds, but also their accurate mass. This information was then used for the identification of the main phenolic components in FII (Tables 2 and 3). Table 2 reports mainly monomeric, dimeric and trimeric structures, whereas Table 3 summarizes the proanthocyanidin polymeric profile.

Two flavone aglycones (luteolin and apigenin) and one flavonol (fisetin) were identified in the fraction. Luteolin, with a molecular ion ($[M-H]^-$) of 285 Da, showed characteristic fragments at *m/z* 151 and *m/z* 133, originated by cleavage of the C-ring through a *retro*-Diels-Alder (RDA) reaction (*8-10*). Other fragments at *m/z* 175, 199, and 217 were attributed to the losses of $-C_3O_2-C_2H_2O$, $-C_2H_2O-CO_2$, and $-C_3O_2$, respectively (*11*). Apigenin (*m/z* 269) also showed the typical fragments of the RDA reaction at *m/z* 151 and 117, and the fragment at *m/z* 183, which corresponds to losses of $-CO-CO-C_2H_2O$ (*8, 9*). Fisetin, also with $[M-H]^-$ of 285 Da, was distinguished from luteolin through the retention time, since in addition to their fragmentation pattern and accurate mass, these aglycones were confirmed by the use of standards. Fisetin gave fragments originated by the RDA reaction and by heterolytic ring fision (*m/z* 149 and 185), and other fragments caused by several losses of CO and H_2O molecules (*m/z* 239 and 229). Several flavonoid derivatives (with pentose or hexose moieties attached to the aglycone) were also identified in the fraction. These compounds showed less fragmentation, being the loss of the sugar moiety the main fragment in the product ion scan spectra. Luteolin hexoside (*m/z* 447) had a neutral loss of a glucose or galactose fragment (162 Da), and luteolin dihexoside (*m/z* 609) yielded two fragments that could be easily attributed to two consecutive losses of hexoside residues (*m/z* 447 and 285). In the same way, quercetin hexoside as well as quercetin pentoside were identified. These two compounds gave extra fragments at *m/z* 300, which corresponded to the aglycone radical ion, commonly observed in the fragmentation of some flavonols (*9*), and another fragment at 271 Da, which corresponded to the loss of CH_2O. Finally, apigenin hexoside could also be identified through the aglycone fragment and the accurate mass measurement.

Ellagic acid and ellagic acid pentoside were also constituents of F II. Ellagic acid (*m/z* 301) was confirmed through a standard, and gave several typical fragments at 283 (loss of water), *m/z* 271 (loss

of a carboxylic acid moiety), and *m/z* 229 (loss of CO) (*12*). The pentoside derivative gave as main fragment ellagic acid, through the loss of a neutral fragment of 146 Da. Cassiaoccidentalin B, a compound usually found in *Cassia* family plant extracts, was also identified in the fraction. The identity of the compound was confirmed by accurate mass measurements, as well as characteristic fragments in the product ion scan spectra at *m/z* 557 (loss of water), 429 (loss of the rhamnose moiety), 411 (loss of rhamnose and water) and 285 (loss of the whole sugar moiety).

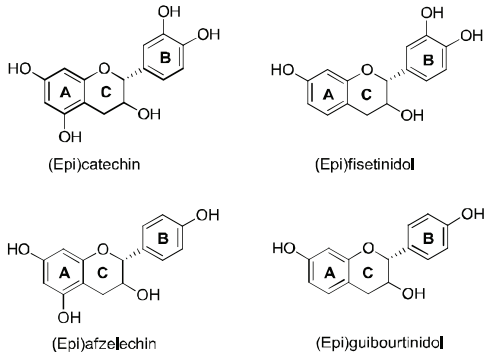


Figure 1. Main monomeric structures found in the extract of *Chamaecrista nictitans* (L).

Oligomeric proanthocyanidins were the most abundant polyphenolic components in the fraction. All the oligomeric and polymeric structures were identified as mixtures of flavan-3-ols. Figure 1 shows the basic monomeric structures found in the extract. Although in some cases fragmentation allows to locate each monomeric unit within the oligomer, in most instances this was not possible. Moreover MS alone does not provide information about epimers (e.g. catechin and epicatechin). Peaks with identical spectra at different retention times observed along the study revealed the presence of stereoisomers.

Dimeric structures (compounds 12-15, Table 2) were identified through the characteristic fragmentation pattern of proanthocyanidins (*13-15*) and also through the confirmation of the molecular formula with the accurate mass measurement. Figure 2 shows as an example the typical fragmentation pattern for a dimeric proanthocyanidin composed of (epi)fisetinidol and (epi)catechin, together with the MS/MS spectra of the compound. The position of the hydroxyl moieties was revealed by the fragmentation of the oligomeric structures and allowed the identification of the monomeric species. For example, RDA losses of 152 Da from the molecular ion were attributed to (epi)fisetinidol and (epi)catechin extension units because these compounds have two hydroxyl groups in the B ring, whereas losses of 136 Da were attributed to (epi)afzelechin or (epi)guibourtinidol, which have only one hydroxyl group in the B ring. In the same way, a heterocyclic ring fision (HRF) with a loss of 110 Da indicated the presence of (epi)fisetinidol or (epi)guibourtinidol, since these compounds have only one hydroxyl group in the A ring, whereas a loss of 126 Da indicated (epi)afzelechin or (epi)catechin in the structure. In addition, the quinone methide (QM) fragmentation (interflavanic bond cleavage) provided the molecular ion of the monomeric units. A-type dimeric proanthocyanidins gave mass spectra very similar to the B-type ones, with the molecular ion and some of the fragments two mass units lower towing to the presence of an extra interflavanic C-C linkage. This extra bond sometimes hampers RDA fragmentation (*14*), which is not always observed.

Several trimeric structures were also identified in the fraction (compounds 16-22, Table 2). All of them were different combinations of (epi)guibourtinidol, (epi)afzelechin, (epi)fisetinidol, and (epi)catechin monomeric units, linked either by B-type or A-type bonds. The monomeric sequence and the position of the A-type bonds were elucidated through the fragment ions shown by the product ion spectra (*16*), since the compounds follow the same elucidation pattern as dimeric structures. As an example, Figure 3 shows the fragmentation scheme and the product ion spectra of compound 18, which is the most abundant polyphenolic compound in the fraction. The fragment at *m/z* 707 is caused by the HRF of the upper (epi)fisetinidol unit (loss of 110 Da), and the fragment at *m/z* 665 is the result of RDA on the (epi)fisetinidol (-152 Da) unit. The HRF of an (epi)fisetinidol unit directly from the molecular ion. The QM reaction gives as a result the fragment at *m/z* 545. This ion can be further fragmented, to obtain a

new fragment at *m/z* 435, result of an HRF of the (epi)fisetinidol unit. RDA reactions of the fragment at *m/z* 545 were also observed, and *m/z* 393 (RDA on the (epi)fisetinidol unit). The further fragmentation of

Table 2: Polyphenolic compounds identified in FII of *Chamaecrista nictitans* (L) by LC-UV-ESI-QTOF analysis.

Id. Nº	Compound	$[M-H]^-$	Product Ions	Acc. Mass	Error (ppm)	MF
1	Luteolin *	285	217, 199, 175, 151, 133, 107	285,0401	-3,02	$C_{15}H_{10}O_6$
2	Luteolin hexoside	447	285	447,0936	-1,09	$C_{21}H_{20}O_{11}$
3	Luteolin dihexoside	609	447, 285	609,1250	0,36	$C_{27}H_{30}O_{16}$
4	Ellagic acid *	301	283, 257, 229, 173	300,9979	-2,93	$C_{14}H_6O_8$
5	Ellagic acid pentoside	433	301	433,0408	-1,73	$C_{19}H_{14}O_{12}$
6	Quercetin hexoside	463	301, 300, 271, 179	463,0882	-3,46	$C_{21}H_{20}O_{12}$
7	Quercetin pentoside	433	301, 300, 271	433,0412	0,12	$C_{20}H_{18}O_{11}$
8	Apigenin *	269	225, 183, 159, 151, 149, 117, 107	269,0456	0,2	$C_{15}H_{10}O_5$
9	Apigenin hexoside	431	269	431,0983	-2,95	$C_{21}H_{20}O_{10}$
10	Fisetin *	285	239, 229, 162, 149, 135, 121	285,0387	-1,62	$C_{15}H_{10}O_6$
11	Cassiaoccidentalin B	575	557, 531, 429, 411, 285	575,1377	0,4	$C_{27}H_{28}O_{14}$
12	(Epi)gui-(epi)afz	529	393, 273, 257, 255, 137	529,149	-2,66	$C_{30}H_{26}O_9$
13	(Epi)fis-(epi)afz	545	435, 409, 393, 273, 271, 137	545,1421	-4,26	$C_{30}H_{26}O_{10}$
14	(Epi)fis-A-(epi)cat	559	449, 289, 287, 273, 271, 161	559,1229	-3,19	$C_{30}H_{24}O_{11}$
15	(Epi)fis-(epi)cat	561	451, 409, 391, 289, 271, 245, 161,137	561,1385	-2,91	$C_{30}H_{26}O_{11}$
16	(Epi)fis-(epi)fis-(epi)gui	801	691, 665, 649, 545, 409, 393, 273, 161	801,2202	2,4	$C_{45}H_{38}O_{14}$
17	(Epi)fis-(epi)fis-A-(epi)fis	815	705, 663, 545, 543, 527, 433, 419, 407, 273, 271, 161			$C_{45}H_{36}O_{15}$
18	(Epi)fis-(epi)fis-(epi)fis	817	707, 665, 545, 435, 409, 393, 271, 161			$C_{45}H_{38}O_{15}$
19	(Epi)fis-(epi)cat-A-(epi)fis	831	721, 679, 561, 559, 435, 407, 287, 271, 161			$C_{45}H_{36}O_{16}$
20	(Epi)afz-A-(epi)fis-A-(epi)cat **	831	695, 679, 543, 433, 271			$C_{45}H_{36}O_{16}$
21	(Epi)cat-A-(epi)afz-(epi)afz **	831	695, 679, 559, 423, 271			$C_{45}H_{36}O_{16}$
22	(Epi)fis-(epi)cat-(epi)fis	833	723, 681, 561, 451, 409, 289, 271			$C_{45}H_{38}O_{16}$

* Identity confirmed through standards.

** Tentatively assigned

the molecule into two monomeric units is possible, giving fragments at *m/z* 273 (not observed) and *m/z* 271.

Many other oligomeric species were detected through the direct infusion of FII into the mass spectrometer. Figure 4A shows the MS spectra in the mass

Table 3: Combinations of monomeric units (G: (epi)guibourtinidol; F: (epi)fisetinidol; A: (epi)afzelechin; C: (epi)catechin) that match the molecular mass of the oligomers (only B-type bonds) found in FII of *Chamaecrista nictitans* (L).

n = 4				n = 5				n = 6			
$[M-H]^-$	G	F/A	C	$[M-H]^-$	G	F/A	C	$[M-H]^-$	G	F/A	C
1025	4	0	0	1281	5	0	0	1537	6	0	0
1041	3	1	0	1297	4	1	0	1553	5	1	0
1057	3	0	1	1313	4	0	1	1569	5	0	1
	2	2	0		3	2	0		4	2	0
1073	2	1	1	1329	3	1	1	1585	4	1	1
	1	3	0		2	3	0		3	3	0
1089	2	0	2	1345	3	0	2	1601	4	0	2
	1	2	1		2	2	1		3	2	1
	0	4	0		1	4	0		2	4	0
1105	1	1	2	1361	2	1	2	1617	3	1	2
	0	3	1		1	3	1		2	3	1
1121	1	0	3		0	5	0		1	5	0
	0	2	2	1377	2	0	3	1633	3	0	3
1137	0	1	3		1	2	2		2	2	2
1153	0	0	4		0	4	1		1	4	1
				1393	1	1	3		0	6	0
					0	3	2	1649	2	1	3
				1409	1	0	4		1	3	2
					0	2	3		0	5	1
				1425	0	1	4	1665	2	0	4
				1441	0	0	5		1	2	3
									0	4	2
								1681	1	1	4
									0	3	3
								1697	1	0	5
									0	2	4
								1713	0	1	5
								1729	0	0	6

range 900-2000 Da, where three different regions, corresponding to tetramers, pentamers and hexamers, respectively can be clearly distinguished. When each of these regions is analyzed individually (see figure 4B, tetramers, and figure 4C, pentamers, as examples), it can be observed that they include series

of peaks differing in 16 Da (one oxygen atom). The molecular weights of the ions corresponding to these peaks match different combinations of (epi)guibourtinidol, (epi)fisetinidol, (epi)afzelechin, and (epi)catechin. In addition, in all cases an increased intensity is also observed for the peaks corresponding to $[M-H-2]^-$, which indicates the presence of oligomers with one A-type bond.

Combinations of these four monomeric species can lead to several oligomers with the same molecular mass. Although the fragmentation pattern of the MS/MS spectra of these peaks corroborates the presence of the four monomeric units, the information provided is insufficient to attempt any accurate assignment as in many occasions the intensity of the signal was too low. This fact,

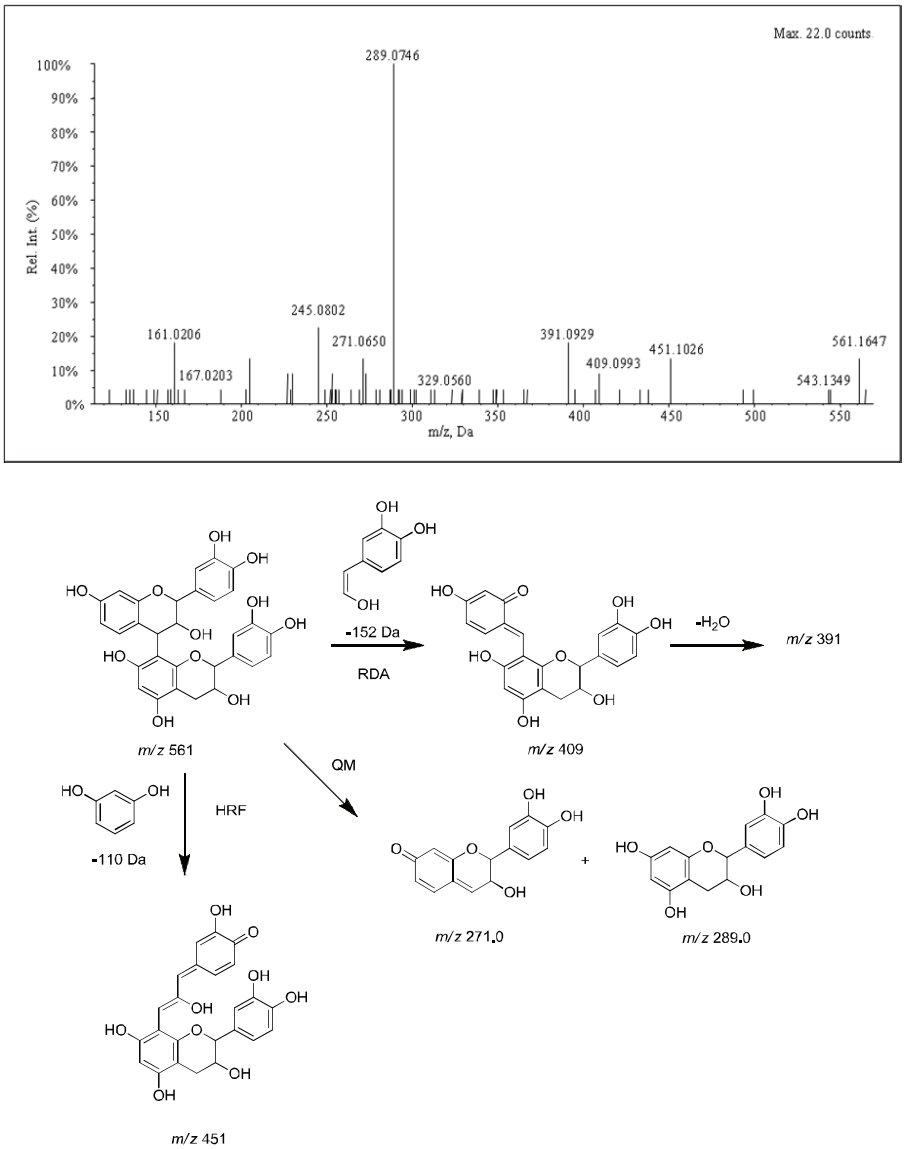


Figure 2: Fragmentation pattern and MS/MS spectra of the dimeric compound (epi)fisetinidol-(epi)catechin (compound 15, *m/z* 561).

together with the absence of standards, makes it very difficult to elucidate the composition and sequence of each of the oligomers, and also the location of the A-bonds. In a few occasions only one combination is possible, as for example the peaks with molecular mass of 1025 Da and 1153 Da, which can be only obtained by the combination of four (epi)guibourtinidol or four (epi)catechin units

respectively. Table 3 provides a list of the possible combinations of monomeric units that match the molecular masses found in the spectra of FII.

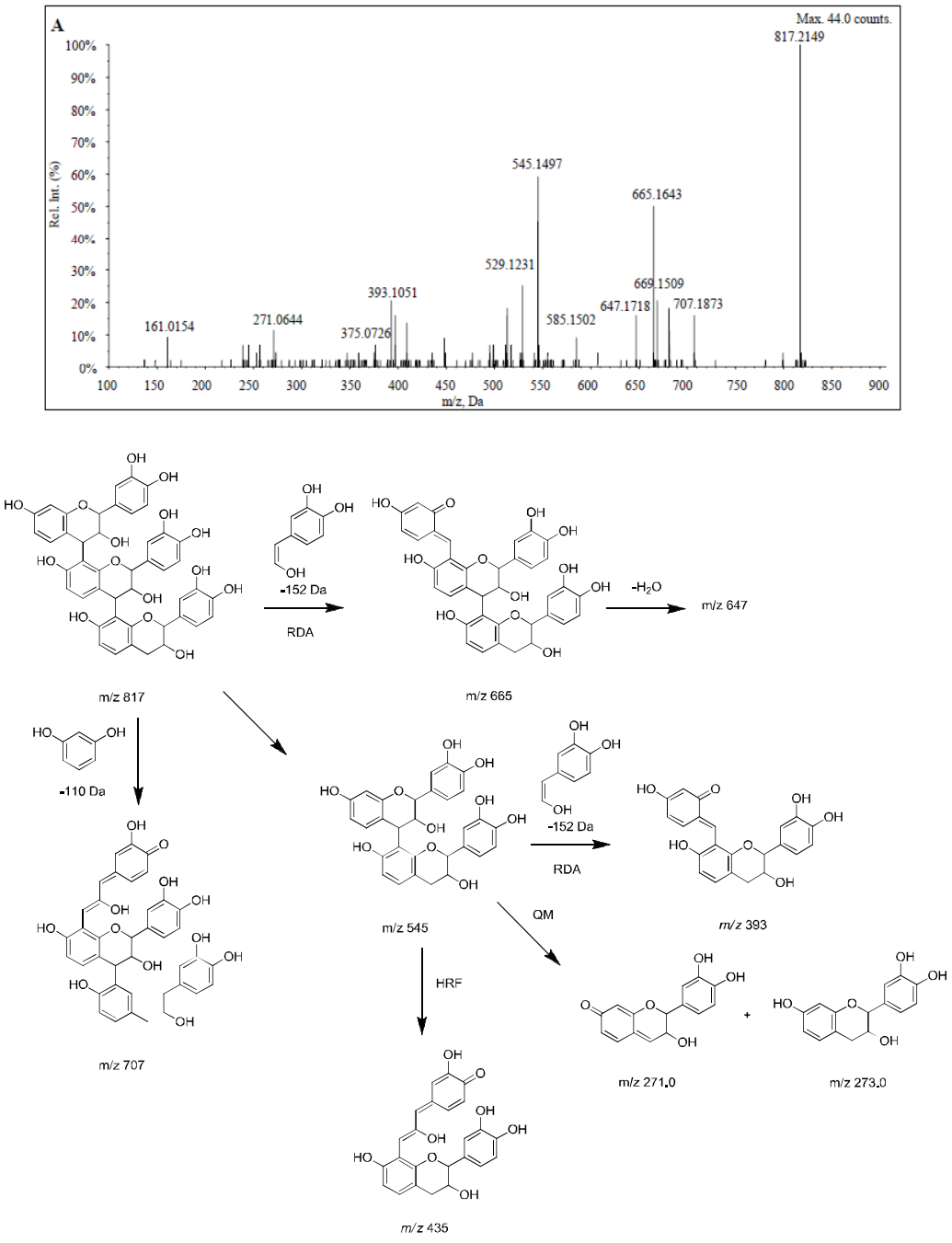
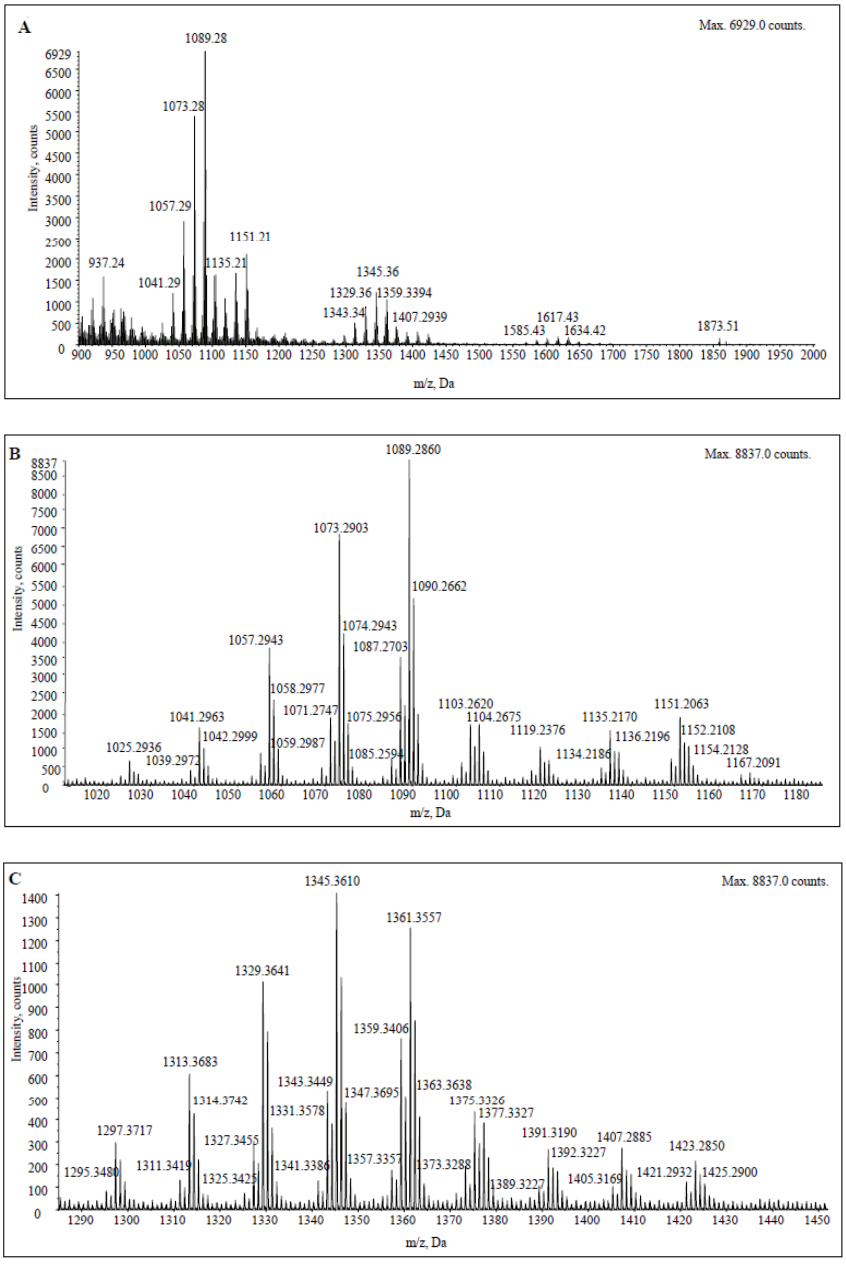


Figure 3: Fragmentation pattern and MS/MS spectra of the trimeric compound (epi)fisetinidol-(epi)fisetinidol-(epi)fisetinidol (compound 18, *m/z* 817).

Structure-activity relationships. In contrast with the proanthocyanidins from commercially available sources such as grape seeds and pine bark that are basically (epi)catechin polymers (*17-19*) *C. nictitans* (L) oligomers are highly heterogeneous, composed of combinations of monomers ((epi)guibourtinidol, (epi)fisetinidol, (epi)afzelechin, and (epi)catechin).

The major compound within the most active fraction (FII) is a trimer: (epi)fisetinidol-(epi)fisetinidol-(epi) fisetinidol. It should be noted that this structure, as well as many other oligomers in the fraction presents an unusual number of monophenols; some of them ((epi)afzelechin, (epi)guibourtinidol) are not even efficient radical scavengers. The herpes reported effect of an epiafzelechin dimer that inhibits simplex virus type 2 replication (*20*) is fully consistent with the multilevel action of our *C. nictitans* (L) extract: the prevention of virus attachment to the cell wall and the inhibition of late transcription of the virus DNA (*3*). The anti-attachment action might be attributed

Figure 4: MS spectra obtained through direct infusions of FII. General polymeric profile (A), polymeric profile for tetramers (B), and polymeric profile for pentamers (C).



to structural features common with other phenolics such as epicatechin and epicatechin-gallate as suggested (*20*) while the monophenols may be more efficient at inhibiting the late transcription. Protease inhibitory activity might be responsible for this late arrest in viral replication as suggested by the effect of fisetin, a monohydroxyflavonol structurally similar to fisetinidol on enterovirus replication (*21*). Another differential feature of *C. nictitans* proanthocyanidins is the presence of A-type oligomers. The A-type linkage have been shown to be systemically bioaccessible after ingestion (*22*) and has been also related to the antiviral activity of proanthocyanidin oligomers (*23*).

In summary, *Chamaecrista nictitans* extracts contain proanthocyanidins with two structural features, namely monophenols and A-type linkages that may be responsible for their antiviral activity. Particularly, a fraction rich in (epi)fisetinidol and (epi)afzelechin oligomers shows potential as preventive agent against herpes simplex virus.

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V. DISCUSIÓN

DISCUSIÓN

La caracterización de la diversidad estructural de proantocianidinas en los alimentos es necesaria para determinar mejor los efectos de estos polímeros sobre la nutrición y la salud. Hoy en día, existen numerosas bases de datos donde se puede consultar la composición y contenido de los principales alimentos. No obstante, es necesario realizar un desarrollo más completo de estas herramientas para obtener unos datos más precisos sobre la ingesta de antioxidantes. La validación y optimización de métodos de análisis para la determinación de componentes bioactivos en productos de origen vegetal es fundamental para conocer su actividad. Los estudios realizados, tanto en animales como en humanos, utilizando productos ricos en proantocianidinas, han indicado que desempeñan una función preventiva asociada con una disminución del riesgo de padecer ciertas enfermedades cardiovasculares y diabetes (Santos-Buelga *et al.* 2000; Zern *et al.* 2005; Serrano *et al.* 2009; Bladé *et al.* 2010; Montagut *et al.* 2010a). Del mismo modo, se ha mostrado una asociación inversa entre la ingesta de proantocianidinas y el riesgo de padecer cáncer colorrectal (Rossi M *et al.* 2010). El cáncer colorrectal es una de las causas más comunes de muerte por cáncer en el mundo occidental (Dimitrios 2006) y parece particularmente probable que las dietas ricas en frutas y verduras puedan reducir el riesgo de la desarrollar la enfermedad (Terry *et al.* 2001). Otros estudios aseguran que las proantocianidinas con enlaces de tipo A están asociados con la prevención de infecciones del tracto urinario (Foo *et al.* 2000a; Foo *et al.* 2000b; Howell *et al.* 2005). No obstante, a pesar del alto contenido de proantocianidinas presente en alimentos, son pocas las investigaciones que han establecido como las proantocianidinas de alto peso molecular y particularmente las que contienen enlaces de tipo A, pueden prevenir a través de la dieta el desarrollo de ciertas enfermedades.

Proantocianidinas en la canela, aplicación de la técnica MALDI-TOF/TOF.

Las proantocianidinas están presentes en alimentos como la canela y la uva. Se cree que parte de las propiedades beneficiosas de las plantas proceden de

las proantocianidinas oligoméricas y poliméricas. En concreto, la canela, ampliamente consumida en numerosos países y culturas, es una importante fuente de proantocianidinas de alto peso molecular. Contiene más de 8% en peso de proantocianidinas totales, mientras que el contenido de monómeros es sólo de 0,024% (Gu *et al.* 2004).

Sin embargo, en los resultados obtenidos en los estudios de caracterización de proantocianidinas de canela usando LC-ESI-MS apenas se encuentran proantocianidinas de alto peso molecular y éstas sólo son detectadas por iones con doble carga. Esto despierta el interés en realizar un estudio de caracterización más pormenorizado, utilizando otras técnicas que permitan realizar un estudio de su composición. Este trabajo (**Publicación 1**, (Mateos-Martín *et al.* 2012a)) es el primer estudio presentado de proantocianidinas de canela determinadas por MALDI-TOF/TOF.

MALDI-TOF/TOF MS es una de las herramientas más recientes para el estudio de la estructura y composición de polímeros (Pasch *et al.* 2001). En primer lugar se realizó la puesta a punto y validación de un método por MALDI-TOF/TOF para la determinación de proantocianidinas en muestras vegetales y estudiar la composición de proantocianidinas en canela. Para confirmar la identidad de los compuestos se realizó un estudio MS/MS de las señales con mayor intensidad. Esta metodología requirió un estudio de diferentes matrices, agentes cationizantes, disolventes, técnicas de preparación de muestra y optimización de los parámetros instrumentales para aumentar la eficacia de la ionización. Para la validación del estudio se utilizó como patrón un extracto de uva con un contenido en proantocianidinas conocido.

El estudio por MALDI-TOF/TOF ha permitido identificar polímeros de proantocianidinas con un alto grado de polimerización. Éstos se han clasificado como procianidinas y prodelfinidinas, con y sin grupos galatos, y propelargonidinas.

Una práctica habitual en MALDI para favorecer la ionización es emplear la adición de algún tipo de sal. En nuestro caso, se utilizó cloruro de sodio

como agente cationizante, aunque durante la preparación de las muestras puede haber una cierta contaminación con iones sodio y potasio. Si se encuentran presentes estos dos iones, se producirán señales de proantocianidinas correspondientes a los iones aductos de éstos que presentan una diferencia de masa atómica de 16 uma, la misma diferencia que una sustitución de un grupo hidroxilo. Una de las principales dudas en el proceso de elucidación estructural fue conocer con certeza si la diferencia de 16 uma entre procianidinas y prodelfinidinas se debía realmente a un grupo hidroxilo o si ésta podía deberse a la formación de aductos de un mismo compuesto con los iones sodio y potasio. Para corroborar la presencia de especies que difieren en el número de grupos hidroxilo se añadió trifluoroacetato de cesio como agente cationizante. El cesio da como resultado la presencia exclusiva de iones moleculares Cs^+, por lo que se utilizó éste para confirmar que las diferencias de 16 uma correspondían a un grupo hidroxilo y no a las diferencias de masa entre iones Na^+ y K^+. Por lo tanto, se registraron las masas correspondientes a los compuestos de interés coordinados con iones de sodio, mostrando la masa del compuesto más 23 Da, e iones de cesio, mostrando la masa del compuesto más 133 Da. Aunque el cesio fue imprescindible para descartar posibles contaminaciones de potasio, los aductos de iones de sodio mostraron mejor resolución.

Las estructuras de todas las especies de alto grado de polimerización determinadas en canela contienen al menos un enlace de tipo A. La presencia de estos enlaces puede ser fácilmente observada en el espectro de masas porque las señales se desplazan dos unidades de masa cuando son comparadas con las señales de proantocianidinas de tipo B, obtenidas para el extracto de uva. Esta diferencia corresponde a los dos átomos de hidrógeno que se pierden en la formación del doble enlace interflaváníco. Las únicas estructuras presentes con enlaces de tipo B fueron las diméricas.

Las procianidinas y propelargonidinas de menor grado de polimerización se han detectado anteriormente en extractos de canela (Lazarus *et al.* 1999; Gu *et al.* 2003a). El uso de MALDI-TOF/TOF permitió detectar la presencia de prodelfinidinas y proantocianidinas con ésteres de galatos, los cuales nunca se habían identificado antes en extractos de canela. Esto puede ser

particularmente interesante y de gran importancia biológica, ya que las unidades de pirogalol de (epi)galocatequinas, que son encontrados por ejemplo en el té, y las proantocianidinas galoizadas son más reactivos que los grupos catecol de (epi)catequinas (Lizárraga *et al.* 2007).

En muchos casos, la caracterización de muestras poliméricas por LC-MS se realiza por inyección directa al espectrómetro de masas ya que los polímeros de proantocianidinas son difíciles de separar por cromatografía líquida en fase inversa. El carácter anfifílico de las proantocianidinas aumenta con el grado de polimerización y la separación es aún más difícil cuando se encuentran presentes grupos galatos (Monagas *et al.* 2010a). Por esta razón la ionización por MALDI juega un papel fundamental, ya que en ésta se produce sólo un ión molecular cargado individualmente para cada molécula parental y permite la detección de la masa con precisión. La naturaleza no destructiva de la ionización por MALDI hace que sea una técnica atractiva para la investigación de mezclas complejas poliméricas (Krueger *et al.* 2000); al encontrarse el polímero protegido con una matriz orgánica que absorbe la mayor parte de la elevada energía implicada en la ionización, se evita la descomposición del mismo. Además, la ionización por MALDI es menos sensible a la presencia de sales y otros contaminantes en el medio que la ionización por ESI. Las proantocianidinas parecen ionizar mejor en el modo positivo con la presencia de una sal. La adición de sal a la muestra no es una práctica común en la ionización por electrospray ya que la adición de una sal no volátil obstruiría rápidamente el capilar a la entrada del analizador. Sin embargo es una práctica común en MALDI que mejora los resultados (Hammerstone *et al.* 1999).

El inconveniente principal de esta técnica es que, debido a que el espectrómetro no está acoplado a un sistema de separación, los espectros se obtienen para toda la muestra sin ninguna separación previa de los componentes de ésta, por lo que los compuestos con la misma masa aparecen juntos en el espectro. La opción de poder realizar experimentos MS/MS es crucial para identificar los diferentes compuestos, siempre que las señales de los iones moleculares tengan una intensidad suficiente como para una correcta elucidación.

El espectro de masas completo obtenido para el extracto de uva fue muy similar al de canela, con la principal diferencia de que las proantocianidinas de uva contienen principalmente enlaces de tipo B, tal como se describe en la literatura (Yang *et al.* 2000). Los experimentos de MS/MS confirmaron la presencia de estas estructuras a partir de la fragmentación característica para este tipo de compuestos.

Para los experimentos de MS/MS se utilizó el modo CID (disociación inducida por colisión) que resultó ser indispensable para la fragmentación de estos compuestos, no observando fragmentación en modo LID (disociación inducida por laser). Los principales fragmentos que se generan durante los experimentos de MS/MS son resultados de reacciones de retro Diels-Alder (RDA), fisión del anillo heterocíclico, escisión de unidades monoméricas, y fragmentos correspondientes a pérdidas de agua. Los fragmentos observados están de acuerdo con los patrones de fragmentación típica ya descritos en la literatura para los compuestos polifenólicos.

Los resultados obtenidos demuestran que la técnica MALDI nos permite obtener un perfil de proantocianidinas en extractos vegetales de forma rápida, con menor cantidad de muestra y con mayor fiabilidad que con LC-ESI-MS. Además, MALDI-TOF/TOF el modo CID permite una elucidación precisa y altamente sensible de compuestos a través de sus espectros MS/MS. Por lo tanto, MALDI-TOF/TOF en el modo de CID es una técnica muy útil para el análisis estructural de polímeros de proantocianidinas de muestras con un alto grado de heterogeneidad y proporciona una resolución superior que otros métodos espectrómetros de masas. Este estudio con canela por MALDI-TOF/TOF está de acuerdo con otros autores que demuestran que la diversidad estructural de proantocianidinas en diversas fuentes naturales es mucho mayor que la que previamente se había descrito por LC-ESI-MS (Krueger *et al.* 2002; Es-Safi *et al.* 2006; Li *et al.* 2010). Además en la mayoría de estos estudios no sólo se identifican proantocianidinas con mayor grado de polimerización, sino también con mayor grado de galoización. Nuestro estudio muestra que la canela contiene estructuras nunca antes descritas para esta fuente y una mezcla muy heterogénea de procianidinas, prodelfinidinas y propelargonidinas, que

incluye ésteres de galato, compuestos identificados por primera vez en canela. Los galatos y el grupo pirogalol de las prodelfinidinas están relacionados con la actividad biológica de los polifenoles de uva y té. Así pues, la presencia de estas subestructuras puede explicar algunas de las propiedades de los extractos de canela.

Metabolización de las proantocianidinas de canela.

Una vez realizado el estudio exhaustivo de las proantocianidinas presentes en la canela de Ceylán (*Cinnamomum zeylanicum* L.) el siguiente objetivo para avanzar en el conocimiento de los posibles efectos de estas proantocianidinas fue obtener el perfil metabólico en detalle, es decir, estudiar las diferentes transformaciones y degradaciones que pueden sufrir una vez ingeridas (**Publicación 2**, (Mateos-Martín *et al.* 2012c)).

Son muy pocos los estudios de biodisponibilidad realizados con proantocianidinas poliméricas. El interés del estudio radica en que las proantocianidinas poliméricas no son directamente biodisponibles (Donovan *et al.* 2002). La mayoría de los trabajos publicados hasta la fecha se han dirigido principalmente a la biodisponibilidad de dímeros o trímeros, mientras que la mayoría de los polifenoles presentes en la dieta están de forma polimérica (Neveu *et al.* 2010). Los flavanoles monoméricos y algunos diméricos, una vez ingeridos, se absorben en el intestino delgado y su metabolitos son conjugados en el hígado, los cuales a continuación pasan a la corriente sanguínea y son posteriormente excretados en la orina, o regresan a través de la bilis al intestino delgado. En un estudio reciente en el que se administró a ratas un dímero de procianidina marcada con ^{14}C, la biodisponibilidad determinada fue de alrededor del 80%, basándose en la búsqueda de ^{14}C en orina (Stoupi *et al.* 2010). Las proantocianidinas que no se absorben en el intestino delgado llegan al colon donde, después de la despolimerización y la fermentación por la microbiota, pueden liberar metabolitos pequeños que son absorbidos, transformados en el hígado, y tienen el mismo destino que los metabolitos derivados de la absorción en el intestino delgado (Appeldoorn *et al.* 2009a; Monagas *et al.* 2010b). Los metabolitos encontrados en la orina indican que la fracción de

proantocianidinas ha estado en contacto con los tejidos a nivel sistémico y que puede haber ejercido un efecto sobre ellos (Touriño *et al.* 2009). Los metabolitos que se excretan en heces están en contacto con el tejido colónico y pueden influir en la salud intestinal (Touriño *et al.* 2011). Estos metabolitos pueden ser responsables de los efectos sobre la salud derivados de alimentos ricos en polifenoles, en lugar de los compuestos originales que se encuentran en los alimentos.

En el presente estudio, se analizaron los metabolitos derivados de proantocianidinas de canela en orina y heces. Se encontraron metabolitos de fase II mono, di y triconjugados de (epi)catequina y más de 20 ácidos fenólicos procedentes de la fermentación microbiana intestinal. Las heces contenían (epi)catequina monomérica y dimérica no conjugadas. Esto sugiere que existen especies captadoras de radicales libres (grupos hidroxilos fenólicos no conjugados) en contacto con las paredes intestinales después de la ingestión de canela que podrían actuar como agentes preventivos contra el cáncer de colon (Lizárraga *et al.* 2007; Chung *et al.* 2009).

El perfil de los metabolitos fenólicos de canela en la orina es compatible con una mezcla de proantocianidinas que se despolimerizan en sus unidades de (epi)catequina para después ser degradadas a unidades más pequeñas como ácidos fenólicos, con la posterior absorción y la conjugación en metabolitos biodisponibles (Rios *et al.* 2003; Touriño *et al.* 2009; Touriño *et al.* 2011). Estos ácidos fenólicos son los principales metabolitos de proantocianidinas poliméricos (Rios *et al.* 2003). Los conjugados microbianos derivados de proantocianidinas que se encuentran en orina nos indican que las proantocianidinas una vez transformadas por la microbiota colónica en ácidos fenólicos se absorben, son conjugados en el hígado y transportados al torrente sanguíneo para finalmente ser excretados en la orina.

Muchos de los metabolitos detectados en el plasma y/u orina después de la ingestión de los productos ricos en polímeros de proantocianidinas son conjugados de monómeros de (epi)catequina que no pueden venir exclusivamente de las pequeñas cantidades de estos monómeros contenidas en el alimento (Touriño *et al.* 2009). Una variedad de pequeños compuestos

fenólicos resultado de la acción de la microbiota intestinal sobre los polímeros o sus unidades despolimerizadas se detectan en plasma varias horas después de la ingestión (Scalbert *et al.* 2000a; Saura-Calixto *et al.* 2010) además de procianidinas diméricas (Sano *et al.* 2003). Otros estudios indican que las proantocianidinas poliméricas aumentan la absorción de dímeros y trímeros (Appeldoorn *et al.* 2009b).

En esta tesis se evalúa por primera vez la biodisponibilidad de proantocianidinas de canela, compuesta principalmente por enlaces de tipo A, después de su ingestión. Se han elucidado una amplia variedad de metabolitos de proantocianidinas, incluyendo metabolitos de (epi)catequina de fase II y derivados de metabolitos microbianos, procedentes de la degradación de polímeros de proantocianidinas. La detección de monómeros y dímeros intactos no conjugados en heces muestra que especies captadoras de radicales libres que provienen de proantocianidinas están en contacto con el tracto intestinal durante horas y podrían contribuir a un efecto antioxidante local. Los metabolitos son absorbidos y entran en contacto con diferentes tejidos durante horas después de la ingesta, y podrían ser responsables de efectos sistémicos. Los resultados obtenidos en el presente estudio con canela están de acuerdo con los obtenidos para otras fuentes ricas en polímeros tales como pulpa de uva y piel de almendra (Touriño *et al.* 2009; Urpi-Sarda *et al.* 2009; Touriño *et al.* 2011), y están en consonancia con la observación de que la canela es una fuente rica en proantocianidinas (Gu *et al.* 2004; Mateos-Martín *et al.* 2012a).

Para el estudio nutricional se suministró una suspensión de canela molida en agua en lugar de los extractos comúnmente utilizados en otros estudios donde sólo se considera la fracción extraíble de proantocianidinas como la única fuente de polifenoles. De hecho, la mayoría de los trabajos realizados con proantocianidinas, incluyendo aquellos que se ocupan de su metabolismo, se han hecho con sobrenadantes obtenidos después de su extracción con acetona, siendo éste el procedimiento más común para su análisis (Gu *et al.* 2004). Las proantocianidinas suministradas en estos estudios en realidad sólo corresponden a las proantocianidinas extraíbles (EPA), que representan sólo una fracción de las proantocianidinas dietéticas.

Últimamente se ha puesto de manifiesto que una proporción considerable de proantocianidinas permanecen en el residuo al realizar las extracciones (Arranz *et al.* 2009) y pueden desempeñar un papel funcional más importante que las EPA; son las proantocianidinas no extraíble (NEPA). Las NEPA se asocian con otros componentes de la matriz alimentaria, principalmente fibra dietética, y de hecho constituyen una parte de ella, de acuerdo con las definiciones actuales de fibra dietética (Goñi *et al.* 2009). Algunos autores sugieren que las NEPA puede ser más abundantes que las EPA en muchos alimentos y, por lo tanto, se ingieren diariamente cantidades significativas de NEPA (Pérez-Jiménez *et al.* 2009a; Saura-Calixto 2012). Hasta la fecha, no existe un método común para determinar NEPA. Por lo general se determinan por métodos espectrofotométricos destructivos y no se ha realizado un análisis estructural significativo (Hellström *et al.* 2008; White *et al.* 2010). Por lo tanto, sigue siendo escasa la evidencia estructural con respecto a la composición de las NEPA como constituyentes esenciales de muchos productos alimenticios. Por otra parte, un análisis que se realiza generalmente después de la extracción para el contenido de polifenoles es la despolimerización catalizada por un ácido en presencia de un nucleófilo, pero los enlaces interflaváni cos de tipo A en proantocianidinas son resistentes por lo cual su detección aún es más complicada y puede conducir a una subestimación del contenido de polifenoles (Thompson *et al.* 1972; Tarascou *et al.* 2010).

Así pues, el hecho de utilizar una suspensión de canela molida sin realizar una extracción previa en nuestro estudio es importante, ya que las proantocianidinas poliméricas son en su mayoría no extraíbles y están presentes en cantidades mayores que las proantocianidinas extraíbles en muchos productos alimenticios (Pérez-Jiménez *et al.* 2009a; Arranz *et al.* 2010). De esta forma garantizamos que fueron administradas tanto la fracción de proantocianidinas extraíbles como las no extraíbles, y por consiguiente, se puede esperar que los metabolitos de proantocianidinas detectados en este estudio procedan de ambos tipos de proantocianidinas. A pesar de la creciente importancia que se otorga a las NEPA nunca se ha examinado su metabolización de forma independiente con respecto a EPA.

Metabolización de proantocianidinas no extraíbles de uva.

La fibra dietética antioxidante de uva (GADF) se prepara a partir de bagazo de uva, subproducto de la industria vitivinícola, con un elevado contenido en fibra y polifenoles. La GADF muestra una alta capacidad antioxidante *in vitro* asociada tanto al sobrenadante como al correspondiente residuo de dichas extracciones (EPA y NEPA) (Saura-Calixto 1998). En estudios *in vivo* realizados tras su ingesta se ha demostrado que se produce un aumento significativo de capacidad antioxidante plasmática y se ha observado una reducción significativa del colesterol total, colesterol por LDL, triglicéridos y tensión arterial en humanos (Pérez-Jiménez *et al.* 2008b; Pérez-Jiménez *et al.* 2009b). El estudio se completó en nuestro laboratorio con una identificación exhaustiva (Touriño *et al.* 2008) y estudio metabólico de los polifenoles de GADF (Touriño *et al.* 2009; Touriño *et al.* 2011).

En esta tesis se han completado los estudios previamente realizados en GADF, caracterizando los metabolitos fenólicos presentes en la orina y las heces de ratas 24 horas después de la ingestión de una fracción rica en NEPA, usando técnicas de cromatografía líquida acoplada a un espectrómetro de masas (**Publicación 3**, (Mateos-Martín *et al.* 2012b)). Éste es el primer estudio de metabolización que se realiza exclusivamente con NEPA.

Los resultados demuestran que las NEPA son parcialmente despolimerizadas durante su tránsito a lo largo del tracto intestinal, como se evidencia por la presencia de monómeros y dímeros de (epi)catequina en las heces y conjugados de (epi)catequina de fase II en la orina. Por otra parte, la NEPA se sigue metabolizando por la microbiota intestinal dando como resultado ácidos fenólicos que son presentes en la orina como compuestos fenólicos libres y/o conjugados con glucuronato o sulfato.

Además de corroborar los resultados de estudios anteriores con canela, por primera vez se han presentado evidencias de que las NEPA se comportan *in vivo* como fuente de compuestos polifenólicos que se liberan progresivamente y proporcionan especies fenólicas que entran en contacto

con las paredes intestinales, siendo sus metabolitos biodisponibles por lo menos durante 24 h después de la ingestión.

Los resultados obtenidos muestran claramente que esta fracción de fibra dietética genera derivados de (epi)catequina biodisponibles. El derivado de (epi)catequina di-glucuronizado detectado en las heces demuestra además que los monómeros liberados de NEPA alcanzan el hígado, sufren conjugación y son transportados de vuelta al intestino través de la bilis. La idea de despolimerización intestinal es consistente con el estudio que indica una alta recuperación de (epi)catequina libre en el contenido cecal de las ratas después de proporcionarles una sola dosis de extracto de almendra rico en proantocianidinas (Jové *et al.* 2011). Por otra parte, más de veinte metabolitos derivados de (epi)catequina detectados en orina son consistentes con la metabolización de estos compuestos.

Mediante el examen del perfil metabólico de NEPA procedente de GADF, se corrobora que las proantocianidinas poliméricas experimentan despolimerización a (epi)catequina durante su tránsito a lo largo del tracto intestinal. Las heces contienen (epi)catequina monomérica y dimérica y la orina contiene 10 metabolitos de (epi)catequina de fase II procedentes de una fracción desprovista de monómeros y dímeros extraíbles.

El aumento de la capacidad antioxidante del plasma después de la ingesta de GADF por sujetos humanos experimenta un retraso en comparación con la observada después de la ingesta de alimentos ricos en EPA, tales como vino tinto (Pérez-Jiménez *et al.* 2009b). Esto se podría deber a que las NEPA se asocian con la matriz alimentaria en los productos alimenticios, en particular con otros polímeros insolubles de la fibra dietética, por lo que su conversión puede ser lenta en comparación con la de la EPA. Esto haría que los metabolitos de NEPA fuesen biodisponibles durante más tiempo después de la ingesta y puedan tener efectos sobre la salud por un mayor tiempo. Además, los metabolitos detectados en las heces prueban que las especies con grupos con capacidad de captación de radicales libres permanecen en contacto con el epitelio del colon durante al menos 24 h después de la ingestión.

En conclusión, se demuestra aquí que las NEPA son una fuente de proantocianidinas poliméricas que se despolimerizan progresivamente durante su tránsito por el tracto intestinal en monómeros y dímeros de (epi)catequina, y son metabolizadas por la microbiota intestinal en unidades más pequeñas. Como resultado, (epi)catequina, ácidos fenólicos y sus metabolitos de fase II están en contacto con el tracto intestinal y biodisponibles durante al menos 24 h después de la ingestión. Esto significa, no sólo que existen metabolitos con capacidad antioxidante en contacto con las paredes intestinales después de la ingestión sino que también encontramos intactos grupos catecol captadores de radicales libres que pueden proteger frente al estrés oxidativo implicado en el inicio de las patologías intestinales. Así, la ingesta de proantocianidinas, y particularmente proantocianidinas con un alto grado de polimerización, se ha asociado con un menor riesgo de padecer cáncer colorrectal (Rossi M *et al.* 2010) y nuestros resultados sugieren que las fuentes de alimentos con NEPA podrían ser en parte responsables de este efecto preventivo.

Actualmente las proantocianidinas no extraíbles se determinan principalmente mediante un método que consiste en la despolimerización de las proantocianidinas por calentamiento y posterior medición espectrofotométrica. Nuestros resultados coinciden con los estudios que afirman que los residuos de la extracción con acetona al 70% contienen cantidades significativas de proantocianidinas con un alto contenido en proantocianidinas no extraíbles, ya que la evidencia directa de la estructura de estos polímeros insolubles es escasa. Este alto contenido en compuestos antioxidantes no extraíbles además podría ser de interés económico para el aprovechamiento de subproductos ricos en polifenoles procedentes de productos agrarios que podrían tener otras aplicaciones.

Proantocianidinas de *Chamaecrista nictitans*.

Los capítulos anteriores se centraron en la caracterización de proantocianidinas de alto grado de polimerización y en la bioaccesibilidad en el organismo de estas proantocianidinas, tanto extraíbles como no extraíbles.

Un cuarto capítulo de la tesis aborda el estudio de proantocianidinas más complejas que han mostrado actividad antiviral (**Publicación 4**).

Entre las infecciones virales, el virus del herpes simple (HSV) es una de las más importantes. Hay dos tipos de HSV, de tipo 1 (HSV-1) y de tipo 2 (HSV-2). El HSV-2 es de vital importancia para la salud humana ya que causa infecciones neonatales e infecciones como la meningitis y el cáncer de cuello uterino.

Hoy en día, en el tratamiento de infecciones por virus de herpes, se utilizan fármacos antivirales como el aciclovir, comúnmente utilizado para tratar el HSV. Aciclovir interrumpe eficazmente la síntesis de ADN viral y la replicación de HSV (Crumpacker *et al.* 1982; Corey *et al.* 1986; Fahad *et al.* 1996). Una preocupación en la lucha contra el HSV es el desarrollo de resistencia viral además de los efectos citotóxicos que pueden tener este tipo de fármacos, por lo que es importante nuevos productos para tratar estas infecciones. Por estas razones, es importante la búsqueda de nuevos fármacos o co-adyuvantes activos contra el HSV. Los estudios con extractos de plantas son especialmente interesantes para la provisión de tratamientos eficaces con menor toxicidad.

La *Chamaecrista nictitans* es una especie de la familia de las Fabaceae distribuida por la zona tropical del continente americano. Hay siete especies de *Chamaecrista* en Costa Rica de las cuales la *Chamaecrista nictitans* es la más común. A un extracto crudo de esta planta se le ha atribuido actividad antiviral contra el virus del herpes simple (Herrero-Uribe *et al.* 2004). La actividad antiviral exhibida por el extracto fue determinada mediante la inhibición total del efecto citopático después de tres días de incubación y la concentración máxima de la fracción positiva que no presentó citotoxicidad. Este resultado indica que el extracto actúa intracelularmente siendo capaz de inhibir la transcripción secundaria. Sin embargo, el extracto permite la transcripción y traducción temprana de proteínas. El virus tratado con aciclovir, fármaco antiviral usado en el tratamiento de este tipo de infecciones, fue capaz de producir un efecto citopático, mientras que el extracto inhibió el virus y no se detectó efecto citopático. Se concluye que el

extracto inhibe la adherencia inicial del virus a las células y los eventos de transcripción secundaria del virus (Herrero-Uribe *et al.* 2004).

El extracto crudo de la planta fue fraccionado y su actividad evaluada. Una fracción del extracto, la fracción II (FII), ha sido identificada como la más activa de todas las fracciones tanto por su función captadora de radicales libres como por ser una potente mezcla antiviral. Por este motivo, se ha analizado la composición polifenólica del extracto y las fracciones por LC-ESI-MS/MS, con el objetivo de establecer relaciones entre sus estructuras y la actividad antiviral, ya que la actividad del extracto de *Chamaecrista nictitans* podría estar relacionada con sus polifenoles.

La caracterización química del extracto demostró la presencia de proantocianidinas. La identificación que se lleva a cabo de las estructuras de proantocianidinas indica que hay una gran heterogeneidad estructural, aumentando esta heterogeneidad con el grado de polimerización. Además, el estudio de la composición revela que éste contiene proantocianidinas principalmente con dos características estructurales que pueden ser responsables de su actividad antiviral: contienen grupos monofenólicos y enlaces de tipo A. En particular, la fracción II del extracto es rica en (epi)fisetinidol y (epi)afzelequina y es la más eficaz frente al virus del herpes simple.

Son pocos los estudios que han investigado la relación entre las propiedades antivirales y la estructura de las proantocianidinas (Gescher *et al.* 2011). Las propiedades de las procianidinas (oligómeros de (epi)catequina) han sido descritas en numerosos artículos, sin embargo, existen otros tipos de proantocianidinas menos conocidas, ya que se encuentran en menor proporción en la naturaleza.

El presente estudio ha mostrado que las proantocianidinas del extracto de *Chamaecrista nictitans* consisten en combinaciones de compuestos con unidades monoméricas de (epi)guibourtinidol, (epi)fisetinidol, (epi)afzelequina y (epi)catequina. El compuesto mayoritario en la fracción II es un trímero de profisetinidina. Cabe señalar que esta estructura, así como

muchos de los otros oligómeros en la fracción, presenta un número inusual de grupos monofenólicos. Compuestos como (epi)fisetinidol, (epi)afzelequina y (epi)guibourtinidol muestran cierta actividad antioxidante aunque no son potentes captadores de radicales libres comparados con homólogos con mayor número de grupos hidroxilos.

La doble acción de las proantocianidinas de *Chamaecrista nictitans* sobre el virus HSV (antiadherente e inhibición de la transcripción de DNA) podría ser debida a unidades estructurales diferentes (Cheng *et al.* 2006). La (epi)catequina y (epi)catequingalato, comunes a otras proantocianidinas podría ejercer principalmente la acción antiadherente tal como se ha sugerido anteriormente. Los monofenoles podrían ser los responsables de la inhibición de la transcripción tardía (Cheng *et al.* 2006). Otra característica diferencial de las proantocianidinas de *Chamaecrista nictitans* es la presencia de oligómeros de tipo A, estando este tipo de enlace relacionados con la actividad antiviral de oligómeros de proantocianidinas (De Bruyne *et al.* 1999b; Xu *et al.* 2010). El grupo OH en posición 4' y la configuración *trans* podrían ser determinantes estructurales necesarios para inhibir la proliferación celular (Stivala *et al.* 2001).

La acción del extracto de *Chamaecrista nictitans* es completamente consistente con el efecto propuesto para el dímero de epiafzelequina, que inhibe la replicación del virus del herpes simple de tipo 2 (Cheng *et al.* 2006). Otros compuestos de configuración similar con grupos monofenólicos como el resveratrol también han mostrado actividad frente al HSV (Docherty *et al.* 1999). Estos estudios demuestran que el resveratrol afecta desfavorablemente a la replicación del HSV durante las primeras 6 horas de infección, inhibiendo de forma reversible el ciclo de crecimiento viral. Además se determinó que el piceatanol, que difiere del resveratrol en que tiene dos grupos hidroxilos en cada anillo de fenilo en lugar de dos en un anillo y uno en el otro, podía afectar a la replicación del HSV, encontrándose que no tienen efecto sobre la replicación de HSV. Aunque el piceatanol y el resveratrol difieren sólo en un grupo hidroxilo, la actividad inhibidora observada del resveratrol sobre la replicación de HSV sugiere un alto grado de especificidad corroborando nuestra teoría. Además, el quebracho,

conocido por ser una fuente rica en profisetinidinas, muestra a concentraciones no tóxicas un porcentaje de inhibición de virus de herpes simple de un 100% (Moreira *et al.* 2005).

Se piensa que el mecanismo biológico de acción de los compuestos polifenólicos está relacionado con su capacidad para actuar como antioxidantes. Sin embargo, los resultados obtenidos sugieren que la actividad antivírica que presentan puede ser también debida a su configuración estructural y no sólo a la capacidad de estos compuestos de ceder electrones. Los compuestos responsables de la actividad antiviral tienen un potencial antioxidante por debajo de otros compuestos los cuales no presentan esta actividad biológica.

VI. CONCLUSIONES

CONCLUSIONES

1.- Se han encontrado subestructuras en canela (*Cinnamomum zeylanicum* L.) nunca antes descritas para esta fuente. Además de (epi)catequina y (epi)afzelequina, las proantocianidinas de la canela contienen unidades de (epi)galocatequina y (epi)catequingalato. La presencia de estas subestructuras puede explicar algunas de las propiedades de los extractos de canela.

2.- MALDI-TOF/TOF es una herramienta eficaz para el análisis estructural de proantocianidinas poliméricas. La técnica combina la alta sensibilidad para compuestos de alto grado de polimerización y galoización con la posibilidad de obtener información estructural a través de los patrones de fragmentación obtenidos a partir de experimentos de MS/MS.

3.- El modo CID resultó ser indispensable para la fragmentación de proantocianidinas por MALDI-TOF/TOF. Los principales fragmentos observados durante los experimentos de MS/MS están de acuerdo con los patrones de fragmentación típica para este tipo de compuestos.

4.- Los polímeros de proantocianidinas se despolimerizan en unidades de (epi)catequina y no sólo se transforman directamente en ácidos fenólicos más pequeños. Sus unidades constituyentes y sus metabolitos son biodisponibles en el organismo como se ha demostrado para proantocianidinas de canela y de bagazo de uva.

5.- Las metabolización de proantocianidinas no extraíbles (NEPA) de uva (*Vitis vinífera*) genera un conjunto de derivados de (epi)catequina y de sus productos de degradación microbiana. Esto constituye la primera prueba de la naturaleza estructural de NEPA como polímeros de (epi)catequina.

6.- Se observan monómeros y dímeros de (epi)catequina intactos en heces tras la ingesta de proantocianidinas extraíbles y no extraíbles. La presencia de estos grupos con capacidad de captación de radicales libres en el intestino

puede proteger frente al estrés oxidativo implicado en el inicio de las patologías intestinales.

7.- El extracto de *Chamaecrista nictitans* (Fabaceae) es una fuente rica en proantocianidinas con un alto grado de polimerización. Los polímeros incluyen unidades monohidroxiladas como (epi)fisetinidol, (epi)afzelequina y (epi)guibourtinidol además de (epi)catequina, común en otras fuentes como la uva o la corteza de pino.

8.- La actividad biológica del extracto de *Chamaecrista nictitans* puede ser debida a su configuración estructural (posición de los grupos hidroxilos) y no sólo a la capacidad de estos compuestos de ceder electrones, ya que los compuestos mayoritarios del extracto son menos activos como captadores de radicales libres que otras proantocianidinas que no presentan actividad antiviral. La actividad antiviral se puede explicar por el efecto antiadherente de las unidades de (epi)catequina y por un posible efecto inhibitorio de la transcripción de ADN vírico ejercida por las unidades monohidroxiladas.

VII. ANEXOS

ANEXOS

1. Otras publicaciones

En este apartado se incluyen publicaciones no contenidas en la tesis doctoral, todas ellas publicadas en revistas del primer cuartil del Science Citation Index.

1.1. Metabolites in contact with the rat digestive tract after ingestion of a phenolic-rich dietary fiber matrix (2009) *Journal of Agricultural and Food Chemistry* 59(11): 5955-5963.

1.2. Hamamelitannin from witch hazel (*Hamamelis virginiana*) displays specific cytotoxic activity against colon cancer cells (2012) *Journal of Natural Products* 75(1): 26-33.

1.3. Punicalagin and catechins contains polyphenolic substructures that influence cell viability and can be monitored by radical biosensors selective to electron transfer (2012) *Journal of Agricultural and Food Chemistry* 60(7): 1659-1665.

1.4. Effect of pressurized hot water extraction on antioxidants from grape pomace before and after oenological fermentation (2013) *Journal of agricultural and food chemistry* 61(28): 6929-6936.

2. Comunicaciones en congresos

En este apartado se incluyen los poster presentados en congresos nacionales e internacionales.

2.1. Relationship between antioxidant/prooxidant activity and structure of punicalagin and punicalagin metabolites. «4th International

Conference on Polyphenols and Health» Harrogate, Universidad de Leeds **2009**.

2.2. Metabolization of non-extractable proanthocyanidins, an underestimated fraction of dietary polyphenols. «34º Congreso Sociedad Española de Bioquímica y Biología Molecular (SEBBM)» Barcelona **2011**.

2.3. Polymeric proanthocyanidin profile in several food matrixes by MALDI-TOF/TOF. «5th International Conference on Polyphenols and Health» Sitges, Universidad de Barcelona **2011**.

2.4. Non-extractable proanthocyanidins generate bioavailable metabolites in rats- a case study with grape antioxidant dietary fibre. «5th International Conference on Polyphenols and Health» Sitges, Universidad de Barcelona **2011**.

2.5. Profile of urinary and fecal proanthocyanidin metabolites after intake of common cinnamon (*Cinnamomum zeylanicum* L.) in rats. «5th International Conference on Polyphenols and Health» Sitges, Universidad de Barcelona **2011**.

2.6. Study of the cytotoxic effect of tannins from *Hamamelis virginiana* on colorectal cancer. «5th International Conference on Polyphenols and Health» Sitges, Universidad de Barcelona **2011**.

1. Otras publicaciones

1.1. Metabolites in contact with the rat digestive tract after ingestion of a phenolic-rich dietary fiber matrix.

Journal of Agricultural and Food Chemistry

***59 (11), 5955-5963,* 2011**

Q1

SONIA TOURIÑO, JARA PÉREZ-JIMÉNEZ, MARÍA LUISA MATEOS-MARTÍN, ELISABET FUGUET, MARÍA PILAR VINARDELL, MARTA CASCANTE & JOSEP LLUÍS TORRES

RESUMEN

El objetivo de este trabajo fue determinar los compuestos fenólicos que entran en contacto con el tejido epitelial del colon después de la ingestión de GADF. Mediante el uso de HPLC-ESI-MS se detectaron metabolitos fenólicos en las heces, contenido cecal y tejido colónico de las ratas. Se detectó (epi)catequina libre en las tres fuentes, y también se detectaron más de 20 metabolitos conjugados de (epi)catequina en las heces. Además, se identificaron 14 metabolitos microbianos fenólicos en las heces, contenido cecal y/o el tejido del colon. Estos resultados muestran que durante el tránsito a lo largo del tracto digestivo, oligómeros y polímeros de proantocianidina se despolimerizan. Después de la ingestión de GADF, (epi)catequina libre y sus conjugados, así como derivados de metabolitos fenólicos microbianos libres y conjugados, entran en contacto con el epitelio intestinal durante más de 24 horas y puede ser en parte responsable de los efectos positivos de la GADF sobre la salud intestinal.

Para este estudio se puso a punto un método por HPLC-ESI-MS/MS optimizando la energía del cono y de colisión para cada grupo de metabolitos (Touriño *et al.* 2011).

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ARTICLE

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Metabolites in Contact with the Rat Digestive Tract after Ingestion of a Phenolic-Rich Dietary Fiber Matrix

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ABSTRACT: Grape antioxidant dietary fiber (GADF) is a phenolic-rich dietary fiber matrix. The aim of this work was to determine which phenolic compounds come into contact with colonic epithelial tissue after the ingestion of GADF. By use of HPLC-ESI-MS/MS techniques phenolic metabolites were detected in feces, cecal content, and colonic tissue from rats. Free (epi)catechin (EC) was detected in all three sources, and more than 20 conjugated metabolites of EC were also detected in feces. Fourteen microbially derived phenolic metabolites were also identified in feces, cecal content, and/or colonic tissue. These results show that during transit along the digestive tract, proanthocyanidin oligomers and polymers are depolymerized into EC units. After ingestion of GADF, free EC and its conjugates, as well as free and conjugated microbially derived phenolic metabolites, come into contact with the intestine epithelium for more than 24 h and may be partly responsible for the positive influence of GADF on gut health.

KEYWORDS: grape antioxidant dietary fiber, polyphenols, proanthocyanidins, epicatechin, metabolites, bioavailability, mass spectrometry, feces, cecal content, colonic tissue

INTRODUCTION

There is mounting evidence that gut physiological status, considered to be the result of the interaction between the epithelial tissue and its associated microbiota, decisively influences the health of whole organisms.[1] In particular, intestinal microbiota may modulate the incidence of certain kinds of cancer such as colon cancer[2] and possibly other cancers that affect organs far from the intestinal tract through the synthesis and absorption of bioactive molecules. Similarly, the recently proposed "gut−heart axis" hypothesis suggests that metabolites formed in the colon may play a significant role in the prevention of cardiovascular disease.[3] Therefore, food components and their metabolites that come into contact with the gut wall and interact with the intestinal microbiota for up to several hours may exert an influence on an organism's overall health that is more important than previously thought.[4]

Dietary fiber is of particular interest in this respect because it is transported largely unaltered along the small intestine all the way to the colon, where it is partially hydrolyzed and absorbed. Dietary fiber consists of two fractions: soluble dietary fiber is constituted mainly of soluble pectins, β-glucans, and gums and may be fermented by intestinal microbiota, thus releasing several beneficial short-chain fatty acids; insoluble dietary fiber is constituted of insoluble pectins, lignin, cellulose, and hemicelluloses and mainly contributes to the bulking effect of dietary fiber.

Interestingly, certain dietary fiber matrices carry putatively bioactive functional components embedded in them, antioxidant compounds in particular, which are gradually released in the intestinal lumen and partly absorbed into gut epithelial cells;[5] such matrices have been called antioxidant dietary fibers.[6] Oligomeric and polymeric proanthocyanidins (PAs) are major components associated with the fiber matrix (celluloses and hemicelluloses) through either weak (hydrophilic/hydrophobic)[7,8] or possibly strong (covalent) interactions[9] and are an object of microbial degradation. The metabolic transformation of PAs appears to be mediated by several bacterial species[10] and to yield the same main metabolites in both rats and humans.[11,12]

Grape antioxidant dietary fiber (GADF), a byproduct of wine production, is a model of a phenolic-rich dietary fiber matrix. Whereas some of the PAs present in GADF are associated with the fiber matrix through weak interactions, and therefore can be extracted by solvents (extractable PAs, or EPAs), most of them remain associated with the fiber matrix after extraction (nonextractable PAs, or NEPAs).[13] Studies in rats have shown that GADF exerts several protective effects on colonic health, including modulation of colonic mucosa apoptosis, modifications in colonic crypts, and increase of cecal free radical scavenging capacity.[14−16] Grape pomace, which is quite similar to GADF, has also been shown to stimulate the growth of beneficial *Lactobacillus acidophilus*.[17]

In previous work[18] we described the metabolic fate of GADF in rat urine, showing that some EPA components are partially depolymerized during transit through the intestinal tract. Furthermore, we reported that the bulkier polymers (NEPAs and the remaining EPAs) are degraded by the intestinal microbiota into smaller compounds such as phenolic acids, which pass into the bloodstream and are finally excreted in urine. As both dietary fiber and phenolic-rich materials have been related to the

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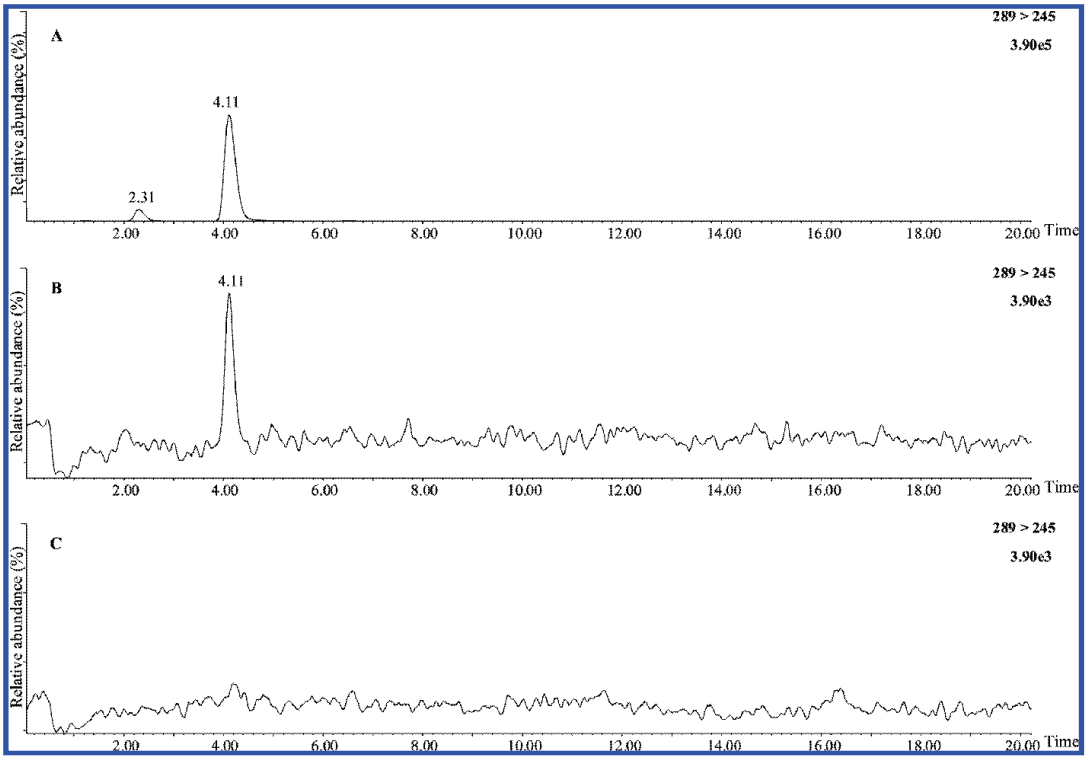

Figure 1. HPLC-ESI-MS profile corresponding to the detection of EC in colonic tissue samples from rats fed (A) EC, (B) GADF, or (C) water. Detection was by multiple reaction monitoring (MRM), transition 289 → 245.

prevention of colorectal cancer[19,20] and may indirectly affect other types of cancer by modulating the gut microbiota, it is important to study the metabolites in feces, cecal content, and colonic tissue as an indication of the type of compounds that come into contact with the colonic epithelial tissue for several hours after GADF ingestion. A number of previous studies have examined the metabolic fate of polyphenols in urine and plasma samples, but they did not directly search for the metabolites within the materials that come into contact with the colon or that can be found in the colonic tissue itself.

In the present work we examine the phenolic composition of feces, cecal content, and colonic tissue in rats 24 h after GADF ingestion. Polyphenols and their metabolites were identified by a combination of mass spectrometry (MS) modalities (multiple reaction monitoring, or MRM, and product ion scan MS/MS experiments) on a triple-quadrupole apparatus. We provide new information regarding components that are in contact with the gut wall and may partly explain the activity of GADF on epithelial cells and on the intestinal microbiota. This will help to complete our understanding of the influence that intestinal transformations of polyphenols have on intestine health and on the status of other parts of the organism.

MATERIALS AND METHODS

Chemicals and Reagents. GADF was obtained from red grapes ('Cencibel' variety) harvested in the vintage year 2005 in the La Mancha region in Spain, as described in a published patent.[21] The percentage composition of GADF used in this work was as follows: dietary fiber, 73.48 ± 0.79 (soluble 15.53 ± 0.11, insoluble 57.95 ± 0.78); polymeric PA associated with insoluble dietary fiber, 14.81 ± 0.19 (measured by the cyanidin method[22]); fat, 7.69 ± 0.49; protein, 11.08 ± 0.46; ash, 5.25 ± 0.19. (−)-Epicatechin (EC) monomer content in GADF was <0.01%.[18] More than 100 phenolic compounds (not associated with dietary fiber) have been detected in GADF.[23] Standards of EC (≥97%), 3- and 4-hydroxyphenylacetic acid (≥98%), 3,4-dihydroxyphenylacetic acid (≥98%), 3- and 4-hydroxybenzoic acid (≥97%), vanillic acid (≥97%), caffeic acid (≥95%), 3,4-dihydroxyphenylpropionic acid (>98%), 4-hydroxyphenylpropionic acid (>98%), protocatechuic acid (≥97%), caffeic acid (≥98%), ferulic acid (≥99%), isoferulic acid (≥97%), *p*-coumaric (≥98%), *m*-coumaric (≥97%), and taxifolin (≥85%) were obtained from Sigma-Aldrich (St. Louis, MO). Methanol (analytical grade) and 37% hydrochloric acid were purchased from Panreac (Castellar del Vallès, Barcelona, Spain). Acetonitrile (HPLC grade) and formic acid (analytical grade) were obtained from Merck (Darmstadt, Germany). Water was purified by using a Milli-Q plus system from Millipore (Bedford, MA) to a resistivity of 18.2 MΩ cm.

Animal Experiments. Female Sprague—Dawley rats (SD, n = 12, body weight = 233 ± 9.3 g, 12 weeks of age) provided by Harlan Interfauna Ibérica SL (Barcelona, Spain) were fed a polyphenol-free diet (TD94048) (from Harlan Interfauna Ibérica SL) and maintained in plastic cages at room temperature (22 ± 2 °C) and 55 ± 10% relative humidity, with a 12 h light/dark cycle for 1 week, in accordance with European Union regulations. The experimental design was essentially the same as that used in our previous study of urine metabolites.[18] The rats were divided into three groups (n = 4) (GADF group, EC group, and control group). The number of animals per group was adequate for qualitative and semiquantitative comparative purposes. Because most PA metabolites are similar to those derived from EC and due to the absence of commercial standards for them, the EC group was included in

Table 1. (Epi)catechin and Conjugated Metabolites in Feces from Rats Fed GADF[a]

metabolite[b]	MRM[c] parent	MRM daughter	MS/MS ions	relative area[d] (%)
(−)-epicatechin[e,f]	289 → 245			16.1
monoconjugated metabolites				
Gluc- EC-1[e]	465 → 289	289 → 245	465; 289; 113	25.7
Gluc- EC-2[e]	465 → 289	289 → 245	465; 289; 245; 205; 143; 125,113	3.2
Gluc- EC-3	465 → 289	289 → 245	465; 327; 289; 245; 203; 175; 151; 113	1.6
Sulf- EC-1[e]	369 → 289	289 → 245	369; 289; 245; 217; 205; 203;151; 137	2.6
Sulf- EC-2[e]	369 → 289	289 → 245	369; 289; 245; 231; 203; 179; 151;137	6.9
Sulf- EC-3	369 → 289	289 → 245	369; 289; 245; 231; 203;179; 151; 137	5.0
GHS EC	594 → 289	289 → 245		0.6
diconjugated metabolites				
Me-Gluc-EC[e]	479 → 303		479.0; 375; 313;303;285;240;235;113	0.2
Me-Sulf-EC-1[e]	383 → 289		383; 321; 303; 285; 217; 137	5.1
Me-Sulf-EC-2[e]	383 → 289		383; 303; 285; 270; 259; 244; 217; 202; 165; 151; 137	1.1
Me-Sulf-EC-3[e]	383 → 289		383; 303; 289; 285; 259; 244; 219; 204; 179; 137	4.9
Me-Sulf-EC-4[e]	383 → 289		383; 303; 285; 259; 245; 219; 204; 165; 137	5.0
di-Gluc-EC	641 → 289			0.4
di-Me-EC	318 → 289			16.6
triconjugated metabolites				
di-Me-Sulf-EC	387 → 289		387;369; 307; 289; 263; 245; 161	1.4
di-Me-Gluc-EC[e]	493→ 289		493.0; 469; 379; 303; 285; 267; 259; 233; 199; 137	0.5
Me-Gluc-Sulf-EC-1[e]	559 → 289		559;313; 289; 231	0.1
Me-Gluc-Sulf-EC-2	559 → 289		625; 335; 289; 193; 175; 159; 113	0.2
Gluc-disulf-EC	625 → 289		625; 335; 289; 193; 175; 159	2.0
di-Gluc-Me-EC-1	655 → 289	289 → 245		0.2
di-Gluc-Me-EC-2	655 → 289	289 → 245		0.4
tri-Sulf-EC[e]	529 → 289			0.3

[a] Metabolites detected only in the GADF group or detected as signals at least 10-fold stronger than those in the control group. [b] EC, (epi)catechin; GHS, glutathione; Gluc, glucuronidated; Me, methylated; Sulf, sulfated. [c] MRM, multiple reaction monitoring. [d] Peak areas from the chromatograms generated by MRM experiments. [e] Metabolites also detected in urine from rats fed GADF.[18] [f] Compound identified by retention time of a standard.

the study to clearly identify the metabolite signals. The animals were housed individually in metabolic cages, and after food deprivation for 12 h with free access to water, the rats were administered the corresponding feed by oral gavage as follows: GADF group, a saturated GADF suspension in tap water (1 g in 6 mL) as a single dose of 1.6 g/kg body weight; EC group, an EC solution in tap water (1 g in 6 mL) as a single dose of 1 g/kg body weight; control group, tap water as a single dose of 10 mL/kg body weight. To facilitate the detection of PA metabolites, the dose of GADF was the highest possible considering both the recommended volume administered via oral gavage on laboratory animals and the dispersibility of GADF in water. Feces were collected over a period of 24 h after administration. Then the animals were killed by an overdose of anesthesia (isoflurane gas) and the colons removed. The cecal contents were collected and weighed, and the colonic wall was washed three times with saline solution (0.9% NaCl in water) to eliminate any residue of cecal contents and weighed. The samples were stored at −80 °C until extraction and analysis. These experimental protocols were approved by the Experimental Animal Ethical Research Committee of the University of Barcelona (Permission DMA3123) in accordance with current regulations for the use and handling of experimental animals.

Sample Processing. The colonic tissue samples (500 mg) were homogenized in 4 mL of cold phosphate-buffered saline (PBS, 0.1 M, pH 7) using an Ultraturrax homogenizer (IKA, Staufer, Germany). After centrifugation (3000*g*, 10 min) and collection of the supernatant, acetonitrile (1.5 mL) was added to precipitate interfering proteins. The pellet was removed and the internal standard (taxifolin, 50 ppm final concentration) added. Samples were concentrated by nitrogen stream, and the residue was taken up in water (1.5 mL) and vortexed.

Samples were then subjected to solid-phase extraction (SPE) to isolate phenolic compounds. An Oasis HLB cartridge from Waters (Milford, MA) was activated with 1 mL of methanol and 2 mL of 1 mM HCl. To remove interfering components, the sample was washed with 9 mL of acid water. Then, samples were eluted with 1 mL of methanol. The eluate was evaporated under nitrogen and the residue reconstituted with 1 mL of 5% methanol in water. The temperature of evaporation was kept under 30 °C to avoid deterioration of the phenolic compounds. The samples were then filtered through a polytetrafluoroethylene (PTFE) 0.45 μm membrane from Waters into amber vials for HPLC-MS/MS analysis.

In the case of feces and cecal content, 500 mg of sample was homogenized in 4 mL of cold PBS using an Ultraturrax homogenizer and centrifuged at 3000*g* for 10 min. Then the same SPE procedure was followed as for the colonic tissue samples. The number of replicates was limited by the amount of sample available (colonic tissue, feces, and cecal content). Four replicate samples (one from each animal) were processed per group.

HPLC-ESI-MS/MS Analysis. A Waters Quattro LC triple-quadrupole mass spectrometer with an electrospray source was used in negative mode to obtain MS and MS/MS data. Liquid chromatography was performed on an Alliance 2695 system from Waters equipped with a Phenomenex (Torrance, CA) Luna C18 (50 × 2.1 mm i.d.) 3.5 μm particle size column and a Phenomenex Securityguard C18 (4 × 3 mm i.d.)

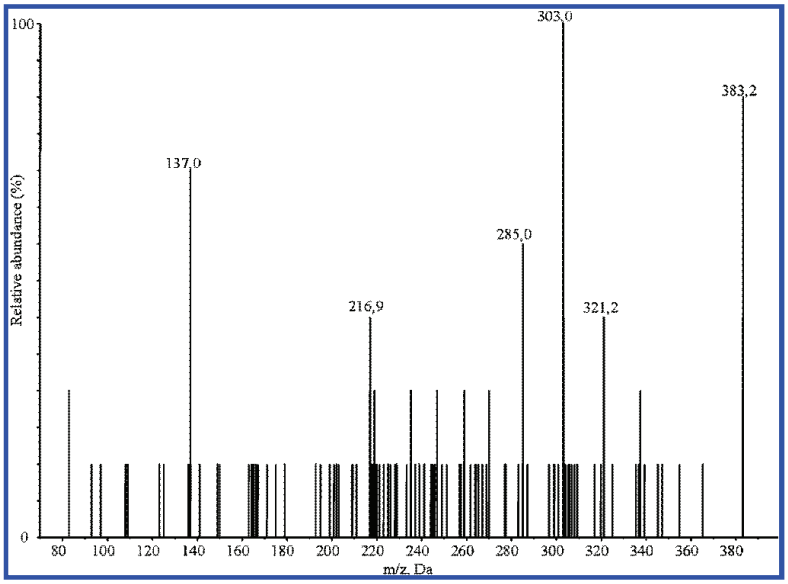

Figure 2. HPLC-ESI-MS/MS product ion scan spectrum of Me-Sulf-EC-1 (m/z 383).

column. Gradient elution was performed with a binary system consisting of (A) 0.1% aqueous formic acid and (B) 0.1% formic acid in acetonitrile. The following increasing linear gradient (v/v) of B was used [t (min), %B]: 0, 8; 10, 23; 15, 50; 20, 50; 21, 100. This was followed by a re-equilibration step.

Metabolites in feces, cecal content, and colonic tissue were identified by (i) MRM transitions of the putative metabolites using a dwell time of 100 ms and (ii) product ion scan MS/MS experiments. The cycle time used was 1.11 s. Cone energy and collision energy in MRM mode were optimized for each group of metabolites: 30 V and 10 eV for taxifolin, 30 V and 15 eV for phenolic acid derivatives, 30 V and 20 eV for EC and PA dimers, and 40 V and 20 eV for EC derivatives. Capillary energy was 2.8 kV. Source temperature and desolvation temperature were 150 and 350 °C, respectively.

The analytical strategy adopted to analyze the samples has been fully described previously.[18] Briefly, the metabolic outcome of GADF in rat feces, cecal content, and colonic tissue was expected to be mainly oligomeric and polymeric EC, so that the fragmentation patterns obtained with pure EC would help to assign the weaker signals from GADF. The search was focused on free EC, conjugated EC metabolites (glucuronidyl, methyl, and sulfate derivatives) and microbially derived phenolic metabolites, mainly phenolic acids (free and conjugated) from intestinal fermentation of EC and PAs.

RESULTS

EC and Its Phase II Metabolites. Free EC (MRM transition 289 → 245) was detected in feces, cecal content, and colonic tissue from the GADF group. Figure 1B shows the HPLC-ESI-MS profile in MRM mode corresponding to the detection of free EC in the colons of the GADF group. No signals corresponding to EC dimers (577 → 289) or to EC dimers with an open ring (581 → 289) were detected in any of the samples.

Most EC in the fecal samples was present as several derivatives, indicating that after absorption (either in the small intestine or in the colon) and hepatic conjugation, some of these metabolites returned to the colon via bile.[24] The conjugates we found included mono-, di-, and triconjugated derivatives resulting from combinations of glucuronidyl, methyl, and sulfate moieties. Table 1 lists all of the EC conjugates detected by HPLC-MS and HPLC-MS/MS in feces from the GADF group. The relative areas of the corresponding MS peaks are given as a preliminary estimation of their relative concentrations. Monoconjugated metabolites detected in the feces of the GADF group included three glucuronidated metabolites (465 → 289) previously detected in urine and three sulfated metabolites of EC (369 → 289), one of them not detected in urine.[18] Additionally, a monoconjugated metabolite with glutathione (GHS) (594 → 289) was detected. No monomethylated EC metabolites were detected in feces. With regard to diconjugated metabolites, the four Me-Sulf-EC metabolites (383 → 289) and the Me-Gluc-EC derivative (479 → 303) previously detected in urine[18] were also identified in feces. Two other peaks were tentatively assigned to other diconjugated (diglucuronidated and dimethylated) metabolites (with MRM transitions of 641 → 289 and 318 → 289, respectively). Several triconjugated metabolites were also detected in feces. These demonstrated the extensive phase II metabolization suffered by EC after absorption. They included EC conjugated with two methyl and one sulfate moieties (di-Me-Sulf-EC) (387 → 289), an EC conjugated with two methyl and one glucuronide moieties (di-Me-Gluc-EC) (493 → 289), a dimethylated glucuronidated sulfated derivative (559 → 289), a glucuronidated disulfated conjugate (625 → 289), and two diglucuronidated methylated derivatives (655 → 289). Additionally, trisulfated EC (529 → 289), previously detected only in urine from the EC group,[18] was identified, although it could not be confirmed either by a second MRM transition or MS/MS fragments. EC metabolites were not detected in either the cecal content or the colonic tissue of the GADF group. Comparison of the MS/MS fragmentation patterns confirmed that some of the metabolites detected in feces were those previously detected in urine. Thirteen EC conjugates detected in the feces of the EC group and seven in their cecal content were not detected in the GADF group.

The MS/MS fragments of EC metabolites (Table 1) provided supporting information on their structures. Besides the characteristic

Table 2. Microbially Derived Phenolic Metabolites in Feces, Cecal Content, and Colonic Tissue from Rats Fed GADF[a]

metabolite	MRM	compound confirmation	feces	relative area[b] (%)	colonic tissue	relative area[b] (%)	cecal content	relative area[b] (%)
valerolactones								
3- or 4-hydroxyphenylvalerolactone	191 → 147	second transition: 191 → 106	X	1.0				
dihydroxyphenylvalerolactone	207 → 163	MS/MS ions: 207; 163; 148; 121; 109	X	47.0			X	49.4
lignans								
enterolactone	297 →253	MS/MS ions: 297; 253; 189; 165; 145; 133; 121; 107	X	25.2				
phenylvaleric acids								
3- or 4-hydroxyphenylvaleric acid[c,d]	193 → 175	MS/MS ions: 193;175;147; 119;107	X	0.3			X	3.9
dihydroxyphenylvaleric acid[e]	209 → 137	MS/MS ions: 209; 163; 144; 117	X[d]	0.04				
phenylpropionic acids								
Sulf-3,4-dihydroxyphenylpropionic acid[c,e]	261 → 181	MS/MS ions: 261; 199; 185; 137	X	0.4				
phenylacetic acids								
3-hydroxyphenylacetic acid[c,d]	151 → 107	standard retention time	X	0.3	X	50.0		
4-hydroxyphenylacetic acid	151 → 107	standard retention time	X	0.2				
homovanillic acid[e]	181 → 121	standard retention time	X	3.4			X	15.7
benzoic acids								
gallic acid[e]	169 → 125	standard retention time	X	13.0			X	30.9
4-*O*-Me-gallic acid[e]	183 →169	MS/MS ions: 183; 124; 106; 95; 78	X	0.2				
sulf-3 or 4-hydroxybenzoic acid	217 → 137	MS/MS ions: 217; 137; 93	X	0.03				
cinnamic acids								
caffeic acid[c]	179 → 135	standard retention time	X	2.4				
m-coumaric acid	163 → 119	standard retention time	X	5.8	X	29.9		
p-coumaric acid[c,e]	163 → 119	standard retention time	X	0.7	X	20.0		

[a] Metabolites not detected in control group or detected at a concentration at least 10-fold higher. [b] Peak areas from the chromatograms generated by MRM experiments. [c] Previously detected in urine from rats fed GADF.[18] [d] Previously detected in supernatant from in vitro fermentation of GADF.[45] [e] Metabolites not detected in samples from rats fed EC.

glucuronide fragment at m/z 113, Gluc-EC-3 yielded a fragment at m/z 327 corresponding to a B-ring fragment plus the glucuronide moiety resulting from retro-Diels–Alder fission of the EC ring and therefore indicating that glucuronidation took place on the B-ring. Sulf-EC-1 yielded sulfated fragments from the A-ring at m/z 217 and 137, whereas the fragment detected at m/z 231 from Sulf-EC-2 and Sulf-EC-3 indicated that in these two metabolites sulfation took place on the B-ring. With regards to the disubstituted metabolites, Me-Sulf-EC-1 and Me-Sulf-EC-2 yielded fragments at m/z 217 and 137, which correspond to sulfation on the A-ring. This fragmentation can be seen in Figure 2, which shows the MS/MS spectrum for Me-Sulf-EC-1. In contrast, Me-Sulf-EC-4 yielded fragments at m/z 245 and 165, corresponding to a B-ring fragment with Me and Sulf moieties and to a B-ring fragment with Me substitution, respectively; this indicates that these substitutions took place on the B-ring.

Microbially Derived Phenolic Metabolites. Both nonconjugated and conjugated microbially derived phenolic metabolites were identified in feces from the GADF group, whereas nonconjugated metabolites were present in their cecal content and colonic tissue. Free microbially derived metabolites come from colonic fermentation of PAs, whereas the conjugated metabolites come from free metabolites that have previously been absorbed, conjugated in the liver, and excreted via bile. Table 2 shows the complete list of microbially derived phenolic metabolites identified in the feces, cecal content, and colonic tissue of the GADF group, including their relative areas from the chromatograms generated by MRM experiments. The assignments were confirmed by the use of standards and/or product ion scan experiments and corresponded to species that were either not detected in control animals or detected at a concentration at least 10-fold lower. We detected some phenolic acids described in the literature as being metabolites of EC (e.g., 3-HBA) that are not included in the list because the signals they yielded were not significantly stronger than those from the control samples obtained from nonsupplemented animals.

Fourteen microbially derived phenolic metabolites were detected in the feces of the GADF group, only 8 of which were detected in the rats fed monomeric EC. The most abundant free phenolic acids previously detected in urine[18] were also detected in feces, including direct products of fermentation, such as hydroxyphenylvaleric acid (193 → 175) and hydroxyphenylacetic acid (151 → 107), as well as acids derived from further transformations in the liver, such as caffeic acid (179 → 135) and

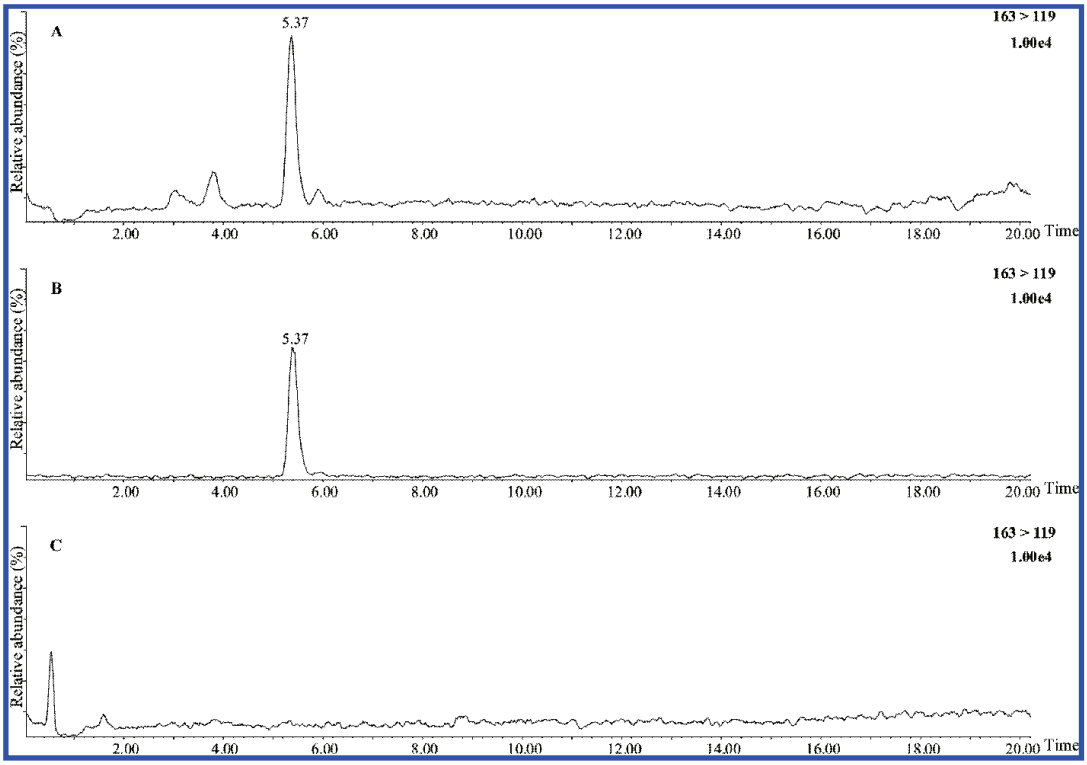

Figure 3. HPLC-ESI-MS profile corresponding to (A) *p*-coumaric acid standard, (B) feces from rats fed GADF, and (C) feces from rats fed EC. Detection was by multiple reaction monitoring (MRM), transition 163 → 119.

m-coumaric acid (163 → 119). Microbial metabolites were excreted via bile after conjugation, as shown by the detection of the derivatives Sulf-3,4-dihydroxyphenylpropionic acid (261 → 181) and Sulf-3- or 4-hydroxybenzoic acid (217 → 137). The metabolites detected only in feces from the GADF group were dihydroxyphenylvaleric acid (209 → 137), Sulf-3, 4-dihydroxyphenylpropionic acid (261 → 181), homovanillic acid (181 → 121), *p*-coumaric acid (163 → 119) (Figure 3), gallic acid (169 → 125), and 4-*O*-methylgallic acid (183 → 169).

Four microbially derived phenolic metabolites, namely, dihydroxyphenylvalerolactone (207 → 163), 3- or 4-hydroxyphenylvaleric acid (193 → 175), homovanillic acid (181 → 121), and gallic acid (169 → 125), were detected in the cecal content of the GADF group, indicating that fermentation of PAs was still taking place 24 h after the intake of the fiber matrix. The nonconjugated phenolic acids 3-hydroxyphenylacetic acid (151 → 107), *m*-coumaric acid (163 → 119), and *p*-coumaric acid (163 → 119) were detected in colonic tissue from the GADF group.

One microbially derived phenolic metabolite detected in the feces of the EC group, five in their colonic tissue, and six in their cecal content were not detected in the GADF group.

MS/MS fragments helped to confirm the identity of some of the microbially derived metabolites. Dihydroxyphenylvalerolatone (*m*/*z* 251), for instance, generated fragments at *m*/*z* 207 and 163, which correspond to the successive loss of two CO_2 molecules. MS/MS spectra of Sulf-3- or -4-hydroxybenzoic acid (*m*/*z* 217) provided signals at *m*/*z* 137 and 93, corresponding to the successive loss of Sulf and CO_2.

Most of the metabolites reported in a given group were detected in all four animals in that group.

DISCUSSION

GADF is a model of a dietary-fiber-rich matrix with associated PAs. It contains a complex mixture of polyphenols including monomers, oligomers, and polymers of EC (PAs), anthocyanins, flavonols, and hydroxycinnamic acids. These are associated with a fiber matrix of both soluble and insoluble polymers such as polysaccharides and lignins, which may influence the absorption of the putatively bioactive GADF components.[13,23] GADF has been used as a supplement in several animal and human studies.[25] It has been shown to have positive effects on colonic health, probably through local action of the dietary fiber and PAs. Moreover, positive modulation of cardiovascular disease risk factors by GADF[13] may also be mediated by metabolites derived from colonic fermentation. We focus here on PAs as the most abundant phenolic constituents of GADF.[13]

After ingestion, PAs are believed to undergo two main processes in the intestinal tract: (1) partial depolymerization into their constituent units, for example, EC, mainly by the gastric milieu[26] coupled with absorption of the free monomers and small oligomers (dimers, trimers) in the small intestine;[27,28] and (2) direct extensive fermentation by the intestinal microbiota into smaller metabolites such as phenolic acids and their subsequent absorption.[29,30] The species absorbed are conjugated into glucuronidyl, methyl, or sulfate derivatives in the small intestine and liver and are transferred either to the bloodstream or back to the colon via the bile.[11,24,31] It is generally accepted that PAs are not depolymerized into their monomeric units in the intestinal tract prior to the microbial transformations. However, this assumption comes from studies of small oligomers or by

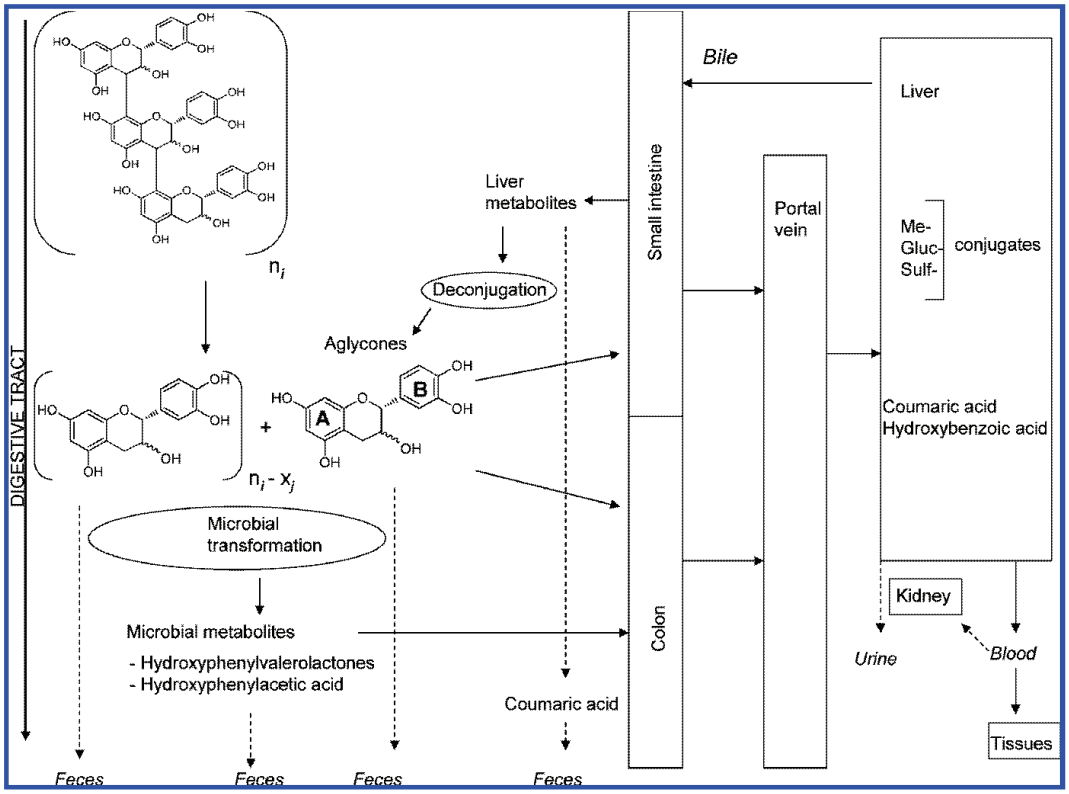

Figure 4. Metabolization of polymeric proanthocyanidins from GADF.

examining the microbial end products of purified oligomers.[11,29] Recently, we have provided indirect evidence that polymeric PAs are depolymerized in the intestinal tract,[18] because the wide variety of EC conjugates that were clearly detected in urine from rats fed GADF could not come only from the minute amounts of free EC present in GADF. By examining the metabolites in feces, cecal content, and colonic tissue, we present here further evidence that PAs are depolymerized during their transit along the intestinal tract. Furthermore, the present work provides novel information about PA-derived species in contact with the intestinal wall and within colonic tissue itself. This information may help to explain the local action of PAs in the colon as well as a possible prolonged systemic action of PA metabolites that results from the host organism and its associated microbiota, causing the slow release of polymers from the fiber matrix.

The feces of the GADF group contained a variety of EC phase II metabolites (Table 1). This corroborates the hypothesis that EC metabolites come from the depolymerization of oligomeric PAs and not only from EC monomers, because the intensities of the MS signals corresponding to EC metabolites are too high to come only from the small amounts (<0.01%) of monomeric EC in GADF.[23] The presence of the same metabolites in both urine and feces indicates that, once EC is conjugated in the liver, a fraction of the phase II metabolites passes into the bloodstream, whereas another fraction returns to the intestine via the bile. Conjugated forms of quercetin, another flavonoid, have been shown to have anti-inflammatory effects,[32] preventive effects on endothelial dysfunction,[33] and effects on apoptosis.[34] Similarly, at least some of the EC derivatives are expected to exhibit activities associated with intact EC, including scavenging activity.[18] No EC derivatives were detected in colonic tissue, indicating that there was no reabsorption of the conjugates. Nonconjugated EC was detected in feces, cecal content, and colonic tissue (Table 1; Figure 1). This again indicates that PAs from GADF are depolymerized and that the monomers that are released are absorbed during transit along the intestine and corroborates our previous suggestion[18] that intact EC is in contact with the intestinal tissue for hours after intake of GADF. Moreover, PAs in the process of being depolymerized must also expose free phenolic groups to the intestinal epithelium.

We also detected a variety of smaller phenolic species in the feces. These products of microbial fermentation included three groups of metabolites: (1) metabolites directly released from PA fermentation, such as 3- or 4-hydroxyphenylvalerolactone, dihydroxyphenylvalerolactone, 3- or 4-hydroxyphenylvaleric acid, dihydroxyphenylvaleric acid, 3- and 4-hydroxyphenylacetic acid, and homovanillic acid; (2) phenolic acids such as caffeic acid and *p*-coumaric acid derived from transformations in the liver of compounds from the aforementioned group after absorption; and (3) conjugated metabolites such as Sulf-3,4-dihydroxyphenylpropionic acid and Sulf-3- or -4-hydroxybenzoic acid generated in the liver by the action of phase II enzymes on compounds from group 1 after absorption. We detected more phenolic acids (e.g., dihydroxyphenylvaleric acid, Sulf-3,4-dihydroxyphenylpropionic acid, homovanillic acid, and *p*-coumaric acid) in the feces of the GADF group than in the feces of the EC group. This indicates that these compounds came from direct fermentation

of PA oligomers and polymers. Similar results were obtained by in vitro fermentation of procyanidin dimer B2 with human microbiota.[35] Most of the products of direct fermentation (group 1) detected in feces were also detected in urine,[18] which means that they are absorbed and bioavailable before being excreted. The metabolites with the longest carbon chains, corresponding to the initial stages of EC fermentation by ring cleavage of EC moieties, that is, hydroxyphenyvalerolactone and dihydroxyphenylvalerolactone, were not detected in urine. This means that these initial products of intestinal fermentation are quickly transformed into smaller units (phenylvaleric, phenylpropionic, and phenylacetic acids), by successive β-oxidations. *p*-Coumaric acid was detected in feces from the GADF group. Because *p*-coumaric acid is quickly absorbed in the small intestine,[36] we concluded that it came from PA fermentation and not directly from GADF. The intestinal metabolites would have been absorbed and further transformed in the liver, and the resulting small species (e.g., *p*-coumaric acid) would have been excreted back to the intestine via bile. Finally, nonconjugated gallic acid and its main metabolite 4-*O*-methylgallic acid were also detected in feces from the GADF group and not in those from the EC group, probably due to the release of gallic acid from gallate esters of catechins. We also detected phenolic acids derived from intestinal fermentation in the cecal content and colonic tissue of the GADF group, which indicates that the process of fermentation, release, and absorption of putatively bioactive compounds was still going on 24 h after the intake of the PA-rich fiber matrix.

The biological activities of the products of microbial fermentation of PAs have not yet been systematically tested, except for a few reports that products of colonic degradation of flavonoids exhibit anti-inflammatory effects[37,38] and antioxidant capacity.[39] It has been pointed out that the scavenging effect of polyphenols in vivo is negligible because they suffer extensive conjugation and subsequent excretion. Whereas studies of the capacity of polyphenols to modify the redox homeostasis of a complex living organism have provided contradictory results,[40−42] local concentrations of intact polyphenolic substructures (catechols, pyrogallols, gallates), for example, in the intestine, may have a significant redox effect, which may be free radical scavenging or generating, depending on the nature and concentration of the polyphenol.[43,44] This might be the case with GADF, which is the object of extensive transformations in the intestinal tract, which include depolymerization into EC units and ring cleaving that lead to smaller phenolic structures, resulting in the release of a large number of putatively bioactive substances over a long period of time.

The picture that arises from our results on GADF metabolization (Figure 4) is that of PA polymers being gradually cleaved into monomeric ECs and smaller phenolics along the intestinal tract for more than 24 h after ingestion. This slow and persistent process provides a variety of phenolics, both free and conjugated, that are in contact with the intestinal epithelial tissue, as proven by the different EC conjugates and microbially derived metabolites found in the feces, cecal content, and/or colonic tissue of rats after GADF supplementation. Many of these phenolic compounds can be absorbed and may exert their action in the colon as well as in other target tissues after absorption.

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ABBREVIATIONS USED

EC, (epi)catechin; EPA, extractable proanthocyanidin; GADF, grape antioxidant dietary fiber; GHS, glutathione; Gluc, glucuronide; Me, methyl group; MRM, multiple reaction monitoring; NEPA, nonextractable proanthocyanidin; PA, proanthocyanidin; PBS, phosphate-buffered saline; PTFE, polytetrafluoroethylene; Sulf, sulfate.

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1.2. Hamamelitannin from witch hazel (*Hamamelis virginiana*) displays specific cytotoxic activity against colon cancer cells.

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Q1

SUSANA SÁNCHEZ-TENA, MARÍA LUISA FERNÁNDEZ-CACHÓN, ANNA CARRERAS, MARÍA LUISA MATEOS-MARTÍN, NOELIA COSTOYA, MARY PAT MOYER, MARÍA JOSÉ NUÑEZ, JOSEP LLUÍS TORRES & MARTA CASCANTE

RESUMEN

La *Hamamelis virginiana* es una fuente rica en taninos condensados e hidrolizables los cuales podrían ejercer una acción protectora contra el cáncer de colon. En el presente estudio se caracteriza diferentes compuestos procedentes del extracto de *Hamamelis virginiana* como agentes citotóxicos selectivos contra el cáncer de colon. Las estructuras seleccionadas para el estudio fueron hamamelitanino y pentagaloilglucosa junto con una fracción rica en proantocianidinas. La pentagaloilglucosa se obtuvo mediante purificación de un extracto de *Hamamelis virginiana* debido a la falta de patrones comerciales.

Como resultado el hamamelitanino resultó ser el más eficiente. Además, no tuvo ningún efecto nocivo en colonocitos normales mientras que la pentagaloilglucosa inhibió el crecimiento de las células cancerosas y normales. En el ensayo con radical TNPTM el hamamelitanino fue más reactivo lo cual puede explicar su eficacia en la inhibición de cáncer de colon (Sánchez-Tena *et al.* 2012).

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Hamamelitannin from Witch Hazel (*Hamamelis virginiana*) Displays Specific Cytotoxic Activity against Colon Cancer Cells

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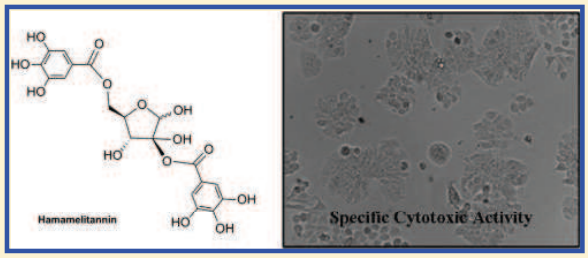
ABSTRACT: *Hamamelis virginiana* (witch hazel) bark is a rich source of condensed and hydrolyzable tannins reported to exert a protective action against colon cancer. The present study characterizes different witch hazel tannins as selective cytotoxic agents against colon cancer. To cover the structural diversity of the tannins that occur in *H. virginiana* bark, the hydrolyzable tannins, hamamelitannin and pentagalloylglucose, together with a proanthocyanidin-rich fraction (F800H4) were selected for the study. Treatment with these compounds reduced tumor viability and induced apoptosis, necrosis, and S-phase arrest in the cell cycle of HT29 cells, with hamamelitannin being the most efficient. Owing to polyphenol-mediated H_2O_2 formation in the incubation media, the antiproliferative effect was determined in the presence and absence of catalase to rule out any such interference. The presence of catalase significantly changed the IC_{50} only for F800H4. Furthermore, at concentrations that inhibit the growth of HT29 cells by 50%, hamamelitannin had no harmful effects on NCM460 normal colonocytes, whereas pentagalloylglucose inhibited both cancerous and normal cell growth. Using the TNPTM assay, we identified a highly reactive phenolic position in hamamelitannin, which may explain its efficacy at inhibiting colon cancer growth.



Several epidemiological studies have indicated that tannins may exert a protective effect against colon cancer, one of the most prevalent neoplastic diseases in the developed world.[1,2] Witch hazel (*Hamamelis virginiana*) bark is a rich source of both proanthocyanidins, or condensed tannins, and hydrolyzable tannins (Figure 1) such as hamamelitannin and pentagalloylglucose,[3] whose capacity to regulate cell proliferation, cell cycle, and apoptosis has attracted much attention.[4] An inverse relation has been reported between proanthocyanidins and colorectal cancer.[5] An in vitro study demonstrated that a grape seed proanthocyanidin extract significantly inhibits cell viability and increases apoptosis in Caco-2 colon cancer cells, but does not alter the viability of the normal colon NCM460 cell line.[6] Other results show that proanthocyanidins from different sources are cytotoxic to human colorectal cells.[7−9] In addition, several in vitro and in vivo studies have shown that hydrolyzable tannins from witch hazel bark exhibit multiple biological activities, which may have potential in the prevention and treatment of cancer. In vivo preclinical studies of pentagalloylglucose, one of the major hydrolyzable tannins in witch hazel, demonstrated inhibition of prostate cancer,[10,11] lung cancer,[12] and sarcoma[13] cells. In vitro inhibition of the growth and invasiveness of breast cancer, leukemia, melanoma, and liver cancer cells has also been reported.[14−17] The other major hydrolyzable tannin in witch hazel, hamamelitannin, inhibits TNF-mediated endothelial cell death and DNA fragmentation in EAhy926 endothelial cells.[18] Since TNFα/TNFR1 signaling may act as a tumor promoter for colon carcinogenesis,[19] the anti-TNF activity of hamamelitannin may indicate a protective effect against colon cancer. Furthermore, hamamelitannin has been described to inhibit 5-lipoxygenase (5-LOX),[20] and given that 5-LOX is an inflammatory enzyme involved in malignant transformation,[21] this inhibition could prevent cancer growth.

Moreover, various studies have analyzed the cytotoxicity and scavenging capacity of *H. virginiana* phenolic compounds. It has been reported that different witch hazel polyphenolic fractions are highly active as free radical scavengers against 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and tris(2,4,6-trichloro-3,5-dinitrophenyl)methyl (HNTTM). They also reduce tris-(2,3,5,6-tetrachloro-4-nitrophenyl)methyl (TNPTM) radical to some extent, which indicates that they contain highly reactive hydroxy groups. In this way, witch hazel fractions protect red blood cells from free radical-induced hemolysis and also inhibit the proliferation of the SK-Mel 28 melanoma tumor cell line.[22] Some of these fractions also inhibited cell proliferation, arrested the cell cycle at the S phase, and induced

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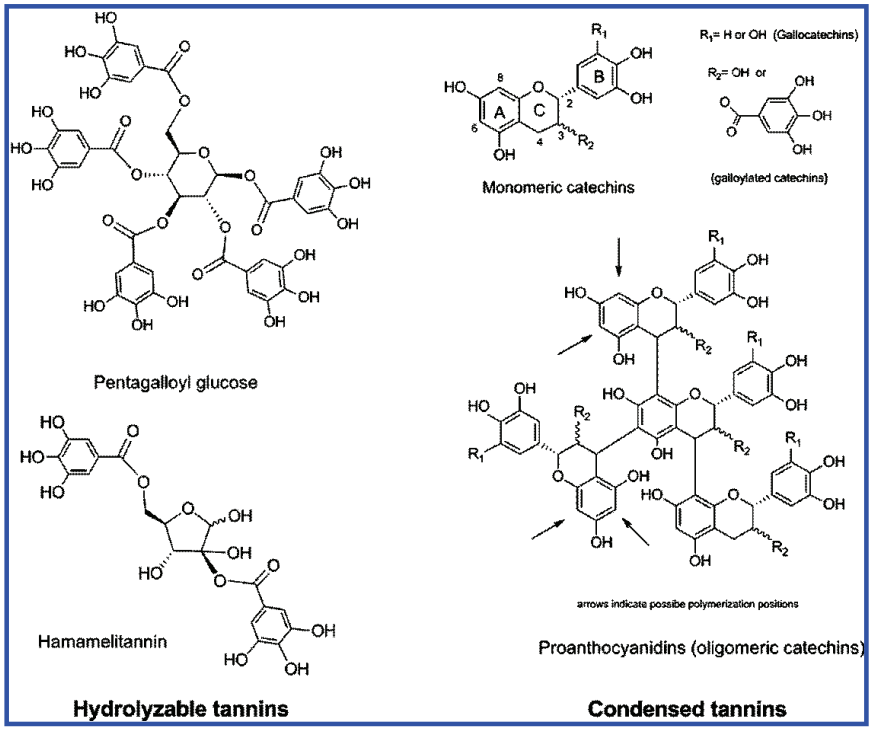

Figure 1. Structures of hydrolyzable and condensed tannins in *Hamamelis virginiana* bark.

apoptosis in HT29 human colon cancer cells.[23] The witch hazel mixtures studied so far include those from highly heterogeneous mixtures containing both hydrolyzable and condensed tannins of low molecular weight, as well as flavan-3-ol monomers;[22,23] however, the activity of oligomeric structures from witch hazel bark has not been evaluated. Furthermore, Masaki et al. reported that hamamelitannin from *H. virginiana* possesses protective activity from cell damage induced by superoxide anion radicals in murine dermal fibroblasts.[24,25]

To advance our understanding of the compounds responsible for the activity of *H. virginiana* bark, we evaluated the behavior of pure hamamelitannin and pentagalloylglucose (hydrolyzable tannins of different size) and a highly purified proanthocyanidin-rich fraction (F800H4). First, we examined the viability, apoptosis, and cell cycle of the human colorectal adenocarcinoma HT29 cell line after treatment with these compounds. To identify products that inhibit cancer cell growth without harming normal cells, the antiproliferative capacity of *Hamamelis* compounds was also measured against the NCM460 cell line (human colonocytes). As several studies have reported that polyphenols can be oxidized under standard cell culture conditions, leading to the production of significant amounts of ROS such as H_2O_2, and that this can modulate cell functions,[26] we supplemented the cell culture medium with catalase, which decomposes polyphenol-generated ROS, thus ruling out this possibility.[27]

RESULTS AND DISCUSSION

Pentagalloylglucose and fraction F800H4 were extracted from the bark of witch hazel, whereas the hydrolyzable tannin hamamelitannin was obtained commercially. Both hydrolyzable tannins presented a purity of 98% or more, as confirmed by HPLC. Once fraction F800H4 was obtained, its polyphenolic composition was characterized to ensure that it possessed a high percentage of condensed tannins. Table 1 summarizes the results of the HPLC analysis after thioacidolysis in the presence of cysteamine (condensed tannins) and direct HPLC analysis (gallic acid, pentagalloylglucose, and hamamelitannin). F800H4 was found to be composed of mostly condensed tannins (83.9% of the total tannins), both monomers and proanthocyanidins [(epi)catechin oligomers and polymers]. It also contained 16.1% hydrolyzable tannins, mainly hamamelitannin. Pentagalloylglucose was not detected in fraction F800H4. The condensed tannins had a mean degree of polymerization (mDP) of 2.6, 35% galloylation and 32% pyrogallol. The total galloylation of the fraction was 45.5%.

Table 1. Polyphenolic Composition of F800H4[a]

Composition of the Condensed Tannins (CTn) 83.9%					
mDP	% G	% P			
2.6	35.0	32.0			
% GC	% EGC	% C	% EC	% EGCG	% ECG
12.4	0.4	29.1	23.0	19.1	15.9
Composition of the Hydrolyzable Tannins (HTn) 16.1%					
% GA		% HT		% PGG	
10.0		90.0		0.0	

[a]mDP, mean degree of polymerization; % G, percentage of galloylation; % P, percentage in pyrogallol; GC, gallocatechin; EGC, epigllocatechin; C, catechin; EC, epicatechin; EGCG, epigallocatechin gallate; ECG, epicatechin gallatel; GA, gallic acid; HT, hamamelitannin; PGG, pentagalloylglucose.

Tannins regulate different cell functions through different actions that may or may not involve redox reactions.[28] Since

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Table 2. Hydrogen Donation and Electron Transfer Capacity

	DPPH			HNTTM			TNPTM		
	EC_{50}[a]	ARP[b]	H/e[c]	EC_{50}[a]	ARP[b]	e[c]	EC_{50}[a]	ARP[b]	e[c]
PGG	23.8	42.0	19.8	54.8	18.2	8.6	2403.9	0.4	0.2
HT	27.8	36.2	8.8	71.2	14.0	3.4	116.2	2.2	1.0
F800H4	39.8	25.1	27.1	66.7	15.0	16.2	1761.6	0.6	0.7

[a]EC_{50}, μg of polyphenol/μmol of radical. [b]ARP, $(1/EC_{50}) \times 10^3$. [c]Number of hydrogen atoms donated or electrons transferred to the stable radical per molecule of polyphenol, calculated as the inverse of 2 × molar EC_{50}.

polyphenols may act as antioxidants and prooxidants, we studied the redox activity of *H. virginiana* compounds and evaluated their free radical scavenging properties using different stable radicals such as DPPH, HNTTM, and TNPTM. DPPH reacts with polyphenols by mechanisms that may include both hydrogen donation and electron transfer,[29] while HNTTM and TNPTM are sensitive only to electron transfer.[30] The reactions with DPPH and HNTTM gave information on the total capacity to scavenge radicals by hydrogen donation or concerted electron proton transfer (DPPH) and by electron transfer (HNTTM). The reaction with TNPTM revealed the presence of highly redox reactive positions. Table 2 summarizes the activities of pentagalloylglucose, hamamelitannin, and the proanthocyanidin fraction F800H4 against the stable free radicals. Overall, pentagalloylglucose, hamamelitannin, and the proanthocyanidin-rich fraction F800H4 showed a similar total scavenging capacity, as their number of phenolic hydroxy groups per unit of mass was similar. Interestingly, differences were detected with TNPTM. While the scavenging capacity of the polyphenols against TNPTM is low because only some of the hydroxy groups are able to donate electrons to this radical, the possible effects of these hydroxy groups may be biologically relevant because they are the most reactive positions. One of the phenolic hydroxy groups in hamamelitannin was reactive enough to transfer its electron to TNPTM, while pentagalloylglucose was much less responsive (Table 2, last column). Hamamelitannin and pentagalloylglucose are structurally similar. In the case of hamamelitannin though, there is a hydroxy moiety geminal to one of the gallate esters, and this might explain the differences detected in the reactivity against the TNPTM radical. The extra hydroxy group might participate in a hydrogen bond with the carbonyl group from the gallate moiety to form a six-membered ring. This could introduce a conformational restriction with loss of planarity and subsequent loss of conjugation within the gallate moiety. The extended conjugation of the carbonyl and aromatic groups is the reason that gallates are less reactive than pyrogallols.[31] The results with TNPTM indicate that hamamelitannin is particularly reactive and may even participate in the formation of ROS through electron transfer to oxygen to form the superoxide radical.

Pentagalloylglucose has been shown to inhibit different malignancies.[10,11,13] Potential mechanisms for its anticancer activity include antiangiogenesis, antiproliferation, S-phase and G1-phase cell cycle arrest, induction of apoptosis, and anti-inflammatory and antioxidative effects . Putative molecular targets include p53, Stat3, Cox-2, VEGFR1, AP-1, SP-1, Nrf-2, and MMP-9. This study reports for the first time the role of pentagalloylglucose in colon cancer. We studied here the viability, the cell cycle, and the apoptosis process in human colorectal adenocarcinoma HT29 cells. In these bioassays, different positive controls were used. Epigallocatechin gallate (EGCG), a major catechin in green tea described to have antitumor activity,[32,33] was used as a standard in the cell viability assays; the cell cycle inhibitor hydroxyurea (HU) was used as a standard in the cell cycle experiments,[34] and staurosporine (ST) was utilized as a positive control in the apoptosis assays.[35] Treatment with pentagalloylglucose reduced the viability of HT29 cells with an IC_{50} value of 28 ± 8.8 μg/mL (Figure 2a) and induced 11% apoptosis compared to control cells, 5% necrosis (Figure 3), and S-phase arrest in the cell cycle with 8% increase in the population of cells in the S phase and a concomitant decrease in the percentage of cells in the G1 and G2 phases (Figure 4). Because pentagalloylglucose inhibits DNA replicative synthesis with greater efficacy than a known DNA polymerase-alpha inhibitor, aphidocolin,[36] this may explain the arrest in the S phase. The antitumor effects of hamamelitannin have not been examined, except for its antigenotoxic action in HepG2 human hepatoma cells reported by Dauer et al.,[37] as well as its anti-TNF[18] and anti-LOX activities.[20] The cellular mechanism that this hydrolyzable tannin induces may be related to the inhibition of the tumor necrosis factor itself and its receptor, which affect apoptosis, necrosis, and cell cycle processes. As a result, after treatment with hamamelitannin, we observed a reduction in the viability of HT29 cells with an IC_{50} of 20 ± 4.5 μg/mL (Figure 2a) and induction of 26% apoptosis, 14% necrosis (Figure 3), and S-phase arrest in the cell cycle with a 16% increase in the population of cells in this phase (Figure 4). With regard to condensed tannins, proanthocyanidins from various sources have been reported to inhibit colon cancer cells.[38,39] Treatment of the human colon adenocarcinoma HT29 cell line with the proanthocyanidin-rich fraction F800H4 extracted from witch hazel bark was less effective at inhibiting cell viability (IC_{50} = 38 ± 4.4 μg/mL; Figure 2a) and inducing apoptosis (9%) and necrosis (6%) (Figure 3) than the same treatment with hydrolyzable tannins. F800H4 had little effect on the normal cell cycle distribution apart from a slight increase in the S and G2 phases (Figure 4).

Overall, the hydrolyzable tannins were more effective than the condensed tannins. Interestingly, hamamelitannin, which includes a highly reactive position, as demonstrated by its reaction with TNPTM (Table 2), showed the strongest inhibition of cell viability, induction of apoptosis and necrosis, and cell cycle arrest in the S phase in HT29 colon cancer cells (Figures 2a, 3, 4). The effect of this reactive position in hamamelitannin may even be prooxidant. The prooxidant effect of some polyphenols has been discussed extensively, and it has been suggested that moderate generation of ROS may produce an antioxidant effect by fostering the endogenous defenses.[40,41] Therefore, in our assays, hamamelitannin may exert its activity, at least in part, by providing mild prooxidant challenges through electron transfer reactions leading to moderate formation of ROS.

On the other hand, since it has been reported that an increase in endogenous ROS levels is required for the transition from the G1 to the S phase of the cell cycle,[42] the cell cycle

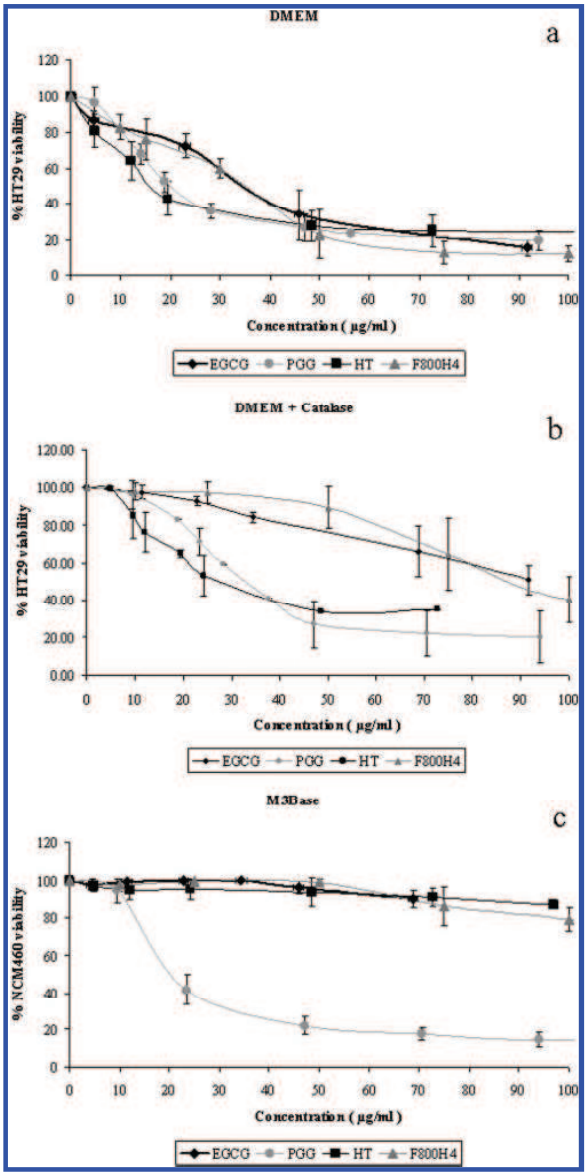

Figure 2. (a) Effect on HT29 cell viability of different concentrations of *Hamamelis virginiana* compounds in DMEM. (b) Effect on HT29 cell viability of witch hazel compounds in DMEM supplemented with catalase (100 U/mL). (c) Effect of Hamamelis products on NCM460 colonocyte growth. In all cases epigallocatechin gallate is used as a standard. Values are represented as mean of percentage of cell viability with respect to control cells ± standard error of three independent experiments.

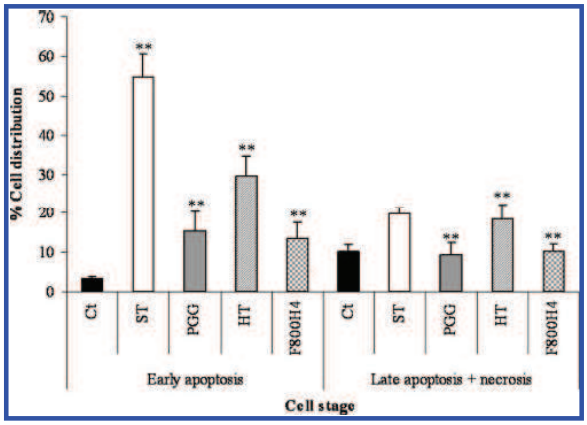

Figure 3. Early apoptotic cells: annexin V+/PI−. Late apoptotic/necrotic cells: annexin V+/PI+ and annexin V−/PI+. Staurosporine is utilized as a positive control. Values are expressed as mean ± standard deviation of three separate experiments. $^{**}p < 0.001$, significant difference with respect to the corresponding value in untreated cells (Ct).

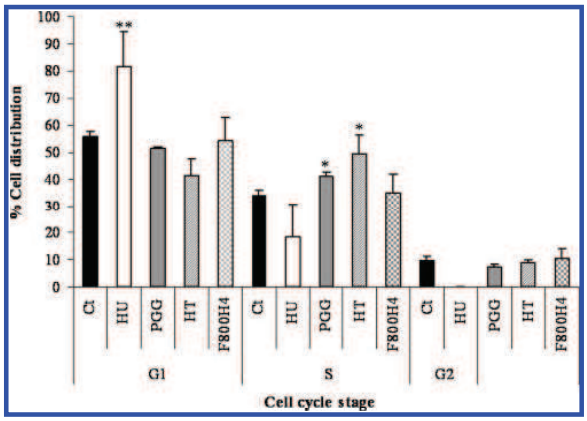

Figure 4. Normalized percentages of cells in different cell stages. Cell phases analyzed: Gl, S, and G2. The cell cycle inhibitor hydroxyurea was used as a standard. Mean ± standard deviation of three separate experiments. $^{*}p < 0.05$; $^{**}p < 0.001$, significant difference with respect to control cells (Ct).

arrest in the S phase induced by witch hazel compounds may be explained to some extent by its ROS scavenging capacity.

In the search for compounds or fractions that inhibit cancer cell growth without harming normal cells, the antiproliferative capacity of pentagalloylglucose, hamamelitannin, and the proanthocyanidin-rich fraction F800H4 was determined in NCM460 human colonocytes. NCM460 are nontumorigenic cells derived from normal colon mucosa that has not been infected or transfected with any genetic information.[43] This is the first comparison of the effects of witch hazel compounds on the growth of nontransformed colonocytes and cancerous colon cells. Our results show that the concentrations of hamamelitannin and F800H4 capable of inducing the death of HT29 cells (Figure 2a) had no harmful effects on normal colon cells (IC_{50} higher than 100 μg/mL for hamamelitannin and F800H4) (Figure 2c), whereas pentagalloylglucose inhibited both cancerous and normal cell growth (Figure 2a, c). Pentagalloylglucose inhibited NCM460 cell viability with an IC_{50} of 23 μg/mL ± 2.4 (Figure 2a, c).

It has been reported that polyphenol-mediated ROS formation in cell culture medium can lead to the artifactual modulation of cytotoxicity attributed to polyphenol exposure. Accordingly, Chai et al. reported that H_2O_2-mediated cytotoxicity, resulting from incubation of PC12 cells with green tea or red wine, was completely prevented by the addition of bovine liver catalase to the culture medium.[44] All *Hamamelis* compounds tested together with the positive

control used (EGCG)[45,46] generated H_2O_2 in a concentration-dependent manner in DMEM (Figure 5a). Hamamelitannin showed the highest H_2O_2 production, at 100 μg/mL. As expected, supplementing the cell culture medium with 100 U/mL catalase resulted in almost complete decomposition of polyphenol-generated H_2O_2 in all cases (Figure 5b). The next step was to study the antiproliferative capacity of *H. virginiana* polyphenolics by co-incubating with catalase. This enzyme had little effect on HT29 cells incubated with hydrolyzable tannins (IC_{50} in DMEM = 28 μg/mL ± 8.8 (Figure 2a)/IC_{50} in DMEM with catalase = 34 μg/mL ± 1.2 (Figure 2b) for pentagalloylglucose and IC_{50} in DMEM = 20 μg/mL ± 4.5 (Figure 2a)/IC_{50} in DMEM with catalase =13 μg/mL ± 4.6 (Figure 2b) for hamamelitannin), whereas F800H4 cytotoxicity was shown to be partially attributable to H_2O_2-mediated modulation (IC_{50} in DMEM = 38 μg/mL ± 4.4 (Figure 2a)/IC_{50} in DMEM with catalase = 95 μg/mL ± 8.7 (Figure 2b)). This effect is probably triggered by the highly reactive pyrogallol moieties in the condensed tannins. Interestingly, the results obtained for the positive control, EGCG, a flavan-3-ol with a pyrogallol B-ring, are in accordance with this hypothesis. Consequently, the difference between the IC_{50} value of F800H4 determined in HT29 cells incubated with catalase (Figure 2b) and the value established in NCM460 cells (Figure 2c) is not as high as when we compared the results obtained for HT29 without catalase (Figure 2a), which were artifactual, with NCM460 (Figure 2c). This demonstrates that, as with pentagalloylglucose, F800H4 is not completely specific against cancer cells. Interestingly, the cytotoxic activity of hamamelitannin was not modified by the addition of catalase to the medium.

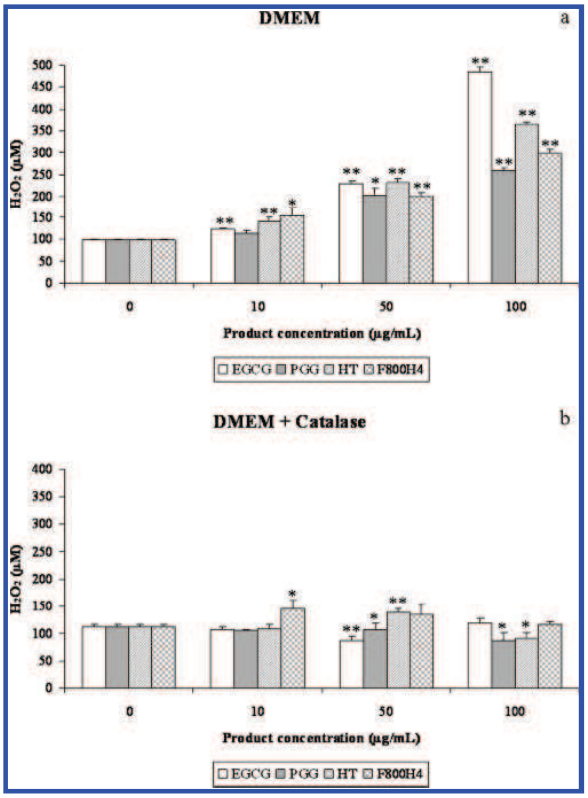

Figure 5. (a) H_2O_2 concentration in cell culture medium (DMEM + 10% FCS + 0.1% streptomycin/penicillin) with pentagalloyl glucose, hamamelitannin, and the proanthocyanidin-rich fraction F800H4 in medium. (b) H_2O_2 concentration produced in DMEM culture medium with catalase (100 U/mL) after incubation with witch hazel compounds. Epigallocatechin gallate is used as a positive control. Mean ± standard deviation of two independent experiments. $^{**}p < 0.001$ and $^{*}p < 0.05$, significant difference with respect to the corresponding value in untreated cells (Ct).

In summary, we conclude that pentagalloylglucose and the proanthocyanidin-rich fraction F800H4 do not show specificity for cancerous cells, whereas hamamelitannin is a promising chemotherapeutic agent, which might be used for the treatment of colon cancer without compromising the viability of normal colon cells. Hamamelitannin appears to contain a highly reactive phenolic position that can be detected by the stable radical TNPTM, which may explain its efficacy at inhibiting colon cancer cell growth. These findings may lead to a better understanding of the structure–bioactivity relationship of tannins, which should be of assistance for formulations of chemopreventive and chemotherapeutic agents.

EXPERIMENTAL SECTION

General Experimental Procedures. UV measurements were made on a Cary 50-Bio UV spectrophotometer (Varian, Palo Alto, CA, USA). Semipreparative chromatography was conducted on a Waters system (Milford, MA, USA) using an X-Terra C_{18} (19 × 250 mm, 10 μm) column. HPLC was carried out on a Hitachi (San Jose, CA, USA) system equipped with a quaternary pump, autosampler, and diode array detector and an analytical Kromasil C_{18} (Teknokroma, Barcelona, Spain) column. All chemicals were purchased from Sigma-Aldrich Co. (St Louis, MO, USA), unless otherwise specified. For extraction, we used deionized water, bulk EtOH (Momplet y Esteban, Barcelona, Spain), bulk acetone (Quimivita, Sant Adrià del Besòs, Spain), and bulk hexane (alkanes mixture) (Quimivita). For purification, deionized water, analytical grade MeOH (Panreac, Montcada i Reixac, Spain), 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 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. TFA (Fluorochem, Derbyshire, UK) biotech grade was distilled in-house. HCl (37%) and HOAc were from E. Merck. Et_3N (E. Merck) was of buffer grade. Deuterated solvents for NMR were from SDS (Peypin, France). DPPH (95%) was from Aldrich (Gillingham-Dorset, UK), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (97%) was from Aldrich (Milwaukee, WI, USA). HNTTM and TNPTM radicals were synthesized as described elsewhere.[30,47] Antibiotics (10 000 U/mL penicillin, 10 000 μg/mL streptomycin) were obtained from Gibco-BRL (Eggenstein, Germany), fetal calf serum (FCS) was from Invitrogen (Paisley, UK), and trypsin EDTA solution C (0.05% trypsin–0.02% EDTA) was from Biological Industries (Kibbutz Beit Haemet, Israel). The annexin V/FITC kit was obtained from Bender System (Vienna, Austria). M3Base medium was purchased from INCELL (San Antonio, TX, USA).

Extraction, Fractionation, and Characterization of F800H4. Polyphenols were obtained from witch hazel bark by extraction with acetone–water (7:3) and fractionation with EtOAc,[22] which produced fraction OWH (polyphenols soluble in EtOAc and H_2O) and fraction AH (polyphenols only soluble in H_2O). To generate fraction F800H4, AH (800 mg) was dissolved in 50% MeOH and fractionated on a Sephadex LH-20 column (50 × 2.5 cm i.d.) using a gradient of MeOH in H_2O and a final step of washing with acetone, as previously reported.[48] Five subfractions (800H1 to 800H5) were collected, and their absorbance was measured at 280 and 400 nm; yield, 8% from

fraction AH; 0.05% from witch hazel bark. Table 1 shows the chemical composition of fraction F800H4, which was estimated as previously described.[22] The content of condensed tannins was estimated by thioacidolytic depolymerization in the presence of cysteamine and HPLC analysis of the cleaved units. The hydrolyzable tannins were determined directly from the fraction by HPLC and standards.

Purification of Pentagalloylglucose. Pentagalloylglucose was purified from fraction OWH by semipreparative chromatography on a Waters system (Milford, MA, USA) using an X-Terra C_{18} (19 × 250 mm, 10 μm) column. A total amount of 2 g of OWH was processed in successive chromatographic runs with loads of 200 mg, 4 mL each, and elution by a binary system [solvent A, 0.1% aqueous TFA; solvent B, 0.08% TFA in H_2O–CH_3CN (1:4)] under the following conditions: 10 min at 16% B and two gradients, 16–36% B over 40 min, and 36–55% B over 5 min, at a flow rate of 10 mL/min with detection at 235 nm. The purity of the pentagalloylglucose was ascertained by HPLC on a Hitachi (San Jose, CA, USA) system equipped with a quaternary pump, autosampler, and diode array detector and an analytical Kromasil C_{18} (Teknokroma, Barcelona, Spain) column under the same elution conditions at a flow rate of 1 mL/min. Pentagalloylglucose was lyophilized, and its identity was confirmed by chromatography coupled to high-resolution mass spectrometry and NMR; purity, 95% by HPLC; yield, 3.8% from fraction OWH, 0.03% from witch hazel bark.

DPPH Assay. The antiradical capacity of the polyphenols was evaluated by the DPPH stable radical method.[49] Fresh MeOH solutions (2 mL) at concentrations ranging from 2 to 30 μM were added to a freshly prepared radical solution (2 mL, 120 μM) in deoxygenated MeOH. The mixture was incubated for 30 min at room temperature in the dark, and the UV absorbance at 517 nm was measured. 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 concentration of DPPH. Each data point was the result of three independent determinations. A dose–response curve was obtained for every sample. The results are expressed as the efficient concentration, EC_{50}, given as the amount of polyphenols that consumes half the amount of free radical divided by the initial amount of DPPH in micromoles. The results are also expressed as antiradical power (ARP), which is the inverse of EC_{50}. UV measurements were made on a Cary 50-Bio UV spectrophotometer (Varian, Palo Alto, CA, USA).

Electron Transfer Capacity against the Stable Free Radicals HNTTM and TNPTM. Fresh solutions of the polyphenols (2 mL) at concentrations ranging from 2 to 62 μM were added to a freshly prepared solution of HNTTM (2 mL, 120 μM) in deoxygenated $CHCl_3$–MeOH (2:1). The mixture was incubated for 7 h at room temperature in the dark, and the UV absorbance was measured at 384 nm. The results are plotted as the percentage of absorbance disappearance $[(1 - A/A_0) \times 100]$ against the amount of sample divided by the initial amount of the radical in micromoles, as described for DPPH. Each data point was the result of three independent determinations. A dose–response curve was obtained for every sample. The results are expressed as the efficient concentration, EC_{50}, and as ARP. The working conditions with TNPTM were essentially those described for HNTTM[30] with some differences. The concentration range was 10–120 μM, the incubation time was 48 h, and the absorbance was measured at 378 nm. The results are plotted as described for HNTTM.

Cell Culture. Human colorectal adenocarcinoma HT29 cells (obtained from the American Type Culture Collection, HTB-38) were grown as a monolayer culture in Dulbecco's modified Eagle's medium (DMEM) in the presence of 10% heat-inactivated fetal calf serum and 0.1% streptomycin/penicillin in standard culture conditions. NCM460 cells, obtained by a Material Transfer Agreement with INCELL, are from an epithelial cell line derived from the normal colon mucosa of a 68-year-old Hispanic male.[43] They were grown as a monolayer culture in M3Base medium (which contains growth supplements and antibiotics) supplemented with 10% heat-inactivated fetal calf serum and 2.5 mM D-glucose (final concentration 5 mM glucose). The cells were cultured at 37 °C in a 95% air, 5% CO_2 humidified environment.

Determination of Cell Viability. The assay was performed using a variation of the MTT assay described by Mosmann.[50] The assay is based upon the principle of reduction of MTT into blue formazan pigments by viable mitochondria in healthy cells. The cells were seeded at densities of 3×10^3 cells/well (HT29 cells) and 1×10^4 cells/well (NCM460 cells) in 96-well flat-bottom plates. After 24 h of incubation at 37 °C, the polyphenolic samples were added to the cells at different concentrations in fresh medium. Some experiments were performed in the presence of catalase (100 U/mL, from bovine liver) to examine the potential influence on extracellular H_2O_2. The use of an antioxidant enzyme in the cell medium allows us to rule out the effects of exogenous H_2O_2 generated during the incubation with polyphenols. The addition of this enzyme does not affect the cellular markers, since it does not enter the cells and is removed after incubation. In all cases the antitumor agent EGCG was used as standard. The culture was incubated for 72 h. Next the medium was removed, and 50 μL of MTT (1 mg/mL in PBS) with 50 μL of fresh medium was added to each well and incubated for 1 h. The MTT reduced to blue formazan, and the precipitate was dissolved in 100 μL of DMSO; absorbance values were measured on an ELISA plate reader (550 nM) (Tecan Sunrise MR20-301, Tecan, Salzburg, Austria). Absorbance was taken as proportional to the number of living cells. The concentrations that caused 50% cell growth inhibition (IC_{50}) were estimated from the dose–viability curves.

Cell Cycle Analysis by FACS. The cell cycle was analyzed by measuring the cellular DNA content using the fluorescent nucleic acid dye propidium iodide (PI) to identify the proportion of cells in each stage of the cell cycle. The assay was carried out using flow cytometry with a fluorescence-activated cell sorter (FACS). HT29 cells were plated in six-well flat-bottom plates at a density of 87×10^3 cells/well. After 24 h of incubation at 37 °C, the polyphenolic fractions were added to the cells at their respective IC_{50} values. We used the G1/S cell cycle inhibitor HU at 1 mM as standard. The cultures were incubated for 72 h in the absence or presence of the polyphenolic fractions. The cells were trypsinized, pelleted by centrifugation (1500 rpm for 5 min), and stained in Tris-buffered saline containing 50 μg/mL PI, 10 μg/mL RNase free of DNase, and 0.1% Igepal CA-630. They were incubated in the dark for 1 h at 4 °C. Cell cycle analysis was performed by FACS (Epics XL flow cytometer, Coulter Corp., Hialeah, FL, USA) at 488 nm.[51]

Apoptosis Analysis by FACS. Double staining with annexin V-FITC and PI measured by FACS was used to determine the percentage of apoptotic cells. Annexin+/PI− cells were considered early apoptotic cells. Annexin+/PI+ and annexin−/PI+ cells were classed together as late apoptotic/necrotic cells, since this method does not differentiate necrotic cells from cells in late stages of apoptosis, which are also permeable to PI. The cells were seeded, treated, and collected as described in the previous section. ST (1 μM) was utilized as a control of apoptosis induction. After centrifugation (1500 rpm for 5 min), they were washed in binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and resuspended in the same buffer. Annexin V-FITC was added using the annexin V-FITC kit. Afterward, the cells were incubated for 30 min at room temperature in the dark. Next, PI was added 1 min before the FACS analysis at 20 μg/mL. Fluorescence was measured at 495 nm (annexin V-FITC) and 488 nm (PI).

Determination of H_2O_2 (FOX Assay). H_2O_2 in the cell culture medium was determined using the ferrous oxidation xylenol orange (FOX) assay.[52] After oxidation of Fe(II) to Fe(III) by H_2O_2, the resulting xylenol orange–Fe(III) complex was quantified spectrophotometrically (560 nm). The cells were incubated for 72 h with a range of concentrations of witch hazel compounds in culture medium (DMEM or M3Base) either alone or in the presence of catalase (100 U/mL, from bovine liver) under cell culture conditions (96-well flat-bottom plate, in the absence of cells). EGCG was used as a positive control in this assay given that it has already been reported that this product generates high levels of ROS in cell culture media. Next, 100 μL of medium was transferred to a new 96-well flat-bottom plate. FOX reagent (900 μL) was added to each aliquot: 100 μM xylenol orange, 250 μM ferrous ammonium sulfate, 25 mM H_2SO_4 and 4 mM BHT in

90% (v/v) MeOH. After 30 min, absorbance at 560 nm was measured in a microplate reader (Tecan Sunrise MR20-301, Tecan). Peroxides were quantified by comparing the absorbance to a standard curve (H_2O_2 concentrations: 0−150 μM).

Data Presentation and Statistical Analysis. Data are given as the means ± SD (standard deviation). For each assay, the parametric unpaired two-tailed independent sample t test was used for statistical comparison with the untreated control cells, and differences were considered to be significant when $p < 0.05$ and $p < 0.001$.

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1.3. Punicalagin and catechins contains polyphenolic substructures that influence cell viability and can be monitored by radical biosensors selective to electron transfer.

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RESUMEN

Los compuestos polifenólicos pueden ser captadores o generadores de radicales libres dependiendo de su naturaleza y de su concentración. Este doble efecto antioxidante-prooxidante, puede contribuir a su influencia sobre la viabilidad celular. Para controlar las propiedades redox de los polifenoles (punicalagina y catequinas) que contienen hidroxilos fenólicos con diferentes capacidades reductoras se utiliza dos radicales estables sintetizados en nuestro laboratorio, el radical tris(2,3,5,6-tetracloro-4-nitrofenil)metilo (TNPTM) y el radical tris(2,4,6-tricloro-3,5-dinitrofenilo) metilo (HNTTM), los cuales sólo actúan en reacciones de reducción de transferencia electrónica. El uso de estos dos radicales revela que subestructuras consistentes en ésteres galato unidos por enlaces C-C procedentes de la punicalagina son más reactivos que los galatos simples y menos reactivos que el grupo pirogalol de las catequinas del té verde. Los grupos más reactivos con TNPTM, están presentes en los compuestos que afectan a la viabilidad de células de cáncer de colon HT-29. El radical TNPTM reacciona con galatos y pirogalol y proporciona una técnica para detectar polifenoles potencialmente beneficiosos a partir de fuentes naturales (Carreras *et al.* 2012).

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Punicalagin and Catechins Contain Polyphenolic Substructures That Influence Cell Viability and Can Be Monitored by Radical Chemosensors Sensitive to Electron Transfer

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Supporting Information

ABSTRACT: Plant polyphenols may be free radical scavengers or generators, depending on their nature and concentration. This dual effect, mediated by electron transfer reactions, may contribute to their influence on cell viability. This study used two stable radicals (tris(2,3,5,6-tetrachloro-4-nitrophenyl)methyl (TNPTM) and tris(2,4,6-trichloro-3,5-dinitrophenyl)methyl (HNTTM)) sensitive only to electron transfer reduction reactions to monitor the redox properties of polyphenols (punicalagin and catechins) that contain phenolic hydroxyls with different reducing capacities. The use of the two radicals reveals that punicalagin's substructures consisting of gallate esters linked together by carbon−carbon (C−C) bonds are more reactive than simple gallates and less reactive than the pyrogallol moiety of green tea catechins. The most reactive hydroxyls, detected by TNPTM, are present in the compounds that affect HT-29 cell viability the most. TNPTM reacts with C−C-linked gallates and pyrogallol and provides a convenient way to detect potentially beneficial polyphenols from natural sources.

KEYWORDS: *punicalagin, catechins, pyrogallol, TNPTM chemosensor, cell viability*

INTRODUCTION

The question of whether natural polyphenols provide benefits in terms of human health is a controversial one among scientists. Ever since Harman published his paper on free radicals and aging,[1] it has been assumed that polyphenols prevent disease and delay aging because they scavenge toxic free radicals, which progressively damage biomolecules in live tissues mainly by oxidation.[2] Because they scavenge potentially oxidizing free radicals, polyphenols are referred to as antioxidants. Nevertheless, although it is true that polyphenols scavenge radicals in solution, their intracellular effectiveness is less obvious, and many authors consider them to be virtually inactive in vivo after oral intake.[3] The reason is that the live organism prevents polyphenols from greatly altering the redox homeostasis by rapidly metabolizing and excreting them, as well as by activating regulatory enzymatic systems. Polyphenols are conjugated into glucuronides, methyl esters, and sulfates mainly in the intestine and liver.[4,5] Most of these conjugates are no longer free radical scavengers, and the very small amounts of remaining intact polyphenolic moieties are very unlikely to modify the redox homeostasis significantly.[3] The skin and intestinal tract may be exceptions to this because local concentrations of intact phenolics may be present in significant amounts in these tissues.[6] Moreover, not only may polyphenols be effective free radical scavengers, they may actually generate free radicals depending on the nature and concentration of the specific polyphenols.[3] This so-called pro-oxidant activity may be behind the moderate toxicity of green tea extracts at very high concentrations[7] and the reason why polyphenols are rapidly transformed and excreted after ingestion. Interestingly, at concentrations that are not so high, this mild pro-oxidant activity may result in an overall antioxidant effect via a mechanism known as hormesis, which can be defined as a low-dose stimulation of defense systems with a subsequent beneficial effect.[8] In the case of foodstuffs in which the redox regulation systems progressively lose their efficiency during the shelf life of the product (e.g., fish rich in PUFA), polyphenols have proven to effectively prevent lipid oxidation.[9] Whatever the case, if polyphenols exert an influence over the redox status of any system, whether it is antioxidant, toxic pro-oxidant, or hormetic pro-oxidant, it is somehow related to the reactivity of the constitutive hydroxyl groups in the polyphenols, the functional groups that first react with oxidants.

Different chemical mechanisms may be involved in the free radical-scavenging and/or free radical-generating effects of polyphenols. To better characterize the scavenging activity of polyphenols, several assays focused on different possible mechanisms of their overall action should be considered.[10] The mechanisms that have been proposed are hydrogen atom transfer (HAT), proton-coupled electron transfer (PCET), and sequential proton loss electron transfer (SPLET), with the generation of a

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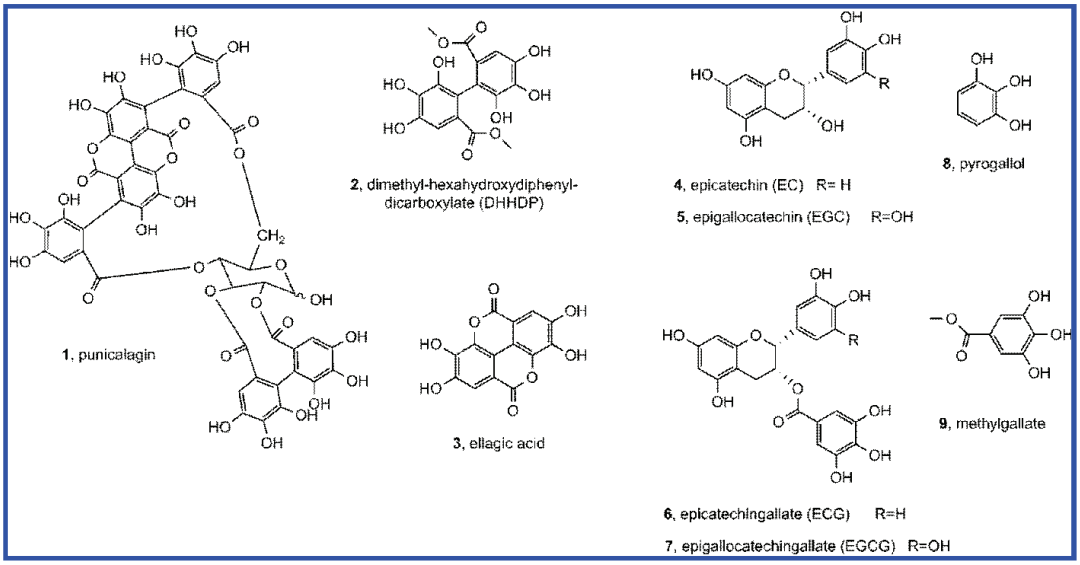

Figure 1. Structures of punicalagin (**1**), related compounds (**2** and **3**), green tea catechins (**4**–**7**), and simple phenols (**8** and **9**).

more stable phenoxyl radical.[11−13] Electron transfer to oxygen generates the superoxide radical $O_2^{\bullet-}$, which is enzymatically converted into hydrogen peroxide[14,15] and ultimately into the deleterious hydroxyl radical in the presence of transition metal cations (e.g., Fe^{2+}).[16] Moreover, the superoxide radical seems to mediate apoptosis.[17,18] Electron transfer appears to be most relevant in the redox cascades involving polyphenols, whether they scavenge or generate reactive radicals. To evaluate the electron transfer capacity of polyphenols, we developed stable radicals of the (2,4,6-trichlorophenyl)methyl (TTM) and perchlorotriphenylmethyl (PTM) series, which react exclusively by electron transfer.[13,19,20] We and others have used these radicals to evaluate the electron transfer capacity of natural and synthetic phenolic scavengers.[21,22] As the activity of the stable radicals of the TTM and PTM series essentially depends on the electron-withdrawing or electron-donating character of the meta- and/or para- substituents introduced into the phenyl rings, radicals with different redox potentials can be designed. The advantage of devising assays using this combination of radicals is that they can discriminate between oxidizing agents by their oxidizing ability, in contrast to the ferric ion reduction method that also operates exclusively by electron transfer processes but measures only the reducing ability based upon the redox potential of the ferric ion. Moreover, the outcome of the ferric ion method is also influenced by binding of the polyphenol to the ion.

Polyphenols may contain more than one reactive polyphenolic substructure. Punicalagin (**1**) (Figure 1), the most abundant polyphenol in pomegranate (*Punica granatum* L.),[23] is a hydrolyzable tannin of the ellagitannin kind because it contains an ellagic acid substructure (**3**). Punicalagin (**1**) releases ellagic acid (**3**) in the small intestine via spontaneous lactonization with later conversion into urolithin A by the gut microbiota.[24] Punicalagin (**1**) also contains in its structure gallate (three geminal phenolic hydroxyls and a carboxylate function) esters linked by carbon−carbon (C−C) bonds either to themselves (hexahydroxy-2,2′-diphenyl, HHDP moiety) or to the ellagic acid substructure. This ensemble of substructures and their metabolites contributes to the bioactivity of the whole molecule. The C−C bond structures constitutive of ellagitannins appear to be important for their activity. Pedunculagin, another hydrolyzable tannin that contains the HHDP moiety, shows higher cytotoxic activity than pentagalloylglucose, a hydrolyzable tannin that contains only simple gallate esters in its structure.[25] Catechins (flavanols of the flavan-3-ol type) are another family of polyphenols that display different polyphenolic substructures and are relevant to dietary considerations. Green tea is a common source of catechins, mainly, in order of abundance, (−)-epigallocatechin gallate (EGCG) (**7**), (−)-epigallocatechin (EGC) (**5**), (−)-epicatechin (EC) (**4**), and (−)-epicatechin gallate (ECG) (**6**) (Figure 1).[26] Tea flavanols scavenge reactive oxygen and nitrogen species, interfere with pro-oxidant processes, or inhibit pro-oxidant enzymes.[27] Polyphenols appear to exert their biological activity through different mechanisms involving redox reactions and protein−ligand interactions. Because the present paper focuses on the redox reactivity of different phenolic moieties and its possible relationship to cell viability in vitro, we selected pomegranate punicalagin (**1**) and green tea flavanols **4**–**7** for our study; together they contain a broad range of polyphenolic substructures. Here, we examine the electron transfer capacity (reducing power) of punicalagin (**1**), and its metabolite ellagic acid (**3**), its related substructure **2**, and green tea flavanols **4**–**7** bearing the catechol, pyrogallol, and gallate moieties, and we evaluate the effect of all these molecules on the viability of colon carcinoma HT-29 cells.

MATERIALS AND METHODS

Tris(2,3,5,6-tetrachloro-4-nitrophenyl)methyl (TNPTM) and tris-(2,4,6-trichloro-3,5-dinitrophenyl)methyl (HNTTM) were synthesized in our laboratory as described previously.[19,20] 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich (St. Louis, MO). Punicalagin (**1**) (≥98% (HPLC)) was obtained from Biopurify (Sichuan, China), ellagic acid (**3**) and the catechins **4**–**7** were from from Sigma-Aldrich, and dimethyl-hexahydroxydiphenyl dicarboxylate (DHHDP, **2**) was synthesized in our laboratory following procedures described elsewhere[28] (see the Supporting Information).

Radical-Scavenging Capacity. The scavenging capacity was determined from mixtures (1:1, v/v) of fresh solutions of stable radicals (TNPTM, HNTTM, DPPH; 120 μM) and fresh solutions of polyphenols **1**–**9** in $CHCl_3$/MeOH (2:1) at different concentrations (1−120 μM) at room temperature. All of the solutions were prepared

and deoxygenated in the darkness. The reactions were monitored by electron paramagnetic resonance (EPR) on an EMX-Plus 10/12 (Bruker BioSpin, Rheinstetten, Germany) after 48 h (TNPTM), 7 h (HNTTM), and 30 min (DPPH). Operating conditions were as follows: center field, 3615 G; scan range, 250 G; microwave power, 5.2 mW; microwave frequency, 9.86 GHz; modulation frequency, 100 kHz; receiver gain, 6×10^3; and time constant, 4.1 s. The scavenging capacity of polyphenols is given as EC_{50}, which corresponds to the amount (micrograms or micromoles) of polyphenol able to consume half the amount of free radical divided by micromoles of initial radical. The results in micrograms per micromole convey the idea of the scavenging capacity of a given amount of polyphenol, and the results in micromoles per micromole provide information about the number of equivalents per molecule. To facilitate the comparison between structures, the results were also expressed as antiradical capacity (ARC), which is the inverse of EC_{50} in micrograms per micromole and hydrogen atoms donated or electrons transferred per molecule of polyphenol (H/e), which is the inverse of $2 \times EC_{50}$ in micromoles per micromole.

Kinetic Measurements. The rate constants of the reactions between TNPTM and polyphenols **2** and **8** were estimated by EPR. Freshly prepared solutions of TNPTM in CH_3Cl/MeOH (2:1) (240 μM) and the polyphenol (48 μM in the same solvent) were mixed (1:1, v/v, molar ratio 5:1), and the decay of the TNTPM band was followed at room temperature. Operating conditions were as follows: center field, 3450 G; scan range, 250 G; microwave power, 1.0 mW; microwave frequency, 9.86 GHz; modulation frequency, 100 kHz; receiver gain, 8.9×10^3; and time constant, 40.96 s. The rate constants and the total number of electrons transferred per polyphenol (n_e) were estimated with a simple and general kinetic model reported by Dangles et al.[29] defined by eq 1. The values for the rate constant, k were calculated from the integrated eq 2.

$$-\mathrm{d}[\mathrm{TNPTM}]/\mathrm{d}t = k \times n[(\mathrm{poly})\mathrm{phenol}][\mathrm{TNPTM}] = k_1[(\mathrm{poly})\mathrm{phenol}][\mathrm{TNPTM}] \quad (1)$$

$$\ln \frac{1 - I_f/I_x}{1 - I_f/I_0} = -\frac{k_1 c}{I_0/I_f - 1} t \quad (2)$$

In eqs 1 and 2, n represents the number of reduced moles of TNPTM per mole of polyphenol; I_0 is the initial intensity of the TNPTM signal in the EPR spectra; I_f is the final visible intensity; and c is the initial concentration of polyphenol. The n_e values of the stoichiometry of the polyphenol were calculated using eq 3; ε is the molar absorptivity characteristic of the stable free radical.

$$n_e = \frac{I_0 - I_f}{\epsilon \times C} \quad (3)$$

Cyclic Voltammetry. Cyclic voltammetries were carried out in a standard thermostated cylindrical, one-compartment, three-electrode cell. A platinum (Pt) disk of 0.093 cm^2 area was used as the working electrode and a Pt wire as the counter electrode. The reference electrode was a saturated calomel electrode (SCE), submerged in a salt bridge of the same electrolyte, which was separated from the test solution by a Vycor membrane. Solutions of polyphenols ($\sim 10^{-3}$ M) in DMF containing tetrabutylammonium perchlorate (0.1 M) as the background electrolyte were studied. The volume of all test solutions was 50 mL. Electrochemical measurements were performed under an argon atmosphere at 25 °C using an Eco Chemie Autolab PGSTA-T100 potentiostat-galvanostat (Autolab, Utrecht, The Netherlands) controlled by a computer with Nova 1.5 software (Autolab). Cyclic voltammograms of all the solutions were recorded at scan rates ranging from 20 to 200 mV s^{-1}.

Cell Culture and Viability Assay. HT-29 human colon adenocarcinoma cells were obtained from the American Type Culture Collection. HT-29 cells were cultured in Dulbeco Modified Eagle's Medium (DMEM with 4500 mg L^{-1} glucose, L-glutamine, and sodium bicarbonate, without sodium pyruvate; Sigma-Aldrich), supplemented with 10% fetal bovine serum (PAA Laboratories, Pasching, Austria) and antibiotics, 100 U mL^{-1} penicillin and 100 mg L^{-1} streptomycin (Invitrogen, Paisley, U.K.), at 37 °C in a humidified atmosphere of CO_2 (5%). The effect of treatment with different polyphenols upon proliferation of HT-29 colon cancer cells was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich) assay, which is based on the ability of live cells to cleave the tetrazolium ring, thus producing formazan, which absorbs at 570 nm. HT-29 cells (3000 cells/well) were grown on a 96-well plate for 24 h and then incubated with the different polyphenols at different concentrations (10−300 μM) in dimethyl sulfoxide (DMSO) (Sigma-Aldrich), except ellagic acid (**3**), which was dissolved in *N*-methylpyrrolidone because of its poor solubility in DMSO. After 72 h, 100 μL of MTT solution (0.5 mg mL^{-1}) was added to each well. After 1 h of incubation, the formazan salt was resuspended in 100 μL of DMSO. Cell viability was measured by absorbance at 550 nm on an ELISA plate reader (Tecan Sunrise MR20-301, TECAN, Austria). The experiments were also run in the presence of catalase (Sigma-Aldrich), 100 U mL^{-1} in DMEM.[30] The results were expressed as IC_{50}.

RESULTS

Radical-Scavenging Capacity of Polyphenols Measured by TNPTM, HNTTM, and DPPH. The scavenging capacity of punicalagin (**1**) and related compounds **2** and **3**, flavanols **4**−**7**, pyrogallol (**8**), and methylgallate (**9**) was measured by making them react with the stable radicals TNPTM, HNTTM, and DPPH in a mixture that includes a polar hydroxylated solvent ($CHCl_3$/MeOH (2:1) (v/v)) and monitoring the decrease of the EPR radical signal. TNPTM and HNTTM are reduced exclusively by accepting electrons, in contrast to DPPH, which reacts by HAT and/or ET depending on the solvent. Table 1 summarizes the results of the scavenging capacity of **1**−**9** against the three radicals.

Punicalagin (**1**), ECG (**6**), and EGCG (**7**) were the most active polyphenols against HNTTM and DPPH. The number of electrons transferred to HNTTM roughly corresponded to the number of putative reactive positions (geminal hydroxyls) of the flavanols except for ECG (**6**), which consumed a larger amount of radical. Surprisingly, DHHDP (**2**) transferred 4.3 electrons instead of 6, and punicalagin (**1**) transferred 14.2 electrons instead of 16. The scavenging capacities of the polyphenols against TNPTM radical were lower than those obtained with HNTTM and DPPH because TNPTM reacts only with the most reactive hydroxyls. One molecule each of EGC (**5**), EGCG (**7**), and pyrogallol (**8**) reacted with 3 molecules of TNPTM (roughly 1 electron transferred from each of the three geminal hydroxyls); 1 molecule of punicalagin (**1**) and its substructure DHHDP (**2**) reacted with 3.3 and 2 molecules of TNPTM, respectively (roughly 1 electron transferred from each C−C linked gallate). In contrast, ellagic acid (**3**), EC (**4**), ECG (**6**), and methylgallate (**9**) did not react at all with TNPTM. Figure 2 shows graphically the selective reactivity of characteristic phenolic moieties with TNPTM, monitored by the decrease of the TNPTM radical EPR signal upon reaction with DHHDP (**2**), pyrogallol (**8**), and methylgallate (**9**).

Kinetic Measurements. To further characterize the scavenging activity of the hexahydroxydiphenyl moiety within punicalagin (**1**) and pyrogallol (**8**), which are the only simple structures that react with TNTPM, we made kinetic measurements of the reactions of DHHDP (**2**) and pyrogallol (**8**) with TNPTM. The course of the reaction was monitored using EPR by recording the decay of the TNPTM signal as a result of the addition of the polyphenol in $CHCl_3$/MeOH (2:1) at a molar ratio TNPTM/polyphenol of 5:1. To calculate the stoichiometric factor, the reaction was monitored to completion over a period of 48 h.

Table 1. Scavenging Capacity of Ellagitannins and Flavanols against Stable Radicals[a]

radical	polyphenol	EC_{50} $\mu g\ \mu mol^{-1}$	EC_{50} $\mu mol\ \mu mol^{-1}$	ARP[b]	e/H[c]
TNPTM	ellagitannins				
	1	50.3 (2.6)	0.15 (0.01)	6.5 (0.3)	3.3 (0.2)
	2	51.2 (0.0)	0.26 (0.00)	3.9 (0.1)	1.9 (0.0)
	3	–[d]	–	–	–
	flavanols				
	4	–	–	–	–
	5	55.2 (6.5)	0.18 (0.02)	5.6 (0.6)	2.8 (0.3)
	6	–	–	–	–
	7	83.3 (5.9)	0.18 (0.01)	5.5 (0.3)	2.7 (0.1)
	simple phenols				
	8	21.7 (1.6)	0.17 (0.01)	5.8 (0.4)	2.9 (0.2)
	9	–	–	–	–
HNTTM	ellagitannins				
	1	38.1 (3.9)	0.04 (0.00)	28.4 (2.7)	14.2 (1.4)
	2	42.2 (5.0)	0.12 (0.02)	8.7 (1.1)	4.3 (0.5)
	3	30.4 (1.1)	0.10 (0.00)	9.9 (0.3)	5.0 (0.1)
	flavanols				
	4	54.0 (4.0)	0.19 (0.02)	5.3 (0.5)	2.7 (0.2)
	5	50.2 (2.2)	0.16 (0.01)	6.2 (0.0)	3.1 (0.1)
	6	24.0 (2.6)	0.05 (0.01)	18.5 (2.0)	9.3 (1.0)
	7	38.3 (3.2)	0.08 (0.01)	11.9 (0.9)	5.9 (0.4)
	simple phenols				
	8	19.7 (1.2)	0.16 (0.01)	6.4 (0.4)	3.2 (0.2)
	9	30.2 (2.5)	0.15 (0.01)	6.5 (0.5)	3.2 (0.3)
DPPH	ellagitannins				
	1	20.0 (1.6)	0.02 (0.00)	55.0 (3.3)	27.5 (1.7)
	2	31.2 (1.6)	0.08 (0.00)	12.2 (0.4)	6.1 (0.2)
	3	22.1 (0.2)	0.07 (0.00)	13.7 (0.3)	6.8 (0.1)
	flavanols				
	4	36.8 (1.6)	0.13 (0.01)	7.9 (0.3)	3.9 (0.2)
	5	31.5 (1.8)	0.11 (0.00)	9.1 (0.3)	4.6 (0.1)
	6	28.9 (3.1)	0.07 (0.01)	15.4 (1.6)	7.8 (0.8)
	7	31.4 (6.1)	0.06 (0.02)	17.3 (3.4)	8.7 (1.7)
	simple phenols				
	8	12.6 (1.2)	0.10 (0.01)	10.0 (0.8)	5.0 (0.4)
	9	31.7 (3.2)	0.17 (0.02)	5.8 (0.6)	2.9 (0.3)

[a]Values are means (standard deviation), $n = 3$. [b]Antiradical power ($1/EC_{50}$ ($\mu g\ \mu mol^{-1}$)). [c]Moles of reduced radical per mole of polyphenol ($1/(2 \times EC_{50})$) corresponding to the number of electrons or hydrogen atoms transferred per molecule of polyphenol. [d]EC_{50} ($\mu g\ \mu mol^{-1}$) $\geq$ 132).

The rate constants and stoichiometric factors for these reactions are given in Table 2. The reaction with pyrogallol (**8**) was faster than that with DHHDP (**2**), and the stoichiometric factors were consistent with those estimated from the concentration/activity curve and shown in Table 1, roughly corresponding to 2 and 3 electrons from DHHDP (**2**) and pyrogallol (**8**), respectively. As commented before, methylgallate (**9**) did not reduce the TNPTM.

Anodic Onset Potentials. To explain why most of the phenolic hydroxyls reacted with HNTTM and only some of them with TNPTM, the anodic onset potentials for the oxidation of DHHDP (**2**), ellagic acid (**3**), pyrogallol (**8**), and methylgallate (**9**) were measured by cyclic voltammetry in DMF solutions. The comparative results obtained at 100 mV s^{-1} are summarized in Table 3. The lower the anodic onset potential, the more reactive the phenolic hydroxyl is. Results in Table 3 show that the compounds reactive against TNPTM (**2** and **8**) possess the lowest anodic onset potentials.

Cell Viability of HT-29 Colon Adenocarcinoma Cells. The influence of polyphenols **1**–**9** on the viability of HT-29 colon cells was measured in regular DMEM and also in the presence of catalase[30] to account for artifactual results due to the formation of H_2O_2 from the superoxide radical generated in the medium by electron transfer to oxygen.[3] The results are presented in Table 4.

The active compounds were those that contained pyrogallol, hexahydroxydiphenyl, or gallate moieties (ellagitannins **1** and **2**; flavanols **5** and **7**; and simple pyrogallol **8**). Polyphenols bearing only two geminal hydroxyls (compounds **3** and **4**) were inactive. The effect on cell viability recorded for pyrogallol and structures containing pyrogallol (compounds **5** and **7**) was, at least in part, artifactual because catalase diminished or eliminated the activity. In contrast, catalase did not influence the activity of ellagitannins **1** and **3**, as well as the related compound **2**, which means that this activity was not due to extracellular hydrogen peroxide.[31]

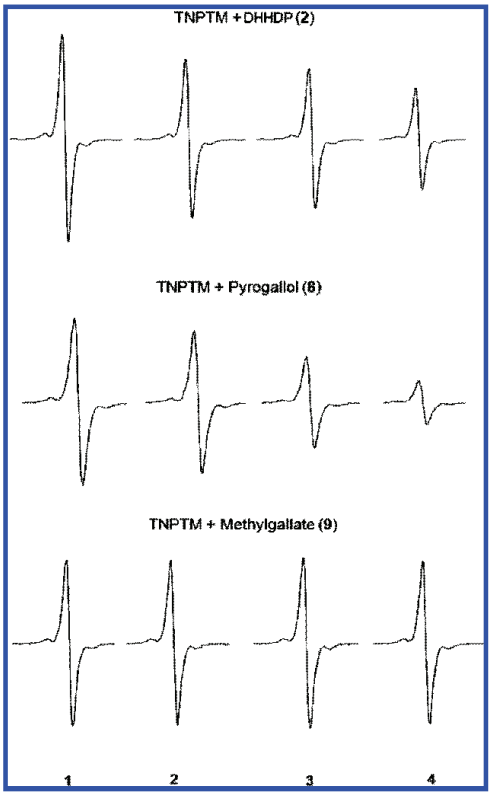

Figure 2. EPR spectra of TNPTM, initial concentration ~120 μM, upon reaction with DHHDP (**2**), pyrogallol (**8**), and methylgallate (**9**) at different initial concentrations: 0 μM (1), 5.7 μM (2), 18.1 μM (3), and 55.1 μM (4) for 48 h. Lande's factor for the TNPTM, g = 2.0026.

Table 2. Rate Constants and Stoichiometric Factors for the Reaction of TNPTM with DHHBD (2), Pyrogallol (8), and Methylgallate (9) in $CHCl_3$/MeOH (2:1)

polyphenol	TNPTM/polyphenol molar ratio[a]	k[b] (M^{-1} s^{-1})	n[c]
2	4.9–4.9	0.115 (0.010)	1.9
8	4.6–4.6	0.338 (0.070)	3.6
9	4.3–4.0	–	–

[a]Range of ratios for a number of experiments between 2 and 5. Initial concentrations around 120 and 24 μM (molar ratio, 5:1) for TNPTM and polyphenol, respectively. [b]Values are means (standard deviation), n = 2–5. [c]Moles of reduced radical per mole of polyphenol corresponding to the number of electrons transferred per molecule of polyphenol.

DISCUSSION

The biological relevance of polyphenols is still a matter of debate, even after decades of intense research. Particularly, the significant structural features behind polyphenol activities have not been satisfactorily established, probably because they interact with live systems in complex ways at different levels including redox reactions and protein–ligand interactions. Polyphenols may modify redox homeostasis by scavenging reactive radicals, by generating reactive radicals, or by a combination of the two. The electron transfer capacity of different phenolic hydroxyl groups determines the kind of effect elicited, if any. Pyrogallol (**8**) (three geminal hydroxyls) and polyphenols such as EGC (**5**) and EGCG (**7**) (gallocatechins), which contain this substructure, may be both

Table 3. Anodic Onset Potential (AOP) of Polyphenolic Moieties

polyphenol	AOP[a] (V vs SCE)
DHHDP (**2**)	0.50
ellagic acid (**3**)	0.64
pyrogallol (**8**)	0.45
methylgallate (**9**)	0.65

[a]10^{-3} M in DMF solutions with 0.1 M Bu_4NClO_4 on Pt at 100 mV s^{-1} and 25 °C.

Table 4. Viability of HT-29 Cells in the Presence of Polyphenols

	IC_{50}[a]	
polyphenol	μg mL^{-1} in DMEM	μg mL^{-1} in DMEM with catalase
ellagitannins and related compounds		
1	21.5 (3.5)	14.4 (0.4)
2	32.5 (3.9)	34.1 (0.5)
3	≥100	≥100
flavanols		
4	≥100	≥100
5	24.1 (2.7)	≥100
6	53.7 (12.0)	58.9 (8.0)
7	17.5 (3.2)	47.9 (8.0)
simple phenols		
8	5.6 (0.5)	71.4 (7.5)
9	24.6 (8.3)	31.6 (1.8)

[a]Cells were treated with the compounds for 72 h, and viability was monitored with MTT. Values are means (standard deviation), n = 2–3

scavengers and generators of free radicals and are among the most biologically active polyphenols. The gallate moiety (pyrogallol with an esterified carboxylate function) is another important structural feature. It has been widely reported that polyphenols that contain pyrogallols and/or gallates lower cell viability either by disrupting the cell cycle and triggering apoptosis or by other effects that involve redox reactions and/or protein–ligand interactions.[32−34] We focus our attention here on the redox reactions of polyphenols by using chemosensors that are able to discriminate between different phenolic hydroxyls according to their redox potentials. The results are compared with the influence on cell viability in vitro. Polyphenols **1**–**9** reacted with HNTTM, whereas only some of them (**1**, **2**, **5**, **7**, **8**) were able to reduce the TNPTM radical. This was expected for the structures containing pyrogallol (**5**, **7**, **8**)[20] and not for the ellagitanin punicalagin (**1**) because ellagic acid (**3**) was inactive against TNPTM. As expected, TNPTM did not react with catechols (two geminal hydroxyls) (**4**) or gallates (**6**, **9**). Punicalagin (**1**) contains an ellagic acid conjugated substructure and other substructures composed of gallate moieties linked by C–C bonds to each other (hexahydroxydiphenyl) or to an ellagic acid moiety. The stable radical TNPTM is reactive against these C–C-linked gallates as proven by the redox behavior of synthetic DHHDP (**2**). This dimeric gallate transferred two electrons to TNPTM, whereas methylgallate (**9**) was unreactive (Tables 1 and 2, last columns). The C–C bond appears to have activated two hydroxyl positions. Inspection of the structure of punicalagin (**1**) and the number of electrons (3.3) transferred to TNPTM (Table 1, last column) leads us to hypothesize that the C–C bond between the gallate moiety and the ellagic acid moiety produces the same hydroxyl activation that we detected for the

hexahydroxydiphenyl substructure. The formation of hydrogen bonds between hydroxyls ortho to the C−C bond may be behind the reactivity of these diphenyl structures.[35] This result was corroborated by measuring the anionic onset potential (AOP) of the gallate conjugates **2**, **3**, and **9** and pyrogallol **8**. The reactivity of polyphenols given by the AOP followed the order **8** > **2** > **3** = **9** (Table 3). These results are also in agreement with the kinetic measurements (Table 2).

The outcome of the cell viability assay cannot be related to the redox behavior of the polyphenolic structures in a straightforward way because polyphenols influence cell functions by more than one mechanism. Whatever the case, our results (Table 3) corroborate that pyrogallols and gallates are active against colon adenocarcinoma cells and suggest that the hydroxydiphenyl substructure of punicalagin may play a role involving a particularly reactive redox position. As some of the effects ascribed to pyrogallols in vitro may be due to the artifactual generation of H_2O_2 in the culture medium,[3,15] we ran the in vitro experiments in the presence of catalase. This resulted in a significant decrease in the activity of the polyphenols that contained pyrogallols in their structure. This does not alter the fact that pyrogallols are the most reactive species, because they must be able to generate the superoxide radical as the first step in the formation of H_2O_2; it just shows that the experimental setup does not adequately mimic the situation in vivo, where the extracellular oxygen concentration is much lower.[3] Punicalagin (**1**) affected cell viability as effectively as gallocatechins. In this case, the effect was not artifactual because it was not affected by the addition of catalase to the medium, which suggests that punicalagin (**1**) did not generate the superoxide radical extracellularly, at least not to a sufficient extent to affect cell viability.

By combining the outcome of HNTTM and TNPTM assays, we may generate a picture of both the total electron transfer capacity of polyphenols and the presence of highly reactive hydroxyls. TNPTM detects the most redox reactive phenolics (e.g., pyrogallols and C−C-linked gallates) and may anticipate their influence on cell viability. Independent of whether these highly reactive positions directly scavenge radicals or trigger antioxidant defense responses, TNPTM is a useful chemical probe that easily detects the presence of some of the most biologically significant phenolic structures. This will be useful when the antioxidant potential of extracts and functional foods as well as new synthetic polyphenolic molecules is examined.

In conclusion, we show here that substructures of punicalagin that contain gallate moieties, linked either to each other (hexahydroxydiphenyl moieties) or to the ellagic acid moiety by C−C bonds, present phenolic hydroxyls that are more redox reactive than those in simple gallates and that these structures can be detected by the stable radical TNPTM. The most reactive polyphenolic structures are also those that have the greatest effect on cell viability in vitro. The chemosensor TNPTM may be a useful tool for detecting other potentially beneficial highly reactive polyphenols from natural sources.

■ ASSOCIATED CONTENT

Supporting Information

IR spectrum of TNPTM; plots of scavenging activity against TNPTM, HNTTM and DPPH; kinetics of the reaction between TNPTM and HDDP/pyrogallol; plots of cell viability on HT-29 cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

DMEM, Dulbecco Modified Eagle's Medium; EGCG, epigallocatechin gallate; EGC, epigallocatechin; EC, epicatechin; ECG, epicatechin gallate; DHHDP, dimethylhexahydroxydiphenyl dicarboxylate; HHDP, hexahydroxy-2,2′-diphenyl; HNTTM, tris(2,4,6-trichloro-3,5-dinitrophenyl)methyl; TNPTM, tris(2,3,5,6-tetrachloro-4-nitrophenyl)methyl; DPPH, 1,1-diphenyl-2-picrylhydrazyl.

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1.4. Effect of pressurized hot water extraction on antioxidants from grape pomace before and after enological fermentation.

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RESUMEN

En este estudio se determinó del contenido y la eficiencia de recuperación de los principales polifenoles de uva, antocianinas, y taninos de una extracción con agua caliente a presión de orujo de uva antes y después de la fermentación. La extracción se realizó a diferentes temperaturas (50-200 °C) y tiempo (5 y 30 min).

El orujo fermentado produjo más antioxidantes totales, actividad antioxidante, y concentración de proantocianidinas, pero un menor número de antocianinas. Al elevar la temperatura de extracción aumentó la concentración de antioxidantes totales y la actividad antioxidante.

En conclusión, la extracción a presión con agua caliente de antioxidantes procedentes de orujo de uva fermentado, en la mayoría de las condiciones de extracción, permite la recuperación de una mayor cantidad de antioxidantes que del orujo de uva sin fermentar. Aunque la mayoría de las antocianinas se eliminaron durante fermentación, con temperaturas moderadas (100 °C) y tiempos de extracción cortos se recuperaron altas cantidades de antocianinas del orujo de uva fermentado. La temperatura de extracción determina el perfil de las proantocianidinas. La mayoría de las proantocianidinas detectadas por MALDI-TOF se recuperaron a 50 y 100 °C. Además, los mayores rendimientos de proantocianidinas y grado de polimerización, tanto de extractos de orujo fermentado y no fermentado, se encontraron después de extracciones de 100 °C y 5 min. Las temperaturas más altas de extracción y mayor tiempo reducen drásticamente el número de proantocianidinas detectadas.

La técnica de MALDI-TOF/TOF ha demostrado ser muy adecuada para el análisis de proantocianidinas en orujo de uva (Vergara-Salinas *et al.* 2013).

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Effect of Pressurized Hot Water Extraction on Antioxidants from Grape Pomace before and after Enological Fermentation

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ABSTRACT: Grape pomace was extracted with pressurized hot water at laboratory scale before and after fermentation to explore the effects of fermentation and extraction temperature (50−200 °C) and time (5 and 30 min) on total extracted antioxidant levels and activity and to determine the content and recovery efficiency of main grape polyphenols, anthocyanins, and tannins. Fermented pomace yielded more total antioxidants (TAs), antioxidant activity, and tannins, than unfermented pomace but fewer anthocyanins. Elevating the extraction temperature increased TA extraction and antioxidant activity. Maximum anthocyanin extraction yields were achieved at 100 °C and at 150 °C for tannins and tannin−anthocyanin adducts. Using higher temperatures and longer extraction times resulted in a sharp decrease of polyphenol extraction yield. Relevant proanthocyanidin amounts were extracted only at 50 and 100 °C. Finally, TA recovery and activity were not directly related to the main polyphenol content when performing pressurized hot water grape pomace extraction.

KEYWORDS: *pressurized hot water extraction, grape pomace, Vitis vinifera, polyphenols, thermal degradation*

INTRODUCTION

Grapes (*Vitis* spp.) are one of the largest fruit crops in the world[1] and are among the highest antioxidant-containing fruits.[2] In 2009, world grape production reached approximately 66.9 million tons, of which 71% corresponded to grapes for winemaking. Consequently, grape byproducts are produced in massive quantities, especially by the winemaking industry. Pomace, a winery byproduct that consists of skins, seeds, and stems remaining after enological fermentation, represents 20% of grapes by weight.[3] Pomace is currently used as a crop fertilizer although with limited success because of its inhibitory effect on plant seed germination due to the high content of polyphenolics.[4] However, the latter contains anthocyanins and condensed tannins (including pigmented polymers and non-pigmented proanthocyanidins), which are high valuable compounds that could significantly benefit health[5] and sensory quality of wine.[6]

Anthocyanins from grape skins are protective against diverse potentially damaging cellular oxidants through different biological mechanisms.[7] Anthocyanins are also used as natural food colorants.[8] Additionally, condensed tannins (proanthocyanidins) are one of the most abundant polyphenols in grapes.[9] Many pharmacological and therapeutic features of grape products such as antioxidant, anti-inflammatory, and antimicrobial activities, as well as cardio-, hepato-, and neuroprotective properties have been primarily attributed to grape tannins content.[10] Moreover, the antioxidant action of low molecular weight polyphenols has been recently questioned because of their low bioavailability.[11] Proanthocyanidins, which are not absorbed and remain in the gut due to their polymeric nature, may have direct effects on the stomach[12] and intestinal mucosa, protecting these tissues from oxidative stress or carcinogen action.[13] Therefore, even though most of the health benefits of wine have been attributed to polyphenols, it is not known how much of the grapes' original polyphenolic content remains in the pomace after enological fermentation. It is known, however, that fermentation favors the breakdown of the cell walls in grapes tissues.[14] Hence, significant amounts of valuable bioactive phenolic compounds could be recovered by applying a clean and effective extraction process after fermentation.

Organic solvents are commonly used to efficiently extract polyphenols from raw plant materials on a large scale.[15] These processes are not environmentally friendly, however, because it is difficult to eliminate all solvent traces from the resulting extracts. In addition, organic solvents substantially increase extraction process costs.

Water is a nonflammable, nontoxic, and readily available solvent. It is safer, cheaper, and more environmentally friendly than organic solvents for grape pomace extraction. Moreover, it is possible to manipulate water's solvent properties to optimize phytochemical extraction by changing the temperature.[16] This involves raising the water temperature to between 100 and 374 °C while applying sufficient pressure to maintain water in a liquid state (i.e., pressurized hot water). Water polarity declines dramatically with increasing temperature due to hydrogen bond dissolution and reaches values comparable to organic solvent−water mixtures.[16] The lower viscosity and surface tension of hot water also increase mass transfer rates of compounds from the

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plant tissue matrix.[16] Both temperature and pressure play significant roles in disrupting water surface equilibrium, thereby lowering the activation energy required for desorption processes.[17] This molecular behavior underscores the basis for using pressurized hot water to replace organic solvents in phytochemicals extraction processes.[16] Furthermore, pressurized hot water extraction (PHWE) is a straightforward scalable process from data gathered in small-scale equipments.[18] Despite several pressurized extraction system designs proposed by researchers[18] and specialized companies,[19] most industrial-scale units are proprietary and therefore detailed information regarding their design and operation are not available in the open literature.[18]

Several studies on PHWE of polyphenols from winery byproducts have been reported. Garcia-Marino et al.[9] studied catechin and proanthocyanidin recovery from grape seeds obtained as winery byproducts, using PHWE in a temperature range of 50−150 °C (at 102 atm) for 30 min. Using pressurized hot water, 2-fold more catechin and epicatechin was recovered versus conventional methanol extraction processes. Aliakbarian et al.[20] studied the effects of different extraction temperatures (100, 120, and 140 °C) and pressures (79, 113, and 148 atm) on total polyphenol and flavonoid recovery, and the radical scavenging capacity, of grape pomace extracts. PHWE was more efficient than a hydroalcoholic mixture at atmospheric pressure for extracting these compounds. However, in these and other similar studies,[21,22] no comparison was made of the polyphenol recovery amount and activity in extracts derived from grape pomace before versus after fermentation. Additionally, previous studies paid little or no attention to the effect of extraction time. During the PHWE of polyphenols from plant materials, diverse phenomena occur including thermal degradation, selective polyphenol extraction, and formation of neo-antioxidant compounds, all of which are highly dependent on extraction temperature and duration.[23,24] Depending on the PHWE conditions used, it is possible to obtain extracts with different chemical compositions and activities and, consequently, different bioactive properties.

In this work, we characterized the polyphenolic content of extractable grape pomace, before and after fermentation, as a preliminary step in determining the content and types of bioactive compounds remaining in this abundant byproduct. We also evaluated the impact of extraction conditions on extract antioxidant activity and on the recovery of total antioxidants (TAs), major polyphenols, anthocyanins, and condensed tannins.

MATERIALS AND METHODS

Chemicals. Reagents and standards used were analytical grade. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Folin−Ciocalteu reagent, and sodium carbonate were purchased from Merck (Germany). Tripyridyl triazine (TPTZ), $FeCl_3(6H_2O)$, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,5-dihydroxybenzoic acid, ascorbic acid, gallic acid, maleic acid−sodium dodecyl sulfate, triethanolamine, iron(III) chloride, bovine serum albumin, sodium hydroxide, hydrochloric acid (37%), glacial acetic acid, and sodium chloride were obtained from Sigma (MO, USA).

Grape Pomace. Cabernet Sauvignon pomace was obtained from Carmen Vineyard, Region Metropolitana, Chile. The prefermentation process was performed at 18 °C for 10 days, and the must was loaded into a 10 m^3 fermentation tank. Fermentation was conducted between 25 and 30 °C for 21 days without pectolytic enzymes. Two samples of the same pomace were taken at different stages of the winemaking process. The first sample was taken at the beginning of the winemaking process just after the must was introduced into the fermentation tank (unfermented pomace). The second sample was taken after the fermentation process had finished (fermented pomace). The samples were dried at ambient temperature for 2 days to reach equilibrium humidity (10% w/w) in a drying cabinet with forced ventilation. Each sample was reduced to a particle size lower than 1 mm diameter by an Oster blender (Sunbeam Products, Inc., Boca Raton, FL) and was frozen to −20 °C until extraction.

Pressurized Hot Water Extraction (PHWE). Fermented and unfermented grape pomace were subjected to PHWE. A 5 g sample (dry weight) of grape pomace was mixed with 100 g of quartz sand to completely fill the 100 mL stainless steel extraction cell and avoid filter clogging. The grape pomace was extracted in an accelerated solvent extraction device (ASE 150, Dionex) with 50 mL of distilled and filtrated (0.22 μm) water to obtain a matrix/extractant ratio of 1:10. A full factorial design with two factors was performed in triplicate at 102 atm. The factors assessed were extraction temperature (50, 100, 150, and 200 °C) and extraction time (5 and 30 min); these values were selected based on previous studies.[24] After extraction, the cell contents were rinsed with 100 mL of distilled and filtrated (0.22 μm) water and purged for 360 s by applying pressurized nitrogen (10.2 atm). Finally, the collected extracts were freeze-dried and stored in amber vials at −20 °C until analysis. Extract solutions of 1 g/L were prepared for analysis.

DPPH Radical Scavenging Activity Determination. Pomace extract antiradical capacity was determined using the DPPH radical-scavenging method.[25] First, 50 μL volumes of extract solutions at different concentrations were mixed with 2 mL of DPPH working solution (50 μM). Bleaching of DPPH was measured at 516 nm (DR 2000 Spectrophotometer; Hach Company, Loveland, CO) until the absorbance remained unchanged (∼30 min) in the dark and at room temperature. The effective pomace extract concentration needed to inhibit 50% of DPPH radical absorption (IC_{50}; mg/L) was calculated. The extract antioxidant capacity was compared with Trolox, using the Trolox equivalent antioxidant capacity (TEAC) equation: TEAC = IC_{50} Trolox/IC_{50} sample.[26] DPPH values were expressed as mg of Trolox equivalent (TE) per gram of dry mass of pomace (dp).

Ferric-Reducing Antioxidant Power (FRAP) Determination. The FRAP test offers a putative index of antioxidant reducing capacity in a sample.[27] A working solution was prepared by mixing 300 mM of acetate buffer (pH 3.6), a solution of 10 mM TPTZ in 40 mM HCl, and a freshly prepared 20 mM $FeCl_3(6H_2O)$ solution in 10:1:1 (v/v/v) proportion. For the assay, 3 mL of working reagent was mixed with 100 μL of sample or calibration standard (ascorbic acid), and absorbance was measured at 593 nm after a 30 min reaction time.[28] A calibration curve was constructed using ascorbic acid (0.1−0.8 mM). The regression coefficient of ascorbic acid was 0.9989. Results were expressed as ascorbic acid equivalent (AAE) per gram of dp.

Total Antioxidant (TA) Determination by Folin Assay. Total antioxidants were determined by Folin assay. Although this method is commonly considered for polyphenol analysis, it indeed determines all compounds in the sample with antioxidant capacity and not only polyphenols.[29] A mixture of 4.25 mL of phenolic extract (1 mg/mL) and 0.25 mL of Folin−Ciocalteu reagent were diluted 1:1 (v/v) with distilled water and mixed with 0.5 mL of a 10% sodium carbonate solution (w/v). Absorbance was measured at 765 nm after a 1 h reaction time at room temperature. A calibration curve was constructed using gallic acid as the calibration standard (20−90 mg/L). The regression coefficient of gallic acid was 0.9987. Results were expressed as gallic acid equivalent (GAE) per g of dp.

Polymeric Pigment and Tannin Assay by Harbertson−Adams. Anthocyanins, condensed tannin, and small and large polymeric pigments (SPPs and LPPs, respectively) content in grape pomace extracts were determined with the Harbertson−Adams assay adapted from the Hagerman and Butler method.[30] Results were expressed as malvidin 3-*O*-glucoside equivalents per g of dp, catechin equivalents (CEs) per g of dp, and absorbance units for anthocyanins, total tannins, and polymeric pigments, respectively.

Qualitative Proanthocyanidin Analysis by Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) Mass

Spectrometry (MS). *Sample Conditioning.* The DHB matrix (2,5-dihydroxybenzoic acid, 10 mg) and the cationizing agent (sodium chloride, 1 mg) were dissolved in 1 mL of 1% aqueous trifluoroacetic acid. First, 1 μL of this solution was mixed with 1 μL of sample solution (1 mg lyophilized pomace extract dissolved in 1 mL of 1% aqueous trifluoroacetic acid), which was then homogenized and deposited (2 μL) on a target plate. After drying at room temperature, the crystals were irradiated in the spectrometer.[31]

Analytical Conditions. A MALDI-TOF/TOF mass spectrometer (AutoFLEX III; Bruker Daltonics GmBH., Bremen, Germany) equipped with a pulsed N_2 laser (337 nm) controlled by the flexControl 1.1 software package (Bruker Daltonics) was used to obtain MS and tandem MS/MS data. The voltage was 20 kV and the reflectron voltage 21 kV. Spectra are the sum of 500 scans with a frequency of 200 Hz.[31] The positive mode was chosen in agreement with the literature for these types of compounds.[32] Proanthocyanidin molecular weights were calculated according to the following equation: $[M + Na^+] = 290.08 \times EC + 274.08 \times AFZ + 306.07 \times EGC + 152.01 \times GAL - 2.02 \times B - 4.04 \times A + 22.99$ where EC, AFZ, EGC, and GAL correspond to the number of (epi)catechin, (epi)afzelechin, (epi)gallocatechin, and galloyl moieties, respectively, and A and B correspond to the number of A and B linkages, respectively.

Statistical Analyses. Extractions and analyses were performed in triplicate with the data presented as mean value ± SD. Statgraphics Plus for Windows, version 4.0 (StatPoint Technologies, Inc., Herndon, VA) was used for statistical analyses. To study the effects of fermentation stage, extraction temperature, and extraction time on overall extraction performance, analysis of variance (factorial) and least significant difference tests were applied to the response variables with p-values $\leq$0.05 considered indicative of statistically significant differences between comparator groups.

RESULTS AND DISCUSSION

To have a common basis for comparison, results of all the analyses of the extracts are expressed in terms of dry mass of pomace (dp) before extraction.

Effect of Fermentation and Extraction Temperature and Time on Total Antioxidant Recovery and Antioxidant Activity. Grape pomace antioxidant activity measured with the FRAP assay was only affected by extraction temperature ($p < 0.001$). The FRAP value increased as the temperature increased, reaching a maximum at 150 °C for unfermented pomace (4.4 mg AAE/g dp) and 200 °C for fermented pomace (4.6 mg AAE/g dp; Figure 1a). Similar studies have shown this positive effect of temperature on the reducing/antioxidant capability of plant extracts.[33] Unexpectedly, for unfermented pomace, a slight decrease in the reducing capacity with increasing temperature from 150 to 200 °C was observed, although this effect was not observed with fermented pomace. Fermentation process degrades the pomace cell structure, increasing the release of numerous pomace-derived compounds including polysaccharides, mannoproteins, seed cuticle, and certainly polyphenols.[14] Therefore, the type and amount of antioxidants extracted could be different when using fermented versus unfermented grape pomace.

Pomace extract antiradical activity assessed with the DPPH assay was significantly affected by the three factors assessed: fermentation, extraction temperature, and extraction time (all p-values <0.001). Fermented pomace extracted at 200 °C for 5 min presented the highest value (184 mg TE/g dp). In most extraction conditions, fermented pomace showed higher antiradical activity than unfermented pomace (Figure 1b). Due to cell wall polysaccharides degradation, the extractability of phenolic compounds in the fermented pomace is enhanced,[34] resulting in extracts with higher antiradical activity. Increasing extraction temperature enhanced antiradical activity,

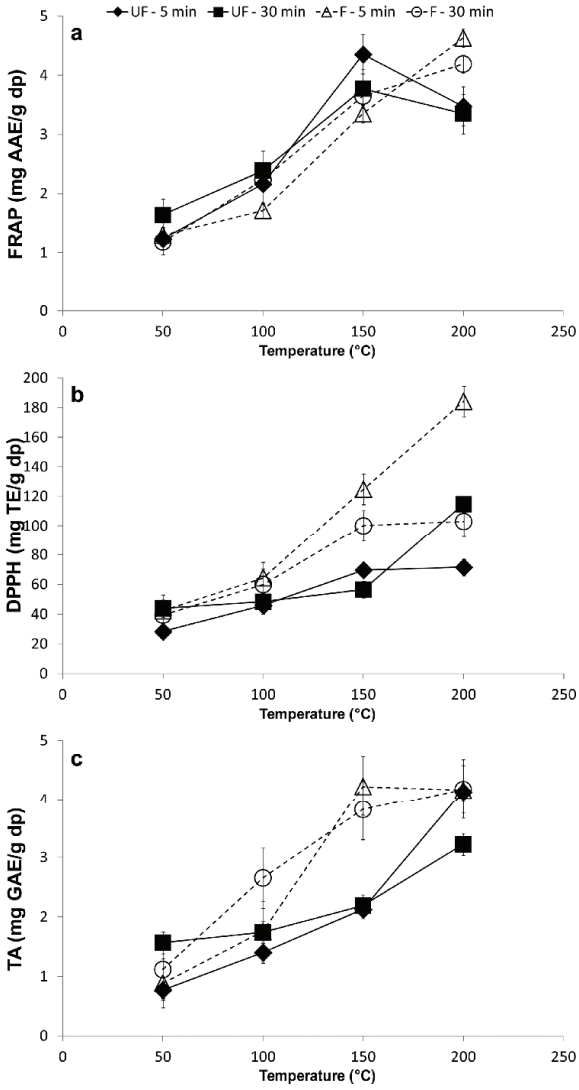

Figure 1. Effects of fermentation and extraction conditions on recovered total antioxidants and antioxidant activity in pressurized hot water extraction (PHWE) grape pomace extracts. (a) Ferric-reducing antioxidant power (FRAP) assay, (b) 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, and (c) Folin assay. Curve symbols ◆ and ■ correspond to unfermented pomace extracted for 5 and 30 min, respectively. Curve symbols △ and ○ correspond to fermented pomace extracted for 5 and 30 min, respectively. UF and F correspond to unfermented and fermented conditions, respectively. Bars represent the upper and lower limits of the 95% confidence interval.

which peaked in the range between 150 and 200 °C (Figure 1b). The temperature effect was more pronounced at temperatures above 100 °C, especially for fermented pomace. The positive effect of temperature on antioxidant PHWE from grape pomace has been reported previously.[20] At 50 and 100 °C, time has no significant effect on extract antiradical activity. At 150 °C, increased extraction time reduced extract antioxidant activity. Additionally, at 200 °C, the antiradical activity of unfermented pomace extracts increased with time while that of fermented pomace extracts decreased. This shows

that the antioxidant profiles of fermented and nonfermented pomace are different.

Total antioxidant extraction was significantly affected by fermentation and temperature (both p-values <0.001). The maximum TA extraction yield was reached at 150 °C and 5 min for fermented pomace (4.2 mg GAE/g dp) and at 200 °C and 5 min for unfermented pomace (4.1 mg GAE/g dp). In most conditions, the fermented pomace extracts had the highest TA values due to the increased release of phenolic compounds during fermentation. The higher the extraction temperature the higher the TA value, which peaked between 150 and 200 °C (Figure 1c). The positive influence of temperature on the PHWE of polyphenols from grape pomace has been reported previously.[20]

Effect of Fermentation and Extraction Temperature and Time on Anthocyanin Extraction. The anthocyanin extraction yield is affected significantly by all three factors assessed, i.e., fermentation ($p < 0.001$), extraction temperature ($p < 0.001$), and extraction time ($p = 0.001$). Figure 2a shows the effects of these factors, where the highest extraction yield was obtained for unfermented pomace extracted at 100 °C for 5 min.

In most cases, higher anthocyanin yields were achieved from unfermented pomace. Anthocyanins are water-soluble pigments in the skin of red grapes and are distributed in vacuoles that are covalently associated with pectins.[35] Anthocyanins are extracted mainly in the aqueous phase during maceration prior to fermentation and at the beginning of alcoholic fermentation.[14] Up to 77% of the anthocyanins are released in this process,[36] resulting in a residual pomace with low content of these pigments. Furthermore, within the anthocyanins family, the structural differences between these compounds results in different extractabilities.[36]

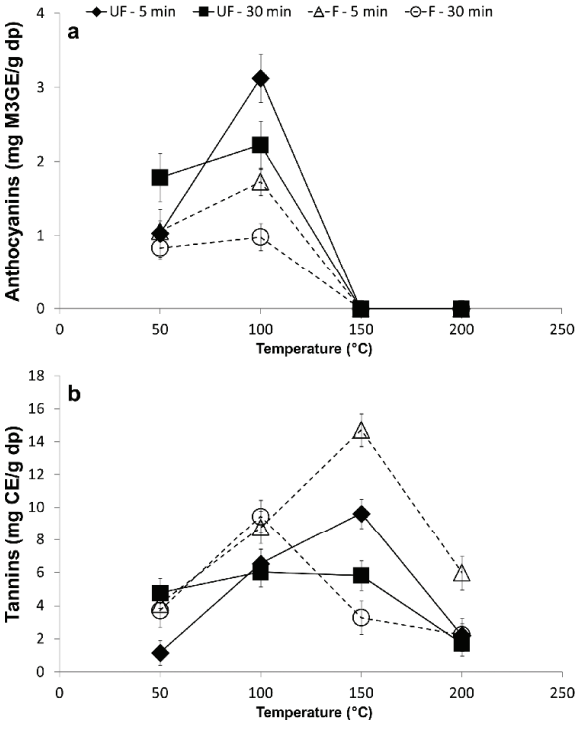

Figure 2. Effects of fermentation and extraction conditions on PHWE recovery of (a) anthocyanins and (b) condensed tannins from grape pomace. Curve symbols ◆ and ■ correspond to unfermented pomace extracted for 5 and 30 min, respectively. Curve symbols △ and ○ correspond to fermented pomace extracted for 5 and 30 min, respectively. UF and F correspond to unfermented and fermented conditions, respectively. Bars represent the upper and lower limits of the 95% confidence interval.

Regarding extraction conditions, an increase from 50 to 100 °C for 5 min increased anthocyanin extraction yield due to increased water solvation power and improved polyphenol solubility.[37] However, at 150 and 200 °C, no anthocyanins were detected in the extracts. Moreover, an increase in extraction time decreased the amount of extracted anthocyanins, which was clearly observable at 100 °C. Both temperature and exposure time have a strong influence on anthocyanin stability. Previous studies have reported that a temperature increase causes a logarithmically increased anthocyanin degradation.[38] Ju et al. also reported that PHWE temperatures above 110 °C decrease individual and total anthocyanins content in dried red grape skin extracts.[39,40] Under excessive heat, grape pomace anthocyanins degrade by opening its pyrilium ring, thereby forming a colorless chalcone equivalent which further degrades to a brown insoluble polyphenolic compound, or by cleaving its sugar moiety to form a more labile anthocyanin aglycon.[41] In our study, these color changes were observed, from red (characteristic of the wine) in the extracts obtained at 50 and 100 °C to brown at 150 and 200 °C. Moreover, the influence of exposure time at high temperatures is very important in anthocyanin degradation. Mishra et al. reported that after 25 min at 126.7 °C grape pomace anthocyanin degradation increased substantially.[41] Additionally, the formation of polymeric pigments (anthocyanins bound to tannins) increases with temperature, especially at temperatures above 100 °C, decreasing the amount of free anthocyanins (monomeric pigments).[40]

Effect of Fermentation and Extraction Temperature and Time on Tannin Extraction. The tannin extraction yield is affected significantly by all three factors assessed: fermentation and extraction temperature and time (all p-values <0.001). The effects of these factors are shown in Figure 2b, where the highest yield was obtained for fermented pomace extracted at 150 °C for 5 min, followed by the unfermented pomace at the same extraction temperature and time.

In most of the extraction conditions tested (except 150 °C and 30 min), the fermented pomace showed higher tannin yields than unfermented pomace. Only a small amount of tannins are released during fermentation, resulting in a fermented pomace with high tannin content and increased tannin extractability. Fournand et al. reported that tannin extraction efficiency from unfermented grape skins in a hydroalcoholic solution similar to wine was lower than 38%.[36] Because tannin−cell wall interactions (hydrogen bonding and hydrophobic interactions) are determined by tannin and cell wall sugar structure and content,[42] the cell wall degradation during fermentation and the PHWE operating conditions enhance tannin recovery.

Higher tannin yields were achieved at 150 °C and 5 min, while at 200 °C the extraction yield greatly decreased from both unfermented and fermented pomace. In PHWE of tannins from grape seeds, where grape tannins are most concentrated,[43] increasing the extraction temperature increases tannin extraction yield, peaking at 150 °C.[9] Likewise, Monrad et al. found

Table 1. Proanthocyanidins in Grape Pomace Extracts Obtained with Different Pressurized Hot Water Extraction (PHWE) Conditions, As Analyzed and Identified by Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry[a]

				50 °C		100 °C		150 °C	
proanthocyanidin subclass	compd	Na adduct (obsd)	Na adduct (calcd)	5 min	30 min	5 min	30 min	5 min	30 min
procyanidins	dimer (B)[48]	601.1	601.0	F		F		F	
	trimer (B)[48]	889.3	889.1	F	F	F	F		
	dimer (B-1 GE)[48]	753.2	753.0	U/F	U/F	U/F	U/F	F	F
	trimer (B-1 GE)[48]	1041.3	1041.1	U/F	U/F	U/F	U/F		
	tetramer (B-1 GE)[48]	1329.4	1329.2	U/F	U/F	U/F	U		
	pentamer (B-1 GE)[48]	1617.4	1617.2		F	F			
prodelfinidins	dimer (A-1 EGC)	615.1	615.0			U			
	dimer (A-2 EGC)	631.1	631.0			U			
	dimer (B-1 EGC)	617.1	617.0	U/F	U	U			
	trimer (B-1 EGC)[47,48]	905.3	905.1	U/F	U/F	U/F	U/F		
	tetramer (B-1 EGC)[47,48]	1193.3	1193.1	U/F	U/F	U/F	U/F		
	pentamer (B-1 EGC)[47,48]	1481.4	1481.2	U/F	U/F	U/F	U		
	hexamer (B-1 EGC)[47,48]	1769.3	1769.3		F	F			
	dimer (B-1 EGC-1 GE)	769.1	769.0	F	F	F			
	trimer (B-1 EGC-1 GE)	1057.2	1057.1	U/F	U/F	U/F			
	tetramer (B-1 EGC-1 GE)[48]	1345.2	1345.2	U/F	U/F	U/F			
	pentamer (B-1 EGC-1 GE)[48]	1633.2	1633.2	U	U/F	U/F			
	dimer (B-2 EGC)	633.1	633.0	U/F	U/F	U	U	U	

[a]obsd, observed; calcd, calculated; A, type-A bonds; B, type-B bonds; GE, galloyl ester; EGC, (epi)gallocatechin; EA, (epi)afzelechin; U, identified in unfermented pomace; F, identified in fermented pomace; U/F, identified in unfermented and fermented pomace. Superscripts indicate the reference in which this compound has been reported in grapes.

that the optimum temperature in the semicontinuous PHWE of grape pomace tannins is 140 °C.[44] To our knowledge, there are few studies about tannin stability at temperatures above 100 °C. However, it has been reported that the onset temperature of degradation of these polyphenols is approximately 150 °C and is dependent on factors such as acetylation and the amount of carbohydrates in the extract.[45]

Varying the extraction time had different effects on tannin recovery, depending on the type of pomace (fermented or unfermented) and the extraction temperature. With unfermented pomace extracted at 50 °C, increasing the extraction time from 5 to 30 min increased the tannin extraction yield. Because at low temperatures the mass transfer rate of tannins is slow, increasing the extraction time results in higher yields. However, in the case of fermented pomace extracted at 50 °C, the time increment produced no change in tannin extraction yield. Pomace degradation during fermentation facilitates the release of compounds reaching solubility equilibrium at short extraction times. In contrast, at 150 and 200 °C, increasing extraction time decreased tannin extraction efficiency for both unfermented and fermented pomace because long exposure times and high temperatures favors polyphenol degradation.[45]

Proanthocyanidin Profiles Observed by MALDI-TOF Analysis. *Compound Identification.* Tentative proanthocyanidin identification was performed by comparing the masses observed on mass spectra with the calculated mass for each compound. For identification, differences less than 0.3 Da between observed and calculated masses were considered acceptable. Proanthocyanidin results are summarized in Table 1.

The proanthocyanidins identified are mainly procyanidins and prodelphinidins with polymerization degrees up to 5 and 6, respectively. These findings are consistent with similar studies performed with positive ion reflectron mode where the polymerization degree ranged between 2 and 6.[32] It should be considered that in grape seeds, highly polymerized procyanidins are generally more abundant than oligomers.[46] The detection limits of the MS and the poor extractability of these large polymers may hamper their identification.

Grape seeds possess significant amounts of procyanidins only, while grape skins and stems also contain (epi)-gallocatechins units and therefore contain both B-type procyanidins and prodelphinidins.[47] Six procyanidins (B-type bond) were identified: a dimer and a trimer of (epi)catechin, and a dimer, trimer, tetramer, and pentamer of (epi)catechin with one gallate. These compounds have been found in grapes previously.[48] Twelve compounds were identified from the prodelphinidin family: two dimers (A-type bond) with one and two (epi)gallocatechins, one dimer (B-type bond) with two (epi)gallocatechins, oligomers from dimer to hexamer (B-type bond) with one (epi)gallocatechin, and oligomers (B-type bond) from dimer to hexamer with one (epi)gallocatechin and one gallate. Prodelphinidins trimers up to hexamers has been previously reported in grapes.[47]

Effect of Extraction Temperature and Time on Proanthocyanidins Profile. Fermentation, extraction temperature, and extraction time caused striking changes in the grape pomace extract proanthocyanidin profiles (Table 1). Procyanidin dimers and trimers were found only in fermented pomace extracts. In fermented extracts, prodelphinidins and procyanidins with one gallate present higher polymerization degrees than those found in unfermented extracts. Additionally, more procyanidins and prodelphinidins with one gallate were identified in the extracts from fermented versus unfermented grape pomace. During fermentation, lower proanthocyanidin extraction yields, especially those with a high degree of polymerization, are observed.[36] These differences in extractabilities result in fermented pomace extracts with different proanthocyanidins profile than unfermented extracts. Moreover, degradation of the cell wall during fermentation[14] facilitates extraction of a greater variety of proanthocyanidins.

Most of the proanthocyanidins detected by MALDI-TOF were recovered at 50 and 100 °C. Additionally, the highest proanthocyanidin yields and polymerization degrees, from both fermented and unfermented pomace extracts, were found after 100 °C and 5 min extractions. Higher extraction temperatures and times dramatically reduced the number of proanthocyanidins detected. At 150 °C, only a few proanthocyanidins were detected, and at 200 °C no proanthocyanidins were recovered.

Effect of Fermentation, Temperature, and Time on Polymeric Pigments. Polymeric pigments consist of anthocyanins (monomeric pigments) bound to tannins or flavan-3-ols such as catechin or epicatechin that are formed in wine after fermentation.[49] Fermentation and extraction temperature and time significantly affected recovery of SPP, LPP, and TPP (all p-values <0.001). The highest SPP extraction yield was obtained in unfermented pomace extracted at 150 °C for 30 min, whereas the highest LPP and TPP extraction yields were obtained in fermented pomace extracted at 150 °C for 5 min.

In most cases, unfermented pomace showed higher SPP values than fermented pomace, especially with 30 min extraction times (Figure 3a). In contrast, higher LPP and TPP yields were obtained with fermented pomace (Figure 3b,c). LPP and TPP showed similar extraction yield patterns because the LPP group is the largest contributor to TPP. In the Harbertson–Adams assay, LPPs represent the colored fraction of the condensed tannins, hence these two values are directly related.[49] Fermented pomace gives higher tannin extraction yields that are representative of higher LPP values.

Increasing extraction temperature increased the recovered SPP and LPP, and therefore the TPP values, peaking at 150 °C and markedly decreasing at 200 °C (except for SPP extracted for only 5 min). Increased extract polymeric color with increasing extraction temperature, especially at temperatures above 100 °C, has been reported previously.[40] The contribution of polymers to extract color indicates that extensive "degradation" of anthocyanins at high extraction temperatures occurred either by thermal degradation or polymeric pigment formation.[40] Extraction time showed no clear impact on polymeric pigment recovery from grape pomace. The highest SPP values were found at extractions times of 30 min in most conditions tested. Longer extraction times, hence, high anthocyanin-tannin reaction times, could favor the formation of SPPs at low temperatures and the breakdown of LPPs at high temperatures, increasing the SPP content in both cases. In contrast, longer extraction times have negative (especially at high temperatures) or no effect on LPP and TPP yields.

Correlation between Polyphenol Subclasses, Total Antioxidants, and Antioxidant Activity. Several different assays were statistically correlated using the Pearson correlation coefficient (Table 2). We calculated correlations after separating the data according to treatment (unfermented or fermented) and extraction time (5 or 30 min). Analysis of antioxidant activity and TA showed a strong positive correlation that was more pronounced with fermented pomace. This observation agrees with previous findings in polyphenol PHWE.[50] However, both antioxidant activity and TA showed strong negative correlations with total recovered anthocyanins, especially for fermented pomace. This is expected because anthocyanins are degraded at temperatures above 100 °C, while antioxidant activity as well as TA increased with temperature and peaked at 150 to 200 °C. At extraction times of 5 min, total tannins showed weak positive correlations with antioxidant activity and TA, while at 30 min these correlations were negative. Increased extraction time decreases tannin extraction efficiency at high temperatures due to thermal degradation,[45] while in most cases the extraction time has little or no effect on antioxidant activity and TAs.

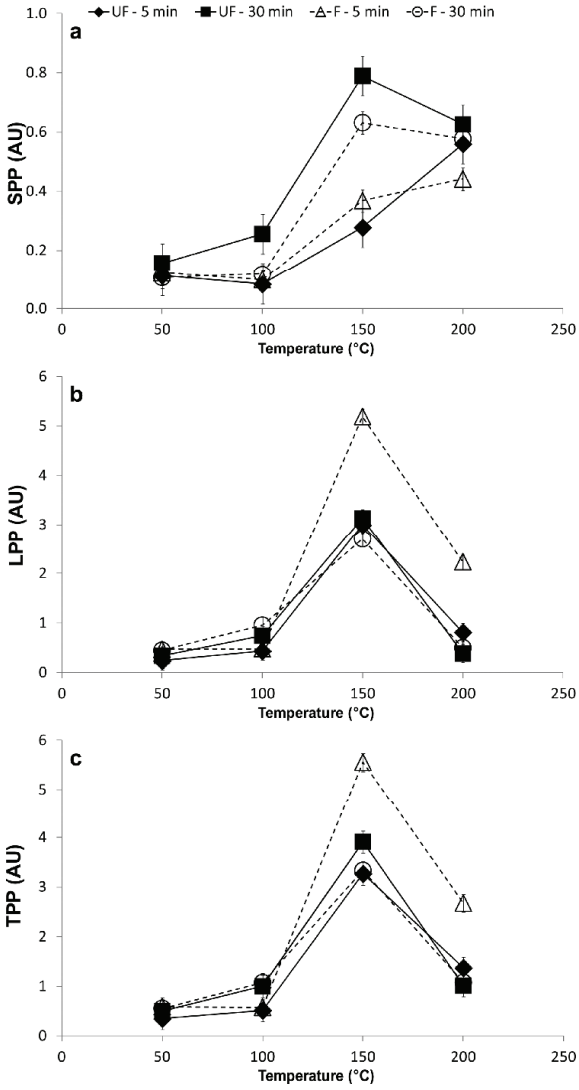

Figure 3. Effects of fermentation and extraction conditions on PWHE recovery of (a) small polymeric pigment (SPP), (b) large polymeric pigment (LPP), and (c) total polymeric pigment (TPP) from grape pomace. Curve symbols ◆ and ■ correspond to unfermented pomace extracted for 5 and 30 min, respectively. Curve symbols △ and ○ correspond to fermented pomace extracted for 5 and 30 min, respectively. UF and F correspond to unfermented and fermented conditions, respectively. Bars represent the upper and lower limits of the 95% confidence interval.

Increasing extraction temperature above 100 °C decreased polyphenol content but increased antioxidant activity and TA. It has been reported that high temperatures favor the formation of derived antioxidant compounds from polyphenols[9,39,51,52] as well as antioxidant Maillard reaction products such as melanoidins.[23]

Table 2. Pearson Product−Moment Correlation Coefficient between the Different Assays of Antioxidants Recovered during Grape Pomace PHWE[a]

	U		F	
assays	5 min	30 min	5 min	30 min
FRAP−DPPH	0.953	0.529	0.999	0.991
FRAP−folin	0.651	0.689	0.931	0.987
DPPH−folin	0.840	0.979	0.918	0.977
FRAP−anthocyanins	−0.596	−0.870	−0.849	−0.894
DPPH−anthocyanins	−0.563	−0.675	−0.821	−0.926
folin−anthocyanins	−0.557	−0.795	−0.859	−0.825
FRAP−tannins	0.637	−0.202	0.240	−0.430
DPPH−tannins	0.451	−0.919	0.213	−0.465
folin−tannins	−0.088	−0.839	0.576	−0.284

[a]Correlation coefficients were calculated with the values of all extraction temperatures separated by treatment (unfermented or fermented) and extraction time (5 or 30 min). U, unfermented pomace; F, fermented pomace; FRAP, ferric-reducing antioxidant power; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical-scavenging assay.

In conclusion, the PHWE of antioxidants from fermented grape pomace, in most of the extraction conditions tested, allows recovery of a greater amount of TAs and antioxidant activity equivalent than from unfermented pomace. In both fermented and unfermented pomace, the highest antioxidant recoveries were obtained at temperatures above 150 °C. Although the majority of anthocyanins were removed during fermentation, high amounts of anthocyanins were recovered from fermented grape pomace using moderate temperatures (100 °C) and short extraction times. Contrary to anthocyanins, high extraction temperatures (about 150 °C) and short times yielded higher amounts of tannins. Extraction temperature determined the proanthocyanidin profile, different in fermented and unfermented pomace, where the greatest amount of these compounds was recovered at lower temperatures (50 and 100 °C). Overall, we found that grape pomace antioxidant activity and TA were not directly related to the main polyphenol content in PHWE extracts. The data obtained here in a laboratory-scale equipment will be useful to develop an industrial scale PHWE processes.

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Notes

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ABBREVIATIONS USED

TA, total antioxidant; PHWE, pressurized hot water extraction; DPPH, 2,2-diphenyl-1-picrylhydrazyl; TPTZ, tripyridyl triazine; Trolox, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; IC_{50}, half-maximal inhibitory concentration; TEAC, Trolox equivalent antioxidant capacity; TE, Trolox equivalent; FRAP, ferric-reducing antioxidant power; AAE, ascorbic acid equivalent; GAE, gallic acid equivalent; TPP, total polymeric pigment; SPP, small polymeric pigment; LPP, large polymeric pigment; CE, catechin equivalent; MALDI-TOF, matrix-assisted laser desorption/ionization-time-of-flight; MS, mass spectrometry; DHB, 2,5-dihydroxybenzoic acid

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2. Comunicaciones en congresos

Relationship between antioxidant/prooxidant activity and structure of Punicalagin and Punicalagin Metabolites.

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Introduction– Ellagitannins are bioactive polyphenols with antioxidant, anti-inflammatory and potential cancer chemopreventive activities. Punicalagin and its metabolites are considered responsible for the bioactivity of Pomegranate (*Punica granatum L.*) extracts. Punicalagin is metabolized to ellagic acid and later to urolithins by gut microbiota [1]. Ellagic acid has antiproliferative properties due to its ability to directly inhibit the binding of DNA to certain carcinogens, including nitrosamines and polycyclic aromatic hydrocarbons [2, 3]. Urolithins A and B (hydroxy-6H-dibenzo[b,d]pyran-6-one derivatives) are biomarkers of human exposure to dietary ellagic acid derivatives. Urolithins may display estrogenic and/or antiestrogenic activity and urolithin A exhibits cancer chemopreventive activities in various cell and animal models [4,5,6].

We have evaluated the radical scavenging activity of punicalagin metabolites against the stable free radicals 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) (hydrogen and electron transfer), tris(3,5-dinitro-2,4,6-trichlorotriphenyl)methyl (HNTTM) (electron transfer exclusively) and tris(4-nitro-2,3,5,6-tetrachlorotriphenyl)methyl (TNPTM) (electron transfer exclusively and selective). Their antiproliferative activity (IC_{50}) has been measured in the cancer cell line HT-29. Also the reactivity of dimethyl 4,4',5,5',6,6'-hexahydroxy-2,2'-diphenoate (DMHD), a synthetic fragment of punicalagin, has been evaluated as potentially responsible moiety for the activity of the whole molecule. Interestingly, DMHD and urolithin A, inhibit cell growth with lower IC_{50} values than ellagic acid. Most probably both compounds exert their effect by different mechanisms which are under study. Catechins were used as positive controls for the artifactual antiproliferative activity of polyphenols.

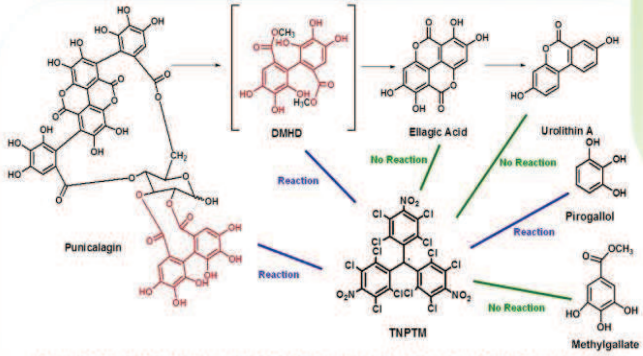


Figure 1. Punicalgin releases ellagic acid in the small intestine and urolithin A is produced by gut microbiota. Selective reaction between TNPTM radical and different punicalagin related structures.

Materials and methods- DMHD, Urolithin A and the stable free radicals HNTTM and TNPTM have been synthesized in our laboratory [7,8,9,10].
The hydrogen or electron transfer capacity of the compounds to DPPH, HNTTM and TNPTM radicals respectively, was measured in $CHCl_3$/MeOH (2:1) at different concentrations of polyphenols and an excess of radical by EPR (Electron Paramagnetic Resonance). The effect of polyphenols on the proliferation of a human cell line (HT-29) using the MTT assay was examined.

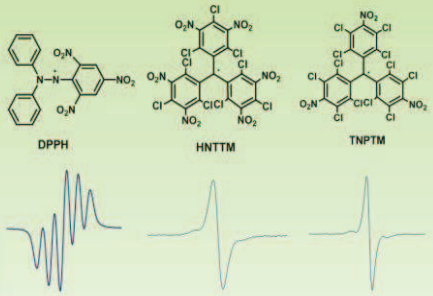
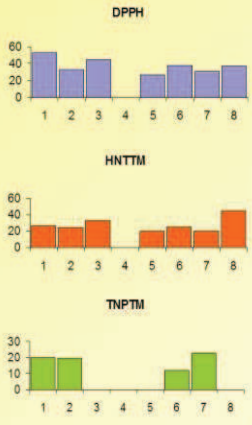


Figure 1. DPPH, HNTTM and TNPTM structures and their respective EPR spectrum.

Radical Scavenging Capacity

Table 1. ED50 µg/ml.Polyphenols react with DPPH, HNTTM and TNPTM radicals in 30 min, 7 h and 48 h, respectively in $CHCl_3$/MeOH(2:1) degassed. ED_{50} (Effective Dose in 50%), S.V (Stechiometric Value), n'H/e (Number of Hidrogens/electrons per molecule)

Radical	Entry	Antioxidant	ED_{50}	S.V	n'H/e
DPPH	1	Punicalagin	18,9	0,04	28,7
	2	DMHD	30,1	0,16	6,10
	3	Ellagic Acid	22,5	0,15	6,70
	4	Urolithin A	n.r	n.r	n.r
	5	EC	38,0	0,26	3,80
	6	EGCG	26,4	0,12	8,70
	7	EGC	32,7	0,21	4,70
	8	ECG	26,7	0,12	8,30
HNTTM	1		38,1	0,07	14,2
	2		42,2	0,23	4,30
	3		30,4	0,20	5,00
	4		n.r	n.r	n.r
	5		51,2	0,35	2,80
	6		40,6	0,18	5,70
	7		50,2	0,32	3,10
	8		22,2	0,10	9,96
TNPTM	1		50,3	0,31	3,30
	2		51,2	0,51	2,00
	3		n.r	n.r	n.r
	4		n.r	n.r	n.r
	5		n.r	n.r	n.r
	6		83,3	0,31	3,30
	7		44,2	0,51	2,90
	8		n.r	n.r	n.r



Graphic 1. Antirradical Power (ARP).

Antiproliferative Activity

Table 2. IC_{50} µg/ml. HT-29 cells were treated with the compounds for 72h, in DMEM[1] and in DMEM with Catalase[2]. Cell viability was measured by the MTT assay.

	IC_{50}[1]	IC_{50}[2]
Punicalagin	16,7	14,4
DMHD	33	34,8
Ellagic Acid	≤ 272	≤ 272
Urolithin A	9,13	10,2
EC	228,8	198
EGCG	20,0	46,0
EGC	23,9	245
ECG	52,4	50,3

Conclusions

1. DMHD reacts with TNPTM radical whereas other punicalagin related structures do not. This means that DMHD include positions with a capacity to reduce radicals higher than catechols and gallates.
2. DMHD is a particularly interesting substructure of Punicalagin with a high electron transfer capacity that may contribute greatly to the overall effect of the whole molecule.
3. Punicalagin, DMHD and Urolithin A do not produce artifactual H_2O_2 while pyrogallol and gallocatechins do.
4. Urolithin A is antiproliferative against HT-29 cells line without any antioxidant and prooxidant capacity.

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Metabolization of non-extractable proanthocyanidins, an underestimated fraction of dietary polyphenols

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Introduction

Proanthocyanidins (PA) are polymers of flavan-3-ols present in many common foods, such as berries, cocoa or certain nuts[1]. Their intake has been related to the prevention of several chronic diseases, including cancer, cardiovascular disease and diabetes[2,3].

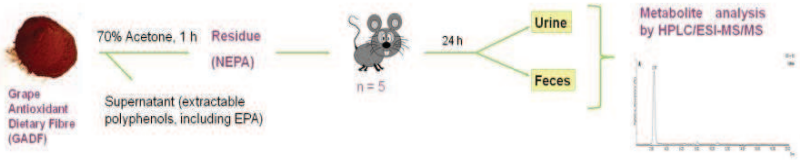
Most studies on PA metabolization assume that the PA in foodstuffs correspond exclusively to the supernatants obtained after extracting the food with 70% acetone, the most common procedure for their analysis[4]. However, these PA would actually only correspond to the extractable proanthocyanidins.

A considerable portion of PA, the non-extractable proanthocyanidins (NEPA) remain in the residue from such extractions[5]. Although few studies have suggested the bioavailability of these polyphenols, this has not yet been proven[6].

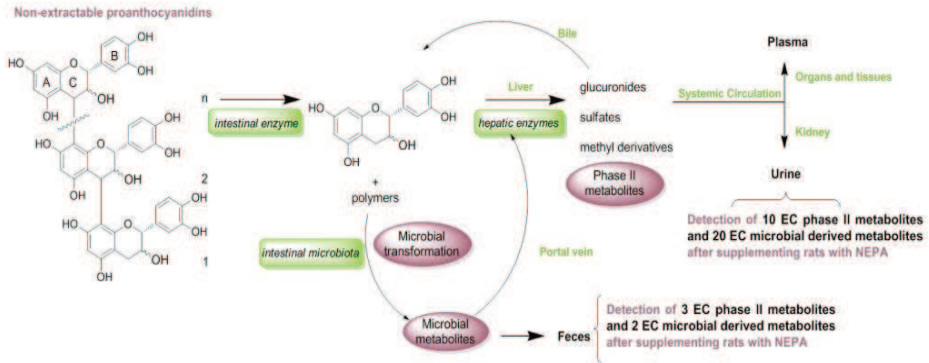
Objetives

The **aim** of this work was to evaluate the metabolic fate of NEPA in rats 24 h after ingestion of a preparation free from any extractable polyphenol, by determining the presence of PA-derived metabolites in urine and feces.

Materials and methods



Results and Conclusions



- This study shows by the first time that NEPA, a fraction of dietary polyphenols present in significant amounts in many common foods, generate phenolic species are bioavailable in rats.
- Polymeric NEPA are progressively depolymerized during their transit along the intestinal tract into (epi)catechin monomers and dimers, and later metabolized by the intestinal microbiota into smaller units.
- The metabolites detected in feces prove that active species remain in contact with the colonic epithelium for at least 24 h after ingestion.
- NEPA should be taken into account as most of the food contain significant amounts of NEPA. Further work is needed, both on the systematic analysis of NEPA in foodstuffs and on the metabolization of NEPA from different food sources, in order to unravel the contribution of this fraction of dietary PA to the health promoting effects of fruit and vegetables.

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Acknowledgements

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Polymeric proanthocyanidin profile in several food matrixes by MALDI-TOF/TOF

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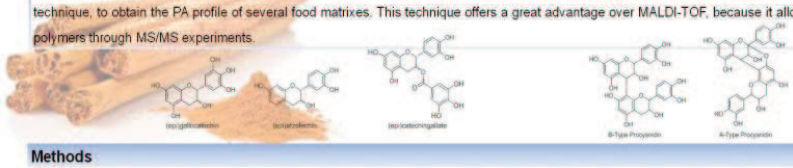
Introduction

MALDI-TOF has been widely applied to the characterization of PA polymeric species in food matrixes. In this work we use MALDI-TOF/TOF, a very powerful technique, to obtain the PA profile of several food matrixes. This technique offers a great advantage over MALDI-TOF, because it allows to confirm the identity of the polymers through MS/MS experiments.



Methods

extracted with hexane

acetone:water:acetic acid (7:2.5:0.5, v:v:v)

1 mg of the extract

Matrix

1 mL of 1% TFA

mixed 1:1, vortexed

Analysis by MALDI-TOF/TOF

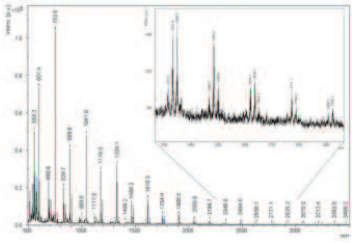
Matrix:
- DHB (10 mg)
- NaCl or Cessium trifluoroacetate (1 mg)
- 1 mL of 1% TFA

Bruker Autoflex III MALDI-TOF/TOF mass spectrometer equipped with:
- pulsed N_2 laser (337 nm)
- accelerating voltage 20 kV
- reflectron voltage 21 kV
- sum of 500 shots with a frequency of 200 Hz
- positive reflectron mode.
- MS/MS spectra were obtained in the collision-induced dissociation (CID) mode using argon as collision gas

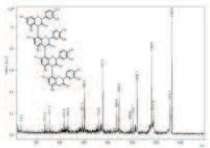
Results

Several PA polymeric series, up to 10 units, were observed in the extracts. Procyanidins and prodelphinidins were detected in the three matrixes; additionally, propelargonidins were detected in cinnamon and almond blanch water.

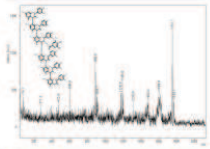
Grape extract
Mass spectrum of 500 – 3500 mass range.



Product ion mass spectra, B-type (epi)catechin tetramer with one (epi)catechin-O-gallate unit ([M+ Na]$^+$ = 1330,0)



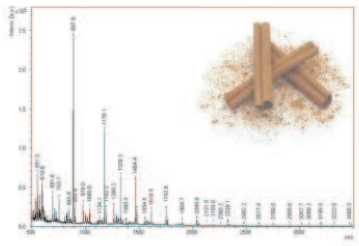
Almond blanch water

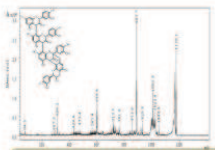


Product ion mass spectra B-type prodelphinidin hexamer ([M+ Na]$^+$ = 1770.1)

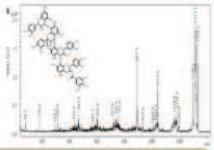
Cinnamon

Mass spectrum of 500 – 3500 mass range and product ion mass spectra, all the PA with one A-type linkage

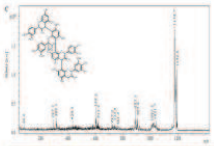




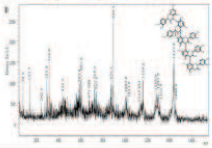
A) (Epi)catechin tetramer ([M+ Na]$^+$ = 1176.1)



B) (Epi)catechin tetramer with one (epi)catechin-*O*-gallate unit ([M+ Na]$^+$ = 1328.2)



C) (Epi)catechin tetramer with one (epi)gallocatechin unit ([M+ Na]$^+$ = 1192.0)



D) (Epi)catechin pentamer with one (epi)afzelechin unit ([M+ Na]$^+$ = 1448.4)

B-type was the most common linkage in grape extract and A-type in cinnamon, while both of them were detected in almond blanch water.

Conclusions

MALDI-TOF/TOF in CID mode is a powerful technique for the structural analysis of polyphenolic polymers. The technique combines high sensitivity for high-molecular-weight compounds with the possibility of extracting structural information via the fragmentation patterns obtained from MS/MS experiments.

The PA profile of several food matrixes as determined by MALDI-TOF/TOF is reported for the first time. The use of this technique has been of main importance for the correct identification of the series, through the fragmentation patterns offered by the MS/MS spectra. A proper characterization of the profile of these compounds may help to elucidate their health-related effects.

Particularly, in cinnamon we have found substructures never described before for this source. Apart from the most common flavanol (epi)catechin and (epi)afzelechin, cinnamon PA contain (epi)catechingallate and (epi)gallocatechin units, the presence of these substructures may explain some of the properties of cinnamon extracts.

Acknowledgements

This work was supported by the Spanish Ministry of Education and Science (AGL2009-12374-C03-03/ALI). J. P-J thanks the Spanish Ministry of Science and Innovation for granting her a *Sara Borrell* postdoctoral contract (CD09/00068). Almond blanch water was kindly provided by Borges S.A. (Reus, Spain).

Profile of urinary and fecal proanthocyanidin metabolites after intake of common cinnamon (*Cinnamomum zelanycum* L.) in rats

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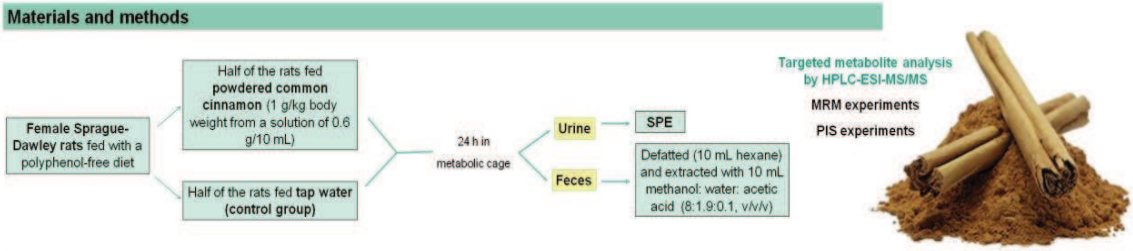
Introduction and objectives

Cinnamon (***Cinnamomum zelanycum*** **L.**) bark is widely used as a spice and in traditional medicine. The positive health effects associated with the consumption of cinnamon (prevention and treatment of type 2 diabetes, anti-inflammatory and cholesterol-lowering effects, among others) are attributed in part to its phenolic compounds, mainly proanthocyanidins (PA: oligomers and polymers of flavan-3-ols). Several studies have revealed that cinnamon PA are mainly oligomeric and polymeric, containing most of them A-type linkages[2]. In addition, a recent study[3] has revealed that cinnamon bark include oligomers and polymers that contain (epi)gallocatechin and (epi)catechin gallate units, which may also contribute to its biological activities.

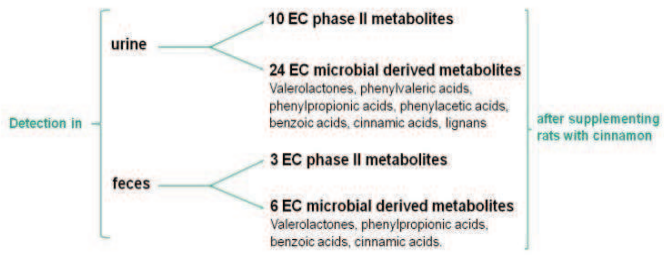
Current works on PA metabolism are focused on dimers and trimers, but studies on the metabolism of polymeric PA are scarce. Indeed, despite cinnamon is one of the most consumed spices and their attributed health effects, the bioavailability of their PA has not been studied.

The aim of the present study was to identify the urinary and fecal PA metabolites derived from cinnamon intake in rats, in order to study cinnamon bioavailability.

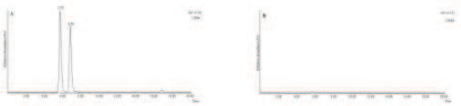
Materials and methods



Results



HPLC-ESI-MS profile corresponding to the detection by multiple reaction monitoring of 3- and 4-hydroxyphenylpropionic acid in urine (transition 165→121)



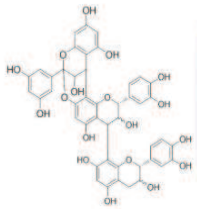
Rats fed cinnamon | Control

Table 1. (−)-Epicatechin and conjugated metabolites in urine and faeces from rats fed with whole cinnamon bark.

Metabolite	MRM	Identification	Urine	Feces
-Epicatechin	289 →245	Standard retention time	X	X
PA Dimer	577 →289	MS/MS: 577; 451; 425; 405; 289; 245		X
Mono-conjugated metabolites				
Gluc - EC-1	465 →289	MS/MS: 465; 289; 245; 163; 113	X	
Gluc- EC-2	465 →289	MS/MS: 465; 289; 245; 113	X	
Gluc- EC-3	465 →289	MS/MS: 465; 289; 245; 217; 113	X	
Sulf- EC-1	369 →289	MS/MS: 369; 289; 245; 113	X	
Sulf- EC-2	369 →289	MRM Daughter 289 →245	X	
GHS EC-1	594 →289	MRM Daughter 289 →245	X	
Di-conjugated metabolites				
Me-Gluc-EC-1	479 →303	MS/MS: 479; 303; 289; 245; 175; 137; 113	X	
Me-Gluc-EC-2	479 →303	MS/MS: 479; 303; 285; 259; 175; 137; 113	X	
di-Gluc-EC-1	641 →289	MS/MS: 641; 465; 289	X	
Tri-conjugated metabolites				
di-Me-Sulf-EC-1	397 →289	MRM Daughter 289 →245		X

Metabolites not detected in the control group or detected from signals at least 10-fold stronger

Conclusions



- A wide variety of PA metabolites, including phase II EC metabolites and microbial-derived metabolites were detected in urine and feces of rats fed cinnamon, in the first study on the bioavailability of this spice.
- Since most PA in cinnamon are type-A polymers, these results evidence the bioavailability of these compounds. Moreover, the detection of free EC and a PA dimer confirm that PA polymers are depolymerized into (epi)catechin units in the intestine and not only directly transformed into smaller phenolic acids.
- The metabolites found in urine indicate the fraction of PA that is bioavailable and that may have an effect in target tissues, while the metabolites excreted in feces are in contact with the colonic tissue and may influence gut health.

References

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Non-extractable proanthocyanidins generate bioavailable metabolites in rats- a case study with Grape Antioxidant Dietary Fibre

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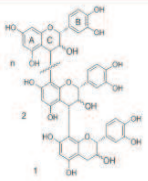
Introduction and objectives

Plant foods

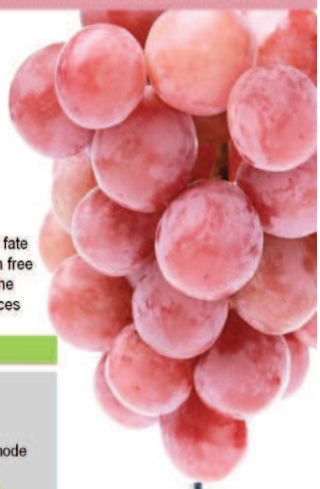
Supernatant → Extractable proanthocyanidins (EPA)
- Studies on content in foods[1] √
- Metabolization studies[2] √

70% Acetone

Residue → Non-extractable proanthocyanidins (NEPA)
- Studies on content in foods[3]
- Metabolization studies ✗



The aim of this work was to evaluate the metabolic fate of NEPA in rats 24 h after ingestion of a preparation free from any extractable polyphenols, by determining the presence of PA-derived metabolites in urine and feces



Materials and methods

NEPA obtention

Grape Antioxidant Dietary Fibre (GADF)

Methanol 50%, pH 2
Acetone 70%

Residue (NEPA)

Animal experiment

NEPA (Devoid of monomers and oligomers) — *n* = 5

Water — *n* = 5 (Intragastric gavage)

24 h in metabolic cage → Feces / Urine

Sample preparation

Urine
IS addition (taxifolin)
SPE

Feces
Defatting
Extraction
IS addition

Sample analysis

HPLC-ESI-MS/MS instrument
Negative ionisation mode

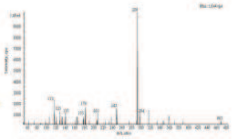
Metabolite detection
MRM experiments

Metabolite confirmation
a) Standard retention time
b) PIS experiments

Results

	Urine	Feces
(Epi)catechin (EC)	-	1
EC dimer	-	1
Phase II metabolites	10	3
Glucuronidated-EC, sulfated-EC, glutathione-EC, methylated-glucuronidated-EC, di-glucuronidated-EC, di-methylated-sulfated-EC		
Microbial derived metabolites	24	6
Valerolactones, phenylvaleric acids, phenylpropionic acids, phenylacetic acids, benzoic acids, cinnamic acids, lignans		

Number of metabolites detected in urine and feces from rats fed NEPA
(non-detected in control group)



HPLC-ESI-MS/MS product ion scan spectrum of GlcA-EC (*m/z* 465)

HPLC-ESI-MS profile corresponding to the detection by multiple reaction monitoring of 4-hydroxybenzoic acid in urine

Conclusions

- This study shows by the first time that NEPA, a fraction of dietary polyphenols present in significant amounts in many common foods, generate phenolic species that are bioavailable in rats.
- Polymeric NEPA are progressively depolymerized during their transit along the intestinal tract into (epi)catechin monomers and dimers, and later metabolized by the intestinal microbiota into smaller units.
- The metabolites detected in feces prove that active species remain in contact with the colonic epithelium for at least 24 h after ingestion.

Mateos-Martín et al. "Non-extractable proanthocyanidins from grape are a source of bioavailable (epi)catechin and derived metabolites in rats" British Journal of Nutrition, *in press*

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Study of the cytotoxic effect of tannins from Hamamelis virginiana on colorectal cancer

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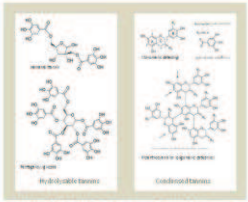
Background and Aims

Witch hazel (*Hamamelis virginiana)* bark is a rich source of condensed and hydrolysable tannins, reported to exert a protective action against colon cancer.

The aim of the present study was the characterization of different witch hazel tannins as putative selective antitumor agents in colon cancer, using a proanthocyanidin-rich fraction (F800H4) as an example of condensed tannins and pentagalloyl glucose and hamamelitannin as an example of hydrolysable tannins.



Structures of hydrolysable and condensed tannins in *Hamamelis virginiana* bark.

Matherials and Methods

To cover the structural diversity of tannins occurring in *Hamamelis* bark, the hydrolysable tannins hamamelitannin and pentagalloylglucose and a proanthocyanidin-rich fraction (F800H4) were selected for the study.

Pentagalloyl glucose and F800H4 fraction were purified from the natural source for this study, while hamamelitannin was acquired commercially. Products composition and purity were evaluated using HPLC, and the viability, cell cycle alterations, apoptosis induction and ROS scavenging activity was evaluated using the HT29 colon cancer cell line and the NCM460 colonocyte cell line.

Results

Treatment with these compounds reduced the viability and induced apoptosis, necrosis and S-phase arrest in the cell cycle of HT29 cells, being hamamelitannin the most efficient compound. Due to polyphenol-mediated H_2O_2 formation in the incubation media, the antiproliferative effect was determined in presence and absence of catalase to rule out the possible artifactual effects. The IC_{50} in presence/absence of catalase only changed significantly for F800H4.

At concentrations inhibiting HT29 cells growth by 50%, hamamelitannin had no harmful effects on NCM460 normal colonocytes whereas pentagalloylglucose similarly inhibited both cancerous and normal cells growth.

Interestingly, using the TNPTM assay, we identified a highly reactive phenolic position in hamamelitanninn which may explain its efficacy inhibiting colon cancer growth, whithout affecting normal cells growth.

Conclusion

In conclusion, our findings support that hamamelitannin is a promising chemotherapeutic agent against colon cancer.

Aknowledgements

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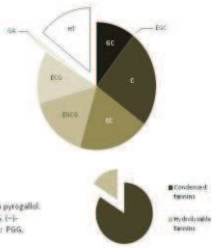
Polyphenolic composition of F800H4

Composition of the condensed tannins (CTn) 83.9%						
[1]mDP	% G	% P				
2,6	35,0	52,0				
[2]% GC	% EGC	% C	% EC	% EGCG	% ECG	
12,4	0,4	29,1	23,0	19,1	15,9	

Composition of the hydrolysable tannins (HTn) 16.1%		
[3]% GA	% HT	% PGG
10,0	90,0	0,0

[1]mDP: mean degree of polymerization; %G, percentage of gallolation; %P, percentage in pyrogallol. [2]GC, (+)-gallocatechin; EGC, (−)-epigallocatechin; C, (+)-catechin; EC, (−)-epicatechin; EGCG, (−)-epigallocatechin gallate; ECG, (−)-epicatechin gallate. [3]GA, gallic acid; HT, hamamelitannin; PGG, pentagalloylglucose.

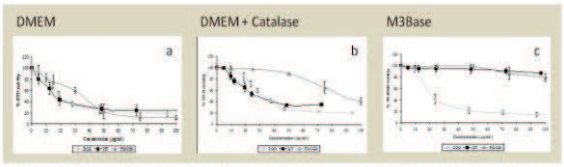


Hydrogen donation and electron transfer capacity

	DPPH			HNTTM			TNPTM		
	EC_{50}[a]	ARP[b]	H/e[c]	EC_{50}[a]	ARP[b]	e[c]	EC_{50}[a]	ARP[b]	e[c]
PGG	23.8	42.0	19.8	54.8	18.2	0.6	2403.9	0.4	0.2
HT	27.8	36.2	8.8	71.2	14.0	3.4	116.2	2.2	1.0
F800H4	39.8	25.1	27.1	66.7	15.0	16.2	1761.6	0.6	0.7

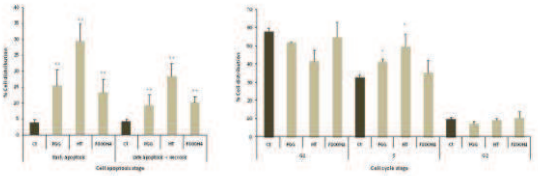
[a]EC_{50} µg of polyphenol/µmol of radical. [b]ARP: $(1/EC_{50}) \times 10^3$. [c]Number of hydrogen atoms donated or electrons transferred to the stable radical per molecule of polyphenol, calculated as the inverse of 2's molar EC_{50}.

Determination of cell viability



[a]Effect on HT29 cell viability of different concentrations of *Hamamelis virginiana* compounds in DMEM. [b]Effect on HT29 cell viability of witch hazel compounds in DMEM supplemented with catalase (100 U/mL). [c]Effect of Hamamelis products on NCM460 colonocyte growth. In all cases values are represented as mean of percentage of cell viability with respect to control cells ± standard error of three independent experiments.

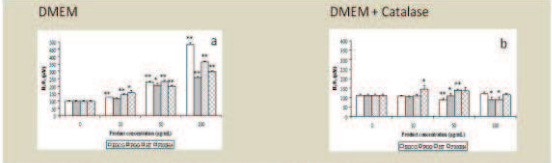
Apoptosis and cell cycle analysis by FACS



Early apoptotic cells: Annexin V+/PI− late apoptotic/necrotic cells: Annexin V+/PI+ and Annexin V−/PI+. Values are expressed as mean ± standard deviation of three separate experiments. *p < 0.05, significant difference with respect to the corresponding value in untreated cells (Ct).

Normalized percentages of cells in different cell stages are shown. Cell phases analyzed were G1, S and G2. Mean ± standard deviation of three separate experiments are shown. *p < 0.05, significant difference with respect to control cells (Ct).

H_2O_2 determination (FOX assay)



[a]H_2O_2 concentration in cell culture medium (DMEM + 10% FCS + 0.1% streptomycin/penicillin) with epigallocatechin gallate (positive control), pentagalloyl glucose, hamamelitannin and the proanthocyanidin-rich fraction F800H4 in medium. [b]H_2O_2 concentration produced in DMEM culture medium with catalase (100 U/mL) after incubation with witch hazel compounds. Mean ± standard deviation of two independent experiments. **p < 0.001 and *p < 0.05, significant difference with respect to the corresponding value in untreated cells (Ct).

VIII. BIBLIOGRAFÍA

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