Perfil metabòlic del resveratrol dietètic i influència de la matriu de l'aliment en la seva biodisponibilitat en humans. Validació de la metodologia per espectrometria de masses (UPLC-MS/MS)

Maria Rotchés Ribalta

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UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA

DEPARTAMENT DE NUTRICIÓ I BROMATOLOGIA

PERFIL METABÒLIC DEL RESVERATROL DIETÈTIC I INFLUÈNCIA DE LA MATRIU DE L'ALIMENT EN LA SEVA BIODISPONIBILITAT EN HUMANS. VALIDACIÓ DE LA METODOLOGIA PER ESPECTROMETRIA DE MASSES (UPLC-MS/MS)

MARIA ROTCHÉS RIBALTA

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VALIDACIÓ DE LA METODOLOGIA PER ESPECTROMETRIA DE MASSES (UPLC-MS/MS)

Memòria presentada per Maria Rotchés Ribalta per a optar al títol de doctor per la Universitat de Barcelona, dirigit per:

Dra. Cristina Andrés Lacueva

Dra. Mireia Urpí Sardà

MARIA ROTCHÉS RIBALTA

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Després de més de 6 anys vivint el món de recerca, crec que és interessant fer una mirada enrere al camí que he recorregut per a arribar fins aquí. I "aquí" és una memòria de gairebé 300 pàgines que, n'estic segura, no resumeix ni tan sols una quarta part de tot el què he après en aquest llarg viatge, que he tingut la sort de compartir amb persones molt i molt especials. Totes i cadascuna d'elles es mereixen un moment de gratitud per part meva, que he volgut deixar plasmat amb el petit relat d'aquesta aventura.

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ABREVIACIONS

ACN	Acetonitril
APCI	Font d'ionització química a pressió atmosfèrica
APPI	Fotoionització a pressió atmosfèrica
CE	Consell Europeu
CID	Dissociació induïda en una cel·la de col·lisió
DAD	Detector de *diode array*
DHR	Dihidroresveratrol
ESI	Font d'ionització per electrospray
FDA	*American Food and Drug Administration*
GC	Cromatografia de gasos
HCl	Àcid clorhídric
HLB	Balanç hidrofílic-lipofílic
HPLC	Cromatografia líquida d'alta eficàcia
IMC	Índex de massa corporal
LC	Cromatografia líquida
LC-MS/MS	Cromatografia líquida acoblada a un espectròmetre de masses en tàndem
LDL	Lipoproteïnes de baixa densitat
LLE	Extracció líquid-líquid
MAX	Mode mixt aniònic
MCX	Mode mixt catiònic
MRM	*Multiple Reaction Monitoring*
MS	Espectrometria de masses
MS/MS	Espectrometria de masses en tàndem

m/z	Relació massa/càrrega
PIS	*Product Ion Scan*
PPT	Precipitació de proteïnes
Q1	Primer quadrupol
Q3	Tercer quadrupol
RV	Resveratrol
SIM	*Selected Ion Monitoring*
SPE	Extracció en fase sòlida
TCA	Àcid tricloracètic
TFA	Àcid trifluoracètic
ToF	Temps de vol
UPLC	Cromatografia líquida treballant a ultra pressió
UV/vis	Detectors de llum ultraviolada/visible
VIH	Virus de la immunodeficiència adquirida
WAX	Intercanvi aniònic dèbil
WCX	Intercanvi catiònic dèbil

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INTERÈS I OBJECTIUS

INTERÈS

El resveratrol és un polifenol representatiu del grup dels estilbens, juntament amb el seu glucòsid, el piceid. En els països occidentals es consumeix principalment a partir de raïm o productes derivats, com ara el most o el vi (Zamora-Ros *et al*., 2008). L'interès del resveratrol va sorgir dels estudis on es va descriure la "paradoxa francesa", en la qual el consum moderat de vi negre es va relacionar amb una reducció del risc cardiovascular i de la mortalitat total (Renaud i de Lorgeril, 1992). Nombrosos treballs han relacionat el consum moderat d'alcohol amb un efecte cardioprotector (Ronksley *et al*., 2011), però sembla ser que la composició polifenòlica del vi aporta certs beneficis addicionals, com una millora de l'activitat enzimàtica antioxidant o una reducció de la pressió arterial (Noguer *et al*., 2012; Chiva-Blanch *et al*., 2012b). De fet, varis estudis han demostrat ja la capacitat del resveratrol per a protegir contra diverses malalties, com el càncer (Kundu i Surh, 2008), les afeccions cardiovasculars (Gresele *et al*., 2011) o neurodegeneratives (Li *et al*., 2012). Més recentment, la capacitat del resveratrol per a mimetitzar els efectes de la restricció calòrica (Wood *et al*., 2004) l'ha relacionat amb un increment de l'esperança de vida o longevitat (Baur i Sinclair, 2006), que només s'ha demostrat en models animals (Baur *et al*., 2006; Howitz *et al*., 2003). Tot i aquests resultats prometedors, les conclusions presentades en el recent "*2nd International Scientific Conference on Resveratrol and Health*", celebrat a Leicester (Regne Unit) del 5 al 7 de desembre de 2012, deixen clar que no hi ha suficient evidència científica per a parlar d'efectes positius en humans. Ara bé, els resultats que s'estan obtenint dels estudis en animals són esperançadors (http://www.resveratrol2012.eu/). Així doncs, el comitè organitzador del congrés recomana l'execució de més estudis sobre els efectes beneficiosos del resveratrol en humans i, sobretot, a llarg termini (més de 6 mesos).

L'interès creixent de la població general per la "medicina natural" ha incrementat la presència en els mercats d'aliments funcionals o nutracèutics, als quals se'ls ha atribuït, directa o indirectament, una funció fisiològica beneficiosa per a la salut humana (Espin *et al*., 2007). El concepte d'aliment funcional defineix qualsevol aliment o ingredient d'aquest que, consumit regularment, ha demostrat científicament que pot proporcionar un efecte beneficiós més enllà de les seves propietats nutricionals, ja sigui en la millora de la salut o en la reducció del risc a emmalaltir (*Functional food science in Europe*, 1998). Els nutracèutics, en canvi, són suplements dietètics que, amb el mateix propòsit, proporcionen una forma concentrada d'un agent bioactiu, que es pot trobar de forma natural en un aliment, present en una matriu no alimentària i en una dosi que excedeix a les que s'aconsegueixen a partir dels aliments normals (Zeisel, 1999). L'interès creixent sobre el consum d'aquest tipus de productes ha suscitat la necessitat d'una regulació legal sobre la comercialització d'aquest tipus de productes. La normativa europea (CE) nº1924/2006 del Parlament Europeu i del Consell de 20 de desembre de 2006, relatiu a les declaracions nutricionals i de propietats saludables en els aliments, indica la regulació per a l'etiquetat i la publicitat dels productes comercialitzats que presenten declaracions nutricionals i de propietats saludables. En aquest document es destaca la necessitat d'un suport científic que recolzi, amb la màxima evidència, les al·legacions que es declaren. A part d'això, una de les condicions generals (Article 5 de la regulació CE nº1924/2006) per a l'autorització d'una declaració d'aquestes característiques és assegurar que el compost actiu sigui disponible en quantitats suficients per a produir l'efecte nutricional o fisiològic declarat en els òrgans i teixits, després del consum en dosis raonables. Aquest aspecte és el que es coneix com a biodisponibilitat que, juntament amb la ingesta dietètica d'un compost, són factors determinants de la bioactivitat d'un compost.

Actualment, nombrosos estudis es focalitzen en la biodisponibilitat del resveratrol, dels quals se'n desprèn la idea de que, malgrat la seva elevada absorció, la seva biodisponibilitat és baixa, ja que es metabolitza ràpidament (Walle, 2011). Això ha generat certa controvèrsia en contra de les propietats beneficioses del resveratrol, però també és cert que és necessària una millor comprensió de l'hipotètic paper dels seus metabòlits en l'activitat biològica d'aquest. De fet, una de les recomanacions de

planteja el comitè organitzador del "*2nd International Scientific Conference on Resveratrol and Health*" (http://www.resveratrol2012.eu/) per a les futures investigacions sobre les propietats beneficioses d'aquest estilbè és l'estudi dels possibles efectes biològics dels metabòlits del resveratrol, sobretot tenint en compte el paper de la microbiota intestinal. Però abans, es requereix informació més concreta sobre la biodisponibilitat del resveratrol i, sobretot, del seu metabolisme després del consum de quantitats moderades d'aliments que continguin aquesta molècula. I és que la majoria d'estudis sobre la biodisponibilitat i farmacocinètica del resveratrol es realitzen administrant elevades dosis del compost pur (Patel *et al*., 2011). Els resultats que deriven d'aquests treballs, no podran ser traslladats als necessaris per a legalitzar una declaració nutricional per als aliments que continguin una font dietètica de resveratrol. Així doncs, calen més estudis enfocats al consum regular d'aquest tipus d'aliments i destinats a la població en general, ja que la majoria de treballs en humans seleccionen només homes, donat que l'efecte estrogènic del resveratrol (Gehm *et al*., 1997) podria condicionar els resultats obtinguts en dones. Un dels factors a tenir en compte en l'estudi de la biodisponibilitat d'aquest compost és l'efecte de la matriu alimentària que el conté. Alguns estudis, per exemple, havien hipotetitzat que l'alcohol del vi podria millorar la biodisponibilitat de compostos fenòlics, augmentant-ne la seva solubilitat (Manach *et al*., 2004). Cal tenir en compte, però, que el vi no podrà presentar declaracions de propietats saludables, per ser un producte amb una graduació d'alcohol superior al 1,2% en volum (Regulació CE nº1924/2006). Per això, recentment, s'han comercialitzat vins desalcoholitzats o extractes de raïm, en els quals podrà figurar una al·legació nutricional, si s'aconsegueix una evidència científica suficient per a legalitzar-lo. No es pot obviar la creixent presència en els mercats dels productes nutracèutics, a partir dels quals s'han dut a terme alguns estudis de biodisponibilitat (Patel *et al*., 2011). Tot i això, la matriu alimentària dels diferents productes que es comercialitzen actualment, podria influenciar la biodisponibilitat dels compostos actius que contenen. Per tant, cal més informació al respecte i, sobretot, en comparació amb els diferents tipus de productes que es troben en el mercat.

HIPÒTESI

La biodisponibilitat del resveratrol dietètic i el seu perfil metabòlic, tant de fase II com l'obtingut per acció de la microbiota intestinal, es veuran afectats per la matriu alimentaria.

OBJECTIUS

L'objectiu principal d'aquesta Tesi Doctoral és conèixer el perfil metabòlic del resveratrol administrat en dosis dietètiques en humans i la influència de la matriu de l'aliment en la seva biodisponibilitat, desenvolupant una metodologia per cromatografia líquida acoblada a espectrometria de masses (UPLC-MS/MS).

En concret, es plantegen els següents objectius:

- Desenvolupar i validar una metodologia òptima per a l'anàlisi del perfil metabòlic del resveratrol per UPLC-MS/MS.
- Identificar i quantificar el perfil metabòlic del resveratrol després del consum moderat de vi i de productes funcionals o nutracèutics.
- Avaluar el comportament farmacocinètic del resveratrol després del consum moderat de vi negre i d'un nutracèutic a base d'extracte de raïm.
- Avaluar el possible efecte de la matriu alimentaria d'un producte nutracèutic a base d'extracte de raïm en front a la del vi negre.
- Definir el possible efecte de l'alcohol del vi en el perfil metabòlic del resveratrol.

ANTECEDENTS BIBLIOGRÀFICS

ANTECEDENTS BIBLIOGRÀFICS

Els antecedents bibliogràfics que es presenten a continuació com a part introductòria de la Tesi Doctoral permeten situar la present en el context científic, abans d'abordar el desenvolupament dels objectius esmentats. Aquests antecedents s'exposen, en part, com a publicacions dutes a terme en el decurs de la Tesi Doctoral: dos capítols de llibre i una revisió bibliogràfica publicada en una revista científica. Poder presentar la part introductòria en aquest format ha esdevingut un repte professional, pel que representa la preparació de la recerca bibliogràfica en format científic i la seva posterior avaluació per un equip editorial internacional.

El primer capítol de llibre representa una introducció sobre els compostos fenòlics, entre els quals es troba el resveratrol. La oportunitat de participar en l'elaboració d'aquest capítol en els inicis de l'etapa predoctoral em va permetre conèixer els polifenols. La meva participació concreta va consistir en dur a terme la recerca bibliogràfica de la química i la classificació d'aquests polifenols (apartat A). A més a més, i donats els objectius sobre els quals es plantejava la Tesi, vaig focalitzar-me en la redacció de l'apartat corresponent a les fonts dietètiques dels estilbens (part de l'apartat C):

> Cristina Andres-Lacueva, Alexander Medina-Remón, Rafael Llorach, Mireia Urpi-Sarda, Nasiruddin Khan, Gemma Chiva-Blanch, Raul Zamora-Ros, Maria Rotchés-Ribalta, Rosa Mª Lamuela-Raventos. *"Phenolic compounds. Chemistry and occurrence in fruits and vegetables",* capítol 2 del llibre *"Fruit and vegetable phytochemicals: Chemistry, nutritional value and stability"* (*Blackwell Publishing,* 2009 – ISBN: 978-0-8138-0320-3).

El segon apartat, que s'exposa com a revisió bibliogràfica, presenta l'interès i la controvèrsia que ha generat aquesta molècula envers a la prevenció de certes

malalties. Amb els objectius que es plantejaven per a la meva Tesi Doctoral, la meva contribució va residir en els estudis que s'havien dut a terme fins al moment sobre la biodisponibilitat del resveratrol, juntament amb l'elaboració de la publicació.

Rafael Llorach, Mireia Urpi-Sarda, Maria Rotchés-Ribalta, Montse Rabassa, Cristina Andres-Lacueva. *"Resveratrol: From dietary intake to promising therapeutic molecule". AgroFood Industry Hi-Tech;* 2010, 21(2): 42-44.

Gràcies a l'experiència adquirida en aquests capítols anteriors, vaig liderar l'elaboració del següent capítol i, conseqüentment en vaig ser la primera autora i la responsable. Aquest capítol presenta els antecedents bibliogràfics sobre l'estudi de la biodisponibilitat i el metabolisme del resveratrol, fent un especial èmfasi en les fonts alimentàries del resveratrol i els estudis previs que tracten l'efecte de la matriu alimentaria en la biodisponibilitat del resveratrol. La recopilació de tota aquesta informació va ésser essencial per al desenvolupament de les publicacions de resultats que constitueixen aquesta Tesi Doctoral. A part de la recerca bibliogràfica vaig elaborar el manuscrit, que va ser aprovat per la resta d'autors.

Maria Rotches-Ribalta, Mireia Urpi-Sarda, Sara Tulipani, Pilar Trespalacios, Olha Khymenets, Montse Rabassa, María Boto-Ordóñez, Rosa Vazquez-Fresno, Mar Garcia-Aloy, Rafael Llorach, Cristina Andres-Lacueva. *"Sources and Food Matrix on the Bioavailability of Resveratrol",* capítol 20 del llibre *"Resveratrol: Sources, Production and Health Benefits"* (*Nova Science Publishers,* 2012 – ISBN: 978-1-62257-072-0).

Finalment, i per acabar la part dels antecedents bibliogràfics de la Tesi Doctoral, s'exposen les diverses tècniques analítiques utilitzades per a l'anàlisi del perfil metabòlic del resveratrol en mostres biològiques, que s'han recollit de la bibliografia en el decurs de l'elaboració d'aquesta memòria.

ELS COMPOSTOS FENÒLICS

QUÍMICA I DISTRIBUCIÓ DELS POLIFENOLS EN ELS ALIMENTS

Els polifenols són metabòlits secundaris de les plantes que generalment s'han associat a la defensa contra la radiació ultraviolada o l'agressió per patògens. Químicament es caracteritzen per un anell aromàtic substituït almenys per un grup hidroxil, que dóna lloc a diferents famílies de compostos amb estructures molt diferents. Principalment, els polifenols es classifiquen en flavonoids (d'estructura C_6-C_3-C_6) i no-flavonoids. Els estilbens formen part del grup de no flavonoids, essent el resveratrol el compost més àmpliament conegut, juntament amb els seu glucòsid, el piceid.

Són nombrosos els mètodes d'identificació i quantificació dels diferents polifenols descrits en la bibliografia. Abans de l'anàlisi pròpiament dit és important dur a terme un tractament de la mostra adequat, que sovint es basa en la extracció de l'analit de la matriu de la mostra. Els mètodes analítics es basen en una separació cromatogràfica seguida de mètodes de detecció, com l'espectrometria de masses.

En general, les fonts dietètiques principals dels polifenols son les fruites i els vegetals, però es pot especificar que les fruites amb major concentració de polifenols són les maduixes, els litxis i el raïm mentre que en les verdures es destaquen les carxofes, el julivert i les cols de Brussel·les. De manera més concreta el capítol exposa les principals fruites i verdures en les que es poden trobar els diferents tipus de polifenols. Pel què fa als estilbens, cal dir que es troben en baixes concentracions en els aliments. El resveratrol i el piceid es troben majoritàriament en productes derivats del raïm, com és el vi.

Tota aquesta informació referent als compostos fenòlics es presenta a continuació sota el títol "*Phenolic compounds. Chemistry and occurrence in fruits and vegetables*",

capítol 2 del llibre "*Fruit and vegetable phytochemicals: Chemistry, nutritional value and stability*" (Blackwell Publishing, 2009 – ISBN: 978-0-8138-0320-3).

Phenolic compounds
Chemistry and occurrence in fruits and vegetables

Cristina Andres-Lacueva*, Alex Medina-Remon, Rafael Llorach, Mireia Urpi-Sarda, Nasiruddin Khan, Gemma Chiva, Raul Zamora-Ros, Maria Rotchés-Ribalta, Rosa M. Lamuela-Raventos.

A. CHEMISTRY AND CLASSIFICATION OF POLYPHENOLS

Polyphenols are the most abundant antioxidants in human diets. They are secondary metabolites of plants. These compounds are designed with an aromatic ring carrying one or more hydroxyl moieties. Several classes can be considered according to the number of phenol rings and to the structural elements that bind these rings.

In this context, two main groups of polyphenols, termed flavonoids and non-flavonoids, have been traditionally adopted. As seen in Figure 1, the flavonoids group comprises the compounds with a C6-C3-C6 structure: flavanones, flavones, dihydroflavonols, flavonols, flavan-3-ols, anthocyanidins, isoflavones and proanthocyanins. The non-flavonoids group is classified according to the number of carbons that they have (Figure 2) and comprises the following subgroups: simple phenols, benzoic acids, hydrolysable tannins, acetophenones and phenylacetic acids, cinnamic acids, coumarins, benzophenones, xanthones, stilbenes, chalcones, lignans and secoiridoids.

Flavonoids

Flavonoids have a skeleton of diphenylpropanes, two benzene rings (A and B) connected by a three-carbon chain forming a closed pyran ring with the benzene A ring (Figure 1).

Flavonoids in plants usually occur as glycosylated mainly with glucose or rhamnose but they can also be linked with galactose, arabinose, xylose, glucuronic acid or other sugars. The number of glycosil moieties usually varies from one to three, nevertheless flavonoids have been identified with four and also with five moieties (Vallejo and others 2004).

Flavonols and *flavones* have a double bond between C2 and C3 in the flavonoid structure and an oxygen atom at the C4 position. Furthermore, flavonols also have a hydroxyl group at the C3 position. *Dihydroflavonols* have the same structure as flavonols without the double bond between C2 and C3.

Flavanones are represented by the saturated three-carbon chain and an oxygen atom in the C4 position.

Isoflavones also have a diphenylpropane structure in which the B ring is located in the C3 position. They have structural analogies to oestrogens, such as estradiol, with hydroxyl groups at the C7 and C4 positions (Shier and others 2001).

Anthocyanins are based on the flavylium salt structure and are water soluble pigments in plants. They are generally found in the form of glycosides in plants and foods of their respective aglycones, called anthocyanidins. The most common sugars encountered are glucose, galactose, rhamnose, xylose, arabinose and fructose and are linked mainly in the C3 position as glycosides and in C3, C5 as diglycosides. Glycosylation at the C7, C3' and C5' positions has also been observed (Clifford 2000).

Flavan-3-ols or *flavanols* have a saturated three-carbon chain with a hydroxyl group in the C3 position. In foods they are present as monomers or as *proanthocyanidins* which are polymeric flavanols (4 to 11 units) known also as condensed tannins. In foods they are never glycosylated.

Non flavonoids

Simple phenols (C6), the simplest group, are formed with an aromatic ring substituted by an alcohol in one or more positions as they may have some substituent groups, like alcoholic chains, in their structure. P*henolic acids* (C6-C1) with the same structure as simple phenols have a carboxylic group linked to benzene. *Hydrolyzable tannins* are

mainly glucose esters of gallic acid. Two types are known: the gallotannins, which yield only gallic acid upon hydrolysis, and the ellagitannins, which produce ellagic acid as the common degradation product.

Acetophenones are aromatic ketones, and *phenylacetic acids* have a chain of acetic acid linked to benzene. Both have a C6-C2 structure.

Hydroxycinnamic acids are included in the phenylpropanoid group (C6-C3). They are formed with an aromatic ring and a three-carbon chain. There are four basic structures: the coumaric acids, caffeic acids, ferulic acids and sinapic acids. In nature, they are usually associated with other compounds such as chlorogenic acid, which is the link between caffeic acid and quinic acid.

Coumarins belong to a group of compounds known as the benzopyrones, all of which consist of a benzene ring joined to a pyrone. They may also be found in nature, in combination with sugars, as glycosides. They can be categorized as simple furanocoumarins, pyranocoumarins and coumarins substituted in the pyrone ring (Murray and others 1982).

Benzophenones and *xanthones* have the C6-C1-C6 structure. The basic structure of benzophenone is a diphenyl ketone, and that of xanthone is a 10-oxy-10H-9-oxaanthracene. Over 500 xanthones are currently known to exist in nature and approximately 50 of them are found in the mangosteen with prenyl substituent.

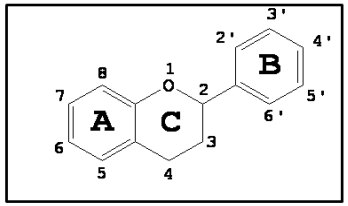
Stilbenes have a 1,2-diphenyl-ethylene as their basic structure (C6-C2-C6). Resveratrol, the most known compound, contains three hydroxyl groups in the basic structure and is called 3,4',5-trihydroxystilbene. In plants, piceid, the glucoside of resveratrol, is the major derivative of resveratrol. Stilbenes are present in plants as *cis* or *trans* isomers. T*rans* forms can be isomerized to *cis* forms by UV radiations (Lamuela-Raventós and others 1995).

Chalcones with a C6-C3-C6 structure are flavonoids lacking a heterocyclic C-ring. Generally, plants do not accumulate chalcones. After its formation, naringenin chalcone is rapidly isomerized by the enzyme chalcone isomerasa to form the flavanone, naringenin. The most common chalcones found in foods are phloretin and its 2'-*O*-glucoside, chalconaringenin and arbutin.

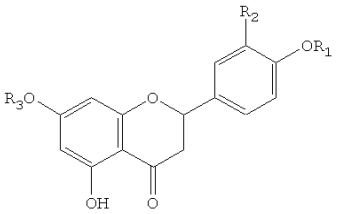
Lignans are compounds derived from two β–β'-linked phenylpropanoid (C6-C3) units and are widely distributed in the plant kingdom. They are classified into eight subgroups: furofuran, furan, dibenzylbutane, dibenzylbutyrolactone, aryltetralin, arylnaphthalene, dibenzocyclooctadiene, and dibenzylbutyrolactol. These subgroups are based upon the way in which oxygen is incorporated into the skeleton and the cyclization pattern. Furthermore, they vary largely in the oxidation levels of both the aromatic rings and the propyl side chains.

Secoiridoids are complex phenols produced from the secondary metabolism of terpenes as precursors of several indole alkaloids (Soler-Rivas and others 2000). They are characterized by the presence of elenolic acid, in its glucosidic or aglyconic form, in their molecular structure. Oleuropein, the most known secoiridoid, is a heterosidic ester of elenolic acid and 3,4- dihydroxyphenylethanol containing a molecule of glucose, the hydrolysis of which yields elenolic acid and hydroxytyrosol (Soler-Rivas and others 2000).

FLAVONOIDS

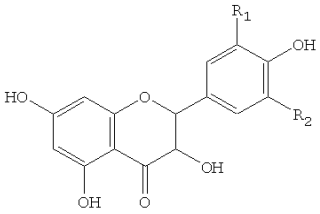


Flavanones



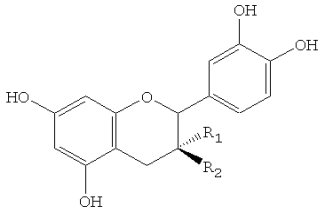
Naringenin: R_1=H, R_2=H, R_3=H
Hesperetin: R_1=CH_3, R_2=OH, R_3=H

Dihydroflavonols



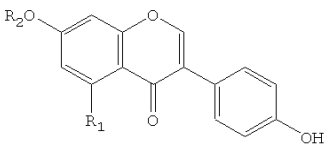
Dihydrokaempferol: R_1=H, R_2=H
Dihydroquercetin: R_1=OH, R_2=H
Dihydromyricetin: R_1=OH, R_2=OH

Flavan-3-ols



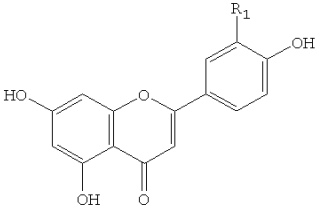
(-)Epicatechin: R_1=OH, R_2=H
(+)Catechin: R_1=H, R_2=OH

Isoflavones



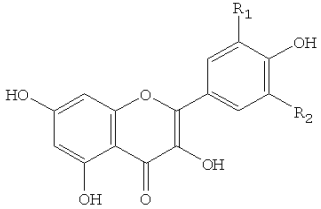
Daidzin: R_1=H, R_2=Glucoside
Daidzein: R_1=H, R_2=H
Genistin: R_1=OH, R_2=Glucoside
Genistein: R_1=OH, R_2=H

Flavones



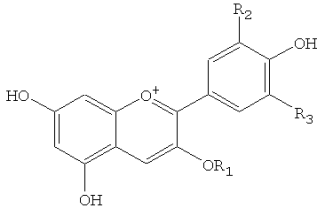
Apigenin: R_1=H
Luteolin: R_1=OH

Flavonols



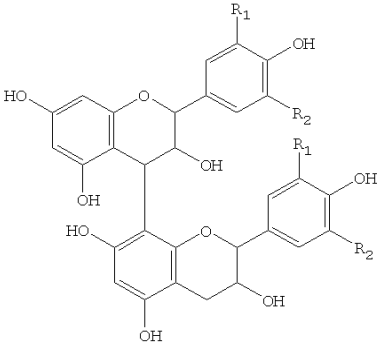
Kaempferol: R_1=H, R_2=H
Quercetin: R_1=OH, R_2=H
Myricetin: R_1=OH, R_2=OH

Anthocyanidins



Cyanidin: R_1=H, R_2=OH, R_3=H
Pelargonidin: R_1=H, R_2=H, R_3=H
Peonidin: R_1=H, R_2=H, R_3=OCH_3

Proanthocyanins



B-type procyanidin dimer:
R_1=OH, R_2=H

Figure 1. Chemical structures of flavonoids

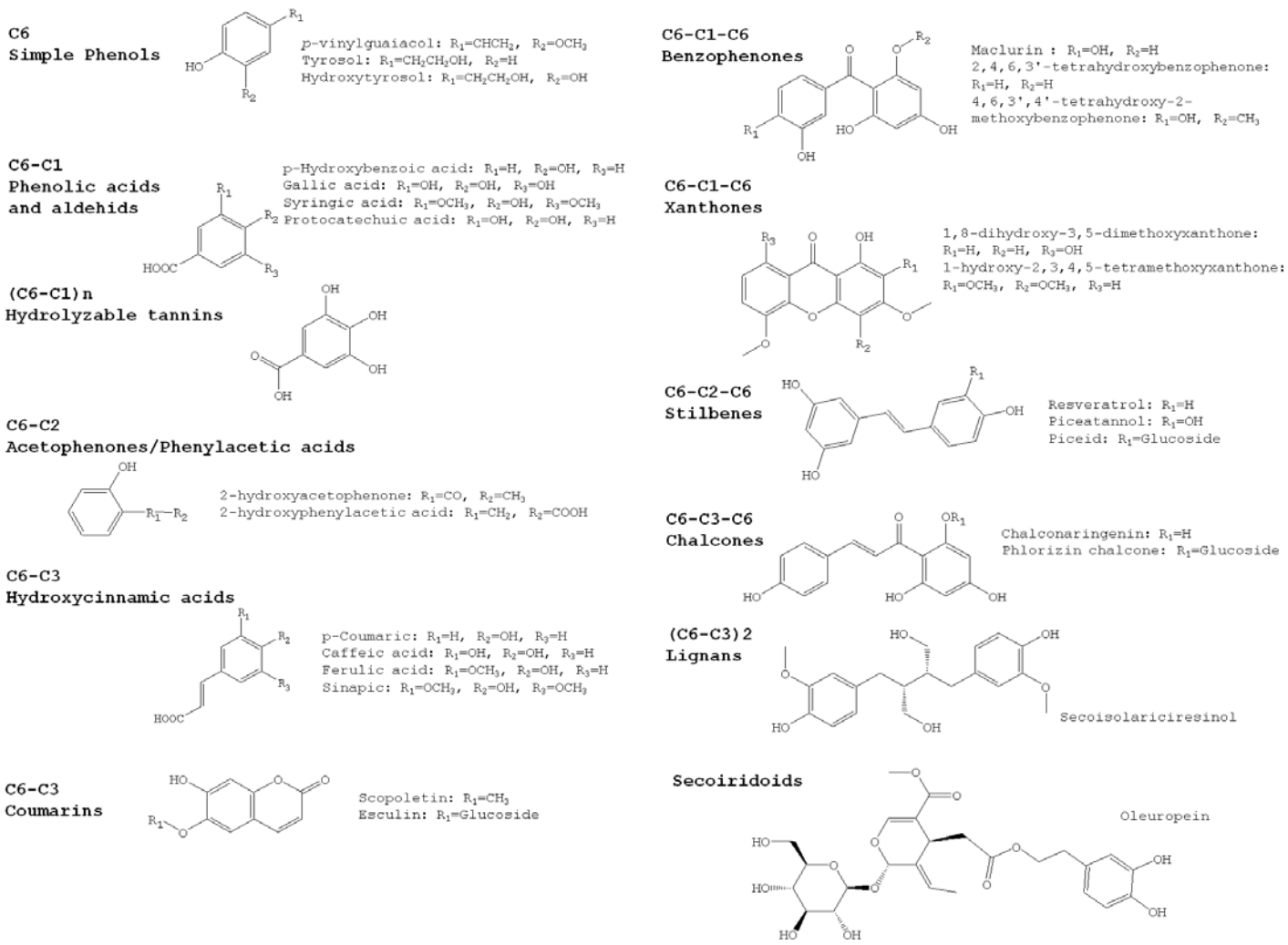


Figure 2. Chemical structures of no flavonoids.

B. METHODS OF IDENTIFICATION AND QUANTIFICATION

Sample Handling

In order to obtain a correct phenolic fingerprint of fruit and vegetables, it is necessary to take into account the complexity and variability of their matrix. This can be grouped into two kinds of factors: the physical and structural aspects and the biological aspects. The first factor is evident when comparing soft fruits such as berries or grapes, with other fruits such as oranges or apples, and also when comparing soft vegetables such as lettuce or watercress, with hard vegetables such as carrot or pumpkin. The structural fragility is apparent in their susceptibility to mechanical damage during handling or manipulation, which is frequently necessary before extraction. Mechanical damage could trigger enzymatic reactions related to the browning that is the consequence of the transformation of phenolic compounds to melanins. Two enzymes, such as polyphenol oxydases (PPO) and peroxidases (POD), are considered important factors in the process of phenolic oxidation (Tomás-Barberán and Espín 2001). Processes like peeling, cutting or crushing, which are fundamental to facilitating the correct extraction of phenolics, might cause alterations that later lead to incorrect identification and quantification of phenolic compounds. Enzymatic inactivation could be achieved using heated solvents, lowering the pH, adding the chemicals and using a high level of organic solvents. In this context, Arts and Hollman (1998) observed a browning in the extract obtained from apple and grape with concentrations of methanol below 40%. Additionally, this low methanol concentration showed a decrease of ~70% of cathechin yield that the authors attributed to the effect of PPO on the phenolic content.

In order to preserve, as much as possible, the phenolic content in fruit and vegetable samples, the literature proposed the application of cold temperatures, even reaching to freezing, when lyophilization is the objective. These procedures also could inactivate the enzymes. The freeze-drying is largely the main preservation technique used in the studies related with to the identification and quantification of the phenolic compound of fruit and vegetables. Asami and others (2003) found that the total phenolic content of freeze-dried samples of marionberries and strawberries was higher than the air-dried samples. In this context, frozen samples should be thawed before phenolic extraction. This procedure could provoke a loss of phenolic compounds due to some of them showing important thermolability, and also the thawing could provoke the activation of the enzyme that alters the phenolic content. Among the different techniques proposed for thawing, the microwave seems to be the most effective. A recent work (Oszmianski and others 2008) looking into the effect of freeze-thaw treatment on the polyphenol content of frozen strawberries showed that, using a microwave thawing for 5 minutes instead of 20h at 20ºC, had some protective effect on many polyphenolic compounds, such as anthocyanins or ellagic acid.

Extraction

The methods of extraction need to be able to produce a correct *photograph* or *fingerprint* of the real phenolic content of samples. In fact, the extraction conditions must be as mild as possible to avoid modifications. In this context several factors, such as the complexity of the matrix, the variation of the solubility of phenolic compounds, the presence of interfering substances, the time of extraction, as well as the temperature, could provoke modifications. Additionally, the fruit or vegetable phenolic profile is a mixture that, from the qualitative point of view, varies from lower mass phenolics, e.g. gallic acid, to highly polymerized phenolics such as procyanidins and tannins and, from the quantitative point of view, varies from traces to several hundreds of milligrams. Some such phenolics have even been detected uniquely in one fruit or vegetable which makes them exclusive. Examples of these exclusive phenolics are dihydrochalcones (e.g. phloridzin a characteristic phenolic compound from apple and its derivatives) (Tomás-Barberán and Clifford 2000), or isoflavones, such as genistin and daidzein, which are restricted to *Fabaceae* (e.g. soja) (Cassidy and others 2000). All of these factors show that a unique solvent and/or method for total phenolic extraction does not exist. Usually, the methods of extraction involve a number of extraction steps with two or more different solvents, or a single extraction step with a mix of organic solvent with water. After this, further steps are required to evaporate, concentrate and possibly purify.

Several extraction methods or techniques have been proposed for phenolic extraction. Often, freeze-dried and frozen samples are subjected to milling, grinding, and homogenization which facilitate the solvent-compounds connection. Recently, extraction protocols have been greatly reviewed (Tura and Robards 2002; Stalikas 2007). For solid samples (e.g. unspoiled fruit and vegetables) the most frequent methods are based on the solid-liquid extraction process and include a soxhlet, sonication, solid phase extraction (Hernández-Montes and others 2006), supercritical fluid extraction and microwave. Devanand (2006) found significant differences in chlorogenic acid extraction from freeze-dried samples of eggplant using sonication, stirring, a shaker and rotator shaker, reflux and pressurized liquid extractor. Herrera and Luque de Castro (2005) applied ultrasound-assisted extraction, subcritical-water, and microwave-assisted extraction to extract the phenolic compounds from strawberries. The ultrasound-assisted extraction was much the fastest and produced less loss of analytes than the other methods. Several studies have shown the effective use of solid-phase extraction as a method to extract the phenolic compounds from raw plant extracts or even biological samples (Michalkiewicz and others 2008; Mezadri and others 2008; Xia and others 2007; Chen and others 2001; Suárez and others 1996).

According to the literature, the most common solvents are water (mainly hot water), methanol, ethanol, acetone and ethyl acetate. It is also common to use a mixture of water and organic solvents. Zhao and others (2008) studied the influence of the different solvents (water, ethanol and acetone), and mixtures of them, on the obtaining of phenolic extracts from raisins. The authors showed that the highest total phenolic content was achieved with the extracts obtained from solvent to water ratios of 60:40 (v/v), this being the extract obtained from ethanol:water (60:40, v/v)

which yielded the highest total phenolic content. On the other hand, Mané and others (2007) found that an acidified mixture of acetone-water-methanol was the best solvent for the simultaneous extraction of major polyphenol groups from different grape parts, including grape skin, pulp and seed.

Other important factors that could lead to error during the identification or quantification of phenolic compounds are the possible artifacts related to the isomerization, hydrolysis and oxidation produced during the extraction. With regard to these factors, light could cause isomerization which is termed photoisomerization. An example of this is the trans-cis photoisomerization of resveratrol. Resveratrol and piceid (resveratol glucoside) are stilbenes present in grape products, where mainly *trans* isomers are detected. The effect of the UV causes a conversion to the cis isomers. In fact, within ten minutes of exposure to sunlight of standard solutions, ~90% of isomerization is achieved (Romero-Pérez and others 1999). The use of a laboratory with UV-filtered light for sample preparation and extraction has been proposed to avoid light degradation (Teow and others 2007). Likewise, some caffeyolquinic derivatives could undergo isomerization in warm aqueous media. The extraction of artichoke by-products with boiling water has been linked to the appearance of different isomers such as 1,3-*O*-dicaffeoylquinic acid (Llorach and others 2003). In this context, the temperature of extraction and the time of extraction have been linked to both a positive effect, due to an increase in the solubility which could facilitate the extraction from the matrix, and a negative effect, due to the higher temperature which could provoke either a loss or a transformation of phenolic compounds. Regarding the oxidation, some chemicals, such as *tert*-butylhydroquinone, 2,6-di-*tert*-butyl-4-methyphenol (BHT), ascorbic acid or sulfites, have been proposed as preventers of oxidation during phenolic extraction (Escribano-Bailón and Santos-Buelga 2003). However, Bradshaw and others (2001) found that ascorbic acid plays a role in inducing browning in catechin.

Separation

Over the past two decades, capillary electrophoresis (CE) and related techniques have rapidly developed for the separation of a wide range of analytes, ranging from large protein molecules to small inorganic ions. Gas chromatography has been considered as a powerful tool due to its sensitivity and selectivity especially when coupled with mass spectrometry. Nevertheless, liquid chromatography is the most used method to separate and analyze phenolic compounds in plant and tissue samples.

Liquid chromatography is carried out in columns. The common columns are packed with reversed-phase C_{18}-bonded columns. Elution systems are usually binary, with one of the solvents being an acidified aqueous solvent. The second is an organic solvent (e.g. methanol), acidified with the same acid used in the aqueous solvent (Ibern-Gómez and others 2002). Usually, it includes gradient elution and, occasionally, isocratic elution. Other aspects, such as the pH of chromatography, or the buffer if it is used, could drastically affect the separation of phenolic compounds and also further ionization.

Mass spectrometry methods

Mass spectrometry has become a very important technique in the identification and quantification of phenolics in fruit and vegetables. Different factors, such as sensitivity and specificity, have been cited to explain the acceptance of this method by the scientific community. Additionally, this technique might easily combine with different separation techniques such as capillary electrophoresis (CE), gas chromatography (GC) and liquid chromatography (LC), including HPLC and UPLC.

An important part of the mass spectrometry methods are the ionization sources and the analyzers. Several ionization sources are available. Among these, electrospray ionization is largely the most used due to the wide range of molecules that it covers. Other important sources are those related to atmospheric pressure ionization, including atmospheric pressure photo-ionization (APPI) and atmospheric pressure chemical ionization (APCI), that have shown interesting results analyzing flavonoids (de Rijke and others 2003). Additionally, the molecules could be ionized by a loss of proton (negative ionization) or by a gain of proton (positive ionization). Both methods have been applied in studies related to phenolic analysis. Other sources of ionization exist, such as fast atom bombardment (FAB) which has been applied successfully in the study of flavonoid glycosides (Stobiecki 2000). In addition, matrix-assisted laser desorption ionization (MALDI) has also been used for the study of polyphenol composition (Prasain and others 2004).

Different analyzers have been used to analyze phenolic compounds. The choice of the MS analyzer is influenced by the main objective of the study. The triple quadrupole (QQQ) has been used to quantify, applying multiple reaction monitoring experiments, while the ion-trap has been used for both the identification and structure elucidation of phenolic compounds. Moreover, time-of-flight (TOF) and Fourier-transform ion cyclotron resonance(FT-ICR) are mainly recommended for studies focused on obtaining accurate mass measurements with errors below 5 ppm and sub-ppm errors, respectively (Werner and others 2008). Nowadays, hybrid equipment also exists, including different ionization sources with different analyzers, for instance electrospray or atmospheric pressure chemical ionization with triple quadrupole and time-of-flight (Waridel and others 2001).

The mass spectrometry applied to characterize phenolic compounds has been largely reviewed (Fulcrand and others 2008; Harnly and others 2007; de Rijke and others 2006; Prasain and others 2004). Therefore, here we describe the most common mass spectroscopic methods used for the analysis of phenolic compounds.

Capillary electrophoresis mass spectrometry

Separation by capillary electrophoresis is based on the differences in electrophoretic motilities in a solution of charged species in an electric field of small capillaries. Its application in the analysis of phenolic compounds in fruit and vegetables is relatively recent compared to gas chromatography or liquid chromatography. Taking into account the format of the buffers used in the capillary, it is possible with this technique to distinguish different CE techniques called capillary zone electrophoresis (CZE), capillary gel electrophoresis and micellar electrokinetic chromatography (MEKC)(Prasain and others 2004). CE as a separation technique has been successfully applied to flavonoid studies (de Rijke and others 2006; Prasain and others 2004). Huck and others (2005) have greatly reviewed the main challenge concerning CE-MS. CE-ESI-MS has been successfully applied to separate and identify the phenolic compounds in olives (Lafont and others 1999). The authors reported that using SIM CE/ESI-MS, the limit of detection in the picogram range may be achieved for some of the detected phenolic compounds.

Gas chromatography-mass spectrometry (GC-MS)

Gas chromatography has been applied over the past 20 years as a separation technique to study phytochemicals. This technique has been considered as a powerful tool due to its sensitivity and selectivity, especially when coupled with mass spectrometry. However, a particular disadvantage of this technique is that the majority of polyphenolic compounds are non-volatiles. Samples used in GC are heavily processed before being ready for analysis. This process includes clean up, solid phase extraction and often a derivatization process. Derivatization is carried out to generate a volatile phenolic compound. A variety of reagents used to derivatize exists, including diazomethane and methyl chloroformate. However, the most frequent derivative is the trialkylsilyl group of which the most common alkyl substituent is the methyl group (trimethylsilyl derivative) (Robbins 2003). According to the literature the *N*,*O*-bis-(trimethylsilyl)acetamide (BSA), *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), and *N*,*O*-bis-(trimethylsilyl)-trifluoroacematide (BSTFA) are the main reagents used in the derivatization process (Robbins 2003). Gas chromatography is often coupled to mass spectrometers. Recently Stalikas (2007) has reviewed the use of this technique in the field of phenolic acids and flavonoids analysis). Traditionally, the studies in GC-MS have been carried out using a flame ionization detector (FID); however, in recent years there have also been studies which used electron impact ionization. Additionally, an important variability has been shown concerning the chromatography conditions, including different kinds of columns and temperature range. The author reported twelve representative studies, including seven related to fruit and vegetables, as examples of sample preparation and gas chromatography methods. These studies include the analysis of phenolic acids, such as gallic acid, vanillic acid or coumaric acid from plant extracts, and flavonoids such as kaempferol or quercetin from *Vitis vinifiera*, or pelargonidin and cyanidin from grapefruit. In this context, the GC-FID has been applied to identify and quantify the phenolic content in mangosteen fruits (Zadernowski and others 2009).

Liquid chromatography mass spectrometry

Liquid chromatography, coupled to the different ionization sources, is generally the technique most used to characterize the phenolic profile in fruit and vegetable products. With regards to the source ionization, it seems that ESI is used more frequently than other sources, such as APCI or APPI. Another important aspect in this technique is the ionization of phenolic compounds. Negative ionization seems to be more suitable than positive; however, positive mode could provide additional information, especially in studies dealing with the identification of unknowns. The LC is frequently coupled to tandem mass (MS-MS), producing a fragmentation of targeted or untargeted compounds and resulting in a different daughter fragment that could be used to correctly identify and also quantify phenolic compounds. A review of the fragment observed in both positive and negative ionization modes for some selected flavonoid classes has been carried out by de Rijke and others (2006).

The main MS/MS techniques are precursor ion, product ion and neutral loss. In addition it is possible to carry out the MS^n experiments using an ion-trap (Kang and others 2007). In this context, de Rijke and others (2003) carried out a study with fifteen flavonoids, comparing different ionization sources and different analyzers. Among the results, the authors showed that the main fragmentations observed in the MS spectra on the ion-trap, or the tandem MS spectra on the triple-quadrupole, were generally the same.

The LC-MS/MS technique has been used to quantify and identify phenolic compounds. In order to quantify, multiple reaction monitoring (MRM), in which there is a combination of precursor ion and one of its daughter fragments, is used to characterize a particular compound. This behaviour should be as specific as possible in samples with a complex mixture of phenolic compounds. This technique has been largely used to quantify phenolic compound metabolites in urine and plasma. (Urpí-Sardà and others 2007; Urpí-Sardà and others 2005). In this context, LC-ESI-MS/MS with negative mode has been applied for the identification of a variety of phenolic compounds in a cocoa sample (Sánchez-Rabaneda and others 2003; Andrés-Lacueva and others 2000).

During the last few years an important challenge of the LC-MS of flavonoids has been to optimize the analytical procedure in order to achieve structure elucidation (Cuyckens and Claeys 2002). Specifically, an important effort has been made to study the glycosilation pattern of flavonoids. Stobiecki reported the application of different MS techniques to flavonoid analysis (Stobiecki 2000). Likewise, Claeys and co-workers carried out an exhaustive study, under different conditions, of the interglycosidic linkage in *O*-diglycosides by tandem MS techniques (MS/MS) (Cuyckens and Claeys 2005; Cuyckens and Claeys 2004; Cuyckens and others 2003). In this context, Ferreres and others (2004) have shown that it is possible to differentiate the (1-->2) and (1-->6) interglucosidic linkages and to discern between the flavonoid isomers with two glucoses, three glucoses and four glucoses. In this context, the

authors have proposed the LC-MS^n as a powerful tool to characterize the C-glycosyl flavones O-glycosilated. The study of the relative abundance of the main ions from the MS2 and/or MS3 fragmentation events allows for the differentiation of the position of the O-glycosylation, either on phenolic hydroxyl or on the sugar moiety of C-glycosylation (Ferreres and others 2007).

Several studies reflect the widespread use of the LC-MS^n for the characterization of phenolic acids, predominantly chlorogenic acids (Clifford and others 2003; Clifford and others 2005; Clifford and others 2006a; Clifford and others 2006b; Clifford and others 2006c). For example, using LC-MS^3 it is possible to discriminate between different isomers of coumaroylquinic acid, caffeoylquinic acid and feruloylquinic acid. In addition, a hierarchical key was proposed to facilitate the process of identification when standards are not available (Clifford and others 2003).

In some studies the LC has been coupled to triple quadrupole and time-of-flight detectors. Moco and others (2006) used this technique to study phytochemicals including flavonoids from tomato samples. In fact, the results have been compiled in a database called "MoTo".

Direct infusion mass spectrometry (DIMS)

In some cases liquid chromatography fails to separate some polyphenol compounds (i.e. proanthocyanidins), hampering their correct analysis. Some studies have suggested direct mass spectrometry as a possible solution. A recent study of the evaluation of the main phenolic components of grape juices was carried out using direct infusion mass spectrometry as well as the ESI-MS in negative mode (Gollücke and others 2008). McDougall and others (2008) reported that DIMS in both negative and positive modes could be applied to rapidly assess differences in the polyphenol content of berries.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF)

The MALDI-TOF technique was first developed for the analysis of large biomolecules (Karas and others 1987). This technique presents some interesting characteristics. Of these, the high speed of analysis and the sensitivity of the technique have been pointed out as important advantages compared with other methods. In MALDI the samples are cocristallized with a matrix that is usually composed of organic compounds, such as 3,5-dimethoxy-4-hydroxycinnamic acid (sinapic acid), 2',4',6'-trihydroxyacetophenone, α-cyano-4-hydroxycinnamic acid (alpha-cyano or alpha-matrix) and 2,5-dihydroxybenzoic acid (DHB). After the cocristallization the laser is fired and the matrix absorbs energy and allows a soft ionization of the samples. Afterwards the ions are analyzed by a TOF mass spectrometer.

This technique has been successfully applied for the analysis of different kinds of polyphenols from different sources. Reed and others (2005) studied the oligomeric polyphenols in foods. Recently MALDI-TOF MS was applied to characterize almond skin proanthocyanidins, revealing the existence of a series of A- and B-type procyanidins and propelargonidins up to heptamers (Monagas and others 2007). The application of MALDI-TOF to soja product provides an isoflavone profile in a few minutes and serves as a powerful tool to identify and study the processing changes of isoflavones in these products (Wang and Sporns 2000b). Likewise, it has been used to characterize flavonol glycosides (Wang and Sporns, 2000a).

Other detection methods

Nuclear magnetic resonance spectroscopy (NMR)

LC-NMR plays a central role in the on-line identification of the constituents of crude plant extracts (Wolfender and others 2003). This technique alone, however, will not provide sufficient spectroscopic information for a complete identification of natural products, and other hyphenated methods, such as LC-UV-DAD and LC-MS/MS, are needed for providing complementary information. Added to this, LC-NMR experiments are time-consuming and have to be performed on the LC peak of interest, identified by prescreening with LC-UV-MS. NMR applied to phenolic compounds includes: ^{1}H NMR, ^{13}C NMR, correlation spectroscopy (COSY), heteronuclear chemical shift correlation NMR (C-H HECTOR), Nuclear Overhauser Effect in the laboratory frame (NOESY), rotating frame of reference (ROESY), total correlation spectroscopy (TOCSY) (Escribano-Bailón and Santos-Buelga 2003), heteronuclear multiple-quantum coherence (HMQC), and heteronuclear multiple-bond correlation (HMBC)(Es-Safi and others 2008).

Electrochemical methods

Electrochemical detection is sensitive, selective, and gives useful information about polyphenolic compounds in addition to spectra obtained by photodiode array detectors. Differences in electrochemically active substituents on analogous structures can lead to characteristic differences in their voltammetric behaviour. Because the response profile across several cell potentials is representative of the voltammetric properties of a compound, useful qualitative information can be obtained using electrochemical detection (Aaby and others 2004).

These methods can be used to determine redox potentials of phenolics, identify the mechanism of oxidation, identify a flavonoid based on comparison with a standard, and determine redox potentials for unknown phenolics (Escribano-Bailón and Santos-Buelga 2003).

Coulometric array detection

The multichannel coulometric detection system serves as a highly sensitive tool for the characterization of antioxidant phenolic compounds because they are electroactive substances that usually oxidize at low potential. The coulometric efficiency of each element of the array allows a complete voltammetric resolution of analytes as a function of their oxidation potential. Some of the peaks may be resolved by the detector even if they coelute (Floridi and others 2003).

Photodiode array (DAD) detectors

Food and plant phenolics are commonly detected using DAD detectors (Tan and others 2008). Photodiode array detection allows collection of the entire UV spectrum during the elution of a chromatographic peak, which makes it possible to identify a phenolic compound by its spectra. Simple phenols, phenolic acids, flavanones, benzophenones, isoflavones and flavan-3-ols have maximum absorbance at 280nm, hydroxycinnamic acids at 320nm, flavonols, flavones and dihydroflavonols at 365nm and anthocyanins have maximum absorbance at 520nm (Ibern-Gómez and others 2002; Howard M. Merken HM and Beecher GR 2000). Hydrolyzable tannins show a characteristic shoulder at 300nm, suitable for identifying them (Arapitsas and others 2008). For stilbenes, maximum absorbance of *trans*-forms are at 306 nm and at 285 nm for *cis*-forms (Lamuela-Raventós and others 1995).

Sectrophotometric assays (classical methods)

A number of spectrophotometric methods for the quantification of phenolic compounds in plant materials have been developed. Based on different principles, these assays are used to determine various structural groups present in phenolic compounds. Spectrophotometric methods may quantify all extractable phenolics as a group (Marshall and others 2008), or may determine a specific phenolic substance such as sinapine (Ferial and Eskin 1979), or a given class of phenolics such as phenolic acids (Brune and others 1989).

Determination of total phenolics

Folin-Denis Assay

The Folin-Denis assay is used as a procedure for the quantification of total phenolics in plant materials, food and beverages. Reduction of phosphomolybdic-phosphotungstic acid (Folin-Denis), reagent to a blue-coloured complex in an alkaline solution, occurs in the presence of phenolic compounds (Folin and Denis 1912).

Folin-Ciocalteu Assay

The Folin-Ciocalteu assay is the most widely used method to determine the total content of food phenolics (Heck and others 2008). Folin-Ciocalteu reagent is not specific and detects all phenolic groups found in extracts, including those found in extractable proteins. A disadvantage of this assay is the interference of reducing substances, such as ascorbic acid (Singleton and others 1999). The content of phenolics is expressed as gallic acid or catechin equivalents.

Determination of proanthocyanins

Vanillin Assay

The vanillin method is based on the condensation of the vanillin reagent with proanthocyanins in acidic solutions. Protonated vanillin, a weak electrophilic radical, reacts with the flavonoid ring at the 6- or 8-position. The vanillin reaction is affected by the acidic nature and concentrations of substrate, the reaction time, the temperature, the vanillin concentration and water content (Sun and others 1998).

Proanthocyanidin Assay

The proanthocyanidin assay is carried out in a solution of butanol-concentrated hydrochloric acid, where proanthocyanidins (condensed tannins) are converted to anthocyanidins (products of autoxidation of carbocations formed by cleavage of interflavanoid bonds) (Matus-Cádiz and others 2008).

Determination of hydrolyzable tannins

The most widely used method is based on the reaction between potassium iodate and hydrolyzable tannins (Hartzfeld and others 2002). This method provides a good estimate for gallotannins, but underestimates the content of ellagitannins.

Other analytical assays proposed for the quantification of hydrolyzable tannins in plant materials include the rhodanine assay for the estimation of gallotannins (Berardini and others 2004) and sodium nitrate for the quantitative determination of ellagic acid (Wilson and Hagerman 1990).

Determination of anthocyanins

Quantification of anthocyanins takes advantage of their characteristic behaviour in acidic media; anthocyanins exist in these media as an equilibrium between the coloured oxonium ion and the colourless pseudobase form. Using an average extinction coefficient, the total content of anthocyanins may also be estimated from the absorption of the total extracts at 520 nm (Moskowitz and Hrazdina 1981).

DMACA (4-(Dimethylamino)-Cinnamaldehyde) Assay

DMCA Assay is used to quantify catechins, and it is based on the formation of a green chromophore between catechin and 4-(dimethylamino)-cinnamaldehyde (DMACA) (Polster and others 2003).

New methods in development

New detection methods of phenolic compounds are being developed. Based on the principle of enzyme-linked immunosorbent assay (ELISA), a method has been developed to quantify phenolic compounds such as isoflavones (Vergne S and others 2007).

C. OCCURRENCE

Polyphenols represent a wide variety of diverse structures from different subclasses, that is why it is difficult to estimate the total polyphenol content in fruit and vegetables. Many phenolic compounds escape HPLC/UV quantification because of the presence of unidentified compounds leading to underestimation of total polyphenol content. The fruits with the highest polyphenol concentrations are strawberries, lychees and grapes (>180mg of gallic acid equivalent (GAE)/100g fresh weight (FW)); the vegetables with the highest concentration are artichokes, parsley and Brussels sprouts (>250mg of GAE/100g FW); melons and avocados have the lowest polyphenol concentration (Brat and others 2006).

Flavonoids

Flavonols

Flavonols are the most frequent flavonoids in foods (Manach and others 2004). Capers are the main source of flavonols (containing up to 490mg/100g FW) (US Department of Agriculture 2007a). Other abundant sources (ranging between 10 and 100mg/100g FW) are onions, kales, berries and some herbs and spices (US Department of Agriculture 2007a). Cocoa, brewed tea and red wine are also good dietary sources of flavonols 30 (Lamuela-Raventós 2001), 4.5 and 3.1mg/100mL FW, respectively (US Department of Agriculture 2007a). Flavonols are mainly accumulated in the outer tissues of fruits and vegetables because their synthesis is stimulated by sunlight (Manach and others 2004).Flavonols are found in glycosylated forms and their bioavailability depends on their sugar moiety (Hollman and others 1997). Quercetin and kaempferol are the main sources of flavonols (Manach and others 2004). The highest dietary sources of quercetin are capers, followed by onions, asparagus, berries and lettuce (Table 1). In many other vegetables and fruits, quercetin is frequently present in low concentrations around 0.1 and 5mg/100g FW (US Department of Agriculture 2007a). Vegetables (0.1-26.7mg/100g FW) and some spices, such as chives, tarragon and fennel (6.5-19mg/100 FW), are characteristic sources of kaempferol, whereas fruits are a poor source (down to 0.1mg/100g) (US Department of Agriculture 2007a). Myrcetin, that is the third most abundant flavonol, is found in some spices, such as parsley, fennel and oregano (2-19.8mg/100g FW) and it is also present in brewed tea (0.5-1.6mg/100mL FW) and red wine (0-9.7mg/100mL FW) (US Department of Agriculture 2007a). In fruits it is only present in high concentrations in berries, whereas in most fruits and vegetables it is found with a content of less than 0.2mg/100g FW (Table 1). Isorhamnetin is the least abundant flavonol; it has been detected in a few foods, such as some spices: fennel 9.3mg/100g FW, chives 5.0-8.5mg/100g FW, tarragon 5mg/100g FW and in almonds it ranged between 1.2 and 10.3mg/100g FW (US Department of Agriculture 2007a). In vegetables and fruits it is only present in onions and pears (Table 1).

Flavones

Flavones are widely present, in a small quantity, in foods of plant origin. Some spices, such as parsley, thyme and oregano, have a range of between 25 to 630mg/100g FW and are the most important sources of flavones (US Department of Agriculture 2007a). Flavones consist basically of apigenin and luteolin glycosides (Manach and others 2004). Apigenin is mainly present in vegetables, such as artichokes, celery, red onion and lettuce (Table 1). However, luteolin is present in both vegetables and fruits (Table 1).

Flavanones

Citric fruits, both raw and as derived products, such as juices and jams, are the main sources of flavonones (Manach and others 2004). In less concentrations we also found eriodictyol in almonds (0.03-0.6mg/100g FW), hesperetin in mint (0-21.9mg/100g FW), and naringenin in artichokes and ripe tomatoes (0-22.9 and 0-1.5mg/100g FW, respectively) (US Department of Agriculture 2007a). Naringenin is the most abundant flavanone present in grapefruit, and hesperitin in lime, whereas orange contains notable amounts of both hesperetin and naringenin. Eriodicyol is the characteristic flavonone of lemon and lemon juice (Table 1). Flavanones are usually glycosylated at position 7 by a disaccharide (neohesperidose, rutinose) or, in a minor percentage, by a monosaccharide (glucose) (Tomás-Barberán and Clifford 2000).

Isoflavones

Isoflavones occur almost exclusively in leguminous plants (Manach and others 2004). Soja bean and its processed products, such as soja milk, tofu, tempeh and miso, are the main source of genistein, daidzein and glycetin (US Department of Agriculture 2007b). We can also find isoflavones in lower concentrations in beans and broadbeans (0.01 to 0.04mg/100g FW) (US Department of Agriculture, 2007b). Isoflavones have not been detected to date in

fruits and vegetables (US Department of Agriculture, 2007b). In foods, isoflavones occur in four forms: aglycone, 7-*O*-glucoside, 6"-*O*-acetyl-7-*O*-glucoside, and 6"-*O*-malonyl-7-*O*-glucoside (Coward and others 1998).

Anthocyanidins

Anthocyanidins provide the characteristic red-blue colours of most fruits and vegetables. Berries are the main dietary source of anthocyanidins (66.8-947.5mg/100g FW) (US Department of Agriculture 2007a).Other fruits such as red grape, cherries and plums, and some vegetables such as red cabbage, red onions, radish and eggplant, are also sources of anthocyanidins, with contents ranging between 2-150mg/100g FW (US Department of Agriculture 2007a). Anthocyanidins are poorly distributed (<10mg/100g FW) in other fruits, such as peaches, nectarines and some kinds of pears and apples (US Department of Agriculture 2007a). The anthocyanidin content increases as the fruit ripens. These polyphenols are found mainly in the skin, except for in berries, where they are present in both skin and flesh (Manach and others 2004).

Berries, such as blueberries, bilberries and blackcurrants, are the main sources of cyanidin, delphinidin, malvidin, peonidin and petunidin. Malvidin is the characteristic anthocyanidin of red grape and red wine. Plums, cherries and red cabbage are rich in cyanidin, whereas eggplant is a good source of delphinidin. Pelargonidin is the most abundant anthocyanidin occurring in strawberries and radishes (Table 1).

Flavan-3-ols

Flavan-3-ols are found in many types of fruit, red wine, beer and nuts, but tea and chocolate are by far the richest sources (Manach and others 2004). A few vegetables present flavan-3-ols contained at very low concentrations (down to 1.5mg/100g FW) (US Department of Agriculture 2007a).In contrast to the main classes of flavonoids, flavan-3-ols are found as aglycones in foods (Manach and others 2004). Catechin and epicatechin are the most frequently occurring flavan-3-ols in foods, such as tea (0-70mg/100mL FW), cocoa powder (19.7-127.7mg/100g FW) (Andres-Lacueva and others 2008), red wine (0.2-55.6mg/100mL FW), nuts (0-4mg/100g FW), beer (0-10mg/100mL FW) and fruits (US Department of Agriculture 2007a). Catechin and epicatechin are present in many fruits at concentrations of 0.5-3 and 0.5-6mg/100mL FW, respectively (Table 1). Epigallocatechin, epicatechin 3-gallate, epigallocatechin 3-gallate, gallocatechin are found in several fruits, such as berries, red grapes, plums, apples and peaches, normally at very low concentrations (less than 1mg/100g FW) (Table 1), but they are present in high amounts in tea (6.8-395mg/100g FW) (US Department of Agriculture 2007a). Chocolate is also a good source of epigallocatechin. Theaflavin (0-5.3mg/100mL FW), thearubigins (7.8-139mg/100mL FW), theaflavin-3-3'-digallate (0-4.9mg/100mL FW), theaflavin-3'-gallate (0-4.1mg/100mL FW) and theaflavin-3-gallate (0-3.2mg/100mL FW) are flavan-3-ols which are only detected in tea (US Department of Agriculture 2007a). These concentrations occur in brewed tea; in tea leaves the content is between 50- and 100- fold more than brewed tea (US Department of Agriculture 2007a).

Proanthocyanidins

Proanthocyanidins (PAs), also known as condensed tannins, are oligomeric and polymeric flavan-3-ols. Procyanidins are the main PAs in foods; however, prodelphinidins and propelargonidins have also been identified (Gu and others 2004). The main food sources of total PAs are cinnamon 8084mg/100g FW and sorghum 3937mg/100g FW. Other important sources of PAs are beans, red wine, nuts and chocolate, their content ranging between 180 to 300mg/100g FW. In fruits, berries and plums are the major sources, with 213.6 and 199.9mg/100g FW, respectively. Apples and grapes are intermediate sources of PAs (60 to 90mg/100g FW), and the content of PAs in other fruits is less than 40mg/100g FW. In the majority of vegetables PAs are not detected, but they can be found in small concentrations in Indian squash (14.8mg/100g FW) (Gu and others, 2004; US Department of Agriculture, 2004).

Table 1 shows the PA content in groups of fruits classified in dimers, trimers, 4-6mers (tetramers, pentamers and hexamers), 7-10mers (heptamers, octamers, nonamers and decamers), and finally polymers (more than 10 monomers). Polymers and 4-6mers are the most common PAs in fruits.

Table 1: Flavonoids in foods

Subclass	Polyphenol	Food	Content (mg/100g FW)	Reference
Flavonols	Quercetin	Onions Asparagaus Berries Lettuce	21.4 (32.3) 12.4 (8.5) 10.7 (6.8) 7.1 (12.8)	(US Department of Agriculture 2007a)
	Kaempferol	Kale Endive Spinach Berries	26.7 (7.2) 10.1 (6.3) 7.6 (13.5) 0.4 (0.6)	(US Department of Agriculture 2007a)
	Myricetin	Berries Grape Red cabbage	5.7 (6.4) 0.4 (0.2) 0.2 (0.4)	(US Department of Agriculture 2007a)
	Isorhamnetin	Onions Pears	5.0 (2.2) 0.3 (0.2)	(US Department of Agriculture 2007a)
Flavones	Apigenin	Artichokes Celery Red onion Lettuce	4.7 (1.1) 2.3 (0.7) 0.4 (0.4) 0.16 (0.3)	(US Department of Agriculture 2007a)
	Luteolin	Pepper Artichokes Red grape Oranges	5.0 (2.6) 2.3 (0.5) 1.3 (1.1) 1.1 (0.5)	(US Department of Agriculture 2007a)
Flavonones	Eriodictyol	Lemon, raw Lemon, juice Orange, juice	21.4 (5.3) 4.9 (4.1) 0.2 (0.5)	(US Department of Agriculture 2007a)
	Hesperetin	Lime, raw Lemon, raw Orange, raw Citric fruit, juice	43.0 27.9 (10.8) 27.3 (10.6) 10.5 (5.4)	(US Department of Agriculture 2007a)
	Naringenin	Grapefruit, raw Orange, raw Artichokes, raw Ripe tomatoes, raw	21.3 15.3 (10.9) 12.5 (6.8) 0.7 (0.4)	(US Department of Agriculture 2007a)
Isoflavones	Genistein	Soja bean Soja milk Tofu	64.8 (32.2) 6.1 (3.4) 13.3 (8.8)	(US Department of Agriculture 2007b)
	Daidzein	Soja bean Soja milk Tofu	34.5 (30.7) 4.5 (2.7) 8.5 (5.9)	(US Department of Agriculture 2007b)
	Glycitein	Soja bean	13.8 (4.2)	(US Department of Agriculture 2007b)

		Soja milk Tofu	0.6 (0.2) 2.3 (1.3)	
Flavan-3-ols	Catechin	Cocoa powder Peaches Berries Red grape Bananas	8.1-44.8 12.2 (5.2) 11.2 (17.5) 10.1 (2.9) 6.1 (3.0)	(Andres-Lacueva and others 2008) (US Department of Agriculture 2007a)
	Epicatechin	Cocoa powder Red grape Apricot Apples	11.6-73.0 8.7 (0.1) 5.5 (2.7) 5.5 (1.1)	(Andres-Lacueva and others 2008; US Department of Agriculture 2007a)
	Epigallocatechin	Peaches Apples Berries	1.1 (0.9) 1.0 (0.8) 0.6 (0.7)	(US Department of Agriculture 2007a)
	Epicatechin 3 gallate	Red grape Plums Apples/Pears	2.8 0.8 (0.5) 0.01 (0.1)	(US Department of Agriculture 2007a)
	Epigallocatechin 3-gallate	Apples Berries Plums	0.5 (0.7) 0.6 (1.0) 0.4 (0.8)	(US Department of Agriculture 2007a)
	Gallocatechin	Berries Plums Pomegranate/persimmons	0.5 (0.6) 0.1 (0.4) 0.2	(US Department of Agriculture 2007a)
Proanthocyanidins	Dimers	Chocolate Berries Plums Apples Peaches/apricot/nectarines Kiwi	22.0-206.5 9.5 (6.9) 30.1 (8.2) 11.3 (2.2) 6.2 (5.5) 1.1 (0.5)	(Gu and others 2004; US Department of Agriculture 2004)
	Trimers	Chocolate Berries Plums Apples Peaches/apricot/nectarines Kiwi	19.3-130.9 7 (5.1) 20.9 (1.8) 7.1 (1.5) 2.3 (1.9) 0.9 (0.4)	(Gu and others 2004; US Department of Agriculture 2004)
	4-6mers	Chocolate Berries Plums Apples Peaches/apricot/nectarines Kiwi	51.4-332.6 23.5 (21.3) 57.8 (12.5) 24.5 (5.1) 9.5 (7.1) 3.2 (1.9)	(Gu and others 2004; US Department of Agriculture 2004)
	7-10mers	Chocolate Berries Plums Apples Peaches/apricot/nectarines	35.3-216.4 21.9 (22.7) 33.8 (11.9) 20.8 (5.4) 5.6 (4.7)	(Gu and others 2004; US Department of Agriculture 2004)

		Kiwi	2.6 (2.4)	
	Polymers	Chocolate Berries Plums Apples Peaches/apricot/nectarines Kiwi	32.8-551.0 151.7 (175.7) 57.3 (24.4) 29.7 (10.3) 10.1 (10.9) 0	(Gu and others 2004; US Department of Agriculture 2004)
Anthocyanidins	Cyanidin	Berries Plums Red cabbage	189.9 (243.8) 12.0 (2.0) 72.9 (22.2)	(US Department of Agriculture 2007a)
	Delphinidin	Berries Eggplant Red grape	97.9 (73.5) 13.8 (13.7) 3.7 (1.5)	(US Department of Agriculture 2007a)
	Malvidin	Blueberries Red grape	61.4 (4.4) 34.7 (5.6)	(US Department of Agriculture 2007a)
	Pelargonidin	Strawberries Radish	31.3 (1.9) 25.7 (2.0)	(US Department of Agriculture 2007a)
	Petunidin	Berries Red grapes	33.9 (36.4) 2.1 (0.2)	(US Department of Agriculture 2007a)
	Peonidin	Berries Cherries Red grape	21.5 (21.4) 4.5 (0.6) 2.9 (0.6)	(US Department of Agriculture 2007a)

Non-Flavonoids

Simple phenols

Before the ageing process wine contains small quantities of volatile phenols, which increase significantly during the time of contact with the wood, especially over the first 12 months. The use of oak wood during the ageing of wines has a great influence on wine composition, especially on volatile substances that are extracted from the wood, affecting its organoleptic properties. Some of these volatile compounds susceptible to migration from oak wood to wine are eugenol, guaiacol and 4-ethylguaiacol (Fernandez de Simon and others 2003). The formation of 4-vinylguaiacol in coffee beans starts immediately at the beginning of the roasting process. The evolution of this compound during roasting is highly dependent on temperature (Baggenstoss and others 2008).

Ferulic acid has been extensively studied as a precursor of *p*-vinylguaiacol, the most detrimental off-flavour that forms in orange juice during storage (Rapisarda and others 1998).

Virgin olive oil contains considerable amounts of simple phenols that have a great effect on the stability/sensory and nutritional characteristics of the product. Some of those most representative are hydroxytyrosol (3,4-dihydroxyphenylethanol), and tyrosol (4-hydroxyphenylethanol); however, phenolic compounds are removed when the oil is refined (Tovar and others 2001). The phenolic content of virgin olive oil is influenced by the variety, location, degree of ripeness and the type of oil extraction procedure used, and that is why hydroxytyrosol can be considered as an indicator of maturation for olives (Esti and others 1998).Hydroxytyrosol concentrations are correlated with the stability of the oil, while those of tyrosol are not (Visioli and Galli 1998).

Phenolic acids

The total phenolic acid content in rowanberry, as determined by HPLC, is 103mg/100g FW. Besides rowanberry, the best phenolic acid sources among berries are chokeberry (96mg/100g FW), blueberry (85mg/100g FW), sweet rowanberry (75mg/100g FW), and saskatoon berry (59mg/100g FW). Among fruits, the highest contents (28mg/100g FW) are determined in dark plum, cherry, and one apple variety (Valkea kuulas). Coffee (97mg/100mL) and green and black teas (30-36mg/100mL) are the best sources among beverages (Mattila and others 2006). Sinapinic acid was notable (4.25mg/100g FW) in Chinese cabbage, and protocatechuic acid had the highest concentration of all the phenolic acids in white wine (Li and others 1993).

Hydrolyzable tannins

The potent antioxidant properties of pomegranate juice have been attributed to its high content of punicalagin isomers which can reach levels >200mg/100mL juice (Cerdá and others 2003a, 2003b). Total hydrolyzable tannins in barley flour, oak wood, and green tea were determined spectrophotometrically as 870, 1120, and 590mg/100g, respectively (Taubert and others 2005). Grape seed extract has a high tannin content of 535.6mg/100g (Hong and others 2002). Persimmon is the edible fruit of a number of species of trees of the genus *Diospyros* in the ebony wood family (Ebenacea). Persimmon seed extract has a tannin content of 577.37 ± 0.66mg/100g (Hong and others 2002). Although citrus fruits do not themselves contain tannins, orange-coloured juices often contain food dyes with tannins. Apple juice, grape juice and berry juices are all high in tannins. Most legumes contain tannins. Red-coloured beans contain the most tannins, and white-coloured beans have the least. Chickpeas, also known as garbanzo beans, have a smaller amount of tannins (Reed 1995).

Acetophenones and phenylacetic acids

Phenylacetic acid has been detected in fermented soja bean made with the strain *Bacillus licheniformis* as a starter, but has not been present in extracts of non-fermented soja bean. The phenylacetic acid produced by *Bacillus licheniformis* during the fermentation of soja bean is one of the main compounds of antimicrobial activity of Chungkook-Jang, a traditional Korean fermented-soja bean food with antimicrobial properties (Kim and others 2004).

Hydroxycinnamic acids

The most common hydroxycinnamic acid derivatives are *p*-coumaric (4-hydroxycinnamic), caffeic (3,4-dyhydroxycinnamic), ferulic (4-hydroxy-3-methoxycinnamic), and sinapic *(*4-hydroxy-3,5-dimethoxycinnamic) acids which frequently occur in foods as simple esters with quinic acid or glucose (Mattila and Kumpulainen 2002).

Hydroxycinnamic acids are found in all parts of fruit and vegetables, even though the highest concentrations are observed in the outer part of mature fruits, although concentration decreases during ripening (Manach and others 2004). An overlong storage period of blood orange fruits induces extensive hydrolysis of hydroxycinnamic derivatives to free acids, and these, in turn, could develop the malodorous vinylphenols, which are an indication of too-advanced senescence in blood orange fruits (Rapisarda and others 2001).

Caffeic acid is generally the most abundant hydroxycinnamic acid in fruit and vegetables. The richest sources are coffee (drink), lettuce, carrots, blueberries, blackberries, cranberries, sweet potatoes (whole, cooked and raw) and potatoes (Table 2). Prunes, peaches, orange juice, apples, tomatoes, grapes and grape products (Betés-Saura and others 1996) also contain small quantities of caffeic acid.

Ferulic acid is the most abundant hydroxycinnamic acid in cereal grains. The content in wheat grain is approximately 800-2000mg/100g DW. It is found chiefly in the outer part of the grain, in the *trans* form, which is transformed into arabinoxylans and hemicelluloses in the aleurone and pericarp (Manach and others 2004). Ferulic acid is much less

common than caffeic acid in fruit and vegetables. Broccoli, eggplant and asparagus are a major source of ferulic acid. Low concentrations of ferulic acid have been reported in blueberries, blackberries, cranberries, orange juice, carrots, potatoes, beetroot, apples, coffee and others (Table 2). The content of *p*-coumaric acid has been reported in high quantities in broccoli, eggplant and asparagus (Yeh and Yen 2005). Other sources of *p*-coumaric acid are blueberries, cranberries, prunes, sweet cherries and orange juice. The highest contents of sinapic acid are found in citrus peel and seeds, and some cranberry varieties. However, the contents in these fruits are clearly higher than the orange juice (Table 2).

The most familiar hydroxycinnamate or ester of hydroxycinnamic acid is chlorogenic acid (5-*O*-caffeoylquinic acid). Other forms of caffeoylquinic acids are also found, namely 3-*O*-caffeoylquinic acid (*neo*- chlorogenic acid) and/or 4-*O*-caffeoylquinic acid (*crypto*- chlorogenic acid) (Mattila and Kumpulainen 2002). Chlorogenic acids are widely distributed in plants. They are a family of esters formed between quinic acid and certain *trans*-cinnamic acids, most commonly caffeic, *p*-coumaric, and ferulic. The coffee bean is remarkably rich, containing at least 30 chlorogenic acids that are not acylated at C1 of the quinic acid moiety (Clifford and others 2006). Coffee drink, apples, prunes, pears and sweet cherries are the highest sources of chlorogenic acids. Moderately good values are found on sour cherries, apricots, nectarines, peaches, quince and potatoes (Table 2). The concentration of chlorogenic acid in the core of pears is greater than that in the peel. However, the mean concentration of chlorogenic acid in the Oriental pear is 16.3mg/100g FW, less than that found in the Occidental pear (30.9mg/100g FW) (Cui and others 2005). Chlorogenic acid is the predominant phenolic in potato tubers, constituting up to 90% of the total phenolic content. Chlorogenic acid in fresh-cut potatoes after six days in cold storage varies between 7 and 30mg/100g FW (Truong and others 2007). For dry lyophilized potato powder, chlorogenic acid levels range from 3.28 to 637mg/100g FW (Im and others 2008). Steam-cooked potato strips retain 42% of the initial chlorogenic acid, while frying only preserves 24%. Boiled and microwaved potato retains 35 and 55% of the original amount, respectively (Tudela and others 2002).

Coumarins

Coumarin content in tonka seeds shows values of up to 300mg/100g FW (Clifford 2000**).** A new coumarin, isoschininallylol, was isolated from the fruits of *Poncirus trifoliata* Raf. (Xu GH and others 2008). Bismurrangatin and murramarin A, two new coumarins, were isolated from the vegetative branches of Murraya exotica (Negi and others 2005). The hydroxycoumarin scopoletin was isolated from seed kernels of Melia azedarach L. from which three other compounds, vanillin, 4-hydroxy-3-methoxycinnamaldehyde, and (+/-) pinoresinol, have also been isolated (Carpinella and others 2005). Coumarins are found at high levels in some essential oils, particularly cinnamon bark oil (700mg/100g), cassia leaf oil (up to 8730mg/100g) and lavender oil. Coumarin is also found in fruits (eg bilberries and cloudberries), green tea and other foods, such as chicory (Lake 1999). Simple coumarins (aesculetin and scopoletin) may also be present in carrots, but in some cases the roots seem to be free from the furanocoumarins (Mercier and others 1993). Parsnip root may contain some 4mg/100g total furanocoumarins (Wawrzynowicz and others 1990). Celery may contain a significant amount (up to 8.5mg/100g) but has a very variable quantity of furanocoumarins, with simple coumarins (aesculetin and scopoletin) contributing a modest 0.1mg/100g (Diawara and others 1995). The total coumarin content (mainly meranzin derivatives) of the flavedo of bitter orange, grapefruit and pomelo are 710, 250 and 510mg/100g, respectively (McHale and others 1987).

Benzophenones

Benzophenones are distributed scarcely in foods. They are mainly present in the *Garcinia* genus, such as in fruits of G*arcinia indica* (Yamaguchi and others 2000) and *Garcinia cambogia* (Masullo and others 2008), characteristic products of India. Recently, benzophenone derivatives have also been identified in several by-products of mango at low concentrations (0-15mg/100g FW) (Barreto and others 2008).

Xanthones

515 xanthones have been identified in 20 families of higher plants, mainly in the Bonnetiaceae and Clusiaceae families (Vieira and Kijjoa A, 2005). Mangosteen fruit, a typical south-east Asian fruit, is the characteristic dietary source of xanthones (less than 7-8mg/100g FW) (Walker 2007). Mangostin is a C-glucosylxanthone that is also found in by-products of mango at high concentration levels (Barreto and others 2008).

Stilbenes

Stilbenes are present at low concentration levels in a few human foods. The most representative stilbenes are resveratrol and its glycoside piceid; both stilbenes can be found in *cis*- and *trans*- forms (Zamora-Ros and others 2007). Resveratrol and piceid are characteristic polyphenols of grape (Table 2) and grape products (Zamora-Ros and others 2007; Manach and others 2004; Waterhouse and Lamuela-Raventós 1994) and their composition is affected by grape variety, degree of maturity at harvest, fungal pressure, climate and wine-making technology (González-Barrio and others 2006; Romero-Pérez and others 2001; Mattivi and others 1995). They are also found in peanuts (0.006mg/100g FW), pistachios (0.007mg/100g FW) and berries (0.008mg/100g FW), but red wines (0.558mg/100mL FW) (Lamuela-Raventós and others 1995) are by far the richest dietary sources (Zamora-Ros and others 2007).

Chalcones

Chalcones and dihydrochalcones have been reported in a restricted number of foods (Robards and others 1999; Tomás-Barberán and Clifford 2000). Chalconaringenin occurs in tomato skin, but the acid extraction conditions of the usual polyphenol analytic converts the chalcone to the corresponding flavanone (naringenin) in the tomato. The most common dihydrochalcones found in foods are phloretin glucoside (phloridzin) and phloretin xylogalactoside, which are characteristic of apples (Table 2) and derived products such as apple juice, cider and pomace (Robards and others 1999; Tomás-Barberán and Clifford 2000).

Lignans

Lignans are a diverse group of plant-derived compounds that form the building blocks for plant cell walls. The richest source of lignans is flaxseed. Flaxseed contains mainly secoisolariciresinol (0.29-0.21mg/100g), but pinoresinol, lariciresinol and matairesinol are also present in substantial amounts (0.55–3.32mg/100g). Lignan content in beverages (wine, beer, tea, coffee) has been found as follows: South African red wine (91.3µg/100mL), French red wine (78.9µg/100mL), Ceylonese black tea (77.1µg/100mL), English-blend black tea (71.2µg/100mL), coffee (18-31µg/100mL), soja milk (37.7µg/100mL), chocolate milk(2.2µg/100mL) (Milder and others 2005).

Secoiridoids

Oleuropein (the most abundant bitter principle) and its analogue ligstroside, both secoiridoid biophenols, were extracted from Hojiblanca black olives (Piperno and others 2004). Secoiridoids are present exclusively in plants of the Olearaceae family (Tripoli E and others 2005). Oleuropein is present in high amounts (6000 ± 90mg/100g DW) in the leaves of the olive tree (Le Tutour and others 1992), but it is also present in all constituent parts of the fruit peel, pulp and seed (Servili and others 1999).

Benzoic aldehyde

Benzoic aldehyde mainly covers syringaldehyde and vanillin. Natural vanilla is prepared from the seeds (beans) of *Vanilla planifolia*, which may contain some 21mg/100g FW total phenols, including the major components vanillin (19.4mg/100mg FW), 4-hydroxybenzaldehyde (1mg/100g FW) and vanillic acid (0.4mg/100g FW) (Clifford 2000). In mango, vanillin has been found as "free" as well as vanillyl glucoside (Sakho and others 1997). It has also been found in lychees (Ong and Acree 1998), and wines (Moreno and others 2007). For analysis of both brandy and wine aged in oak barrels, the limits of detection were found to be 27.5, 14.25, 14.75, and 19.75µg/100mL for syringaldehyde, coniferaldehyde, sinapaldehyde and vanillin, respectively, which is acceptable (Panossian and others 2001).

Table 2: Non-flavonoids in foods.

Subclass	Polyphenol	Foods	Content (mg/100g FW)	Reference
Phenolics alcohols	Tyrosol	Olive oils	0.29-2.44[£]	(De la Torre-Carbot and others 2005)
	Hidroxytyrosol	Olive oils	0.70-6.35[£]	(De la Torre-Carbot and others 2005)
Hydroxycinnamic acid	*P*-coumaric acid	Blueberries	2.4-15.8	(Sellappan and others 2002)
		Cranberries	2.2-25.4	(Zuo and others 2002)
		Sweet cherries	1.0-6.8	(Kim and others 2005)
		Sour cherries	0.9-4.1	(Kim and others 2005)
		Orange juices	7.9-4.46[£]	(Rapisarda and others 1998)
		Citrus[§]	1.8-19.3[¥]	(Bocco and others 1998)
		Broccoli	130.6 ±42	(Yeh and Yen 2005)
		Eggplant	173.3 ±40	(Yeh and Yen 2005)
		Asparagus	18.3 ±16	(Yeh and Yen 2005)
	Caffeic acid	Blueberries	0-6.32	(Sellappan and others 2002)
		Blackberries	1.38-3.64	(Sellappan and others 2002)
		Cranberries	0.38-15.6	(Zuo and others 2002)
		Potatoes	3.6	(Im and others 2008)
		Sweet potato	0.3-2.2	(Truong and others 2007)
		Carrot	14	(Mattila and Kumpulainen 2002)
		Lettuce	4-55	(Llorach and others 2004)
		Coffee drink	96	(Mattila and Kumpulainen 2002)
	Ferulic acid	Blueberries Blackberries Cranberries	3.02-16.97	(Sellappan and others 2002)
		Orange juices	2.99-3.51	(Sellappan and others 2002)
		Citrus[§]	0.8-8.8	(Zuo and others 2002)
		Carrot	3.07-6.37[£]	(Rapisarda and others 1998)
		Beet root	3.6-158	(Bocco and others 1998)
		Coffee drink	1.57	(Kang and others 2008)
		Broccoli	1.3-14.3	(Ng and others 1998)
		Eggplant	9	(Mattila and Kumpulainen 2002)
		Asparagus	105.9	(Yeh and Yen 2005)
			93.6	(Yeh and Yen 2005)
			46.1	(Yeh and Yen 2005)
	Sinapic acid	Cranberries	0-21.18	(Zuo and others 2002)
		Orange juices	0.78-3.59[£]	(Rapisarda and others 1998)
		Citrus[§]	3.0-95.4[¥]	(Bocco and others 1998)

Hydroxycinnamates or Esters of hydroxycinnamic acid	Chlorogenic acids	Sweet cherries	3.2-12.0	(Kim and others 2005)
		Sour cherries	0.6-5.8	(Kim and others 2005)
		Apricot	3.0-16.5	(Ruiz and others 2005)
		Nectarines	2.3-27.7	(Tomás-Barberán and others 2001)
		Peaches	2.4-24.2	(Tomás-Barberán and others 2001)
		Prunes	41.1-43.6	(Donovan and others 1998)
		Apples	1.93-119.5	(Wojdylo and others 2008)
		Pear	6 -59	(Cui and others 2005)
		Quince pulp	0.56-18.56	(Silva and others 2002)
		Potato	0.35-18.71	(Im and others 2008)
		Sweet potato	4.6-13.6	(Truong and others 2007)
		Coffee drink	96	(Mattila and Kumpulainen 2002)
		Green coffee beans	1158-2741[¥]	(Guerrero and others 2001)
		Tomato	8.5	(Buta and Spaulding 1997)
Volatile phenols	Eugenol	Passion fruit Juice	92	(Chassagne and others 1997)
		Passion fruit peel	172	(Chassagne and others 1997)
		Wines	0.0035	(Fernandez de Simon and others 2003)
			0.0089[£]	
	Guaiacol	Wines	0.002-0.0043[£]	(Fernandez de Simon and others 2003)
	4-Vinylguaiacol	Raw Coffee	0.0117	(Czerny and Grosch 2000)
		Roasted coffee	3.9	(Czerny and Grosch 2000)
	4-Ethylguaiacol	Raw coffee	0.0021	(Czerny and Grosch 2000)
		Roasted coffee	0.406	(Czerny and Grosch 2000)
		Wines	0.0018-0.0029[£]	(Fernandez de Simon and others 2003)
	Methyl salicylate	Passion fruit juice	0.076	(Chassagne and others 1997)
Stilbenes	*trans*-Resveratrol	Red grapes	0.25	(Zamora-Ros and others 2007)
		White grapes	0.07	
	trans-Piceid	Red grapes	0.06	(Zamora-Ros and others 2007)
		White grapes	0.025	
Chalcones	Chalconaringenin	Cherry tomatoes	15.3 (8.3)	(Slimestad and Verheul 2005)
Dihydro-chalcones	Phloretin xylogalactoside	Apples	2.1 (0.9)	(Burda and others 1998)
	Phloridzin	Apples	1 (0.3)	(Burda and others 1998)
Secoiridoids	Oleuropein aglycon	Olive fruit	1.45 (0.05)	(Gómez-Caravaca AM and others 2005)
	Ligstroside aglycon	Olive fruit	0.66 (0.004)	(Gómez-Caravaca AM and others 2005)
Coumarin	Scopoletin	Noni fruit juice	6100[£]	(Surono IS and others 2008)

Lignan	Lariciresinol	Brassica Curly kale Apricot Strawberry Peach Pear	0.599 0.972 0.105 0.117 0.080 0.155	(Milder IE. and others 2005)
	Pinoresinol	Brassica Curly kale Apricot Strawberry Peach Pear	0.1691 0.315 0.314 0.212 0.186 0.034	
	Secoisolarici-resinol	Brassica Pear Curly kale Strawberry Peach Apricot	0.019 0.004 0.038 0.005 0.027 0.031	
	Matairesinol	Brassica	0.012	
Hydrolyzable tannins	Ellagitannins	Pomegranate juice Strawberry Blueberry Raspberry Blackberry Longan seed	156.1[£] 19.8±0.2 0.9±0.1 17.9±0.3 42.4±0.4 23.3	(Navindra P and others 2006) (Mertens-Talcott SU and others 2003) (Mertens-Talcott SU and others 2003) (Mertens-Talcott SU and others 2003) (Mertens-Talcott SU and others 2003) (Soong YY and others 2006)
	Gallotannins	Longan seed	156	(Soong YY and others 2006)
		Mango kernel	1550	(Berardini N and others 2004)
Benzoic aldehyde	Syringaldehyde	Ripe walnut fruit	33.83	(Colaric M and others 2005)
	Vanillin	Orange Tangerine Lemon Lime Grapefruit juice	0.02 0.35 0.041 0.035 0.06	(Goodner KL and others 2000)
Benzoic Acid	Ellagic acid	Strawberries	0.4-2.9	(Da Silva-Pinto M and others 2007)
		Muscadine grapes	66.7	(Pastrana-Bonilla E and others 2003)
	Gallic acid	Muscadine grapes	8.6	(Pastrana-Bonilla E and others 2003)
	4-Hydroxybenzoic acid	White currant	1.8	(Maatta K and others 2001)
		Redcurrant	0.3	
	Protocatechuic acid	Raspberry	6-10	(Macheix JJ and Fleuriet A 1990)

FW: fresh weight; [£]mg/100ml, [¥] mg/100g dry material, [§]Citrus peel and seeds

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EL RESVERATROL

RESVERATROL: DE LA INGESTA DIETÈTICA A MOLÈCULA PROMESA

La ingesta de resveratrol ha estat associada amb diferents activitats biològiques relacionades amb la millora de la salut (Baur i Sinclair, 2006). Per això, en el últims anys, ha esdevingut una "molècula promesa" per al possible desenvolupament de futurs tractaments contra malalties cardiovasculars i aquelles relacionades amb l'envelliment. Tot i aquestes implicacions, altres aspectes, com són la ingesta dietètica o la biodisponibilitat, han de ser considerats abans de fer recomanacions nutricionals o de proposar pautes de tractament. La revisió que es presenta a continuació és un recull de les evidències científiques publicades en aquests aspectes, proposant possibles directrius per al futur amb l'objectiu d'obtenir un coneixement més ampli dels vincles entre el consum de resveratrol i la salut.

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Resveratrol
From dietary intake to promising therapeutic molecule

RAFAEL LLORACH, MIREIA URPI-SARDA, MARIA ROTCHES-RIBALTA, MONTSE RABASSA, CRISTINA ANDRES-LACUEVA*

*Corresponding author

University of Barcelona, Nutrition and Food Science Department, XaRTA, INSA
Igenio-Consolider Program, Fun-c-food (CSD2007-063)
Pharmacy Faculty, Av. Joan XXIII s/n, Barcelona, 08028, Spain

ABSTRACT: Resveratrol (a natural stilbene) intake has been associated with several health-promoting activities. In recent years it has become a promising molecule, mainly for the possible development of future treatments for cardiovascular and aging-related diseases. Despite its health implications, other aspects such as dietary intake and bioavailability should be considered before making nutritional recommendations or proposing treatment guidelines. In this context, this review will attempt to provide evidence of these aspects and will also propose possible approaches for the future towards gaining an understanding of the links between resveratrol intake and health.

KEYWORDS: Resveratrol, dietary intake, bioavailability, health, biomarkers, moderate wine consumption, therapeutic molecule, anti-aging, mimetic of the caloric restriction, metabolomics.

INTRODUCTION

Resveratrol is a phenolic compound belonging to the stilbene group (Figure 1) which can present two isomers *cis*- (Z) and *trans*- (E). This molecule occurs naturally as glucosides (piceid) or polymers (viniferins). Resveratrol and piceid (thereafter R&P) are present mainly in grape and grape products, and also in small amounts in peanuts, pistachios, some berries, tomato skin and chocolate. Resveratrol content in grape-derived foods is highly affected by several factors, such as agronomics, grape genotype, environmental conditions and wine-production technology. This article will review different aspects, such as dietary intake and bioavailability, which have been identified as important modulators of resveratrol bioactivity. Intervention and epidemiological studies have provided evidence of the health benefits of resveratrol intake and resveratrol-rich food consumption (i.e. moderate consumption of red wine). In this context, resveratrol metabolites (RMs) have been proposed as nutritional biomarkers of wine intake in free-living subjects.

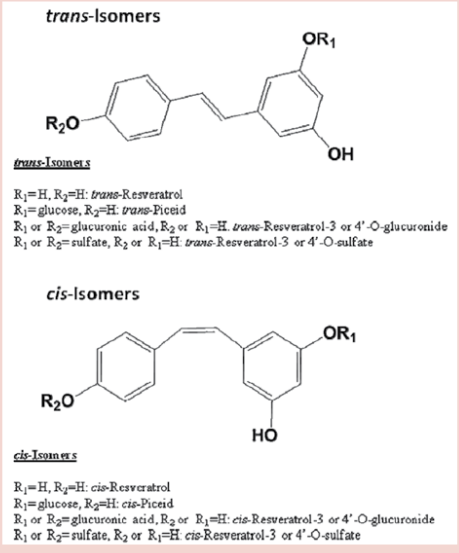

Figure 1. Chemical structures of RMs.

RESVERATROL: DIET COMPOSITION AND DIETARY INTAKE

Resveratrol and other stilbenes have been identified in a large number of plants. However, it has been found in a limited number of dietary components, the most significant being those derived from grape products (i.e. red wine). To our knowledge, little information is available on either the content of resveratrol in the diet or on the estimation of its dietary intake. In this context, Zamora-Ros et al. (1) carried out an attempt to calculate the content of resveratrol and its derivatives in foods in a Spanish cohort (European Prospective Investigation into Cancer and Nutrition (EPIC)-Spain cohort). In addition, this study also estimated the dietary intake of resveratrol. The results showed that the main dietary source of R&P was wine (98.4 percent) and grape and grape juices (1.6 percent), whereas peanuts, pistachios and berries contributed less than 0.01 percent. Considering separately the R&P intake of the two isomeric forms (*cis* and *trans*), wine provides 95 and 98.7 percent of the total dietary intake of *trans*-resveratrol and *trans*-piceid respectively, and 99.9 and 99.7 percent of the total dietary intake of *cis*-resveratrol and *cis*-piceid respectively. Other dietary components, such as grapes, provided 3.8 percent of *trans*-resveratrol.

RESVERATROL BIOAVAILABILITY

The biological activities of resveratrol depend on their dietary intake and bioavailability. Bioavailability has been defined by US FDA's Guidance for industry as the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action (2). In addition, food matrix plays an important role in bioavailability. Due to this possible influence, resveratrol bioavailability has been assayed using different ways of administration. These experiments could be grouped in: ingestion of grape juice, oral administration of resveratrol dissolved in different matrices such as white wine, grape juice and vegetable juice, ingestion of red wine, oral administration of resveratrol and intravenous labelled

resveratrol (3, 4). Despite these considerations, R&P have been assayed in several biological samples such as plasma or serum, LDL, feces and urine.

According to the ingested dose, some differences in absorption between resveratrol and piceid have been detected. At nutritional doses resveratrol has great absorption, while at pharmacological doses similar percentages of resveratrol and piceid were recovered (3, 5). Resveratrol is mainly metabolized to mono- and di-glucuronides and mono- and di-sulfates derivatives (5, 6) (Figure 1). In terms of the maximum concentrations, (C_{max}) resveratrol and piceid differed. Whereas piceid showed its maximum at 6 h (5), resveratrol peaked around 30-60 min (7). As other polyphenols, resveratrol and its metabolites (6, 8) can be transported bound to proteins such as albumin (5) or lipoproteins (i.e. LDL). Recently, Almeida et al. (9) conducted a study to evaluate the pharmacokinetics of *trans*-resveratrol in a rising multiple-dose study, where eight healthy volunteers consumed 25, 50, 100 or 150 mg of *trans*-resveratrol, six times/day, for thirteen doses. The results depicted an important circadian variation in bioavailability, being higher after morning administration. Moreover, repeated oral administration of high daily doses of trans-resveratrol produced relatively low plasmatic concentrations of trans-resveratrol, despite the relatively high doses and frequent dosing regimen used.

As for other polyphenols, such as proanthocyanidins or cholorogenic acid, ingested R&P could be metabolized by the gut microbiota during their passage through the gut. It has been demonstrated that gut microbiota has the ability to reduce resveratrol, producing dihydroresveratrol (3)

Dietary intake and bioavailability as key factors to understand resveratrol health implications

HEALTH RELEVANCE OF RESVERATROL

Several studies have positively associated the consumption of resveratrol with the prevention of a number of diseases. Tables 1 and 2 show scientific evidences in cardiovascular diseases and cancer, respectively. In addition to these studies, a recent review suggested that resveratrol could be an excellent molecule in the treatment of several cardiovascular complications (myocardial ischemia, hypercholesterolemia, and diabetes) due to its ability to regulate several target molecules implied in the protection of myocardium against ischemic injury (10). In the field of cancer, Kundu and Surth (11) have reviewed the potential of resveratrol as a cancer chemopreventive and therapeutic molecule, indicating that it has been shown to inhibit or retard the growth of various cancer cells in culture and implanted tumours *in vivo*. Another aspect to note is the ability of resveratrol to mimic the effects of dietary restrictions in lower organisms and also in mice (12). In fact, elderly mice fed resveratrol reveal an important modification in signs of aging, including decreased inflammation, increased aortic elasticity, greater motor coordination, reduced cataract formation, and preserved bone mineral density (12). In addition to this potential anti-aging activity, resveratrol has been associated with preventing neurodegenerative diseases, probably by activating sirtuin-1, a protein implicated in pathological and physiological states(13). These two factors make resveratrol a very interesting molecule in the development of future treatments for neurodegenerative diseases through the activation of this sirtuin (14).

System	Mechanism of action	Reference
Platelet aggregation	↓thrombin ↓platelet activating factor ↓lipooxygenase and PG H synthetase ↓COX 1 and ~COX 2	(20, 21)
Vasodilation	Stimulate channels K+/Ca2+ ↓COX ↓NADH/NADPH oxidase	(22, 23)
↓ lipid peroxidation	↑ catalase, SOD, glutathion ↓ NAD(P)H oxidase, NO oxidase	(24)
Antiinflammatory	VCAM-1, ICAM-1 ↓ Interleuquin-8 ↓ COX	(25)

Table 1. Scientific evidence of the relevance of resveratrol in cardiovascular diseases.

System	Mechanism of action	Reference*
↓Carcinogenesis	↓ COX ↓ Ornithine decarboxylase (mediator ↓ protein kinase C)	(26, 27)
↓Angiogenesis	Directly ↓COX and ODC	(28, 29)
Metabolism cancer agents	↓cytochrome P450	(30)

Table 2. Scientific evidence of the relevance of resveratrol in cancer.

RESVERATROL AS NUTRITIONAL BIOMARKER OF WINE INTAKE

Epidemiological studies have evidenced widely variable results about the health benefits of moderate wine consumption, probably due to the difficulty of accurately assessing dietary habits (including alcoholic beverage consumption) and physical activity in these studies. This may mean that there are many uncertainties associated with the dietary assessment methods currently used in epidemiological studies to evaluate the intake of red wine. As a result it is assumed that nutritional biomarkers have several advantages over dietary self-reported data, because they reflect a more objective assessment of nutrient intake (15). As described by Spencer et al. (15), robust methodology, sensitivity, bioavailability and specificity are the criteria that must be considered in the development of nutritional biomarkers.

With regards to the methodology, Urpi-Sarda et al. (6, 8) proposed a robust method to characterize the complete profile of resveratrol metabolism in human LDL and urine, using LC-MS/MS. Evaluation of the sensitivity of RMs as nutritional biomarkers of wine intake has been assayed in two randomized crossover studies (n=10 healthy men and n=10 healthy women) and in a pilot cohort study on the effects of a Mediterranean diet on the primary prevention of cardiovascular disease with 52 participants (the PREDIMED Study) (16). In this study, the authors were able to differentiate wine consumers from abstainers with a sensitivity (the proportion of consumers correctly classified) of 72 percent (95 percent confidence interval (CI) 60-84 percent)

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and a specificity (the proportion of non-consumers properly classified) of 94 percent (CI 87-100 percent), using an RMs cut-off of 90 nmol/g creatinine. Recently, this sensitivity has been estimated in 1000 free-living participants in the same PREDIMED study (17), and using a cut-off of 411.4 nmol/g creatinine, the measurement of urinary RMs could discriminate wine consumers from non-wine consumers with a sensitivity of 93.3 percent (CI 91.5-94.7 percent) and a specificity of 92.1 percent (CI 90.2-93.7 percent) (17). Overall, these data indicate that urinary RMs fulfil the criteria to be considered as nutritional biomarkers of wine consumption in a short intervention and a large sample of free-living subjects.

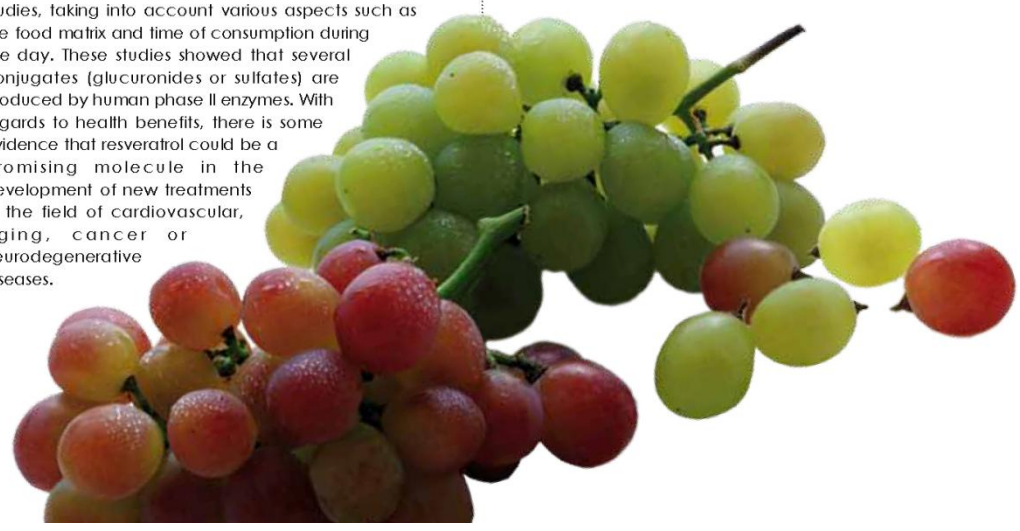
Resveratrol has demonstrated to be great biomarker of moderate wine consumption

FUTURE TRENDS

Despite the significant amount of literature that provides evidence of the benefits of regular intake of resveratrol, some important questions such as individual differences, recommended intake, interactions with other dietary components or the role of microbiota on the metabolism of resveratrol remain unanswered. Further human studies to evaluate the impact of synergistic or antagonistic effects between R&P and other dietary components should be considered, mainly when red wine (i.e. alcohol intake) has been identified as an important dietary source of this molecule. In this context, it is also necessary to investigate through *in vivo* studies the real role of microbiota on the metabolism and absorption of resveratrol. The application of a new approach termed metabolomics could be an interesting and promising move towards addressing these questions. Metabolomics aims to provide a comprehensive picture of the chemical composition of a biological sample (metabolome). An important challenge of metabolomics or metabonomics is to obtain biomarkers that reflect the intake of dietary components and the expected or unexpected effect of their consumption. In this context, metabolomics has been successfully applied to evaluate the effect of the intake of pure polyphenols (18) and also polyphenol-rich food, such as tea, cocoa or wine, (19) on the metabolome.

Future investigations about individual differences, interactions with other dietary component or microbiota role on the metabolism of resveratrol a necessary way to establish possible resveratrol intake recommendations

In summary, dietary intake of resveratrol has been significantly associated with red wine consumption and hardly at all with other possible dietary sources. The bioavailability of resveratrol has been reviewed in different studies, taking into account various aspects such as the food matrix and time of consumption during the day. These studies showed that several conjugates (glucuronides or sulfates) are produced by human phase II enzymes. With regards to health benefits, there is some evidence that resveratrol could be a promising molecule in the development of new treatments in the field of cardiovascular, aging, cancer or neurodegenerative diseases.



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BIODISPONIBILITAT DEL RESVERATROL

CONCEPTE DE BIODISPONIBILITAT

Segons la *Food and Drug Administration* (FDA), la biodisponibilitat es pot definir com la velocitat d'un component actiu i de la quantitat que, absorbit a partir de la forma de dosificació que el conté, es fa disponible en el lloc d'acció (Chen *et al*., 2001). Quan un compost actiu és administrat per via intravenosa, la totalitat de la dosis administrada accedeix al corrent sanguini, essent biodisponible en un 100%. Ara bé, quan el compost actiu és administrat per altres vies, com la oral, l'accés del compost a la circulació sistèmica tindrà lloc després d'un procés d'absorció, en el qual es podrà veure alterat químicament per l'efecte hepàtic de primer pas, que implica la seva metabolització; reduint-se, així, la seva biodisponibilitat. Amb aquesta definició és fàcil veure que seran nombrosos els factors que poden influenciar la biodisponibilitat d'un compost actiu; ja des de la seva estructura química i solubilitat, fins a l'efecte matriu de la font alimentària que el conté, tenint en compte l'edat, gènere, possibles afectacions gastrointestinals de l'individu, etc.

L'estudi de la biodisponibilitat d'un compost actiu és d'elevat interès abans de poder extrapolar certa activitat biològica observada *in vitro* a un organisme *in vivo*. De fet, ja la normativa europea (CE nº1924/2006) contempla aquest paràmetre, requerit en l'estudi previ a la sol·licitud d'al·legacions nutricionals d'un producte alimentari; ja que es demana que una quantitat suficient del compost actiu arribi a ser disponible en els teixits per a exercir la seva activitat biològica després del consum d'una dosi raonable del producte en qüestió.

Absorció, metabolisme i excreció

Després de l'administració oral d'un compost actiu, aquest pot ser absorbit per l'organisme després d'un procés d'alliberació de la matriu alimentària que el conté i de la seva digestió a l'estómac i al tracte gastrointestinal. Durant aquest procés d'absorció a l'enteròcit, el compost actiu podrà patir biotransformacions (Manach *et al*., 2004). Els compostos que no romanguin a l'intestí podran ser metabolitzats per acció microbiana abans de la seva absorció (Manach *et al*., 2004). Un cop al corrent sanguini el compost serà dirigit al fetge des d'on es distribuirà cap als teixits, en els que podrà exercir l'efecte biològic (Figura 1). Abans, però, aquest podrà patir l'efecte de primer pas, que defineix l'alteració química resultant de la biotransformació d'un xenobiòtic abans de que arribi al sistema circulatori i que dóna lloc als metabòlits (Manach *et al*., 2004). Aquestes biotransformacions podran ser de fase I: oxidació, reducció o hidròlisi, on el compost actiu perdrà part de la seva estructura base, o biotransformacions de fase II, anomenades també conjugacions, que impliquen la formació de noves substàncies més hidrosolubles per addició d'un grup sulfat, glucurònid o metil i on el compost original forma part de la nova molècula (Manach *et al*., 2004). El resultat d'aquesta biotransformació pot ser la inactivació completa o parcial de l'activitat del compost (Miksits *et al*., 2009), l'augment o activació d'aquesta o el canvi per un nou efecte biològic depenent de les característiques de la substància biotransformada.

Els metabòlits formats seran eliminats de l'organisme mitjançant dues vies majoritàries d'excreció: hepàtica i renal. Principalment, els compostos suficientment hidrosolubles es deriven cap als ronyons, essent excretats en orina. En canvi, els compostos liposolubles o de major pes molecular són excretats en la bilis, arribant a l'intestí gruixut on pot sofrir una recirculació enterohepàtica, tornant a ser absorbits, o bé ser finalment eliminats en la femta.

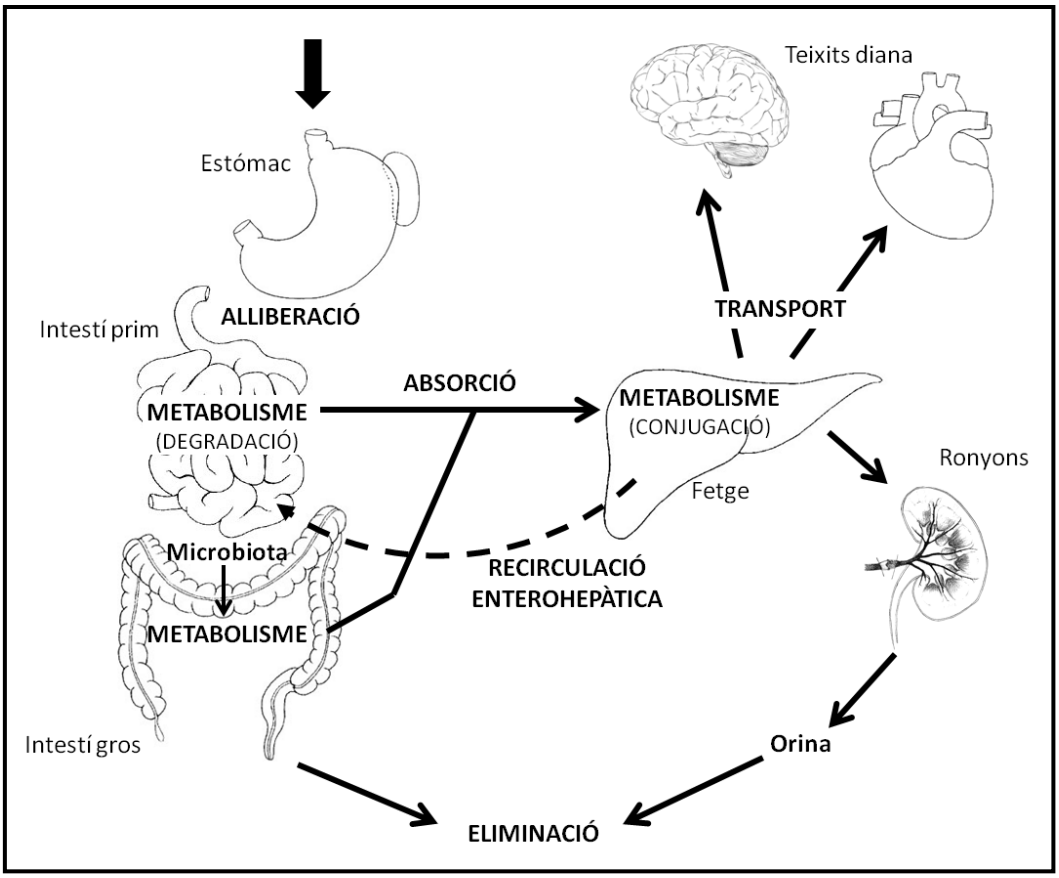


Figura 1. Esquema del procés d'absorció, metabolisme i excreció.

FONTS ALIMENTÀRIES I EFECTE MATRIU EN LA BIODISPONIBILITAT DEL RESVERATROL

El resveratrol i altres estilbens, com ara el piceid, s'han identificat en un gran nombre de plantes, tot i que formen part de pocs components de la dieta, essent els derivats del raïm i el vi negre les principals fonts de consum en la societat occidental (Zamora-Ros *et al.*, 2008). El consum de resveratrol s'ha associat positivament amb la prevenció d'una sèrie de malalties, donada l'activitat antioxidant i els efectes antiinflamatoris i antitumorals que se n'han descrit. Tot i això, pocs estudis s'han realitzat en éssers humans i els resultats que se'n deriven són objecte de controvèrsia, donada la baixa biodisponibilitat que se n'ha reportat. I és que abans de conèixer els efectes del consum d'aquesta molècula és important tenir en compte que el resveratrol es metabolitza ràpidament i, per tant, la bioactivitat d'aquest es relaciona amb els seus metabòlits. Així doncs, la identificació i quantificació de les formes metabòliques són

requisits essencials per a la comprensió del paper d'aquests compostos, ja que són els que arribaran a la circulació sistèmica i als teixits on podrien exercir el seu efecte biològic. Els metabòlits de fase II del resveratrol han estat àmpliament estudiats, però pocs estudis se centren en el metabolisme microbià de resveratrol. A més, la matriu en què es lliura el resveratrol podria tenir un paper important en la seva biodisponibilitat.

El capítol titulat "*Sources and Food Matrix on the Bioavailability of Resveratrol*" que es presenta a continuació forma part del llibre "*Resveratrol: Sources, Production and Health Benefits*" (Nova Science Publishers, 2012 – ISBN: 978-1-62257-072-0) i és un recull bibliogràfic de la biodisponibilitat i metabolisme *in vivo* del resveratrol així com del seu perfil metabòlic descrit fins al moment. A més a més, es recullen els estudis sobre l'efecte de la matriu alimentaria en la biodisponibilitat del resveratrol, un cop presentades les fonts alimentaries que el contenen.

Chapter 20

SOURCES AND FOOD MATRIX ON THE BIOAVAILABILITY OF RESVERATROL

Maria Rotches-Ribalta[a,b#], Mireia Urpi-Sarda[c,d], Sara Tulipani[a,b], Pilar Trespalacios[a,b], Olha Khymenets[a,b], Montse Rabassa-Bonet[a,b], Maria Boto-Ordóñez[c,d], Rosa Vazquez-Fresno[a,b], Mar Garcia-Aloy[a,b], Rafael Llorach[a,b] and Cristina Andres-Lacueva[a,b*]

[a]University of Barcelona, Av Joan XXIII, s/n, Barcelona, Spain.
[b]Ministry of Science and Innovation, Barcelona, Spain
[c]Institut d'Investigació Biomèdica August Pi i Sunyer, Barcelona, Spain
[d]Instituto de Salud Carlos, Barcelona, Spain

ABSTRACT

Resveratrol consumption has been positively associated with the prevention of a number of diseases, mainly due to its reported antioxidant activity and anti-inflammatory and antitumor effects, and has, therefore, the potential to be evaluated for therapeutic use. However, scarce studies have been carried out in humans and the results yielded are still controversial. Before making nutritional recommendations regarding this promising molecule, other aspects such as dietary intake and bioavailability should be considered.

Resveratrol and other stilbenes, such as piceid, have been identified in a large number of plants, although they have been found in a few dietary components, with red wine and grape products as the major sources. Therefore, urinary resveratrol metabolites have been described as nutritional biomarkers of wine consumption, providing an additional tool for clinical and epidemiological studies, since they reflect a more objective assessment of nutrient intake than self-reported dietary data. Its estimated intake or exposure is essential in order to relate resveratrol consumption with its possible health effects *in vivo*. However, resveratrol is rapidly metabolized, and therefore the bioactivity of this compound will be related to its metabolites. Identification and measurement of metabolic forms are key prerequisites for understanding the role of these compounds, since they will be the ones that reach the peripheral circulation and tissues where they could exert their biological effect. Phase II metabolism of resveratrol has been widely studied, with the glucuronide and sulfate forms being the main resveratrol conjugates. However, few studies have focused on the microbial metabolism of resveratrol, which leads to dihydroresveratrol formation,

[#] [a]Nutrition and Food Science Department, XaRTA-INSA. Pharmacy Faculty, University of Barcelona, Av Joan XXIII, s/n, Barcelona, Spain.
[b]Ingenio-CONSOLIDER program, FUN-C-FOOD, Spain CSD2007-063. Ministry of Science and Innovation, Barcelona, Spain.
[c]Department of Internal Medicine, Hospital Clinic, Institut d'Investigació Biomèdica August Pi i Sunyer (I. D. I. B. A. P. S.), Villarroel 170, 08036 Barcelona, Spain.
[d]CIBER 06/03: Fisiopatología de la Obesidad y la Nutrición, Instituto de Salud Carlos III.
[*] E-mail: *candres@ub.edu.*

although this could give a better idea about effects of long-term resveratrol consumption. Furthermore, the matrix in which resveratrol is delivered could play an important role in its bioavailability.

This chapter, therefore, provides an overview of the metabolism of resveratrol after short- and long-term consumption and the microbiota and food matrix effects on its bioavailability. This needs to be considered in order to gain a better understanding of the links between resveratrol intake and health.

INTRODUCTION

Interest in resveratrol has increased in recent years due to its well-known health-related properties [1]. The protective effect of red wine consumption against cardiovascular diseases was first linked to resveratrol antioxidant activity, along with its anti-inflammatory effect, in the early 90s [2], as highlighted in the "French Paradox" [3]. After that, several studies focused on this molecule, which also demonstrated chemopreventive effects with antitumor activity [4]. Neuroprotective properties of resveratrol have also been observed [5], with a slow the progression of Alzheimer's disease [6]. More recently, resveratrol has been identified as a sirtuin-activating compound, since it is able to mimic caloric restriction [7]. Indeed, resveratrol has been related to an enlargement of the lifespan of several species [8, 9]. With all these biological activities leading to health improvement, resveratrol has become a promising molecule, although its low bioavailability has generated some criticism.

In this chapter, several bioavailability studies on resveratrol, as well as its glucoside, are reviewed in order to better understand the first steps towards their health-promoting activities. Bioavailability is defined as the rate and extent to which the active compound is absorbed and becomes available at the site of action [10], where it could exert its potential beneficial effect. Thus, health effects related to resveratrol would be influenced by its bioavailability, along with the food matrix, which plays an important role in this process. For this reason, this review focuses on food matrix effect studies, which should be considered before making nutritional recommendations regarding resveratrol. In fact, the use of supplements could be an interesting way to ingest resveratrol and take advantage of its biological activities, since chronic and non-moderate alcohol intake from wine has been associated with certain diseases [11].

RESVERATROL SOURCES

Resveratrol, or 3,4',5-trihydroxystilbene, is the most representative stilbene in natural products, together with its glucoside, called piceid [12]. Although these bioactive compounds have been found in many different plants [13], they are part of only a few dietary components with low and variable concentrations, since resveratrol is a phytoalexin [14]. The main resveratrol and piceid food sources are grapes and grape products, such as wine, although their content is highly affected by grape variety, climate, fungal pressure, degree of maturity at harvest and wine-making technology [15]. Resveratrol has also been found in peanuts, pistachios, berries, tomatoes, chocolate, beer, etc. [1, 15-18] but, when their serving size was considered, these become minor food sources (Table 1). Itadori Tea, which is prepared from *Polygonum cuspidatum Sieb. et Zucc.* [19], could be considered a good source of resveratrol bearing in mind its content and serving size. However, red wine is widely consumed around the world and contributes 98% of the resveratrol and piceid consumption in the Spanish population [15]. With these intake values, resveratrol metabolites have been proposed as nutritional biomarkers of wine intake [20, 21]. Indeed, their urinary values fulfill the criteria that must be considered in the development of such biomarkers: robust methodology, sensitivity, specificity and bioavailability [22]. Nutritional biomarkers, which reflect a more objective

assessment of nutrient intake than self-reported dietary data, may be a better measure of dietary exposure and therefore could be used in clinical and epidemiological studies.

As well as all these previously reported resveratrol and piceid sources, resveratrol nutraceuticals cannot be ignored, as they have become important sources of this compound since its many health benefits have been known. These supplements are mainly prepared from *Vitis vinifera* L. or grape pomace extracts, although many of them are enriched with purified resveratrol from the root of the Japanese knotweed *Polygonum cuspidatum Sieb. et Zucc* [23].

Table 1. Mean values of *trans*- and *cis*-resveratrol and *trans*- and *cis*-piceid content of different foods, together with their total values. Values are expressed as µg per liter or kilogram and total values also per serving

Food	Total Resveratrol			*trans*-Resveratrol	*cis*-Resveratrol	*trans*-Piceid	*cis*-Piceid	References
	(µg/kg)	(µg/serving)	serving	(µg/kg)	(µg/kg)	(µg/kg)	(µg/kg)	
Red grapes	3100	310	100 mL	2500	tr	600	-	[15]
White grapes	930	93	100 mL	680	tr	250	-	[15]
Grape juice	890	222	250 mL	100	tr	360	430	[15, 16]
Red wine	8470	2118	250 mL	1810	440	4950	1270	[15, 16]
White wine	740	185	250 mL	100	160	260	220	[15, 16]
Peanuts, toasted	55	1.7	30 g	55	-	-	-	[1]
Peanuts, butter	790	24	30 g	650	-	140	-	[15]
Pistachios, toasted	70	2.1	30 g	70	-	-	-	[15, 16]
Berries, not specified	80	3.2	40 g	80	-	-	-	[15, 16]
Tomatoes[a]	13	1.3	100 mL	11	2	tr	tr	[16, 18]
Chocolate[b]	3325	133	40 g	692	-	2633	-	[16, 17]
Beer	14.5	4.8	330 mL	9	3.1	0.5	1.9	[16]
Itadori tea	9740	2435	250 mL	680	-	9060	-	[1]

tr, traces.

[a]Skin tomato values (5% of dry weight and 92% humidity) from MicroTom, Beafsteak, UglyRipe, Heirloom and PlumTom varieties.

[b]Including cocoa powder, unsweetened, semi-sweet, dark and milk chocolate, and chocolate syrup.

RESVERATROL BIOAVAILABILITY

In vivo studies on resveratrol bioavailability date back to 1996, when several studies led by Bertelli were published [24-27]. In these works, resveratrol absorption and its bioavailability in rats was evaluated after both a single and a long-term red wine intake, also taking tissue accumulation into consideration. For the single-dose study, 86 µg/kg of total resveratrol from red wine were administered to 36 male Wistar rats by gastric intubation, while 6 more were considered as control. Resveratrol was first observed in plasma 30 minutes after this consumption, reaching maximum concentrations 1 hour after red wine intake. At this time the highest concentrations were already reached in both liver and kidneys, which are the main excretion route for this polyphenol. Resveratrol concentrations were also observed in heart, although up to 2 hours were needed to reach the maximum values [25, 27]. For the regular-dose study, a total resveratrol amount of 43 µg/kg from red wine was intragastrically administered every day to 36 male Wistar rats for 15 days. Long-term administration of red wine could lead to an increased resveratrol concentration in different

tissues, since the amount of resveratrol found after a punctual intake was lower than that required for pharmacological activity [24, 26]. However, only free *trans*- and *cis*-resveratrol were considered in these previous results, which could be conjugated rapidly after absorption, mainly to sulfate and glucuronide forms [28]. In fact, it was reported that free resveratrol in plasma either represented less than 2% of the total resveratrol consumed or was not detectable at all [29].

Despite the extensive metabolism of resveratrol, there are some other studies also focused on the analysis of free aglycone in biological samples, ignoring completely its metabolic profile. Soleas et al. in 2001 focused their analyses on free *trans*-resveratrol after a low ingested dose of this compound, obtaining urine recoveries of up to 14.7% with low plasma concentrations from 2.5 to 5.7 μg/L (Table 2) [30]. Lower *trans*-resveratrol concentrations of 550 ng/L were obtained in the plasma of Sprague Dawley rats administered with 2 mg/kg [31]. Some years later, Chen et al. carried out a pharmacokinetic study after intravenous administration of both aglycone isomers, *trans*- and *cis*-resveratrol, which were only analyzed in rat blood, with approximately concentrations of 8 μg/L [32]. Only piceid was found in biological samples of rats after its high dose administration (50 mg/kg), obtaining also low plasma concentrations of 0.36 μg/L [33]. Some studies, looking only for the free aglycone, were also performed in humans after the intake of *trans*-resveratrol capsules [34-36], or the pure compound [37], underestimating its urine and plasma concentrations (Table 3).

The resveratrol metabolic profile after its consumption was first studied by Yu et al. in Sprague Dawley rats (20 mg/kg) and in Balb/c mice (20 and 60 mg/kg) [38]. Only *trans*-resveratrol-3-*O*-glucuronide (Figure 1) was identified in rat urine, although *trans*-resveratrol-3-*O*-sulfate (Figure 1) was even identified in the serum of mice (Table 4), as well as in rat hepatocytes. The resveratrol sulfate showed higher concentrations than those obtained for the glucuronide conjugate, defining sulfation as the main way of resveratrol metabolism. Both conjugates were also identified in some tissues of mice after the intake of 240 mg/kg of *trans*-resveratrol [39], although the exact isomer could not be distinguished. After a single oral dose of a P. cuspidatum extract, containing over 10 mg/kg of resveratrol, unchanged aglycone was observed in rat urine, as well as in bile, with a 24-h excretion value of 0.59% of the dosage, since it was found mainly as a glucuronide and a sulfate phase II metabolites [40]. The glucuronide conjugate was also identified by Asensi et al. in rat urine after consumption of 20 mg/kg of resveratrol [41], together with another unidentified peak, which could be a sulfate conjugate [38]. In this work, a pharmacokinetic study of resveratrol was also performed in rats, mice and rabbits with plasma concentrations of *trans*-resveratrol from 159.6 to 592.8 μg/L (Table 2). Although only free resveratrol was determined, metabolic profile was also considered, since plasma samples were pretreated with β-glucuronidase [41]. Resveratrol glucuronides were also determined after hydrolysis with β-glucuronidase of plasma samples from Sprague Dawley rats after administration of 50 mg/kg of *trans*-resveratrol, with 16-fold higher concentrations than those obtained for the aglycone [42]. With this pretreatment, resveratrol glucuronide was determined indirectly as aglycone by subtracting the concentrations of the sample without hydrolysis from the concentrations measured in pretreated samples. This procedure was also applied by Meng et al. in the urine samples of rats receiving oral doses of 2 or 5 mg/kg of pure resveratrol [43] in order to quantify resveratrol glucuronides (Table 3), which represents more than 89% of total resveratrol quantified. Sulfates were also quantified using this procedure, as carried out by Juan et al., where plasma and tissues from Sprague Dawley rats, obtained after 15 mg/kg of resveratrol intravenously administered, were incubated with β-glucuronidase or sulfatase to quantify both resveratrol conjugates, together with the aglycone [44]. Glucuronide conjugates were almost 4-fold higher than the algycone and also than the sulfate metabolites, disagreeing previously reported results [38].

Table 2. Resveratrol bioavailability studies in animal models

Species	Source	Oral Dosage	Maximum Plasma Concentration	Urinary Excretion (Time)	Studied Tissues	Studied compounds	References
Wistar rats.	Red wine.	4 ml * 6.5 mg/l	20.5 µg/L	---	Heart, liver and kidney.	Free *trans*-/*cis*-resveratrol.	[24]
		2 ml * 6.5 mg/l * 15 days	6.8 µg/L	66.1% (24h)			
Wistar rats.	[^{3}H] *trans*-resveratrol in 10% (v/v) ethanol, V-8 homogenized vegetable or white grape juice.	120 nCi	~600 dpm/mL	49-61% (24h)	Liver, kidney, heart and spleen.	Radioactivity.	[30]
	trans-resveratrol.	0.5 mg 1.5 mg 2.5 mg	2.5 µg/L 3.6 µg/L 5.7 µg/L	2.5% (24h) 7.4% (24h) 14.7% (24h)	---	Free *trans*-resveratrol.	
C57BL/6J mice.	*trans*-resveratrol.	20 mg/kg	592.8 µg/L	2% (24h)	Liver, kidney, lung and brain.	Resveratrol metabolites (Table 4).	
Wistar rats.	*trans*-resveratrol.	20 mg/kg	159.6 µg/L				
ESD NZW rabbits.	*trans*-resveratrol.	20 mg/kg	250.8 µg/L				
Sprague Dawley rats.	*trans*-resveratrol.	2 mg/kg	550.0 ng/L	---	---	Resveratrol metabolites (Table 4).	[31]
Sprague Dawley rats.	*trans*-resveratrol.	50 mg/kg	1.5-24 mg/L	---	---	Resveratrol and its glucuronides by hydrolisis.	[42]
Sprague Dawley rats.	Resveratrol.	20 mg/kg	---	n.q.	---	Resveratrol metabolites (Table 4)	[38]
Balb/c mice.	Resveratrol.	20 mg/kg and 60 mg/kg	1.1-3.0 mg/L	---	---		
CF-1 mice.	Grape preparation (38 µmol of total resveratrol).	9.2, 18.4, and 36.8%	---	0.9-2.3%	---	Resveratrol metabolites by hydrolisis.	[43]
Wistar rats.	Resveratrol.	2 or 5 mg/kg	9.1-25 µg/L	---			
C57BL/6J mice.	*trans*-resveratrol.	240 mg/kg	7.3 mg/L	---	Liver, kidney, heart, small intestine, colon, lung and brain.	Resveratrol metabolites (Table 4).	[39]
	trans-3,4,5,4'-tetramethoxystilbene.	240 mg/kg	1.5 mg/L	---			
Balb/c mice.	[^{14}C]-*trans*-resveratrol.	7.4, 74 and 92.5 kBq	$1.25 \cdot 10^{3}$ dpm/mL	$2.86 \cdot 10^{3}$ dpm/mL (3h)	Duodenum, colon, liver, heart, spleen, kidney, lung, testis and brain.	Radioactivity.	[70]
Sprague Dawley rats.	*trans*-resveratrol.	20 mg/kg	---	n.q.	---	Resveratrol metabolites (Table 4).	[48]

Table 2. (Continued)

Species	Source	Oral Dosage	Maximum Plasma Concentration	Urinary Excretion (Time)	Studied Tissues	Studied compounds	References
Wistar rats.	*trans*-resveratrol.	300 mg/kg daily * 8 weeks	0.4-7.5 µg/L	54% (after treatment).	Liver and kidney.	Resveratrol metabolites (Table 4).	[45]
		50 mg/kg daily * 8 weeks	n.d.	15% (after treatment).			
Sprague Dawley rats.	*trans*-resveratrol + [^{3}H] *trans*-resveratrol.	50 mg/kg + 1.85 MBq	0.48% dpm	3.3% (18h)	Liver, kidney, spleen, heart, small intestine, large intestine, lung and brain.	Radioactivity + *trans*-resveratrol and metabolites.	[71]
Wistar rats.	Piceid.	50 mg/kg	0.36 µg/L	---	Heart, liver, spleen, lung, kidney, stomach, small intestine, brain and testis.	Piceid.	[33]
Sprague Dawley rats.	Piceatannol. Pinosylvin Rhanpontigenin.	10 mg/kg	~10 µg/L ~5 µg/L ~8 µg/L	1-1.6 mg 0.3-1 mg 50-200 mg	---	Free aglycones and their glucuronides after hydrolisis.	[64]
Sprague Dawley rats.	*trans*- and *cis*-resveratrol.	5.13 mg/kg and 4.87 mg/kg, respectively (i.v)	~8 µg /L	---	---	*trans*- and *cis*-resveratrol.	[32]
Wistar rats.	Piceid.	150 mg/kg	0.8-26.2 mg/L	---	---	Piceid, Resveratrol and Resveratrol-glucuornide.	[62]
Sprague Dawley rats.	*Smilax china* extract.	180 mg/kg Oxyresveratrol 80 mg/kg Resveratrol	 5.35 µg/L 2.19 µg/L	---	---	Oxyresveratrol and resveratrol.	[68]
Sprague Dawley rats.	*Polygonum cuspidatum* extract.	20 mg/kg	---	0.3-0.8% (24h)	Brain, heart, lungs, liver, spleen, stomach, small intestine and kidneys.	Resveratrol metabolites (Table 4).	[40]
Pigs (25% Landrace, 25% Large White, 50% Duroc).	Resveratrol.	6.25 mg/kg	0.2-2.2 µg/L	---	---	Resveratrol metabolites (Table 4).	[46]
Sprague Dawley rats.	*trans*-resveratrol.	15 mg/kg (i.v.)	17.8 mg/L	---	Brain, testes, liver, lungs and kidney.	Resveratrol metabolites using hydrolisis.	[44]
Sprague Dawley rats.	Dihydroresveratrol.	60 mg/kg	50.2-7524 µg/L	---	---	Dihydroresveratrol and metabolites (Table 4).	[49]

Table 2. (Continued)

Species	Source	Oral Dosage	Maximum Plasma Concentration	Urinary Excretion (Time)	Studied Tissues	Studied compounds	References
CD rats.	*trans*-resveratrol. Pterostilbene.	50 mg/kg/day * 2weeks 150 mg/kg/day * 2weeks 56 mg/kg/day * 2weeks 168 mg/kg/day * 2weeks	0.2-2.0 mg/L 2.6-10.5 mg/L 0.5-2.0 mg/L 2.0-2.4 mg/L	---	---	Resveratrol, pterostilbene and its metabolites.	[69]
Sprague Dawley rats.	Piceid.	20 mg/kg	---	n.q.	---	Resveratrol metabolites (Table 4).	[63]

n.q.: Not Quantified; n.d.: Not Detected; i.v.: Intravenous.

Table 3. Resveratrol bioavailability studies in humans

Source	Dosage	Studied compounds	Maximum Plasma Concentration	Urinary Excretion (Time)	References
Pure *trans*-resveratrol in 120 mL white wine.	25 mg	Resveratrol and its metabolites by hydrolisis.	338 µg/L	24.6% (24h)	[51]
Pure *trans*-resveratrol in 100 mL white wine.	25 mg	Resveratrol and its metabolites by hydrolisis.	416 µg/L	17.0% (24h)	[50]
Pure *trans*-resveratrol in 100 mL vegetable juice.			471 µg/L	16.8% (24h)	
Pure *trans*-resveratrol in 100 mL grape juice.			424 µg/L	16.0% (24h)	
Pure *trans*-resveratrol in 5:50 (v/v) whiskey:water.	0.03 mg/kg	Resveratrol and its glucuronides by hydrolisis.	n.d.	52% (24h)	[43]
	0.5 mg/kg		n.d.	34% (24h)	
	1.0 mg/kg		n.d.	26% (24h)	
200 mL grape juice.	0.32 mg		n.d.	n.d.	
400 mL grape juice.	0.64 mg		n.d.	n.d.	
600 mL grape juice.	0.96 mg		n.d.	< 1%	
1200 mL grape juice.	1.92 mg		n.d.	< 1%	
^{14}C-*trans*-resveratrol.	25 mg	Radioactivity.	491 µg/L	53.4–84.9% (72h)	[54]
Pure *trans*-resveratrol.	100 mg	Resveratrol metabolites (Table 4).	< 5 µg/L	22-44% (12h)	
300 mL Red wine + standard meal.	0.25 mg	Resveratrol metabolites (Table 4).	0.16 µg/L	---	[58]
600 mL Red wine.	1.92 mg		0.90 µg/L		

Table 3. (Continued)

Source	Dosage	Studied compounds	Maximum Plasma Concentration	Urinary Excretion (Time)	References
600 mL Red wine + fat/lean meal.	0.48 mg		0.54 μg/L		
250 mL Red wine.	5.38 mg	Resveratrol metabolites (Table 4).	112 ng/mg LDL protein	---	[59]
300 mL/day sparkling wine * 4 weeks.	0.357 mg	Resveratrol glucuronides.	n.d.	~112 nmol/g creat. (MU)	[20]
200 mL/day red wine * 4 weeks.	2.56 mg		n.d.	~610 nmol/g creat. (MU)	
200 mL/day white wine * 4 weeks.	0.398 mg		n.d.	~280 nmol/g creat. (MU)	
Resveratrol capsules.	1000 mg	Resveratrol metabolites (Table 4).	---	58% (4h)	[56]
Resveratrol caplets.	500 mg	Resveratrol metabolites (Table 4).	1.1 mg/L	77% (4h)	[55]
	1000 mg		2.1 mg/L		
	2500 mg		2.8 mg/L		
	5000 mg		4.3 mg/L		
250 mL Red wine.	5.38 mg	Resveratrol metabolites (Table 4).	45 ng/mg LDL protein	~11 μmol/g creat. (4h)	[60]
Pure *trans*-piceid in 100 mL ethanol (15%) and 400 mL low-fat milk (1.5%).	85.5 mg	Resveratrol metabolites (Table 4).	2.9 μg/L	35.7% (48h)	[57]
Resveratrol capsules in fasting conditions.	400 mg	Free resveratrol.	47.3 μg/L	---	[34]
Resveratrol capsules + high fat meal (>850 kcal).			42.2 μg/L		
Resveratrol capsules every 4h for 48h.	25 mg	Free resveratrol.	3.89 μg/L	---	[36]
	50 mg		7.39 μg/L		
	100 mg		23.1 μg/L		
	150 mg		63.8 μg/L		
Resveratrol capsules.	200 mg	Free resveratrol.	28 μg/L	---	[35]
Resveratrol capsules every 8h for 3 days.	200 mg		34.5 μg/L		
Pure *trans*-resveratrol + standard meal (500 kcal) every day * 1 week.	2000 mg	Free resveratrol.	1274 μg/L	---	[37]
Pure *trans*-resveratrol + high-fat meal (750 kcal) every day * 1 week.			689 μg/L		
Pure *trans*-resveratrol + quercetin (500 mg) twice a day * 1 week.			1272 μg/L		
Pure *trans*-resveratrol + quercetin (500 mg) + 100 mL alcohol (5%) twice a day * 1 week.			1296 μg/L		
250 mL Red wine.	2.1 mg	Resveratrol and its metabolites by hydrolisis.	29.2 μg/L	26.4% (24h)	[52]
1 L Grape juice.	1.16 mg		32.1 μg/L	14.2% (24h)	
Resveratrol tablets.	1.14 mg		6.2 μg/L	5.1% (24h)	

Table 3. (Continued)

Source	Dosage	Studied compounds	Maximum Plasma Concentration	Urinary Excretion (Time)	References
375 mL Red wine. 15 Grape extract tablets.	6.5 mg 4.9 mg	Resveratrol metabolites (Table 4).	296.3 μg/L 85.7 μg/L	96.3 mg (24h) 125.2 mg (24h)	[73]
272 mL Red wine. 272 mL Dealcoholized red wine.	6.2 mg 6.3 mg	Resveratrol metabolites (Table 4).	---	6.0 μmol (24h) 6.3 μmol (24h)	[74]

n.d.: Not Detected.
MU: Morning Urine

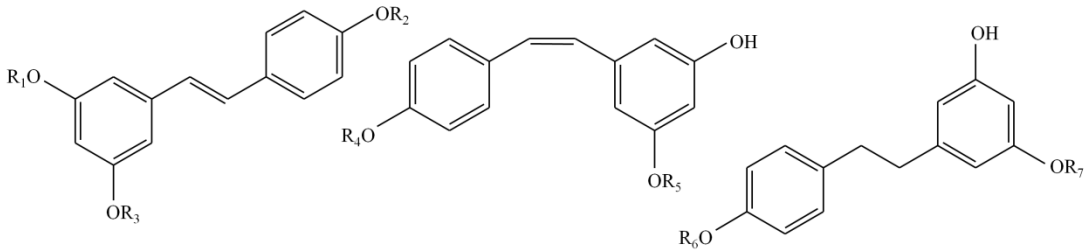
Table 4. Resveratrol and its derivates identified in both animal models and humans

Resveratrol and derivates.		Animal models			Humans	
		Plasma, blood or serum	Urine	Tissues	Plasma	Urine
Resveratrol aglycone.		[24, 30, 31, 39, 42, 43, 46, 69]	[24, 30, 40, 43, 45, 63]	[24, 39-41, 45]	[34-37, 43, 50-52]	[43, 50-52, 55, 56]
	trans-resveratrol.	[32, 38, 41, 44, 62, 68]	[38, 41]	[44]	[58] [59, 60]*	
	cis-resveratrol.	[32]				
Resveratrol glucosides.		[33]		[33]		
	trans-piceid.	[62]			[73] [59]*	[61, 73]
	cis-piceid.				[73]	[61, 73]
	Piceid-glucuronide.		[63]		[73]	[61, 73]
	Piceid-sulfate.		[63]			[61, 73]
Resveratrol phase II metabolites.						
	Resveratrol-glucuronides (not specified).	[31, 42-44, 46, 69, 71]	[40, 41, 43, 48, 71]	[39, 40, 44, 71]	[50, 51]	[43, 50, 51, 54-56]
	trans-resveratrol-3-*O*-glucuronide.	[38, 45, 62]	[38, 45]	[45]	[57, 58, 73] [59, 60]*	[20, 21, 60, 61, 73, 74]
	trans-resveratrol-4'-*O*-glucuronide.				[57, 58, 73] [59, 60]*	[60, 61, 73]
	cis-resveratrol-3-*O*-glucuronide.				[73] [59, 60]*	[20, 21, 60, 61, 73, 74]

	cis-resveratrol-4'-*O*-glucuronide.				[73]	[21, 60, 61, 73, 74]
	Resveratrol-diglucuronides.	[46]				
	trans-resveratrol-3,4'-*O*-diglucuronide.				[57]	
	trans-resveratrol-3,5-*O*-diglucuronide.				[57]	
	Resveratrol-sulfates (not specified).	[31, 44, 46, 69]	[48]	[39, 44]	[50, 51]	[43, 50, 51, 54-56]
	trans-resveratrol-3-*O*-sulfate.	[38, 45]	[45]	[45]	[57] [60]*	[21, 57, 60, 61, 73, 74]
	trans-resveratrol-4'-*O*-sulfate.			[45]	[60]*	[21, 60, 61, 73, 74]
	cis-resveratrol-3-*O*-sulfate.				[60]*	[21, 60, 61, 73, 74]
	cis-resveratrol-4'-*O*-sulfate.				[60]*	[21, 60, 61, 73, 74]
	Resveratrol-disulfates (not specified).			[45]		[55, 56]
	trans-resveratrol-3,4'-*O*-disulfate.	[45]	[45]		[57]	[61]
	trans-resveratrol-3,5-*O*-disulfate.	[45]	[45]		[57]	
	Resveratrol-trisulfates.					
	trans-resveratrol-3,4',5-trisulfate.	[45]	[45]			
	Resveratrol glucuronide-sulfate.	[46]				[55, 56, 61]
Resveratrol microbial metabolites.						
	Dihydroresveratrol.	[49]	[48]			[52, 61]
	Dihydroresveratrol-glucuronide.	[49]			[73]	[54, 61, 73]
	Dihydroresveratrol-sulfate.	[49]	[48, 63]		[73]	[54, 61, 73]
	Dihydroresveratrol glucuronide-sulfate.	[46]				[61]

* Metabolites identified in LDL samples.

Apart from these reported resveratrol glucuronide and sulfate metabolites, two disulfates and a trisulfate conjugates (Figure 1 and Table 4) were identified in the urine of male Wistar rats in a study with higher amounts of resveratrol administered (50 and 300 mg/kg) [45]. *trans*-Resveratrol-3-*O*-glucuronide was the main metabolite found in samples after both experiments, for which excretion recoveries were 15 and 54%, respectively (Table 2) [45]. Azorín-Ortuño et al. identified one resveratrol diglucuronide and two sulfoglucuronide isomers, together with two resveratrol glucuronides, a resveratrol sulfate and traces of the aglycone in the plasma of pigs (25% Landrace, 25% Large White, 50% Duroc), with values up to 2.2 µg/L after administering 6.25 mg/kg of resveratrol [46]. Dihydroresveratrol and its sulfate conjugate (Figure 1), metabolites derived from the intestinal microbiota [47], were identified by Wang et al. in rat urine after administration of 20 mg/kg of resveratrol (Table 2) [48]. Dihydroresveratrol was also identified by Juan et al. in plasma after its oral administration (60 mg/kg) to Sprague Dawley rats, together with its glucuronide and sulfate conjugates [49]. In contrast and also according to results cited above for the resveratrol aglycone, the glucuronide form was excreted in higher amounts compared to sulfate conjugates (2.8 and 5.2-fold) and also to dihydroresveratrol (11.9 and 152-fold) [49]. Therefore, the knowledge of which metabolite is major after administration of resveratrol and its analogues still remains a conundrum, since contradictory results have been obtained. Different doses and foods delivered to different animal studies, the lack of available pure standards of metabolites and the different methodologies used to determine resveratrol metabolites could explain all these contradictory data.



	R1	R2	R3	R4	R5	R6	R7
trans-Resveratrol	H	H	H	---	---	---	---
cis-Resveratrol	---	---	---	H	H	---	---
trans-Piceid	Glucose	H	H	---	---	---	---
cis-Piceid	---	---	---	H	Glucose	---	---
trans-Resveratrol-3-*O*-glucuronide	Gluc. Ac	H	H	---	---	---	---
cis-Resveratrol-3-*O*-glucuronide	---	---	---	H	Gluc. Ac	---	---
trans-Resveratrol-4'-*O*-glucuronide	H	Gluc. Ac	H	---	---	---	---
cis-Resveratrol-4'-*O*-glucuronide	---	---	---	Gluc. Ac	H	---	---
trans-Resveratrol-3,4'-*O*-diglucuronide	Gluc. Ac	Gluc. Ac	H	---	---	---	---
trans-Resveratrol-3,5-*O*-diglucuronide	Gluc. Ac	H	Gluc. Ac	---	---	---	---
trans-Resveratrol-3-*O*-sulfate	SO_3H	H	H	---	---	---	---
cis-Resveratrol-3-*O*-sulfate	---	---	---	H	SO_3H	---	---
trans-Resveratrol-4'-*O*sulfate	H	SO_3H	H	---	---	---	---
cis-Resveratrol-4'-*O*-sulfate	---	---	---	SO_3H	H	---	---
trans-Resveratrol-3,4'-*O*-disulfate	SO_3H	SO_3H	H	---	---	---	---
trans-Resveratrol-3,5-*O*-disulfate	SO_3H	H	SO_3H	---	---	---	---
trans-Resveratrol-3,5,4'-*O*-trisulfate	SO_3H	SO_3H	SO_3H	---	---	---	---
Dihydroresveratrol	---	---	---	---	---	H	H
Dihydroresveratrol-3-*O*-glucuronide	---	---	---	---	---	H	Gluc. Ac
Dihydroresveratrol-4-*O*-glucuronide	---	---	---	---	---	Gluc. Ac	H
Dihydroresveratrol-3-*O*-sulfate	---	---	---	---	---	H	SO_3H
Dihydroresveratrol-4'-*O*-sulfate	---	---	---	---	---	SO_3H	H

Figure 1. Chemical structures of resveratrol and its derivates found in biological samples.

Early studies in humans measured resveratrol metabolites indirectly after enzymatic hydrolysis [43, 50-52], which results in free aglycone [53]. However, better analytical methods have been described in order to study the metabolic profile of resveratrol. Walle et al. were the first to identify a total of five resveratrol metabolites in human urine: two resveratrol glucuronides, a dihydroresveratrol glucuronide, resveratrol sulfate and a dihydroresveratrol sulfate (Figure 1), after 25 mg of resveratrol intake. Urine recoveries 12 hours after resveratrol intake were 24% for sulfate conjugates and 13% for the glucuronide ones, with low plasma concentrations (Table 3) [54]. Dihydroresveratrol was also found in a human pharmacokinetic study after 250 mL of red wine consumption, but after hydrolysis, so its conjugates could not be identified. Its urine kinetics showed a delayed excretion of this microbial metabolite compared to those of the resveratrol aglycone, which could give a better idea of long-term resveratrol consumption [52]. Two resveratrol sulfates, a resveratrol disulfate, two glucuronides and a glucuronide sulfate of resveratrol were identified (Table 4) in a phase I dose escalation study where 0.5, 1, 2.5 and 5 g of resveratrol were administered (Table 3) [55] and after administration of a single oral dose of 1 g [56] (Table 3). The highest maximum concentrations were obtained for resveratrol-3-sulfate, more than 3-fold higher than glucuronide concentrations [55]. Two resveratrol disulfates and two diglucuronide conjugates (Figure 1) were identified by Burkon and Somoza [57] after piceid administration (85.5 mg/70 kg), together with one resveratrol sulfate and two glucuronide metabolites. In this study, also higher concentrations of trans-resveratrol-3-*O*-sulfate was found at early times (1h), although even higher concentrations were obtained for trans-resveratrol-3,5-*O*-disulfate at 6-8 hours after piceid administration [57].

Most of this previous literature cited has described resveratrol metabolic profile after the intake of the pure compound. In fact, few studies have focused on the metabolic profile of resveratrol after the consumption of natural sources, such as red wine. In 2005, a study by Vitaglione et al. could only identify trans-resveratrol-3 and 4'-*O*-glucuronides, with concentration values of up to 0.9 µg/L, along with traces of free aglycone, in the serum samples of human volunteers after consumption of several wines (Table 3), with total resveratrol doses from 0.25 to 1.96 mg [58]. trans- and cis-Resveratrol-3 and 4'-*O*-glucuronides, trans- and cis-resveratrol-3 and 4'-*O*-sulfates (Figure 1), together with resveratrol glucosides were determined in LDL samples after red wine consumption [59, 60]. The higher concentration was obtained for trans-resveratrol-3-*O*-glucuronide with a mean concentration of 45.13 ng/mg LDL protein [59, 60]. Resveratrol metabolites were also identified and quantified in urine samples of volunteers after consumption of 250 mL of red wine (equivalent to 5.4 mg of total resveratrol) [60]. The highest excreted metabolite was cis-resveratrol-4'-*O*-sulfate with a concentration value of 2.9 mg/g creatinine. The same resveratrol metabolites were quantified in the morning urine of 1000 free-living participants the PREDIMED Study (Table 4). This measure could discriminate moderate daily wine consumers from non-wine consumers fullfilling the criteria that resveratrol metabolites were considered as nutritional biomarkers of wine consumption [21]. The recent availability of purified standards of resveratrol metabolites by our group, enabled improvements of methodology for resveratrol disulfate and sulfoglucuronide metabolites together with their accurate quantification (results under revision) [61]. Up to 21 resveratrol derivates were identified using this analytical methodology in human urine after moderate red wine and the same dealcoholized beverage intake, including: trans- and cis-resveratrol-4'-*O*-glucuronide, trans- and cis-resveratrol-3-*O*-glucuronide, trans- and cis-resveratrol-4'-O-sulfate, trans- and cis-resveratrol-3-*O*-sulfate, trans-resveratrol-3,4'-*O*-disulfate, resveratrol sulfoglucuronide, trans- and cis-piceid, piceid glucuronide, two piceid sulfates, dihydroresveratrol, two dihydroresveratrol glucuronides, two dihydroresveratrol sulfates and dihydroresveratrol sulfoglucuronide (Table 4) [61].

The bioavailability study of resveratrol also includes the administration of related compounds, such as its glucoside [33, 57, 62, 63], called piceid, and other natural or synthetic stilbenes [39, 64], both in human and animal models. When piceid was administered, resveratrol was found in several biological samples, together with its metabolic profile [57, 62, 63], as happens when wine is consumed, since it is the main stilbene in this product [65]. In fact, piceid is absorbed by the active transporter sodium-dependent glucose transporter 1 (SGLT1) [66] and, within enterocytes, it might be enzymatically hydrolyzed by cytosolic-β-glucosidase [67]. This glucoside could also be hydrolyzed by the membrane-bound lactase phlorizin hydrolase resulting in the formation of resveratrol aglycone [67], which could, then, be absorbed using the passive transport [66]. Despite both absorption mechanisms, piceid could also be found in biological samples in intact form after the pure standard administration [33, 62, 63], together with its glucuronide and sulfate conjugates [63], and after red wine intake [59]. Resveratrol bioavailability in mice was also compared to that of 3,4,5,4'-tetramethoxystilbene (Figure 2 and Table 2), a resveratrol analogue with up to four methoxy groups. Resveratrol was found to be more readily available in the plasma, liver, kidney, lung and heart, although the analogue has better bioavailability in the brain and intestinal and colonic mucosa [39]. Three different resveratrol analogues – piceatannol, pinosylvin and rhanpontigenin (Figure 2) – were also studied in rats, with poor bioavailability observed (Table 2) [64]. The pharmacokinetic parameters of oxyresveratrol were also studied and compared to those of resveratrol, by administering *Smilax china* Linn. extract to Sprague Dawley rats. Both compounds were rapidly absorbed and bioavailable for 6 hours [68]. Recently, resveratrol bioavailability was compared to that of its dimethylether analogue in CD rats (Figure 2 and Table 2), which demonstrates greater bioavailability [69].

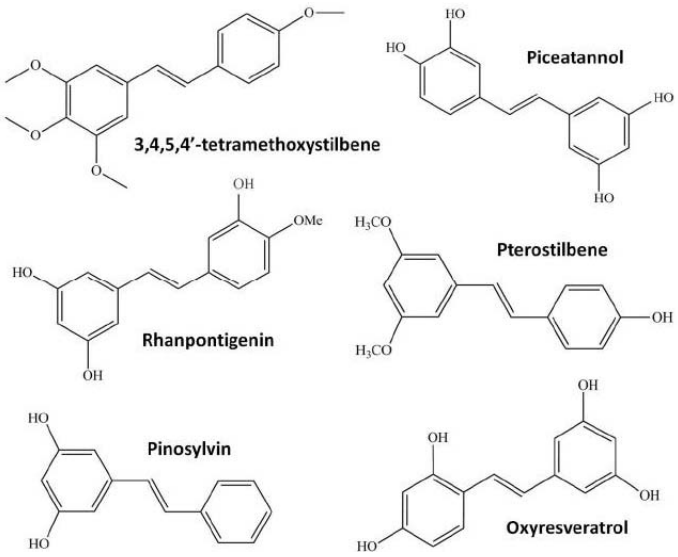


Figure 2. Chemical structures of resveratrol analogues administered in several trials.

Although human bioavailability studies will always be more reliable, studies based on animal models are very useful in order to gain a better knowledge, for instance, of tissue distribution, since invasive techniques are required. When an active compound reaches the target tissue, it is considered bioavailable [29], so several studies have been performed in this way, mainly in rats and mice [24, 38, 40, 41, 45, 70, 71]. The main tissues studied were the liver and kidney [24, 33, 38, 40, 41, 45, 70, 71] followed by the brain, heart and lung [33, 39-41, 70, 71], and also the spleen, stomach, intestines and testes [33, 39, 40, 70, 71] (Table 2). In

general, higher concentrations of resveratrol and/or its metabolites were found in the liver [24, 39-41], where hepatocytes metabolized the absorbed aglycone [38], and in the kidney [39, 44], since this is the main elimination way [53]. Surprisingly, the lung also showed high resveratrol concentrations [39-41], although the highest values were found in the stomach and intestine 10 minutes after resveratrol or piceid intake [33, 39], since these were not yet totally absorbed. Low concentrations, but not negligible, were observed in the brain [39, 41, 44] and heart [24, 33, 39], where it could exert its biological effect.

The administration of labeled resveratrol is also used in this way to gain a better and easy understanding of the absorption, distribution and metabolism of the active compound. The first bioavailability study on radiolabeled [^{3}H] *trans*-resveratrol was performed by Soleas et al. with male Wistar rats in short-term (2h) and long-term (24h) experiments (Table 2) [30]. Results showed that *trans*-resveratrol was absorbed up to 77-80%, obtaining urine recoveries of up to 61% over the 24-hour period. The same product was administered, together with 50 mg/kg of pure *trans*-resveratrol, to Sprague Dawley rats with only 3.3% recovered in urine 18 hours post-administration, although absorption was almost 90% [71]. The authors put these differences down to the loss via perspiration or respiratory water and to the accumulation in other tissues that were not considered. A multiple single oral dose of ^{14}C-*trans*-resveratrol, consisting of 7.4, 74 and 92.5 kBq, was administered to Balb/c mice and nearly complete absorption was obtained (Table 2) [70], according to these previous works [30, 71]. In all these studies, radioactivity was found in different organs, such as the brain, lung, heart, liver and kidney, although the two first studies only found trace amounts 24 and 18 hours after intake of labeled *trans*-resveratrol, respectively [30, 71]. Vitrac et al. found high radioactivity values in the duodenum after 1.5 and 3 hours of ^{14}C-*trans*-resveratrol administration and not negligible amounts in the kidney, lung and liver (Table 2) [70]. Labeled resveratrol (25 mg of ^{14}C-*trans*-resveratrol) was also administered to humans in a bioavailability study (Table 3) [54]. Recovered radioactivity in urine was from 53.4 to 84.9% with variable values in feces. In plasma, two concentration peaks of ^{14}C-*trans*-resveratrol were observed at 1 and 6 hours after its intake. The second one could be explained by enterohepatic recirculation of conjugated metabolites, previously demonstrated by Marier et al., who established a linked-rat model consisting of bile-donor and bile-recipient rats [42]. With all these studies, important information is now available on the study of the resveratrol bioavailability (Figure 3). However, the results obtained are not yet conclusive due to the high variability related to this kind of studies and, thus, further investigation is still necessary.

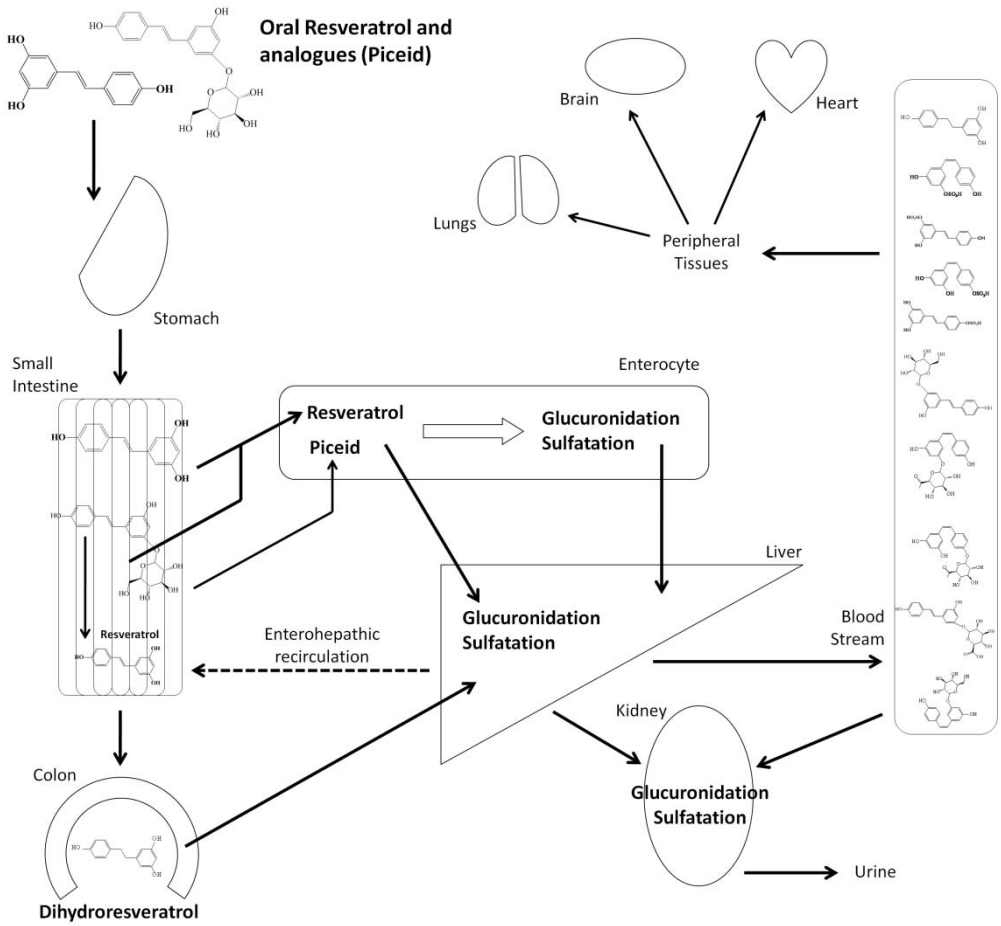


Figure 3. Absorption, distribution, metabolism and excretion of resveratrol and piceid.

FOOD MATRIX EFFECT

As previously stated, bioavailability studies were carried out by administering different sources, such as the pure compound, sometimes as tablets or caplets [34-36, 55, 56] or dissolved in several matrices [30, 37, 50, 51, 54, 57], red wine [20, 58-60] or other naturally rich products [40, 43, 52, 68], which implies a large range of ingested doses. With all these different study designs, conflicting data could be obtained from all the resveratrol bioavailability studies already performed. Moreover, it should be borne in mind that food matrix could influence the bioavailability of an active compound, since other compounds present in food might affect its absorption, distribution and elimination. Therefore, the effect of food matrices should always be considered in bioavailability studies, in order to interpret the results obtained. Several studies, mainly performed in humans, have focused on the effect of food matrix. In animal models, however, only Bertelli et al. have studied resveratrol bioavailability after red wine consumption [24], while the remaining works were carried out using the pure standard (Table 3), except for the studies performed using extracts of grape [43], P. cuspidatum [40] and S. china [68], mentioned above. Soleas et al. performed a study with rats administering pure radiolabeled resveratrol dissolved in different matrices – 10% (v/v) ethanol, homogenized vegetable or white grape juice – observing no differences between them [30].

The effect of food matrix has already been the focus of one of the early resveratrol bioavailability studies in humans [50], in which absorption of pure *trans*-resveratrol standard was compared when dissolved in three different matrices: grape juice, white wine and vegetable juice (Table 3). The results obtained showed similar absorption curves among all these matrices, with variations in resveratrol excretion of 1% [50]. So resveratrol absorption seemed not to be influenced by these matrices, which differed mainly in alcohol content. With regard to alcohol, it is interesting to have a look at the work performed by la Porte et al., in which pure *trans*-resveratrol together with 500 mg of quercetin were administered to human subjects, with or without 5% of alcohol (100 mL), and was compared to a single resveratrol intake (Table 4) [37]. Quercetin and the combined intake with alcohol did not improve *trans*-resveratrol absorption, when compared with the single resveratrol intake, failing to support the authors' hypothesis that quercetin would inhibit resveratrol metabolism [72]. Both studies administered the pure resveratrol standard, whose absorption was previously compared to that of a natural product, such as grape juice, taking different oral doses into consideration (Table 3) [43]. The results showed that resveratrol excretion after grape juice consumption was less than 10% of the values obtained when the standard was administered [43]. However, a recent work concluded that resveratrol is better absorbed (6-fold higher) from natural products [52] in a resveratrol bioavailability study in humans after red wine or grape juice intake in order to be compared with tablets of red wine extract enriched with *trans*-resveratrol (Table 3). Similar concentration values were obtained after administration of both natural sources of resveratrol; so no influence on alcohol matrix was observed [52]. With these conflicting results, the contradictory data now available have been revealed. However, it should be borne in mind that one of these studies only looked for resveratrol aglycone [37], although in the other works glucuronide and sulfate conjugates were considered after enzymatic hydrolysis [43, 50, 52]. With regard to the whole reported metabolic profile of resveratrol (Table 4) it is important to state that resveratrol absorption and its bioavailability might be underestimated.

Our group has recently focused the studies on matrix effect, taking the metabolic profile of resveratrol into consideration. A pharmacokinetic study was carried out after the intake of red wine compared to the same total resveratrol dose as grape extract tablets. The results obtained suggested that tablets may delay absorption, giving more time for the intestinal microbiota to extensively metabolize resveratrol, since higher excreted amounts of resveratrol microbial metabolites were observed after administration of tablets [73]. Another work of our group focused the resveratrol bioavailability study on the effect of the alcohol matrix on a long-term consumption, administering the same red wine 'as is' or without alcohol [61, 74]. Excretion of resveratrol metabolites were not statistically different after red wine or dealcoholized red wine interventions (Table 3). Thus, alcohol seems not to affect resveratrol bioavailability [61, 75].

Apart from the study of the effect of the source matrix, several reports studied the effect of the food matrix with the intake of resveratrol [34, 37] or red wine [58]. The first of these studies, performed in 2005, administered red wine, together with a standard, high-fat or lean meal, to human subjects (Table 3). With the results obtained, authors were able to conclude that the meal could not influence the bioavailability of *trans*-resveratrol from red wine, or the kind and/or the quantity of lipids in this meal [58]. However, when the pure compound was administered it seemed that a high-fat meal may delay and/or decrease its absorption [34, 37]. In fact, it should be borne in mind, as previously commented, that the bioavailability of resveratrol is considerably different depending on whether it is administered as its usual form or through natural products. Moreover, both studies with the pure standard intervention determined only the free aglycone in plasma samples [34, 37], while in the study with red wine administration, considered some resveratrol metabolites, which could explain the differences in the results obtained [58].

CONCLUSIONS

Research into the bioavailability of resveratrol has been studied for some time and has already provided important information (Figure 3). Differences in resveratrol administration, doses and assay methods make data difficult to compare, although the development of recent databases, such as phenol-explorer.eu, could improve their interpretation [76]. However, most of the studies concluded that resveratrol is highly absorbed, although it has low bioavailability, since it is rapidly metabolized. In fact, free resveratrol in biological samples was found to constitute less than 2% of the total resveratrol consumed [29]. Therefore, the bioactivity of resveratrol would be related to its metabolites, since would be those that reach the peripheral circulation and tissues where they could exert their biological effect. So, identification and measurement of this metabolic profile is necessary in order to understand the role of these compounds in providing health benefits.

The main reported conjugates of resveratrol were glucuronide and sulfate forms [31, 39, 40], although disulfates, trisulfates and diglucuronides were also identified [45, 55, 57] (Table 4). Special attention should be paid to the less reported microbial metabolites of resveratrol, dihydroresveratrol and its conjugates [48, 49, 54], which seem to be related to long-term resveratrol consumption [52]. Pharmacokinetics show maximum concentrations of these compounds within 4 hours of consumption, considering also a second concentration peak due to enterohepatic recirculation [42]. Resveratrol, together with its metabolites after hydrolysis, was found to be distributed extensively to different tissues, mainly to the liver and kidney, but also the lung, heart and brain, although the main concentrations were found in the intestine [39]. These compounds were mainly excreted in urine, with recoveries of up to 85%, although they were also found in feces.

Bioavailability studies must cope with significant limitations, such as individual variability, as well as the matrix in which resveratrol is delivered, since these could play an important role. Up to now, studies carried out on the food matrix effect have been inconclusive, although it seems that alcohol and the presence of other phenolic compounds do not modify resveratrol absorption after a single intake [37, 50, 52] or after long-term administration of red wine [61,74]. Moreover, the intake of resveratrol as a pure standard within a high-fat meal might reduce its absorption [34, 37], but no influence has been observed when resveratrol is administered in red wine [58]. Further investigation is necessary into the influencing factors of resveratrol bioavailability, such as the food matrix effect, prior to studying the health-promoting activities of resveratrol, in order to make nutritional recommendations regarding this promising molecule. In fact, recent studies have already focused on the improvement of this bioavailability [77, 78].

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ESTUDIS RECENTS SOBRE LA BIODISPONIBILITAT DEL RESVERATROL

Els treballs més recents sobre el tema que ens ocupa s'han focalitzat en l'estudi de la biodisponibilitat del resveratrol amb el principal objectiu de millorar-la. De fet, dues revisions recents recullen els treballs sobre noves formulacions amb sistemes d'alliberació modificada que permeten minimitzar les limitacions de la baixa biodisponibilitat del resveratrol (Amri *et al*., 2012; Santos *et al*., 2011). També s'han dut a terme estudis en els quals es pretén millorar la biodisponibilitat inhibint les rutes metabòliques del resveratrol, combinant la seva ingesta amb la d'altres compostos, com la piperina (Johnson *et al*., 2011). Una altra estratègia seria l'administració de compostos anàlegs, la qual ja es comenta en el capítol presentat anteriorment (Rotches-Ribalta *et al*., 2012c); tot i que, s'han publicat nous treballs en aquest sentit. La recerca més recent s'ha focalitzat en els compostos metoxilats del resveratrol, en diferent grau de conjugació, més concretament del *trans*-3,5,4'-trimetoxiestilbè (Lin i Ho, 2011) i del *trans*-2,4,3',4',5'-pentametoxiestilbè (Lin *et al*., 2012a); a part del pteroestilbè (3,5-dimetoxi-4'-hidroxi-*trans*-estilbè) (Kapetanovic *et al*., 2010) i el 3,4,5,4'-tetrametoxiestilbè (Sale *et al*., 2004), que ja havien estat objecte d'interès. En els dos estudis més recents (Lin *et al*., 2012a; Lin i Ho, 2011), la biodisponibilitat del compost estudiat va ser negligible, tot i que es va poder millorar amb la presència de ciclodextrina metilada. De totes maneres, la metilació de la molècula del resveratrol en totes les opcions possibles (2,4,3',4',5'-pentametoxiestilbè) en limita molt la seva biodisponibilitat (Lin *et al*., 2012a). Així doncs, i fent un cop d'ull a tota la bibliografia disponible en aquest camp, podem dir que la biodisponibilitat del resveratrol només es veurà millorada amb la metilació de dos dels substituents, és a dir, amb el pterostilbè (Kapetanovic *et al*., 2010).

Centrant-nos en el resveratrol pròpiament dit, cal destacar el treball d'Azorín-Ortuño, *et al*., on es va ampliar el perfil metabòlic del resveratrol fins a 19 compostos (Taula 1) (Azorin-Ortuno *et al*., 2011). La presència d'aquests es va estudiar en 47 mostres de teixits, òrgans i fluids de porcs, 6h després del consum de *trans*-resveratrol. Tot i que la identificació es va dur a terme en espectrometria de masses utilitzant analitzadors de triple quadrupol, temps de vol i, fins i tot una trampa de ions, la quantificació es va realitzar mitjançant detectors de llum ultraviolada (UV) i triple quadrupol. Amb això es

va descriure al resveratrol-3-*O*-glucurònid com el metabòlit més abundant en òrgans i fluids, tot i que el derivat de la microbiota dihidroresveratrol va esdevenir el principal metabòlit en el cec, còlon i el recte (Azorin-Ortuno *et al*., 2011). Posteriorment, en un estudi de col·laboració dut a terme entre el grup de recerca on s'ha realitzat la Tesi Doctoral i el grup de recerca del Departament de Farmàcia i Ciències dels Aliments de la Universitat del País Basc, liderat per la Dra. Maria Puy Portillo, es va descriure un perfil metabòlic de 8 metabòlits de resveratrol, incloent tant metabòlits intestinals com els derivats de la microbiota intestinal (Taula 1). Aquests es van quantificar en fetge, teixit adipós i múscul de rates Sprague-Dawley després del consum d'una dosi creixent de 6, 30 o 60 mg/Kg de *trans*-resveratrol durant 6 setmanes (Andres-Lacueva *et al*., 2012). Amb aquest disseny d'estudi es va poder descriure que els compostos sulfats tenien un comportament dosi-resposta, mentre que el metabolisme dels glucurònids se saturava.

Seguint amb els estudis en animals, cal destacar un estudi farmacocinètic de resveratrol dut a terme en gossos Beagle administrant dosis de 200, 600 i 1200 mg/kg (Muzzio *et al*., 2012); considerant, a part de l'aglicona, el corresponent glucurònid i sulfat (Taula 1). Com ja s'ha descrit en estudis anteriors, les quantitats analitzades dels metabòlits de resveratrol van ser superiors a les del resveratrol lliure, les quals es van descriure com a dosi-depenent (Muzzio *et al*., 2012). Els conjugats glucurònid i sulfat han sigut els metabòlits de resveratrol més descrits en els estudis de biodisponibilitat del resveratrol (Rotches-Ribalta *et al*., 2012c). De fet, Iwuchukwu, *et al*., van sintetitzar el resveratrol-3- i -4'-*O*-glucurònid i el resveratrol-3- i -4'-*O*-sulfat per tal de desenvolupar un mètode enfocat a l'anàlisi dels paràmetres farmacocinètics, obtinguts després de l'administració de resveratrol en ratolins (Iwuchukwu *et al*., 2012). També van ser els glucurònids i sulfats, obtinguts després d'un procés d'hidròlisi enzimàtica selectiva, els considerats en un estudi farmacocinètic en rates que van consumir *Polygonum Cuspidatum* (Taula 1) com a font alimentària de resveratrol (Lin *et al*., 2012b). També recentment, s'han creat models matemàtics per tal de descriure la farmacocinètica del *trans*-resveratrol en plasma i dels seus conjugats glucurònid i sulfat (Colom *et al*., 2011). Un dels estudis més recents, de fet, ha comparat la farmacocinètica, basada en models matemàtics, del glucurònid i sulfat de resveratrol formats *in vivo* amb la dels compostos administrats com a tal, observant diferències considerables (Sharan *et al*., 2012).

Amb totes aquestes noves dades, cada vegada es té més coneixement de la biodisponibilitat del resveratrol, la qual s'ha vist que es troba limitada pel seu ampli metabolisme. Tot i això, encara cal més informació, sobretot en humans i considerant sempre aquells aspectes que puguin tenir una major influència en aquesta paràmetre, per tal de discernir sobre els efectes beneficiosos descrits per al resveratrol (Smoliga *et al*., 2011). En aquest sentit, i com ja s'ha plantejat en el "*2nd International Scientific Conference on Resveratrol and Health*" (http://www.resveratrol2012.eu/), cal disposar de dades que puguin aclarir la possible activitat biològica dels metabòlits de resveratrol. I és que, actualment, els pocs treballs que es focalitzen en aquest aspecte es basen principalment en estudis *in vitro* (Hoshino *et al*., 2010; Calamini *et al*., 2010; Miksits *et al*., 2009; Wang *et al*., 2004). Els metabòlits més estudiats han sigut els sulfats de resveratrol (Hoshino *et al*., 2010; Calamini *et al*., 2010; Miksits *et al*., 2009), que han evidenciat una pobre activitat biològica, en comparació amb la que ha demostrat tenir el resveratrol. De totes maneres, cal tenir en compte que l'activitat dels metabòlits observada *in vitro* no ha de reflectir necessàriament la seva funció *in vivo*, ja que les omnipresents sulfatases podrien convertir, de nou, els metabòlits en aglicona ja en el teixit diana, on aquesta podria exercir la seva activitat biològica (Miksits *et al*., 2009). Queda palès, doncs, la necessitat de més estudis sobre els efectes biològics dels metabòlits del resveratrol; focalitzats, també, a avaluar les interaccions entre el resveratrol i la microbiota intestinal (http://www.resveratrol2012.eu/).

Taula 1. Estudis recents sobre la biodisponibilitat del resveratrol.

Espècie	Dosi de producte administrat	Valor màxim en plasma	Excreció urinària	Teixits estudiats	Compostos estudiats	Referència
Porcs	6.25 mg/kg RV	3,87 mmol/L de metabòlits totals (obtinguts en vena porta)	7,7% del ingerit	Estómac, duodè, jejú, il·li, cec, còlon, recte, nòduls limfàtics, cervell, cerebel, cor, fetge, ronyó, pulmó, melsa, pàncrees úter, bufeta, ovaris, teixit adipós i múscul esquelètic.	*trans*- i *cis*-RV, RV-3- i -4'-*O*-glucurònid, 2 RV-diglucurònids, RV-sulfat, RV-disulfat, RV-trisulfat, 2 RV-sulfoglucurònids, RV-ribosil-sulfat, dihidroresveratrol (DHR), DHR-3-*O*-glucurònid, DHR-diglucurònid, DHR-sulfat, DHR-disulfat, DHR-trisulfat, DHR-sulfoglucurònid	(Azorin-Ortuno *et al*., 2011)
Rates	2, 10 o 20 mg/Kg *trans*-RV	~ 10 µmol/L	---	---	*trans*-RV, RV-glucurònid i RV-sulfat	(Colom *et al*., 2011)
Ratolins	100 mg/kg RV	0,2 i 17,5 mg/L	---	---	RV i RV-3-*O*-β-D-glucurònid (respectivament)	(Johnson *et al*., 2011)
	100 mg/kg RV + 10 mg/kg piperina	35,2 i 32,4 mg/L				
Rates	5, 10, 15 o 20 mg/kg *trans*-3,5,4'-trimetoxiestilbè	2,3 mg/L	---	---	*trans*-3,5,4'-trimetoxiestilbè	(Lin i Ho, 2011)
Rates	6, 30 o 60 mg/kg RV	---	---	Fetge, teixit adipós i múscul	*trans*-RV-3-*O*-glucurònid, *trans*-RV-4'-*O*-sulfat, *trans*- i *cis*-RV-3-*O*-sulfat, trans-RV-3,4'-disulfat, DHR, DHR-glucurònid, DHR- sulfat	(Andres-Lacueva *et al*., 2012)
Ratolins	60 mg/kg RV	~ 100 µmol/L	---	---	RV, RV-3- i -4'-*O*-glucurònid, RV-3- i -4'-*O*-sulfat.	(Iwuchukwu *et al*., 2012)
Rates	4 mg/kg *trans*-2,4,3',4',5'-pentametoxiestilbè	53,2 µg/L	---		*trans*-2,4,3',4',5'-pentametoxiestilbè	(Lin *et al*., 2012a)
Rates	2 i 4 g/Kg Polygonum Cuspidatum	~ 30 nmol/L totals	---	Cervell, fetge, pulmó, cor i ronyó	RV, RV-glucurònids i RV-sulfats obtinguts per hidròlisi	(Lin *et al*., 2012b)
Gossos	200, 600 i 1200 mg/kg RV	1,7 – 9,9 mg/L 3,8 – 210 mg/L 17 – 30 mg/L	---	---	RV, RV-glucurònid i RV-sulfat (respectivament)	(Muzzio *et al*., 2012)

RV: resveratrol; DHR: dihidroresveratrol.

TÉCNIQUES ANALÍTIQUES UTILITZADES PER A L'ANÀLISI DEL PERFIL METABÒLIC DEL RESVERATROL EN MOSTRES BIOLÒGIQUES

Tal com s'ha descrit anteriorment, la biodisponibilitat del resveratrol és molt baixa malgrat l'elevada absorció oral que presenta, ja que és metabolitzat ràpidament. Amb aquesta premissa és fàcil concloure que l'estudi de la biodisponibilitat del resveratrol requereix tècniques analítiques d'elevada sensibilitat i selectivitat, precedides d'una bona preparació de la mostra a analitzar, ja sigui plasma, orina o, fins i tot, òrgans o teixits.

A continuació s'exposen els mètodes més utilitzats a la bibliografia per a l'estudi de la biodisponibilitat del resveratrol, tant pel què fa a la preparació de la mostra, com a la tècnica d'anàlisi utilitzada. Alhora, es pretén explicar el fonament de cadascuna de les metodologies i, sobretot de les utilitzades en els estudis que constitueixen la present Tesi Doctoral.

PREPARACIÓ DE LA MOSTRA

Les mostres biològiques solen tenir un elevat contingut proteic i, en alguns casos també lipídic. L'eliminació d'aquestes fraccions és molt important per a poder obtenir una mostra més lliure de matriu que faciliti el seu posterior anàlisi, sobretot quan aquest té lloc en un espectròmetre de masses; ja que, sense un tractament previ, les mostres podrien provocar l'anomenat efecte matriu, que defineix a la disminució o supressió de la ionització dels analits en una mostra. L'analit, doncs, ha de ser degudament extret de la matriu que constitueix la mostra, per a una correcta identificació i quantificació.

L'aïllament d'aquest analit se sol dur a terme mitjançant tècniques d'extracció o pretractament de mostra, ja sigui en fase sòlida (SPE), líquid-líquid (LLE) o bé per precipitació de proteïnes (PPT). Abans d'aquest procés, però, alguns estudis realitzen un pretractament d'hidròlisi enzimàtica amb l'objectiu de determinar, tot i que de manera indirecta, la quantitat de resveratrol conjugat.

Hidròlisi enzimàtica

En alguns estudis, la determinació del resveratrol en les seves formes conjugades es realitza de manera indirecta, analitzant el resveratrol lliure obtingut mitjançant un procés d'hidròlisi enzimàtica (Ortuño *et al*., 2010; Juan *et al*., 2010b; Zhou *et al*., 2007; El-Mohsen *et al*., 2006; Meng *et al*., 2004; Walle *et al*., 2004; Goldberg *et al*., 2003; Marier *et al*., 2002; Soleas *et al*., 2001). El tractament enzimàtic es realitza incubant les mostres, el temps suficient a una temperatura i pH adequats, amb els enzims β-glucuronidasa o arilsulfatasa, o bé amb una mescla d'ambdós com a extracte d'*Helix Pomatia,* que provoca el trencament dels enllaços que formen els conjugats del resveratrol alliberant l'aglicona. Amb aquesta metodologia es pot donar un valor de resveratrol total absorbit, però també de la quantitat de resveratrol lliure detectat en les mostres sense aplicar hidròlisis enzimàtica i, de la mateixa manera, de la quantitat total de sulfats o glucurònids, comparant el valor de concentració de resveratrol lliure determinat en les mostres tractades amb un o altre enzim.

Si bé és cert que la majoria d'estudis realitzen una hidròlisi de tipus enzimàtic, no podem obviar el procés d'hidròlisi àcida que prèviament s'havia descrit (Morand *et al*., 2001). En aquest sentit, Ortuño, *et al*., va comparar l'eficiència del mètode d'hidròlisi àcida amb el tractament enzimàtic, estudiant la recuperació del resveratrol lliure a partir dels seus conjugats addicionats a una orina sintètica (Ortuño *et al*., 2010). La incubació de les mostres durant 3h a 55ºC amb *Helix Pomatia* es va comparar amb la incubació amb àcid clorhídric 6 mol/L a 100ºC durant 20 minuts. La hidròlisi enzimàtica es va definir com el millor compromís en termes d'eficiència per ambdós tipus de conjugats (Ortuño *et al*., 2010). De totes maneres, es van obtenir recuperacions del 71% per als glucurònids i del 48% per als sulfats (Ortuño *et al*., 2010). A part de la baixa eficiència,

amb aquest procés es perd la possibilitat de conèixer el perfil metabòlic del resveratrol i, per tant, es pot perdre certa informació que podria ajudar a entendre millor la biodisponibilitat d'aquest i els mecanismes d'acció per a dur a terme els efectes beneficiosos descrits.

Precipitació de proteïnes (PPT)

La PPT és un procés simple de tractament de la mostra, que serveix per eliminar les interferències proteiques d'aquesta. Consta de l'addició d'un dissolvent a la mostra que provoca la precipitació de la part proteica, la qual s'elimina per centrifugació. El principal dissolvent utilitzat per a provocar la precipitació de la part proteica (PPT) és l'acetonitril (ACN) (Azorin-Ortuno *et al*., 2011; la Porte *et al*., 2010; Azorin-Ortuno *et al*., 2010), tot i que alguns estudis treballen també amb metanol (Iwuchukwu *et al*., 2012); a vegades acidificats (Zhou *et al*., 2007; Boocock *et al*., 2007a), permetent la ruptura de l'enllaç proteïna-polifenol. Cal tenir en compte que, en el cas del resveratrol, molts dels estudis que parlen, simplement, d'extracció de l'analit de la matriu de la mostra, utilitzen aquests dissolvents (Taula 2, pàgina 107) (Wenzel *et al*., 2005; Sale *et al*., 2004; Marier *et al*., 2002). L'ús d'àcids forts, com l'àcid tricloracètic (TCA) també podria ser una bona opció per a la PPT (El-Mohsen *et al*., 2006), però no se solen utilitzar pel fet que podrien provocar la hidròlisi d'alguns compostos conjugats.

Després de la centrifugació, la fase orgànica superior o sobrenadant serà analitzat per el mètode cromatogràfic seleccionat (Chen *et al*., 2007; Boocock *et al*., 2007a; Lv *et al*., 2006; Meng *et al*., 2004; Marier *et al*., 2002; Yu *et al*., 2002). Ara bé, en molts altres estudis, aquest és evaporat fins a sequedat, per tal de ser reconstituït en un dissolvent molt més adient per al seu posterior anàlisi (Azorin-Ortuno *et al*., 2011; Azorin-Ortuno *et al*., 2010; Burkon i Somoza, 2008; Zhou *et al*., 2007; El-Mohsen *et al*., 2006; Sale *et al*., 2004; Asensi *et al*., 2002). En alguns casos, la mostra reconstituïda és centrifugada de nou (Muzzio *et al*., 2012; Kapetanovic *et al*., 2010; Boocock *et al*., 2007b), o bé filtrada (Huang *et al*., 2008; Wenzel *et al*., 2005), abans de ser analitzada.

Extracció líquid-líquid (LLE)

La LLE s'utilitza per separar components d'una mostra en funció de la seva polaritat, mitjançant la mescla de dues fases líquides immiscibles. S'aconsella especialment per a compostos lipofílics, tot i que també s'ha utilitzat per a determinar compostos polifenòlics. Per a dur a terme aquesta LLE, se sol utilitzar acetat d'etil (Lin *et al*., 2012b; Vitaglione *et al*., 2005; Soleas *et al*., 2001) i, fins i tot, mescles amb els dissolvents descrits per a la PPT, com l'ACN (Ortuño *et al*., 2010). La LLE, però, és poc utilitzada pel fet que és una tècnica molt laboriosa.

En aquests estudis, un cop assegurada la mescla de la mostra amb el dissolvent de l'extracció, aquesta és centrifugada per tal de separar fàcilment la fase on es trobaran els analits, la qual és evaporada fins a sequedat, essent reconstituïda en un dissolvent molt més adient per al seu posterior anàlisi (Lin *et al*., 2012b; Ortuño *et al*., 2010; Soleas *et al*., 2001). Abans de ser analitzada, però, la mostra reconstituïda pot ser filtrada (Vitaglione *et al*., 2005).

Extracció en fase sòlida (SPE)

La SPE és un mètode ràpid que permet eliminar sals i proteïnes de la mostra que podrien interferir en l'analítica d'aquesta. Tot i així, l'eficàcia de la SPE dependrà del tipus de sorbent (o fase sòlida) utilitzat, del volum i pH de la mostra, dels solvents utilitzats, i el seu volum, en cada una de les etapes de la SPE; proporcionant-li una àmplia versatilitat. De fet, s'han extret diverses conclusions d'estudis que comparen la SPE amb la LLE. Un dels primers treballs del grup de recerca on s'ha dut a terme la present Tesi Doctoral va posar a punt un mètode per a l'anàlisi de resveratrol, piceid i alguns metabòlits en mostres de LDL humanes, obtingudes després d'un consum puntual de vi negre (Urpi-Sarda *et al*., 2005). En aquest treball, es van comparar tres mètodes de preparació de la mostra, àmpliament utilitzats per a l'anàlisi de resveratrol: SPE, LLE amb acetat d'etil i PPT amb metanol acidulat. Les millors recuperacions, determinades per LC-UV-DAD, es van obtenir, primer per la PPT i després per la SPE, descartant la LLE, ja que va presentar una recuperació del 65% (Urpi-Sarda *et al*., 2005). Tot i els valors de recuperació, i donat l'anàlisi posterior de la mostra per LC-MS/MS, es

va elegir la SPE, ja que va mostrar una menor supressió iònica de la mostra, obtenint una recuperació del 85%, en comptes del 57% obtingut en la PPT (Urpi-Sarda *et al*., 2005). Ara bé, més recentment, Azorín-Otruño, *et al*. en el seu estudi amb porcs, va comparar la PPT amb ACN o metanol acidulat i la SPE, obtenint millors recuperacions amb la PPT amb ACN (99%) i amb la SPE (95%), tot i que els autors es van decantar per la PPT, donada la seva rapidesa (Azorin-Ortuno *et al*., 2010). Cal tenir en compte, però, que, quan es treballa amb estudis clínics en humans, es disposa de volums de plasma o sèrum molt menors al que es pot obtenir a partir d'estudis amb animals, com els porcs. A més a més, les dosis administrades en els estudis clínics són normalment molt menors a les utilitzades en estudis amb animals d'experimentació, de manera que la concentració final dels analits en la mostra serà més baixa. És per això, doncs, que es requereixen tècniques de preparació de la mostra i anàlisis més sensibles i acurades. Tot i això, avui en dia, es disposa de cartutxos per a SPE en sistemes de plaques, on es pot processar un total de 96 mostres, aconseguint un anàlisi més ràpid i utilitzant menys volum dels solvents i, el que és més important, de mostra; permetent, a més a més, una concentració dels analits que no seria possible amb una PPT (Urpi-Sarda *et al*., 2007).

L'aïllament de l'analit a partir de la SPE pot realitzar-se amb dos objectius ben diferenciats: retenir l'analit en la fase sòlida o cartutx, deixant escapar les interferències, o bé retenir les substàncies interferents, mentre els analits flueixen a través de la fase sòlida. Tot i això, per a la determinació del resveratrol s'ha treballat amb la primera opció; sovint precedida de l'acidificació de la mostra, per tal de trencar les unions entre els polifenols i les proteïnes plasmàtiques o les que constitueixen un teixit (Andres-Lacueva *et al*., 2012; Urpi-Sarda *et al*., 2007; Urpi-Sarda *et al*., 2005). El procés de SPE comença amb el precondicionament i/o equilibrat del cartutx, seguit de la càrrega de la mostra (Figura 2). En aquest moment, les molècules de l'analit són adsorbides en la fase sòlida d'aquest i es requereix un procés de neteja en el qual s'eliminen les molècules interferents i aquells components de la matriu de la mostra que no són retinguts en el cartutx. Finalment, l'analit és eluït de l'adsorbent amb l'aplicació del solvent adequat (Figura 2) el qual sol ser evaporat per tal de concentrar la mostra, la qual es reconstituirà en un petit volum del solvent utilitzat per a l'anàlisi cromatogràfic.

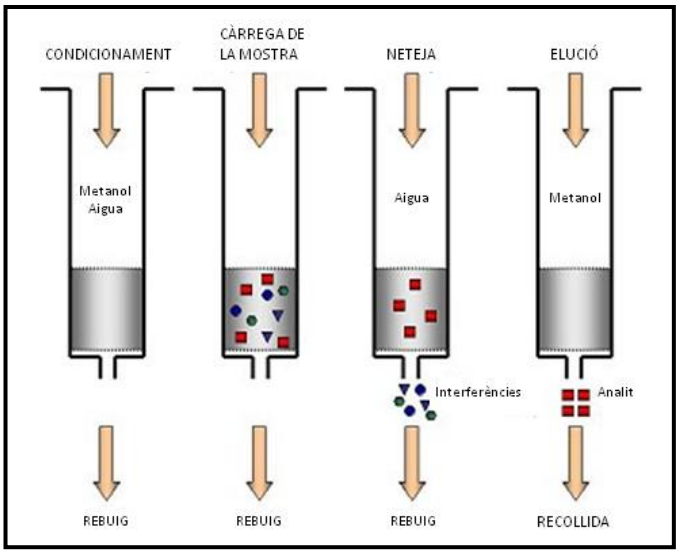


Figura 2. Procediment estàndard de la extracció en fase sòlida (SPE).

La figura 2 mostra gràficament les diferents etapes de la SPE, amb el procediment estàndard pel que fa als solvents utilitzats en els estudis de la biodisponibilitat del resveratrol (Wang *et al*., 2011; Juan *et al*., 2010a; Juan *et al*., 2010b; Wang *et al*., 2005; Juan *et al*., 2002). Alguns estudis, però, han modificat lleugerament els solvents utilitzats en cada una de les etapes. L'addició d'un petit percentatge (< 15%) de metanol en el procés de neteja (Andres-Lacueva *et al*., 2012; Almeida *et al*., 2009; Nunes *et al*., 2009; Vaz-da-Silva *et al*., 2008; Urpi-Sarda *et al*., 2007; Zamora-Ros *et al*., 2006; Urpi-Sarda *et al*., 2005; Walle *et al*., 2004), sovint ha estat utilitzat; de la mateixa manera que ho ha estat l'acidificació d'algun solvent (Andres-Lacueva *et al*., 2012; Urpi-Sarda *et al*., 2007; Zamora-Ros *et al*., 2006; Urpi-Sarda *et al*., 2005). Pel que fa a la fase sòlida, cal destacar l'ús d'empaquetaments de fase reversa, ja sigui d'octadecilsilà (Juan *et al*., 2010a; Juan *et al*., 2010b; Juan *et al*., 2002), més conegut com C18, o bé els presentats per la casa comercial Waters sota el nom d'Oasis® HLB (Andres-Lacueva *et al*., 2012; Almeida *et al*., 2009; Nunes *et al*., 2009; Vaz-da-Silva *et al*., 2008; Urpi-Sarda *et al*., 2007; Zamora-Ros *et al*., 2006; Urpi-Sarda *et al*., 2005; Walle *et al*., 2004); tot i que alguns treballs utilitzen cartutxos de poliamida (Wang *et al*., 2013; Wang *et al*., 2008; Wang *et al*., 2005).

En els estudis presentats en aquesta Tesi Doctoral s'ha treballat amb el producte Oasis® HLB, les sigles del qual indiquen que es tracta d'un sorbent amb balanç hidrofílic-lipofílic (HLB) de fase reversa. La relació entre els monòmers N-vinilpirrolidona (hidrofílic) i divinilbenzè (lipofílic) proporciona al farciment dels cartutxos una qualitat superior de

fase reversa amb una afinitat superior amb les substàncies neutres-polars. Cal destacar, també, la presència en el mercat dels cartutxos Oasis® amb intercanvi catiònic (MCX i WCX) o aniònic (MAX i WAX), àmpliament utilitzats en l'anàlisi de compostos fenòlics, tot i que no s'ha descrit el seu ús en la determinació del resveratrol i el seu perfil metabòlic. La figura següent (Figura 3) mostra les característiques del farciment que presenten els diferents cartutxos Oasis®, així com el tipus de substància d'anàlisi a la que estan destinats.

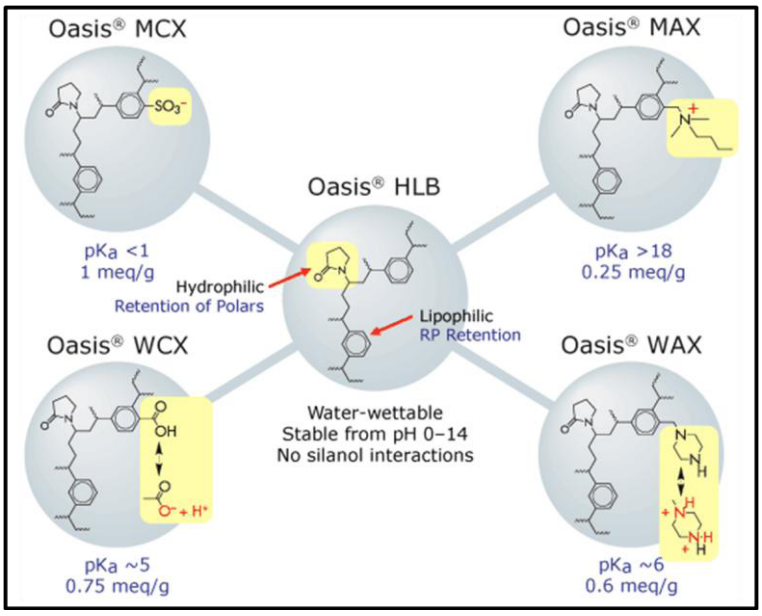


Figura 3. Tipus de farciment dels cartutxos Oasis® de Waters.

TÈCNIQUES INSTRUMENTALS D'ANÀLISI

La cromatografia és la tècnica més utilitzada per a la separació dels analits abans de la seva detecció i, tot i que normalment es treballa amb cromatografia líquida, la cromatografia de gasos també ha sigut utilitzada. La detecció dels analits sí que és més divergent, encara que es basa en tècniques d'espectrometria de masses o per espectrofotometria ultraviolada/visible (UV/Vis).

Separació cromatogràfica

La cromatografia permet separar els components d'una mostra en funció de l'afinitat que aquests presentin a una fase estacionària, segons polaritat, solubilitat relativa o adsorció. Així, cada compost assolirà diferents velocitats de pas a través del sistema, essent detectat en un "temps de retenció" característic, que ajuda en la seva identificació. En funció de l'estat físic en el qual es troba la mostra, parlarem de cromatografia líquida (LC) o de gasos (GC). La GC sol estar destinada a l'anàlisi de compostos volàtils, tot i que també és utilitzada per a la determinació de compostos de major pes molecular, després de sotmetre la mostra a un procés de derivatització en el qual una reacció química transforma l'analit en un derivat més fàcil d'analitzar. En l'estudi de la biodisponibilitat del resveratrol les metodologies descrites solen utilitzar la LC (Taula 2, pàgina 107), tot i que alguns treballs han utilitzat la GC (Ortuño *et al*., 2010; Goldberg *et al*., 2003; Soleas *et al*., 2001).

La cromatografia líquida d'alta eficàcia (HPLC) és la tècnica més utilitzada per a l'anàlisi del resveratrol i els seus metabòlits, per al qual se solen utilitzar columnes de fase reversa. La llargària de la columna i les fases mòbils, utilitzades amb un gradient adequat, seran decisius per a definir el temps de cromatograma. Els solvents utilitzats en la fase mòbil solen ser de tipus binari (Taula 2, pàgina 107), on un dels solvents és aigua, que pot ser acidulada o tamponada amb sals d'amoni, i l'altre de naturalesa orgànica i de polaritat intermèdia (metanol, ACN, etc).

En l'estudi del perfil metabòlic del resveratrol, cal tenir en compte que els conjugats sulfats presenten una baixa resolució cromatogràfica, que dificulta la seva caracterització i quantificació, donada la major amplitud dels pics cromatogràfics (Walle, 2011). L'ús d'una fase mòbil ternària amb acetona va solventar aquest inconvenient en un dels mètodes desenvolupats en el grup de recerca on s'ha dut a terme la Tesi Doctoral (Urpi-Sarda *et al*., 2007).

Espectrofotometria ultraviolada/visible

El resveratrol, al igual que la resta de compostos fenòlics, presenten la capacitat d'absorció de la llum ultraviolada, de manera que la seva detecció mitjançant tècniques espectrofotomètriques dins l'espectre UV/vis, comptant amb detectors de llum UV/vis o de *diode array* (DAD), ha sigut àmpliament utilitzada. Aquestes tècniques permeten quantificar la radiació d'una longitud d'ona determinada absorbida per l'analit, la qual serà proporcional a la seva concentració. En la majoria dels sistemes utilitzats, l'absorció de llum UV/Vis es mesura de manera continua durant el pas d'una mostra, obtenint l'espectre d'absorció de l'analit, el qual pot ser més o menys característic d'aquest i, per tant, facilitant-ne la identificació. Tot i això, és necessari disposar de l'estàndard pur de l'analit en qüestió, per tal de definir l'espectre d'absorció i el temps de retenció, que seran claus per la seva identificació.

Un inconvenient d'aquests detectors és la baixa sensibilitat que presenten, essent difícil la detecció de concentracions baixes d'analits, les quals s'obtenen al treballar en estudis clínics d'intervenció on s'administren dosis moderades de l'aglicona i/o del seu glucòsid; ja sigui per a detectar el resveratrol lliure o el seu perfil metabòlic. De fet, els estudis que utilitzen aquesta tècnica (Taula 2, pàgina 107), administren dosis elevades, les quals poden arribar a ser 20 vegades superiors a les que es poden ingerir normalment en un aliment. Realment, els detectors UV-DAD no són suficientment sensibles per a detectar el resveratrol, el piceid i els respectius metabòlits en mostres biològiques, per als quals es requereixen tècniques d'elevada sensibilitat.

Espectrometria de masses

L'espectrometria de masses és una de les tècniques més utilitzades en la investigació biomèdica, donada la seva elevada sensibilitat, especificitat i la seva fàcil combinació amb tècniques cromatogràfiques, que possibilita la identificació, determinació i quantificació d'un ampli ventall de compostos. Aquesta tècnica identifica les molècules en funció de la relació massa/càrrega (*m/z*) del seu ió, generat en el mateix espectròmetre. Amb aquest tipus de detecció es pot conèixer la massa molecular d'un compost i, fins i tot, en el cas de l'espectrometria de masses en tàndem, permet obtenir-

ne informació estructural. A més a més, inclou la capacitat de distingir entre dos pics cromatogràfics amb masses diferents solapats en el temps. L'elevada sensibilitat i selectivitat d'aquesta tècnica la fa extremadament interessant per a l'estudi de la biodisponibilitat del resveratrol, considerant també el seu perfil metabòlic, donades les extremadament baixes concentracions en les que es troben en les mostres biològiques. Tot i això, són pocs els treballs que utilitzen aquesta tècnica per a la quantificació del resveratrol i/o els seus conjugats (Taula 2, pàgina 107), essent el seu objectiu la identificació del perfil metabòlic del resveratrol (Wang *et al*., 2005; Meng *et al*., 2004; Walle *et al*., 2004; Asensi *et al*., 2002). Sovint, l'espectrometria de masses s'acobla a un sistema cromatogràfic amb DAD, per tal d'assegurar la identificació dels conjugats de resveratrol, que seran quantificats amb les dades d'absorció a l'UV/vis (Wang *et al*., 2011; Azorin-Ortuno *et al*., 2011; Azorin-Ortuno *et al*., 2010; Juan *et al*., 2010a; Juan *et al*., 2010b; Wang *et al*., 2008; Boocock *et al*., 2007a; Boocock *et al*., 2007b; El-Mohsen *et al*., 2006; Vitaglione *et al*., 2005). I és que la quantificació del perfil metabòlic del resveratrol a partir del seu anàlisi per espectrometria de masses és susceptible d'errors quan no es disposa d'estàndards purs que es puguin utilitzar com a referència. Tot i això, alguns treballs s'han dut a terme quantificant els conjugats com a equivalents de l'aglicona (Kapetanovic *et al*., 2010; Urpi-Sarda *et al*., 2007; Zamora-Ros *et al*., 2006; Urpi-Sarda *et al*., 2005), el qual és útil com a primera aproximació, ja que la ionització dels metabòlits podria ser diferent de l'aglicona. Si bé és cert que la tècnica de detecció de llum UV/vis pot evitar aquest inconvenient, la necessitat d'alguns estudis de treballar amb metodologies més sensibles, donades les baixes concentracions que s'obtenen en estudis nutricionals on s'administren dosis moderades, fa obviar el contratemps que presenta l'espectrometria de masses. Alguns estudis intenten evitar-lo amb la hidròlisi enzimàtica de les mostres (Taula 2, pàgina 107), tot i que també presenta els seus inconvenients. Això explica la divergència de metodologies utilitzades en l'estudi de la biodisponibilitat del resveratrol, per al qual cal arribar a un compromís entre els avantatges i inconvenients que presenten les tècniques descrites, sempre tenint en compte l'objectiu de l'estudi.

Taula 2. Metodologia utilitzada en els diferents estudis sobre la biodisponibilitat del resveratrol.

Espècie	Dosi de producte administrat	Mostra	Hidròlisi	Extracció	Cromatografia	Sistema de detecció	Compostos d'interès	Referència
Humans	25 mg *trans*-resveratrol en vi	Sèrum i orina	Si	LLE (acetat d'etil)	GC	MS (mode SIM)	Metabòlits després d'hidròlisi	(Goldberg *et al*., 2003; Soleas *et al*., 2001)
Rates, ratolins i conills	20 mg/kg *trans*-resveratrol	Plasma i teixits Orina	No No	LLE (acetat d'etil) Filtració	LC[a]: àcid acètic en aigua vs. ACN	UV/vis MS/MS (Identificació)	Aglicona lliure Perfil metabòlic (Només identificació)	(Asensi *et al*., 2002)
Rates	2 mg/kg *trans*-resveratrol	Plasma	No	SPE	LC: àcid acètic en aigua vs. ACN:aigua	DAD	Aglicona lliure	(Juan *et al*., 2002)
Rates	50 mg/kg *trans*-resveratrol	Plasma i orina	Si	PPT (metanol)	LC: acetat amoni en aigua vs. metanol	MS/MS	Metabòlits després d'hidròlisi	(Marier *et al*., 2002)
Rates i ratolins	20, 60 mg/kg Resveratrol	Sèrum i orina	Si[b]	PPT (ACN)	LC: àcid fòrmic en aigua vs. ACN	UV/DAD-MS/MS	Perfil metabòlic	(Yu *et al*., 2002)
Humans, rates i ratolins	Extracte de raïm o 2, 5 mg/kg Resveratrol	Plasma i orina	Si No	PPT (metanol) LLE (CH_2Cl_2)	LC: TFA en aigua vs. metanol	*Electrode Array* MS/MS (Identificació)	Metabòlits després d'hidròlisi	(Meng *et al*., 2004)
Ratolins	240 mg/kg *trans*-resveratrol	Sèrum	No	PPT (ACN)	LC: acetat amoni en aigua vs. propan-2-ol & ACN	UV/vis	Perfil metabòlic	(Sale *et al*., 2004)
Humans	100 mg *trans*-resveratrol	Plasma Orina	Si No	SPE	LC: TFA en aigua vs. metanol	UV/vis MS/MS (Identificació)	Metabòlits després d'hidròlisi Perfil metabòlic	(Walle *et al*., 2004)
Humans Humans	250 mL de vi negre 200 i 300 mL de vi x 4 setmanes	LDL Plasma i Orina	No	SPE	LC: àcid acètic en aigua vs. ACN	MS/MS (Triple quadrupol	Perfil metabòlic	(Urpi-Sarda *et al*., 2005) (Zamora-Ros *et al*., 2006)
Humans	300 mL de vi negre 600 mL de vi negre	Sèrum Sèrum	Si No	LLE (acetat d'etil) LLE (acetat d'etil)	LC: fosfat sòdic en aigua vs. ACN LC: àcid acètic en aigua vs. ACN	UV/vis + MS (Identificació) DAD + MS/MS (Identif.)	Metabòlits després d'hidròlisi Perfil metabòlic	(Vitaglione *et al*., 2005)

Rates	20 mg/kg *trans*-resveratrol	Orina	No	SPE	LC: aigua vs. metanol:aigua	MS/MS (Identificació)	Perfil metabòlic (Només identificació)	(Wang *et al*., 2005)
Rates	50, 300 mg/kg *trans*-resveratrol x 8 dies	Plasma, orina, femtes i teixits	No	PPT (ACN)	LC: formiat amoni en aigua vs. metanol	DAD	Perfil metabòlic	(Wenzel *et al*., 2005)
Humans	85.5 mg *trans*-piceid	Plasma i orina						(Burkon i Somoza, 2008)
Rates	50 mg/kg *trans*-resveratrol	Plasma, orina i teixits	Si	PPT (TCA) + PPT (metanol)	LC: metanol:aigua:HCl vs. ACN:aigua:HCl	DAD MS/MS (Identificació)	Perfil metabòlic (Només identificació)	(El-Mohsen *et al*., 2006)
Rates	50 mg/kg Piceid	Plasma i teixits	No	PPT (metanol)	LC: aigua vs. ACN	UV/vis	Piceid	(Lv *et al*., 2006)
Humans	0.5, 1, 2.5, 5 g Resveratrol	Plasma i orina	No	PPT (metanol) + SPE	LC: acetat amoni en aigua + propanol vs. metanol + propanol	UV/vis + MS/MS (Identificació)	Perfil metabòlic	(Boocock *et al*., 2007a; Boocock *et al*., 2007b)
Rates	~5 mg/kg *trans*- and *cis*-resveratrol	Plasma	No	PPT (ACN)	LC: àcid acètic en aigua vs. metanol	UV	Aglicona lliure	(Chen *et al*., 2007)
Humans	250 mL de vi negre	Orina i LDL	No	SPE	LC: àcid acètic en aigua vs. ACN:acetona	MS/MS (Triple quadrupol)	Perfil metabòlic	(Urpi-Sarda *et al*., 2007)
Rates	150 mg/kg Piceid	Plasma	Si	PPT (ACN acidulat)	LC: àcid fòrmic en aigua vs. ACN	UV	Perfil metabòlic	(Zhou *et al*., 2007)
Rates	80 mg/kg Resveratrol *en Smilax china*	Plasma	No	LLE (acetat d'etil)	LC: àcid acètic en aigua vs. ACN	DAD	Aglicona lliure	(Huang *et al*., 2008)
Rates	20 mg/kg *Polygonum cuspidatum*	Orina, bilis i teixits	No	SPE	LC: aigua vs. ACN	UV MS/MS (Identificació)	Perfil metabòlic	(Wang *et al*., 2008)
Humans	Càpsules de resveratrol (dosi 25 – 400 mg)	Plasma	No	SPE	LC: aigua vs. metanol	MS (mode SIM)	Aglicona lliure	(Almeida *et al*., 2009; Nunes *et al*., 2009; Vaz-da-Silva *et al*., 2008)
Porcs	6.25 mg/kg Resveratrol	Plasma	No	LLE (ACN)	LC: àcid fòrmic en aigua vs. ACN	DAD-MS/MS (Identificació amb trampa d'ions)	Perfil metabòlic	(Azorin-Ortuno *et al*., 2010)
Rates	15 mg/kg (i.v.) *trans*-resveratrol	Plasma i teixits	Si	SPE	LC: àcid acètic en aigua vs. ACN	UV/vis MS/MS (Identificació)	Metabòlits després d'hidròlisi	(Juan *et al*., 2010b)

Rates	60 mg/kg Dihidroresveratrol	Plasma	No	SPE	LC: àcid acètic en aigua vs. ACN	UV/vis MS/MS	Perfil metabòlic (Només identificació)	(Juan *et al*., 2010a)
Humans	2 g *trans*-resveratrol	Plasma	No	PPT (ACN)	LC: àcid acètic en aigua vs. metanol	MS/MS (Triple quadrupol)	Aglicona lliure	(la Porte *et al*., 2010)
Humans	250 mL de vi negre 1 L suc de raïm Capsules	Plasma i orina	Si	LLE (acetat d'etil + ACN)	GC	MS (mode SIM)	Aglicona lliure	(Ortuño *et al*., 2010)
Porcs	6.25 mg/kg Resveratrol	Fluid biològics i teixits	No	PPT (ACN)	LC: àcid fòrmic en aigua vs. ACN	DAD-MS/MS (Identificació amb trampa d'ions, triple quadrupol i temps de vol)	Perfil metabòlic	(Azorin-Ortuno *et al*., 2011)
Rates	50, 150 mg/kg *trans*-resveratrol * 15 dies	Plasma	No	PPT (ACN)	LC: acetat amoni en aigua + isopropanol vs. metanol:isopropanol	MS/MS (Triple quadrupol)	Perfil metabòlic	(Kapetanovic *et al*., 2010)
Rates	20 mg/kg Piceid	Orina	No	SPE	LC: àcid acètic en aigua vs. metanol	UV/vis MS/MS (Identificació)	Perfil metabòlic	(Wang *et al*., 2011)
Ratolins	60 mg/kg resveratrol	Plasma	No	PPT (metanol)	LC: acetat amoni en aigua vs. metanol	MS/MS (Triple quadrupol)	Perfil metabòlic	(Iwuchukwu *et al*., 2012)
Gossos	200, 600 i 1200 mg/kg resveratrol	Plasma	No	PPT (ACN)	LC: acetat amoni en aigua + isopropanol vs. metanol:isopropanol	MS/MS (Triple quadrupol)	Perfil metabòlic	(Muzzio *et al*., 2012)
Rates	6, 30 o 60 mg/kg Resveratrol	Teixits	No	SPE	LC: àcid acètic o acetat amoni en aigua vs. ACN:acetona	MS/MS (Triple quadrupol)	Perfil metabòlic	(Andres-Lacueva *et al*., 2012)

[a]S'especifiquen les fases mòbils de la LC. Tots els àcids i sals anomenats es troben en solució aquosa.
[b]El procés d'hidròlisi serveix únicament per a confirmació dels diferents metabòlits identificats

Donat el fet que l'espectrometria de masses és la que s'utilitza en els diferents estudis que constitueixen la Tesi Doctoral, es creu interessant incloure un capítol sobre el fonament de les instrumentacions analítiques que pot contemplar aquesta tècnica, la qual consta d'un procés amb dos punts clau: la font de ionització i l'analitzador de masses.

Font de ionització

La font de ionització ha de ser capaç de ionitzar els analits, ja sigui en positiu o en negatiu. Aquesta tècnica dependrà de l'estructura, polaritat i pes molecular del compost; de manera que, donada la gran varietat estructural dels compostos presents en les mostres biològiques, no existeix una font adequada per a l'anàlisi de tots els compostos. En l'espectrometria de masses lligada a la LC existeixen tres tècniques de ionització que treballen a pressió atmosfèrica i que cobreixen l'ampli rang de polaritats i pesos moleculars:

- Font d'ionització per electroesprai (ESI), la qual realitza una ionització més suau, destinada a l'anàlisi de compostos polars i iònics, com ho són el resveratrol i el seu perfil metabòlic. És per això que és la tècnica més utilitzada en l'estudi de la biodisponibilitat del resveratrol, normalment treballant en mode negatiu.

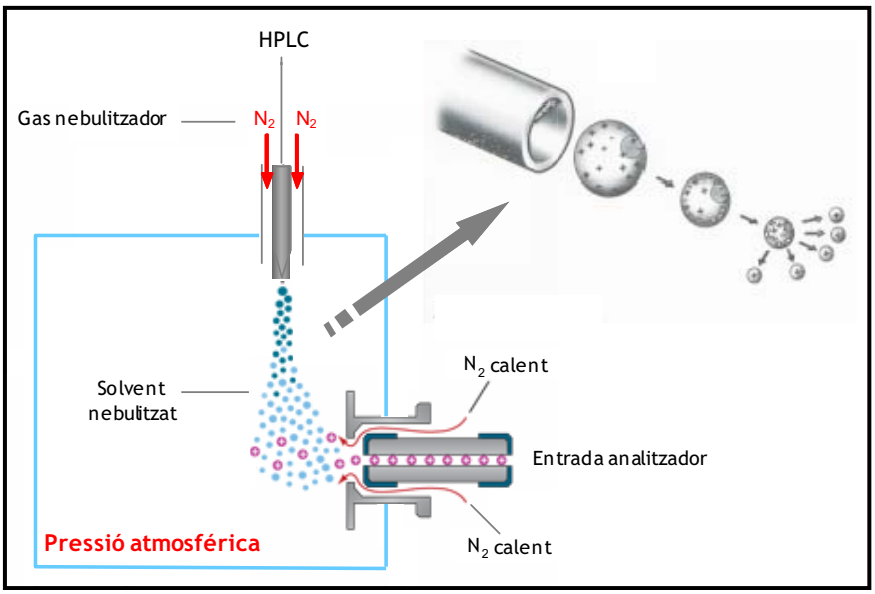


Figura 4. Esquema de la ionització per electrospray i detall de la dissociació d'ions.

En l'electrospray, la mostra dissolta en la fase mòbil entra en una cambra a pressió atmosfèrica, on es nebulitza transformant-se en un tipus d'esprai abans d'arribar a l'analitzador de masses (Figura 4). Unes fortes condicions electrostàtiques dissocien les molècules de l'analit, mentre la presència de gas a elevada temperatura va evaporant el solvent de la fase mòbil en petites gotes. A mesura que la mida de les gotes disminueix, la seva càrrega augmenta, afavorint així, la dissociació dels diferents ions en la nova fase gasosa.

- Font d'ionització química a pressió atmosfèrica (APCI) que presenta una major tolerància per a les sals i per a l'efecte de la matriu de la mostra, comparada amb l'anterior.
- Fotoionització a pressió atmosfèrica (APPI), la qual presenta un rang d'aplicació similar al del APCI, tot i que es pot extendre a compostos no polars.

Analitzadors de masses

La funció d'un analitzador de masses és la separació dels ions en funció de la relació *m/z* que presenten, la qualitat de la qual es caracteritza per el grau en el que poden arribar a separar valors de *m/z* molt pròxims. Existeixen 4 tipus diferenciats d'analitzadors de masses, amb diferent resolució:

- Quadrupol, anomenat també "filtre de masses", és un dels analitzadors de baixa resolució, ja que determina els ions amb una relació *m/z* < 1000. Està format per quatre cilindres paral·lels formant un quadrat (Figura 5), als quals se'ls aplica un potencial de manera combinada, que genera un camp electromagnètic. Aquest ambient permet fixar els ions en funció de la relació *m/z* en el centre del quadrupol, podent arribar així al detector. Amb aquest analitzador es pot operar amb el mode d'escanejat *full scan,* el qual analitza totes les masses en un rang entre dos valors determinats, o bé seleccionant i monitoritzant un ió en concret (*Selected Ion Monitoring*, SIM).

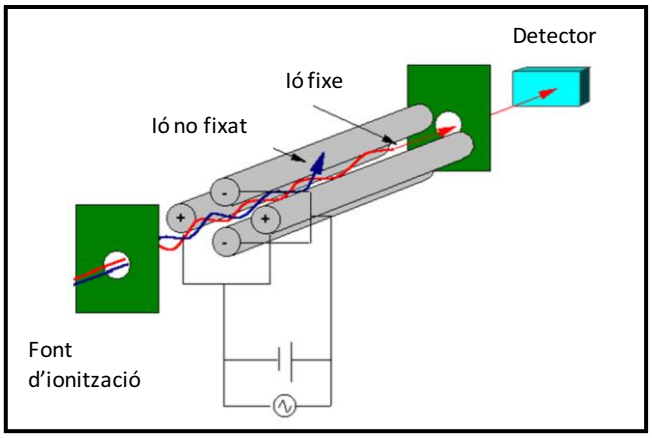


Figura 5. Diagrama esquemàtic d'un quadrupol.

- Temps de vol (ToF), que treballant amb *m/z* < 15000, té una resolució superior al presentat anteriorment, ja que permet donar una mesura molt més exacte de les masses dels ions (fins a 4 decimals). En aquest tipus d'analitzador s'aplica un força electromagnètica uniforme a tots els ions al mateix temps, provocant una acceleració en el seu pas a través d'un tub anomenat "tub de vol". Els ions més lleugers viatgen més ràpid i arriben abans al detector, de manera que les relacions *m/z* estaran condicionades i es determinaran en funció del temps d'arribada.

- Trampa d'ions, la qual treballa amb *m/z* < 1000, presentant, també, una baixa resolució. Està format per una cambra amb 2 elèctrodes i dues peces de finalització, que atrapen els ions gràcies a una sèrie de camps electromagnètics. L'avantatge de la trampa d'ions és la possibilitat de fer un escanejat de masses (MS^n), molt útil per a estudiar el perfil de fragmentació dels compostos a analitzar.

- Transformadors de Fourier, que determinant ions de *m/z* < 50000, presenten una resolució molt superior.

Dissociació induïda en una cel·la de col·lisió (CID)

Les tècniques d'ionització per electrospray són suaus i generen ions moleculars de càrrega positiva o negativa, adductes simples o ions que representen pèrdues moleculars simples, com d'aigua. Aplicant diferents voltatges a aquestes molècules

s'incrementa l'energia provocant una major fragmentació, que permet conèixer, no només el pes molecular de la molècula, sinó també la seva estructura. Aquesta tècnica de dissociació o de fragmentació múltiple sol estar lligada a vàries etapes d'espectrometria de masses (MS/MS) unint més d'un analitzador, tot i que també es poden dur a terme en una sola etapa, ja sigui en un quadrupol senzill o en un ToF. Quan la CID té lloc en una etapa de MS, la cel·la de col·lisió és la mateixa font de ionització, on els ions procedents de la mostra ionitzada s'acceleren i col·lisionen amb molècules residuals neutres, formant els fragments anomenats "ions producte". Amb aquesta tècnica no es pot conèixer de quin ió precursor procedeix cada fragment generat, de manera que fa difícil la identificació de compostos en mostres molt complexes.

Els experiments en tàndem (MS/MS o MS^n) són els que realitzen una CID en múltiples etapes, essent realment útils per a determinar l'estructura dels analits, en aparells que contenen, com a analitzadors, un triple quadrupol (Figura 6), o bé, un quadrupol/ToF. La dissociació té lloc en el segon quadrupol, generant-se l'espectre d'ions en el tercer quadrupol o en el ToF. El primer quadrupol pot ser un seleccionador d'ions o estar estàtic, segons el tipus d'experiment que s'hi dugui a terme.

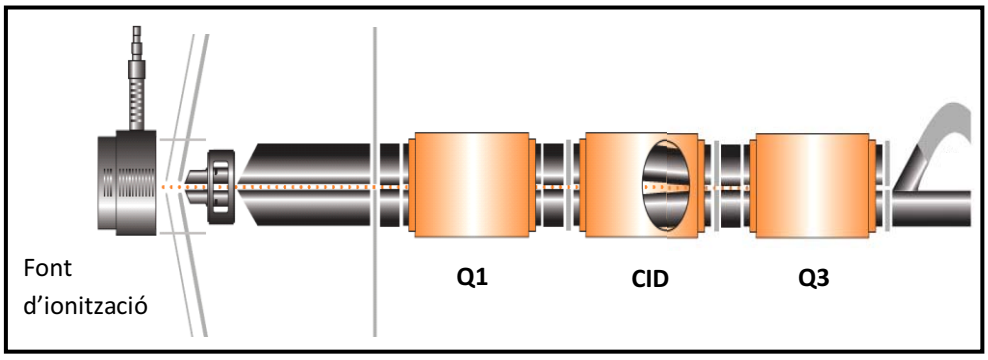
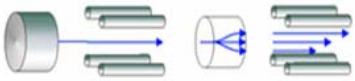
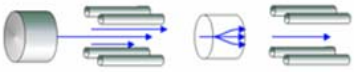
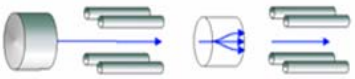
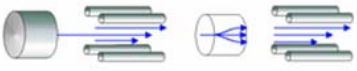


Figura 6. Esquema d'un triple quadrupol.

En el cas del triple quadrupol, que és l'aparell utilitzat en els estudis que formen part d'aquesta Tesi Doctoral, es poden realitzar fins a quatre tipus d'experiments en MS/MS. En qualsevol d'aquests experiments, les condicions del primer i tercer quadrupol (Q1 i Q3, respectivament) estan controlades i tenen una funció concreta (Taula 3):

- *Product Ion Scan* (PIS), en el qual es determinen tots els fragments (fills) que provenen d'un ió precursor (pare) concret. Permet confirmar la identitat d'un compost determinat.
- *Precursor Ion Scan*, que determina els ions pare (precursors) d'un fragment fill concret. Se sol utilitzar quan es busca un compost concret o una família específica de compostos.
- *Multiple Reaction Monitoring* (MRM), que busca un ió concret determinant les masses, tant dels ions pares com dels fills. S'utilitza per a la quantificació dels diferents analits, ja que permet obtenir límits de detecció més baixos.
- Neutral Loss Scan, el qual busca tots els parells de ions, precursors i productes, que discrepen per una massa neutre constant, prèviament determinada. Ideal si es busca una família específica de compostos o un sol compost.

Taula 3. Condicions per a cada un dels experiments MS/MS en triple quadrupol.

Experiment	Q1	CID	Q3
Product Ion Scan	Estàtic (selecció del precursor)		Escanejat
			
Precursor Ion Scan	Escanejat		Estàtic (selecció del fragment)
			
Multiple Reaction Monitoring	Estàtic (selecció del precursor)		Estàtic (selecció del fragment)
			
Neutral Loss Scan	Estàtic (sincronitzat amb Q3)		Estàtic (sincronitzat amb Q1)
			

METODOLOGIA

METODOLOGIA

DISSENY D'ESTUDIS

ESTUDI 1: ESTUDI CLÍNIC ALEATORITZAT D'INTERVENCIÓ EN HUMANS AMB UN CONSUM PUNTUAL DE VI NEGRE I COMPRIMITS D'EXTRACTE DE RAÏM

El primer treball consisteix en un estudi farmacocinètic de resveratrol, piceid i els seus respectius metabòlits en humans després del consum moderat de vi, com a font natural de resveratrol, per a ser comparat amb un estudi pilot després del consum d'extracte de raïm, com a producte nutracèutic.

Per aquest estudi, es van reclutar 10 homes sans, d'entre 24 i 35 anys i amb un índex de massa corporal (IMC) de 24,9 ± 3,3 kg/m^2, 7 dels quals van ser seleccionats aleatòriament per a consumir el vi, mentre que els altres 3 van realitzar l'estudi pilot amb l'extracte de raïm. Els voluntaris van iniciar l'estudi amb un període de neteja (*wash-out*) en el qual se'ls va restringir el consum de productes derivats del raïm durant 3 dies i de qualsevol aliment ric en polifenols el dia previ i durant l'estudi. Després d'aquest període de neteja, els voluntaris van ser citats a l'Hospital Clínic de Barcelona en condicions de dejú on se'ls va fer la primera extracció de sang i recol·lecta d'orina. Seguidament, els voluntaris van consumir una dosi similar de resveratrol total en dues formes alimentàries diferents: 375 mL de vi negre o 15 comprimits d'extracte de raïm que van prendre acompanyats de 400 mL d'aigua. Sis hores després de la intervenció, tots els voluntaris van rebre el mateix dinar i van romandre a l'hospital durant 5 hores més. Els voluntaris van tornar a l'hospital al matí següent per al mostreig a 24-hores i els participants de l'estudi pilot, en el qual van

ingerir el comprimit, s'hi van estar tot un segon dia, ja que estudis preliminars havien suggerit que els comprimits podrien retardar l'absorció de resveratrol i, per tant, el temps necessari per a ser totalment eliminat. Es va subministrar el mateix sopar a tots els voluntaris pel dia previ a la intervenció i pels dies de l'estudi.

La figura 7 mostra un esquema del disseny de l'estudi, on es poden veure els diferents temps en els quals es van realitzar les extraccions de sang i les recol·leccions d'orina.

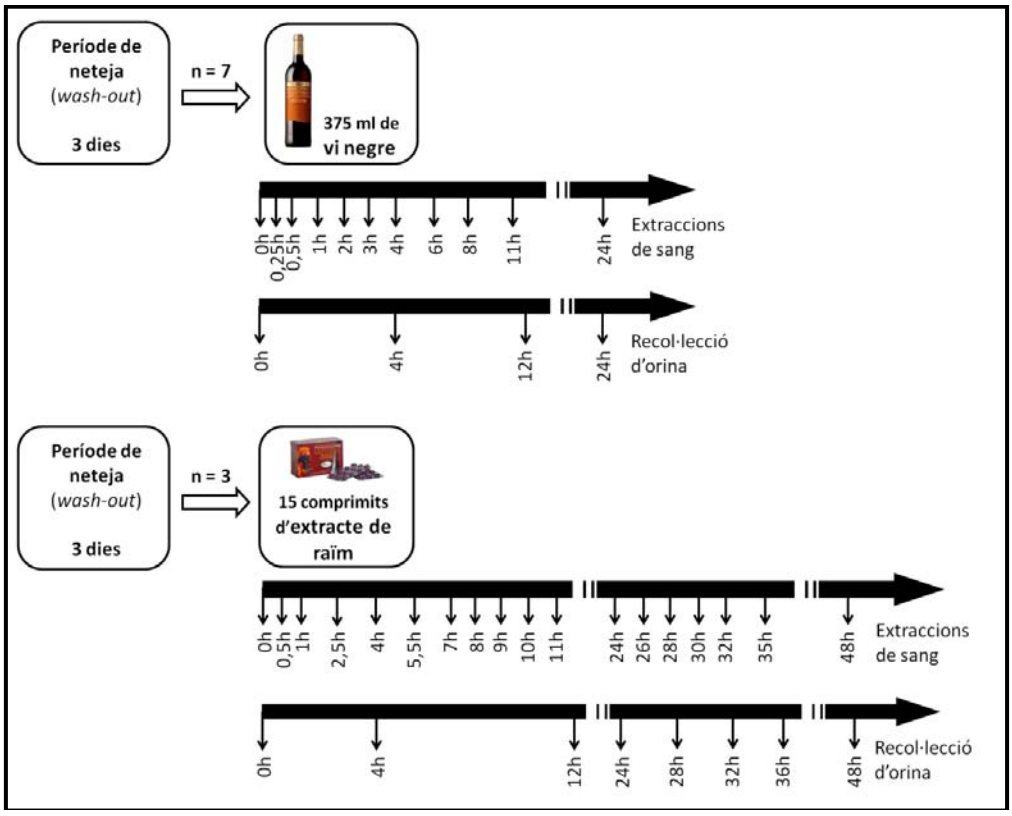


Figura 7. Disseny de l'estudi clínic aleatoritzat d'intervenció en humans amb un consum puntual de vi negre i comprimits d'extracte de raïm (estudi 1).

ESTUDI 2: ESTUDI CLÍNIC ALEATORITZAT I CREUAT D'INTERVENCIÓ EN HUMANS AMB UN CONSUM REGULAR DE VI NEGRE I EL MATEIX VI DESALCOHOLITZAT

L'estudi clínic corresponent a l'estudi 2 es va dissenyar per tal d'avaluar els efectes dels polifenols i/o alcohol del vi negre en la malaltia cardiovascular (Chiva-Blanch *et al.*, 2012a). En la present Tesi Doctoral, aquest disseny d'estudi va servir per avaluar el possible efecte de l'alcohol en la biodisponibilitat del perfil metabòlic del resveratrol.

Un total de 73 individus amb elevat risc cardiovascular i edats compreses entre els 55 i 75 anys, van ser reclutats per a l'estudi a l'Hospital Clínic de Barcelona, tot i que per a l'estudi de biodisponibilitat es va analitzar l'orina disponible de 59 voluntaris. Els 73 voluntaris es van seleccionar per ser consumidors moderats d'alcohol (1 – 3 copes al dia) i tenir diabetis o 3 o més dels següents factors de risc de la malaltia cardiovascular: addicció al tabac, hipertensió, concentracions de colesterol LDL plasmàtic ≥160 mg/dL, concentracions de colesterol HDL plasmàtic ≤35 mg/dL, sobrepès o obesitat (IMC ≥25 kg/m^2) i/o antecedents familiars de malalties cardiovasculars. Els criteris d'exclusió van ser el coneixement de malalties cardiovasculars prèvies, embòlia o malaltia vascular perifèrica, infecció del VIH, malaltia hepàtica alcohòlica, desnutrició i neoplàsia o malalties infeccioses agudes.

L'estudi d'intervenció, obert, aleatoritzat i creuat, es va dur a terme en dos períodes de 4 setmanes (Figura 8). Prèviament, els voluntaris van seguir un període de rentat durant 2 setmanes, en el qual se'ls va demanar que seguissin una dieta sense begudes alcohòliques ni productes derivats del raïm. Després d'això, es va demanar als voluntaris que consumissin 272 mL al dia de vi negre (30 g d'alcohol/dia) o del mateix vi desalcoholitzat, durant 4 setmanes, seguint les mateixes limitacions dietètiques que en el període de rentat. Un cop passades aquestes quatre setmanes, la intervenció es va repetir en l'ordre invers (Figura 8). Els voluntaris van recollir la orina durant les últimes 24 hores de cada un dels tractaments i del període de rentat.

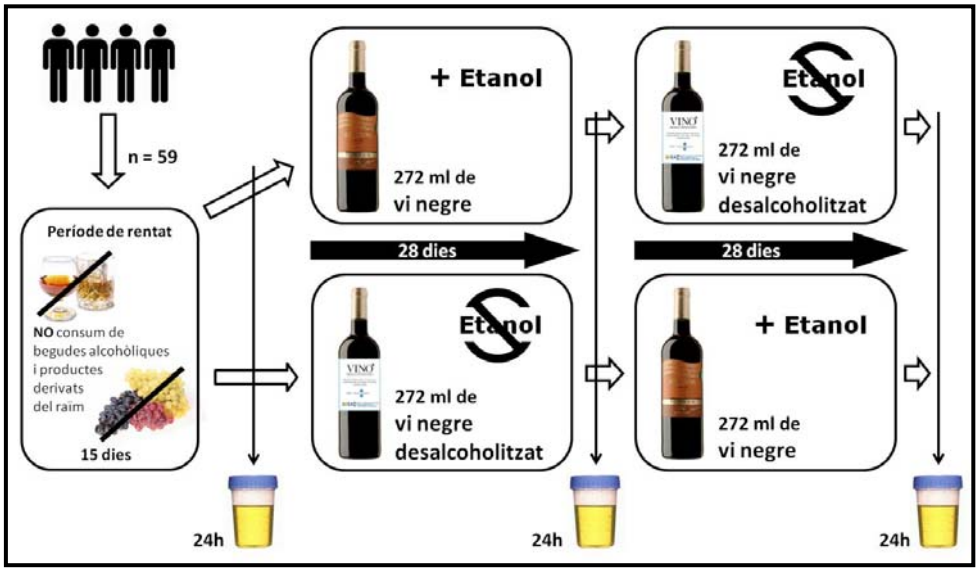


Figura 8. Disseny de l'estudi aleatoritzat i creuat d'intervenció en humans amb un consum regular de vi negre i el mateix vi desalcoholitzat (estudi 2).

ESTUDI 3: ESTUDI CLÍNIC ALEATORITZAT, CREUAT I CONTROLAT D'INTERVENCIÓ EN HUMANS AMB UN CONSUM PUNTUAL I REGULAR D'UNA BEGUDA FUNCIONAL A BASE D'EXTRACTE DE RAÏM

La biodisponibilitat d'un compost actiu pot variar enormement en funció de nombrosos factors, com poden ser la matriu de l'aliment en el qual es consumeix el compost, el tipus de dosificació, el gènere dels individus, etc. En l'estudi 3 es va estudiar l'efecte de la ingesta aguda i regular d'una beguda funcional d'extracte de raïm, alhora que se'n determinaven les possibles diferències presents entre gèneres.

Amb aquest objectiu, un total de 31 voluntaris sans, amb edats compreses entre 25 i 55 anys, es van reclutar per aquest estudi clínic d'intervenció, aleatoritzat, creuat i controlat, que va tenir lloc a l'Hospital Universitari La Paz de Madrid. Com que 5 voluntaris van abandonar l'estudi abans de completar totes les seves etapes, 26 individus, 13 homes i 13 dones, van ser inclosos. Al tractar-se d'un estudi amb persones sanes, no es van incloure persones amb malalties declarades, com diabetis, hipertensió, obesitat, demències o malalties mentals, etc., ni tampoc dones embaraçades o en període de lactància. Després d'un període de rentat de 7 dies, en el qual els voluntaris van seguir una dieta amb baix contingut en polifenols, van recollir la orina de les 24 hores prèvies a la intervenció. Un cop a l'hospital els voluntaris van ingerir, de manera aleatòria, 187 mL d'una beguda funcional a base d'extracte de raïm, o bé, de la beguda control o placebo, juntament amb un esmorzar estàndard (pa blanc, formatge i iogurt). Fins a 4 hores després de la ingesta, els voluntaris van estar recollint la orina per a l'estudi puntual (Figura 9). Per a l'estudi de consum regular, els voluntaris van ingerir 187 mL de la mateixa beguda que en l'estudi puntual, dos cops al dia (dinar i sopar), seguint les mateixes limitacions dietètiques que les establertes durant el període de rentat. Al final d'aquest període els individus van recollir la orina corresponent a les últimes 24 hores d'estudi (Figura 9). El mateix procediment es va repetir per els estudis puntual i regular, tenint en compte l'aleatorització de la beguda ingerida, després d'un període de rentat de 7 dies, igual al que va tenir lloc a l'inici de l'estudi.

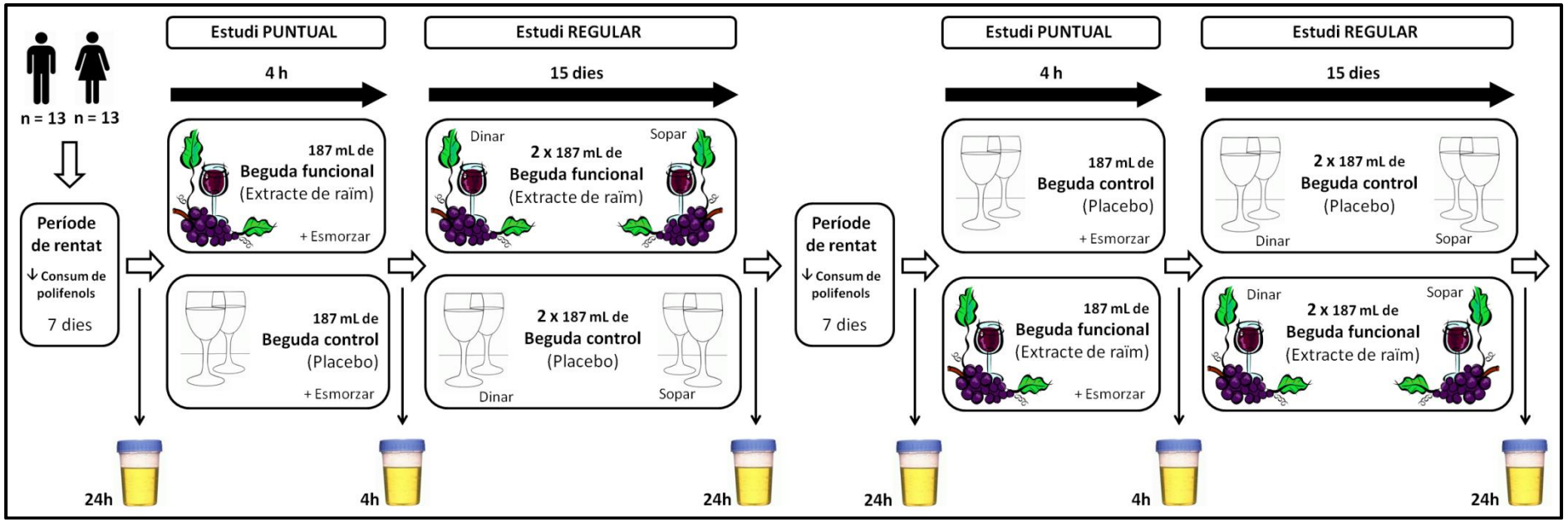


Figura 9. Disseny de l'estudi clínic aleatoritzat, creuat i controlat d'intervenció en humans amb un consum puntual i regular d'una beguda funcional a base d'extracte de raïm (estudi 3).

METODOLOGIA ANALÍTICA

L'anàlisi de les mostres biològiques provinents dels estudis presentats anteriorment es va realitzar seguint la metodologia desenvolupada per Urpi-Sarda, *et al*. (2007) i optimitzada i validada a la present Tesi Doctoral. El mètode consisteix en un procés d'extracció en fase sòlida (SPE), com a preparació de la mostra biològica per tal d'eliminar possibles interferències, i l'anàlisi instrumental per cromatografia líquida acoblada a un espectròmetre de masses en tàndem (LC-MS/MS), concretament a un API 3000 (Applied Biosystems, PE Sciex; Concord, Ontario, Canada). En aquesta tècnica i quan s'utilitzen columnes analítiques de fase reversa amb fases mòbils acidificades, els conjugats sulfatats mostren una pobre resolució cromatogràfica (Walle, 2011), ben al contrari al comportament que presenta l'aglicona i els conjugats glucosilats i glucuronidats. Tot i que en el mètode publicat anteriorment (Urpi-Sarda *et al*., 2007), utilitzat per a l'anàlisi de les mostres de plasma i orina del primer estudi, se solucionava aquest problema amb l'addició d'acetona a la fase mòbil orgànica per a donar-li més apolaritat (Urpi-Sarda *et al*., 2007), la detecció dels disulfats i sulfoglucurònids no va poder ser efectuada. Per això i gràcies a la disponibilitat comercial d'estàndards purs d'alguns metabòlits del resveratrol, la metodologia d'anàlisi del perfil metabòlic del resveratrol va poder ser optimitzada i validada, com a objectiu de la present Tesi Doctoral, en l'estudi 2; essent aplicada a les mostres d'orina provinents dels estudis 2 i 3. A més a més, la metodologia optimitzada es va adaptar, alhora, a un cromatògraf d'ultra pressió (Waters Acquity UPLC system; Milford, Massachusetts, Estats Units) per tal de reduir els temps d'anàlisi.

ESTÀNDARDS

Per a l'anàlisi de les mostres biològiques es va disposar d'estàndards comercials de *trans*-resveratrol (puresa 99%) i *trans*-piceid (puresa 97%), provinents de Sigma-Aldrich (St. Louis, Missouri, Estats Units). Per al primer estudi clínic també es va disposar dels isòmers *trans*- de resveratrol-3-*O*-sulfat, resveratrol-4'-*O*-sulfat, resveratrol-3-*O*-glucurònid, resveratrol-4'-*O*-glucurònid, per a la seva identificació, purificats a partir de mostres de bilis de rates obtingudes després d'una perfusió de resveratrol (Urpi-Sarda *et al*., 2007).

Per a la optimització i validació de la metodologia presentada a la present Tesis Doctoral i l'anàlisi de les mostres d'orina dels estudis 2 i 3, es va poder comptar, a més a més, amb els estàndards comercials purs de *cis*-resveratrol (puresa 97%), *trans*- i *cis*-resveratrol-4'-*O*-glucurònid (puresa 98%), *trans*- i *cis*-resveratrol-3-*O*-glucurònid (puresa 98% i 96%, respectivament), *trans*-resveratrol-3-*O*-sulfat (puresa 98%) i *trans*-resveratrol-$^{13}C_6$, per a ser utilitzat com a patró intern, adquirits a l'empresa Toronto Research Chemicals Inc. (North York, ON, Canada). També es va poder comptar amb estàndards de *trans*-resveratrol-4'-*O*-sulfat (puresa >95%) i *trans*-resveratrol-3,4'-*O*-disulfat (puresa >91%), obtinguts com es va descriure prèviament (Miksits *et al*., 2005). L'estándar de dihidroresveratrol va ser sintetitzat seguint el treball de Thakkar, *et al*. (Thakkar *et al*., 1993), confirmant-se la seva estructura per ressonància magnètica nuclear i determinant-ne la puresa, que es va esdevenir del 80% per a la quantificació; tot i que es va observar una puresa relativa, respecte a la matèria d'origen (*trans*-resveratrol), superior al 97%.

PREPARACIÓ DE LES MOSTRES DE PLASMA I ORINA

Les mostres de plasma provinents de l'estudi clínic 1 es van pretractar amb 20 µL d'àcid orto-fosfòric, seguit de 2 minuts de mescla en vòrtex, per tal de trencar les unions dels analits amb les estructures proteiques. Les mostres d'orina dels tres estudis van requerir una centrifugació a 10000g durant 3 minuts, sempre mantenint la refrigeració (4ºC). El procés d'extracció en fase sòlida d'aquestes mostres biològiques es va realitzar en plaques Waters Oasis® HLB (30 mg) de 96 pous (Milford,

Massachusetts, Estats Units), en les quals es pot dur a terme el procés d'SPE de manera simultània en un elevat nombre de mostres. Abans de la càrrega de les mostres, els cartutxos van ser degudament condicionats amb l'addició d'un mL de metanol seguit d'un mL d'una solució aquosa d'àcid acètic 2 mol/L per a equilibrar el cartutx. Seguidament, 1 mL d'orina o plasma pretractat es van carregar a la placa, juntament amb 100 µL de patró intern. Els cartutxos es van netejar amb 1 mL de la solució aquosa d'àcid acètic 2 mol/L, seguit d'un mL d'àcid acètic 2 mol/L en aigua/metanol (85:15, v/v). L'elució dels metabòlits va ser lleugerament diferent en cada un dels procediments. La metodologia prèvia utilitzava 0,5 mL d'una solució metanòlica d'àcid acètic 1mol/L, seguit de dues etapes de 0,75 mL d'àcid acètic 1mol/L en acetat d'etil (Urpi-Sarda *et al*., 2007). Amb el mètode analític optimitzat en aquesta Tesi es va afegir una etapa al final de l'elució amb 0,5 mL d'amoni al 5% (v/v) en metanol, un cop reduït el volum de la solució d'àcid acètic 1mol/L en acetat d'etil a dues etapes de 0,5 mL. El total dels 2 mL eluïts en ambdós procediments van ser evaporats fins a sequedat, sota corrent de nitrogen, i reconstituïts amb 100 µL d'una solució de taxifolina en fase mòbil.

ANÀLISI INSTRUMENTAL PER HPLC o UPLC-MS/MS

Separació cromatogràfica

La separació cromatogràfica de les mostres biològiques del primer disseny d'estudi va tenir lloc en un sistema Agilent 1200 (Waldbronn, Germany) equipat amb una bomba quaternària, un injector automàtic de plaques refrigerat i amb una columna analítica Phenomenex Luna C_{18} [50 × 2,0 mm i.d., 5 µm] (Torrance, CA, USA), que es va mantenir a una temperatura de 40 ºC (Urpi-Sarda *et al*., 2007). El volum injectat per mostra va ser de 15 µL a un flux de 0,5 mL/min amb àcid acètic 0,05% (v/v) en aigua, com a fase mòbil A i acetona/ACN (70:30, v/v) amb 0,04% d'àcid acètic com a fase mòbil B, seguint un gradient lineal (Taula 4):

Taula 4. Gradient cromatogràfic utilitzat en l'anàlisi de mostres per HPLC.

Temps (min)	0	0,5	5	5,6	5,7	8
% Fase Mòbil A	90	85	0	0	90	100
% Fase Mòbil B	10	15	100	100	10	0

Després d'aquest gradient la columna es va equilibrar durant 3 minuts.

La fase mòbil utilitzada en la separació cromatogràfica té una elevada influència en la ionització dels compostos a analitzar. La disponibilitat dels estàndards purs d'alguns dels metabòlits del resveratrol va permetre optimitzar les condicions cromatogràfiques per al seu anàlisi, examinant el seu comportament en vàries fases mòbils aquoses: la utilitzada en el mètode presentat anteriorment (0,05% d'àcid acètic en aigua) i una solució tampó amb acetat amoni (10 mmol/L) a diferents pH (3, 4 i 5).

El mètode instrumental presentat anteriorment (Urpi-Sarda *et al*., 2007) es va adaptar a un cromatògraf d'ultra pressió (Waters Acquity UPLC; Milford, Massachusetts, Estats Units), amb el qual es va reduir el temps d'anàlisi i, per tant, costos, tant de solvents com de residus; essent, molt més respectuós amb el medi ambient. La separació cromatogràfica d'un volum de mostra injectat de 5 µL es va aconseguir amb una columna Acquity UPLC BEH C18 (Waters; Milford, Massachusetts, Estats Units), a 40ºC, a un flux d'un mL/min, el qual va haver de ser dividit (1:1) abans d'entrar a l'espectròmetre de masses. Després d'examinar les diferents fases mòbils, se'n van utilitzar dues per a una separació cromatogràfica òptima de tot el perfil metabòlic: 0,05% d'àcid acètic en aigua i una solució tampó d'acetat amoni (10 mmol/L) a pH 5, mantenint la fase mòbil B del mètode que el precedeix (acetona:ACN, 70:30 – v/v) (Urpi-Sarda *et al*., 2007) i amb el gradient lineal que mostra la taula 5.

Taula 5. Gradient cromatogràfic utilitzat en l'anàlisi de mostres per UPLC.

Temps (min)	0	1	2	2,3	2,31	3
% Fase Mòbil A	90	70	0	0	90	90
% Fase Mòbil B	10	30	100	100	10	10

Espectrometria de masses

La identificació i quantificació dels metabòlits de resveratrol va tenir lloc en un espectròmetre de masses API 3000 de triple quadrupol d'Applied Biosystems (Pe Sciex, Concord, Ontario, Canada), equipat amb una font d'ionització (ESI) Turbo IonSpray, treballant en mode negatiu. Els paràmetres de MS i MS/MS es van optimitzar per a cadascun dels estàndards en la fase mòbil adient en experiments d'infusió, obtenint els valors que mostra la taula 6.

Taula 6. Paràmetres de MS i MS/MS utilitzats en l'anàlisi de mostres biològiques per HPLC i UPLC.

Voltatge capil·lar	-3500 V
Gas nebulitzador, N_2	10/12* unitats arbitràries
Gas cortina, N_2	12 unitats arbitràries
Gas de col·lisió, N_2	4 unitats arbitràries
Potencial d'enfocament	-200 V
Potencial d'entrada	-10 V
Potencial de desagrupament	Segons compost
Energia de col·lisió	Segons compost
Temperatura del gas d'assecat	400ºC
Gas auxiliar	6000 cm^3/min

*Mètode per HPLC-MS/MS, 10 unitats de gas nebulitzador; 12 unitats per al mètode en UPLC-MS/MS

Els metabòlits del resveratrol i piceid van ser identificats comparant el temps de retenció i la fragmentació amb els estàndards disponibles o mitjançant experiments de *Product Ion Scan* (PIS). Per a la quantificació d'aquests en mostres biològiques es va treballar amb *Multiple Reaction Monitoring* (MRM), pel qual es van definir les transicions que exposa la taula 7, considerant el resveratrol i els seus metabòlits de fase II, els glucòsids de resveratrol i els seus metabòlits de fase II i els derivats de la microbiota. Les transicions utilitzades per a l'anàlisi del perfil metabòlic es mostren separades en funció de la fase mòbil més adient per a la seva determinació amb la metodologia optimitzada en aquesta Tesis Doctoral, tot i que les mateixes transicions es van utilitzar en el mètode utilitzat a l'estudi 1 (Rotches-Ribalta *et al.*, 2012a), excepte pel disulfat i sulfoglucurònids que van ser identificats posteriorment:

Taula 7. Transicions utilitzades en l'anàlisi de mostres per UPLC.

Compost	Transició MRM
Fase mòbil A: Àcid acètic 0,05%	
Resveratrol	227/185
Resveratrol-glucurònids	403/227
Piceid	389/227
Piceid-glucurònids (eq. *trans*-piceid)	565/227
Dihidroresveratrol	229/123
Dihidroresveratrol-glucurònids (eq. Dihidroresveratrol)	403/229
Fase mòbil B: Acetat amoni 10 mmol/L a pH 5	
Resveratrol- sulfats (*cis*-sulfats: eq. del respectiu isòmer *trans*-)	307/227
Resveratrol-disulfat	387/227
Resveratrol-sulfoglucurònid (eq. Resveratrol-disulfat)	483/307
Piceid-sulfats (eq. *trans*-piceid)	469/227
Dihidroresveratrol-sulfats (eq. Dihidroresveratrol)	309/229
Dihidroresveratrol-sulfoglucrònid (eq. Dihidroresveratrol)	485/309
trans-Resveratrol-$^{13}C_6$ (Patró intern)	233/191

L'anàlisi quantitatiu de les mostres de plasma i orina obtingues en l'estudi 1 amb la metodologia prèvia (Rotches-Ribalta *et al*., 2012a; Urpi-Sarda *et al*., 2007) es van realitzar corbes de calibratge de *trans*-resveratrol i *trans*-piceid en plasma blanc i orina sintètica, per tal d'estimar les concentracions dels metabòlits analitzats. Concretament, els metabòlits de fase II del resveratrol i els derivats de l'acció microbiana van ser quantificats com a *trans*-resveratrol i expressats com a equivalents d'aquest; mentre que els glucòsids de resveratrol es van expressar com a equivalents de *trans*-piceid, després de ser quantificats amb la seva corba de calibratge.

Per a la determinació quantitativa de les mostres d'orina dels estudis 2 i 3, analitzades amb el mètode optimitzat en la present Tesis Doctoral, es van realitzar corbes de calibratge en orina blanca humana, obtinguda després d'una setmana de dieta sense polifenols, per a cadascun dels estàndards disponibles. Els metabòlits dels quals no es disposava estàndard, es van estimar amb la corba del compost més similar, sempre

tenint en compte la fase mòbil utilitzada en el seu anàlisi i, per tant, expressat com al corresponent equivalent.

RESULTATS

RESULTATS

ESTUDI 1

FARMACOCINÈTICA DEL PERFIL METABÒLIC DEL RESVERATROL EN HUMANS SANS DESPRÉS DEL CONSUM MODERAT DE VI NEGRE I COMPRIMITS D'EXTRACTE DE RAÏM.

Els resultats obtinguts en l'estudi 1 sobre la farmacocinètica del perfil metabòlic del resveratrol en humans sans després del consum moderat de vi negre i comprimits d'extracte de raïm, han estat publicats en una de les revistes considerada pel *Science Citation Index*, amb un índex d'impacte de 4,436 i situada en el primer quartil de la categoria *Pharmacology & Pharmacy* (34 de 261).

Publicació 1: Maria Rotches-Ribalta, Cristina Andres-Lacueva, Ramon Estruch, Elvira Escribano, Mireia Urpi-Sarda. Pharmacokinetics of resveratrol metabolic profile in healthy humans after moderate consumption of red wine and grape extract tablets. *Pharmacological Research,* 2012;66(5):375-382.

Resum

La biodisponibilitat del resveratrol s'ha vist altament influenciada per nombrosos factors, entre els quals s'hi destaca la matriu de l'aliment que el conté. Aquest factor és el que es va estudiar en aquest primer treball, que consisteix en un estudi farmacocinètic de resveratrol, considerant el seu ampli perfil metabòlic en humans després del consum moderat d'una font natural de resveratrol, com és el vi negre, per

a ser comparat amb la ingesta d'un producte nutracèutic a base d'extracte de raïm, en un estudi pilot. Les mostres d'orina i plasma obtingudes en diferents punts de temps després del consum d'ambdós productes es van analitzar com s'ha exposat anteriorment, identificant fins a 17 metabòlits de resveratrol i piceid, considerant, també, els obtinguts per acció de la microbiota intestinal.

Els glucòsids de resveratrol es van detectar en les mostres de plasma en la seva forma intacta, aconseguint concentracions màximes 1 hora després de la ingesta dels dos productes. Es van observar majors concentracions (C_{max}) pels glucurònids de resveratrol amb temps màxims (t_{max}) més tardans, els quals van ser encara superiors per als conjugats derivats de l'acció microbiana, com els dihidroresveratrol-glucurònids. La mateixa tendència es va poder observar per a la quantitat excretada en les mostres d'orina.

Comparant les dues intervencions, es van obtenir diferències estadísticament significatives per alguns metabòlits, les quals van ser atribuïdes a la diferent composició de resveratrol i piceid en ambdós productes; fet que va permetre proposar possibles estructures químiques. De totes maneres, l'efecte de la matriu alimentaria es va poder observar donada la formulació del producte nutracèutic, la qual podria retardar l'absorció del resveratrol, afavorint el temps de permanència en l'intestí i augmentant el seu metabolisme microbià. De fet, l'excreció urinària dels metabòlits microbians va ser de fins a 6 vegades superior entre les 12 i 24 hores després del consum de l'extracte, comparat amb el de vi negre. Per tant, la ingesta de suplements dietètics rics en resveratrol podria ser també una bona manera d'aportar els beneficis d'aquesta molècula a la salut humana.

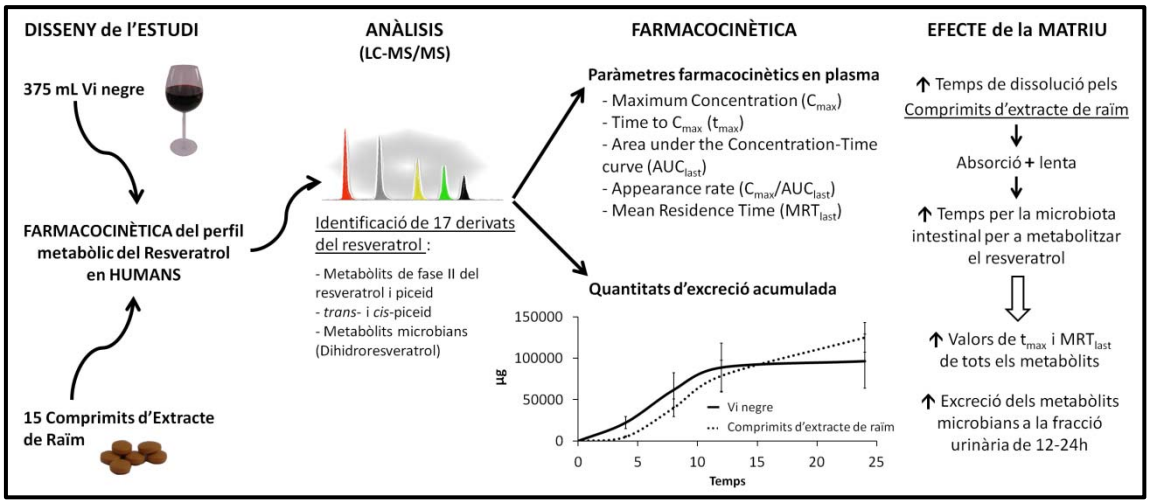


Figura 10. Gràfic resum de l'estudi farmacocinètic del perfil metabòlic de resveratrol en humans sans després del consum moderat de vi negre i comprimits d'extracte de raïm (estudi 1).

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Pharmacokinetics of resveratrol metabolic profile in healthy humans after moderate consumption of red wine and grape extract tablets

Maria Rotches-Ribalta[a,b], Cristina Andres-Lacueva[a,b], Ramon Estruch[c], Elvira Escribano[d], Mireia Urpi-Sarda[a,*]

[a] Nutrition and Food Science Department, XaRTA-INSA, Pharmacy Faculty, University of Barcelona, Av Joan XXIII, s/n, Barcelona, Spain
[b] Ingenio-CONSOLIDER program, FUN-C-FOOD, CSD2007-063, Ministry of Science and Innovation, Barcelona, Spain
[c] Department of Internal Medicine, Hospital Clinic, Institut d'Investigació Biomèdica August Pi i Sunyer (IDIBAPS), University of Barcelona, CIBER 06/03: Fisiopatología de la Obesidad y la Nutrición, Instituto de Salud Carlos III, Villarroel 170, 08036 Barcelona, Spain
[d] Biopharmaceutics and Pharmacokinetics Unit and Institute for Nanoscience and Nanotechnology, Pharmacy Faculty, University of Barcelona, Av Joan XXIII, s/n, Barcelona, Spain

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ABSTRACT

A pharmacokinetic study of the metabolic profile of resveratrol has been performed in healthy men after moderate red wine (RW) consumption. The bioavailability of resveratrol is highly influenced by several factors such as the food matrix and, therefore, this study has been compared with a pilot study in which men ingested grape extract (GE) tablets as a nutraceutical, containing similar total amounts of resveratrol than RW. Blood and urine samples were taken before and at several time points after intervention and then analyzed by SPE and LC–ESI–MS/MS. Up to 17 resveratrol and piceid derivatives were identified, including those formed by the intestinal microbiota. Resveratrol glucosides were found in plasma as intact forms and reached the lowest maximum concentrations 1 h after both interventions. Higher plasma concentrations and longer times (t_{max}) were observed for resveratrol glucuronides due to phase II metabolism and even higher values for conjugates derived from microbiota, such as dihydroresveratrol-glucuronides. The same trend was observed for total excreted amounts in urine samples. When both treatments were compared, statistically significant differences for some metabolites were obtained, which may be due to the different composition of resveratrol and piceid in both sources. However, GE formulation seems to delay resveratrol absorption, staying longer in the gut where could be metabolized to a greater degree, since 2.1–3.6-fold higher urinary concentrations of microbial metabolites were observed after GE intervention at 12–24 h urinary fraction. Therefore, supplement intake could be also a way to bring resveratrol benefits to human health.

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1. Introduction

Resveratrol is a natural stilbene with health-related properties, mainly present in grapes and its derivates [1]. The interest of this compound began after the studies on the "French Paradox" [2], in which cardioprotective effect of wine [3] is not only due to the alcohol content, since its polyphenolic composition, including resveratrol [4,5], showed additional properties in this way. In addition, several studies have reported that it could prevent some kinds of cancer [6,7] and slow the progression of Alzheimer's disease [8], due to its neuroprotective properties [9], as well as giving protection against obesity and its associated diseases [10]. Recent studies have also linked resveratrol to an enlargement of the lifespan of a number of species [11,12], due to its capacity to mimic caloric restriction [13]. Consequently, resveratrol has the potential to be evaluated for therapeutic use. However, although oral absorption of *trans*-resveratrol is high, it shows low bioavailability [14], since pharmacokinetic studies indicate that resveratrol is rapidly metabolized [15]. Therefore, the conjugated forms will be those that reach tissues where they could exert their possible biological effect, although the reported lower in vitro activity of the metabolites than resveratrol [16–18] may not necessarily reflect their in vivo function, since human enzymes could convert the metabolites back to resveratrol in humans [18]. The identification and measurement of conjugates are thus key prerequisites for understanding the role of these compounds. However, some bioavailability studies have only looked for free resveratrol [19–21] and many others have applied enzymatic hydrolysis [22–25], measuring resveratrol metabolites indirectly as free aglycones. Additionally, many studies have focused on the metabolic

Abbreviations: AUC, area under the plasma concentration–time curve; C_{max}, peak plasma concentration; DHR, dihydroresveratrol; GE, grape extract; MRM, multiple reaction monitoring; MRT, mean residence time; PIS, product ion scan; RW, red wine; t_{max}, time of peak plasma concentration.

* Corresponding author. Tel.: +34 93 4024508.
E-mail address: murpi@ub.edu (M. Urpi-Sarda).

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profile of resveratrol [26–30], where its glucuronide and sulfate conjugates have been the most frequently reported metabolites [14,31]. But little is known about resveratrol glucosides, their conjugates and those derived from microbiota metabolism such as dihydroresveratrol (DHR), which could be produced by hydrogenation of the aliphatic double bond of *trans*-resveratrol in the gut [32].

Bioavailability studies of resveratrol in humans vary enormously in the choice of the administered source and in the wide range of doses applied (5–5000 mg), including moderate consumption of red wine (RW) [25–27,33], grape juice [24,25], resveratrol capsules [19,25,29], pure piceid [30] and high doses of pure *trans*-resveratrol dissolved in different matrices [22,24,28], such as white wine, grape juice, vegetable homogenate, and diluted whiskey or ethanol. However, previous pharmacokinetic studies of resveratrol in humans have mainly been carried out with pure resveratrol and have only looked for its free form with or without hydrolysis [19–21,34]. Only one study has focused on the pharmacokinetics of three resveratrol metabolites: two monoglucuronides and resveratrol-3-sulfate, after pure resveratrol tablets [29]. More recently, Ortuño et al. [25] compared the plasma pharmacokinetics for *trans*- and *cis*-resveratrol after hydrolysis in men, but after natural products (RW and grape juice) and grape extract (GE) tablets enriched with *trans*-resveratrol. Therefore, to our knowledge, no pharmacokinetic studies of the metabolic profile of resveratrol in humans after moderate RW consumption have been performed despite this being the main food source of resveratrol and piceid [35]. However, the use of resveratrol as a nutraceutical is growing in importance since it has great potential, as seen in the growing number of scientific reports [36]. But there are conflicting views about the effect of resveratrol consumption in food as opposed to in pill form and the effects of long-term consumption are still uncertain [37]. The first approach would be to test its bioavailability after different administration forms, since previous studies have reached conflicting conclusions when different resveratrol sources were compared [22,24,25].

Therefore, the aim of this report is to study the pharmacokinetics of resveratrol, piceid and their respective metabolites in men, considering the parent compounds and phase II metabolites, including those formed by the intestinal microbiota, after moderate RW consumption, being compared with a pilot pharmacokinetic study in which men ingested grape extract tablets as a nutraceutical.

2. Materials and methods

2.1. Standards and reagents

Standards of *trans*-resveratrol (99% purity) and *trans*-piceid (97% purity) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Resveratrol-3-*O*-sulfate, resveratrol-4′-*O*-sulfate, resveratrol-3-*O*-glucuronide, resveratrol-4′-*O*-glucuronide and dihydroresveratrol (DHR) were obtained as previously described [27,38] for identification purposes. Methanol and acetonitrile were purchased from Scharlau S.L. (Barcelona, Spain), acetone from SDS (Peypin, France) and glacial acetic acid, ethyl acetate and *o*-phosphoric acid (purity 85%) from Panreac (Barcelona, Spain). Synthetic urine was prepared as previously described [39] and blank human plasma was purchased from Sigma–Aldrich (St. Louis, MO, USA). Ultrapure water (Milli-Q) was obtained from Millipore System (Bedford, MA, USA).

RW was kindly donated by Miguel Torres, S.A. (Vilafranca del Penedès, Spain). The Merlot variety used was selected because of its high content of total resveratrol compared to other red varieties [40]. One box of 60 GE tablets (Resverasor®, Soria Natural, S.A.) was purchased.

Table 1
Intake of both isomers of resveratrol and piceid and the total resveratrol by the participants in this study.

	RW	GE
trans-Resveratrol (mg)	0.64 ± 0.04	2.49 ± 0.06
cis-Resveratrol (mg)	0.98 ± 0.07	0.30 ± 0.01
trans-Piceid (mg)	3.30 ± 0.20	1.91 ± 0.01
cis-Piceid (mg)	1.39 ± 0.08	0.02 ± 0.004
Total resveratrol (mg)	6.30 ± 0.09	4.72 ± 0.07

Values are mean ± SD.

2.2. Subjects and study design

Ten healthy men (24–35 years old) with a BMI of 24.9 ± 3.3 kg/m^2 were recruited for the study. Seven of them were selected randomly to drink RW and the other three were chosen for the pilot study in which they consumed the GE tablets. All of them were declared healthy after anamnesis and clinical examinations, were non-smokers and had stable alimentary habits. The study protocols were approved by the Ethics Committee of the University of Barcelona (IRB00003099) and informed consent was obtained from all participants.

Subjects started with a washout period in which they were requested to avoid consuming grape products for 3 days and polyphenol-rich foods the day before and throughout the study period. On the morning of the intervention day, and after the collection of fasting urine and plasma, subjects consumed a similar amount of total resveratrol in two forms (Table 1): 375 mL of RW or 15 GE tablets with 400 mL of water consumed within 10 min. After 6 h, all subjects received the same meal and stayed in hospital for another 5 h. The volunteers returned to hospital the next morning for the 24-h blood sampling and those who had consumed GE stayed there throughout the second day. Preliminary tests suggested that GE consumption could delay resveratrol absorption and the time required to be totally eliminated, so additional collection points were introduced. The same dinner was provided for the volunteers on the day before the intervention and on the days of the study.

Blood samples from the RW consumers were taken before intervention and at 0.25, 0.5, 1, 2, 3, 4, 6, 8, 11 and 24 h, and samples from the GE consumers were taken before intervention and at 0.5, 1, 2.5, 4, 5.5, 7, 8, 9, 10, 11, 24, 26, 28, 30, 32, 35 and 48 h. These samples were collected in 4 mL vacuum tubes containing EDTA and centrifuged for 15 min at 3000 rpm to obtain plasma, which was immediately stored at −80 °C. Urine samples were collected at the following intervals: baseline, 0–4 h, 4–8 h, 8–12 h, 12–24 h and, additionally, samples from the GE consumers were collected at: 24–28 h, 28–32 h, 32–36 h and 36–48 h. Urines were acidified to 200 mM HCl and were frozen at −80 °C.

2.3. Resveratrol content in red wine and grape extract tablets

Resveratrol and piceid from the GE tablets and RW were analyzed by HPLC–DAD as previously described [40]. Before that, 1 g taken from 10 homogenized GE tablets from the same box as the subjects' intake was mixed with 10 mL of methanol/water (80:20 v/v) for 15 min. RW and diluted tablet extract (1:20, v/v) were filtered through 0.45 μm waters PTFE filters (Milford, MA, USA) before the HPLC–DAD analysis. RW and GE contained respectively: 8.8 ± 0.5 mg/L and 127.5 ± 0.5 μg/tablet of *trans*-piceid, 3.7 ± 0.2 mg/L and 1.2 ± 0.3 μg/tablet of *cis*-piceid, 1.7 ± 0.1 mg/L and 166.1 ± 4.3 μg/tablet of *trans*-resveratrol and 2.6 ± 0.2 mg/L and 20.1 ± 0.8 μg/tablet of *cis*-resveratrol. The corresponding intake of these stilbenes is shown in Table 1.

2.4. Extraction of resveratrol metabolites from urine and plasma samples

The extraction of resveratrol metabolites from plasma and urine was carried out following a validated and published method [27]. Briefly, 1 mL of urine or plasma, pretreated with 20 μL of *o*-phosphoric acid, were loaded onto a preconditioned Waters Oasis® HLB 96-well plate for solid-phase extraction (Milford, MA, USA). Metabolites were eluted with acidified methanol and ethyl acetate after washing the plate. Eluates were evaporated to dryness and reconstituted with mobile phase. All samples and standards were handled with no exposure to light.

2.5. Analysis of resveratrol metabolites by LC–MS/MS

Plasma and urine samples were analyzed by LC–ESI–MS/MS. LC analyses were performed in an Agilent 1200 system equipped with a quaternary pump and a refrigerated autosampler plate (Waldbronn, Germany). A Phenomenex Luna C_{18} analytical column [50 × 2.0 mm i.d., 5 μm] (Torrance, CA, USA) was used, at 40 °C. The volume injected was 15 μL at a flow rate of 0.5 mL/min with water containing 0.05% (v/v) acetic acid as mobile phase A and 70% acetone and 30% acetonitrile with 0.04% acetic acid as mobile phase B [27]. A linear gradient profile was applied with the following proportions (v/v) of phase B [*t*(min),%B]: (0,10), (0.5,15), (5,100), (5.6,100), (5.7,10), (8,10). The column was re-equilibrated for 3 min. Mass spectrometry (MS) and MS/MS data were obtained, with the parameters previously described [27], in an Applied Biosystems API 3000 triple quadrupole mass spectrometer (Pe Sciex, Concord, Ontario, Canada) equipped with a Turbo IonSpray source, ionizing in the negative mode. Phase II and microbial metabolites of resveratrol and piceid were identified by comparing the retention time and ion fragmentation of available standards or by product ion scan (PIS). For quantification purposes, the following MRM transitions were used: 227/185 for resveratrol, 403/227 and 307/227 for its glucuronide and sulfate conjugates, respectively; 389/227 for resveratrol glucoside, 565/227 and 469/227 for piceid glucuronides and sulfates, respectively; 229/123 for DHR, 405/229 and 309/229 for its glucuronide and sulfate conjugates, respectively.

For quantitative analysis, calibration curves were prepared in blank plasma and synthetic urine. Concentrations of resveratrol and DHR metabolites were estimated using the *trans*-resveratrol standard curve and expressed as resveratrol equivalents. Resveratrol glucosides and their metabolites were expressed as piceid equivalents after being quantified with the *trans*-piceid standard curve.

2.6. Pharmacokinetic analysis

Pharmacokinetic parameters were determined by means of a non-compartmental analysis using the WinNonlin® Professional software 2.0 (Pharsight Corporation, USA). The peak plasma concentration [C_{max} (ng/mL)], and the time of peak plasma concentration [t_{max} (h)], were determined by visual inspection of the experimental data. The linear trapezoidal method was used to calculate the area under the plasma concentration–time curve [AUC_{last} (ng h/mL)] from time 0 until the last detectable concentration. Mean residence time [MRT_{last} (h)] was estimated by means of the ratio AUMC/AUC, where AUMC is the first-moment curve. The absorption/appearance rate parameter [C_{max}/AUC_{last} (h^{-1})] was also calculated.

2.7. Statistical analysis

A Mann–Whitney *U* test was performed to compare the pharmacokinetic parameters and the urinary excretion values between

Table 2
Pharmacokinetic parameters of resveratrol derivatives after RW and GE interventions.

	*n*RW/*n*GE	t_{max} (h)		C_{max} (ng/mL)		AUC_{last} (ng h/mL)		C_{max}/AUC (h^{-1})		MRT_{last} (h)	
		RW	GE	RW	GE	RW	GE	RW	GE	RW	GE
t-resv-4′-*O*-gluc	6β	2.00 (1.00–4.00)	7.00* (4.00–7.00)	23.73 (0.00–197.56)	14.83 (9.97–42.19)	90.58 (0.00–679.48)	112.12 (40.87–207.84)	0.23 (0.00–0.45)	0.20 (0.13–0.24)	3.14 (0.00–4.80)	5.78* (5.66–6.28)
t-resv-3-*O*-gluc	6β	2.50 (0.50–4.00)	4.00* (4.00–5.50)	42.48 (8.02–101.79)	35.23 (27.78–87.23)	103.51 (56.27–544.64)	175.32 (149.25–365.69)	0.20 (0.14–0.64)	0.20 (0.19–0.24)	3.95 (1.43–4.56)	5.24* (4.39–5.62)
c-resv-4′-*O*-gluc	6β	2.50 (0.50–8.00)	4.00 (4.00–5.50)	65.78 (34.66–115.22)	19.36* (10.32–27.86)	232.08 (140.34–477.76)	91.22 (37.09–371.76)	0.22 (0.14–0.57)	0.21 (0.07–0.28)	3.74 (1.90–5.88)	5.13 (4.52–12.12)
c-resv-3-*O*-gluc	7β	2.00 (0.50–8.00)	4.00 (4.00–5.50)	130.19 (74.19–244.94)	29.57* (16.93–55.72)	534.33 (183.62–1656.16)	143.03 (78.55–395.88)	0.23 (0.13–0.61)	0.21 (0.14–0.22)	3.43 (1.65–5.55)	5.04 (4.83–9.27)
trans-piceid	5β	0.75 (0.00–2.00)	1.00 (0.50–1.00)	0.58 (0.00–9.47)	3.82 (1.68–11.55)	1.04 (0.00–15.68)	38.04 (6.41–44.19)	0.47 (0.00–1.21)	0.26 (0.10–0.26)	1.56 (0.00–6.88)	18.30* (4.93–21.34)
cis-piceid	7β	1.00 (0.50–2.00)	1.00 (1.00–1.00)	2.66 (1.27–3.23)	0.68 (0.60–2.44)	4.12 (2.03–10.27)	2.42 (1.01–7.93)	0.67 (0.31–0.79)	0.31 (0.28–0.59)	1.57 (1.16–4.34)	2.14 (1.22–4.23)
Piceid-gluc	7β	2.00 (1.00–3.00)	1.00 (1.00–4.00)	8.74 (1.38–25.02)	0.82* (0.32–1.25)	40.59 (6.78–58.12)	4.74* (0.97–5.38)	0.21 (0.15–0.61)	0.23 (0.17–0.32)	3.77 (3.09–4.69)	3.10 (2.90–4.36)
DHR-gluc 1	5β	4.00 (0.00–8.00)	7.00[a]	53.02 (0.00–158.83)	23.06[a]	386.42 (0.00–672.13)	90.97[a]	0.18 (0.00–0.30)	0.23[a]	5.72 (0.00–6.59)	6.87[a]
DHR-gluc 2	7β	6.00 (3.00–8.00)	8.00 (7.00–10.00)	296.26 (47.28–1368.93)	85.70 (53.75–586.43)	1575.65 (207.31–6870.04)	1303.91 (747.22–2181.66)	0.19 (0.11–0.35)	0.07 (0.07–0.27)	7.49 (4.05–9.62)	11.66 (7.26–15.30)

Values are median and range.
*n*RW/*n*GE, number of volunteers in which the kinetic parameters were calculated for the metabolite. t_{max}, time of peak plasma concentration. C_{max}, peak plasma concentration. AUC, area under the plasma concentration–time curve. C_{max}/AUC, absorption/appearance rate. MRT_{last}, mean residence time. *t*-resv, *trans*-resveratrol. *c*-resv, *cis*-resveratrol. gluc, glucuronide.

[a] Value of one volunteer.

* $P < 0.05$ vs. RW group.

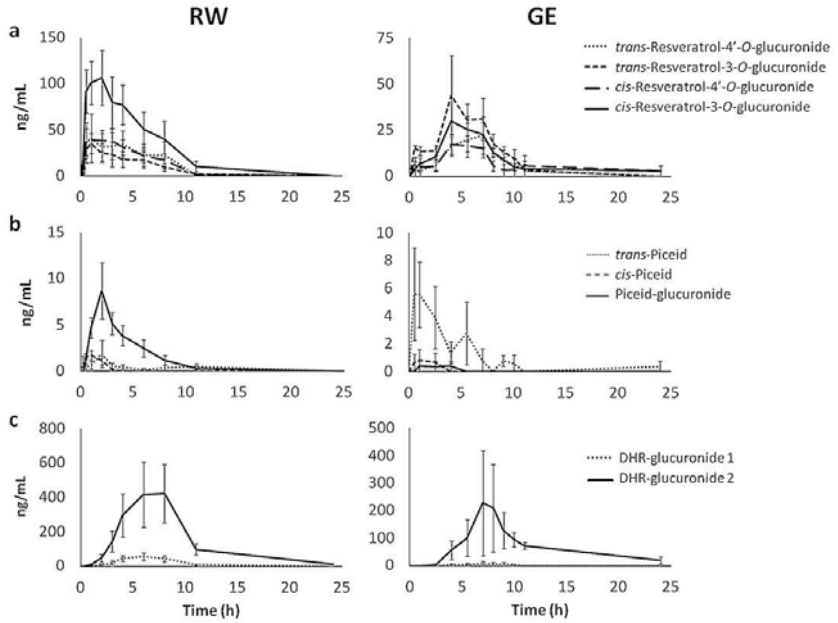

Fig. 1. Plasma concentration–time curves for (a) resveratrol-glucuronides, (b) *trans*- and *cis*-piceid and the glucuronide conjugate, (c) DHR glucuronides after consumption of RW and GE tablets. Values are mean ± SE.

GE consumption and RW intake, since data were skewed. Moreover, in order to consider any differences between the excretion of the sum of resveratrol phase II metabolites and the microbial ones, a Wilcoxon test was carried out. Differences were statistically significant at $P < 0.05$. PASW® Statistics 18.0 (SPSS Inc., Tokyo, Japan) was used.

3. Results and discussion

3.1. Identification of metabolites

In this report, 17 metabolites, including conjugates of resveratrol, piceid and DHR, from the intestinal microbiota, have been identified in humans after RW consumption and GE tablet intake (Supplemental Fig. S1 and Fig. S2). Previous studies on the metabolic profile of resveratrol were mainly focused on the study of resveratrol phase II metabolites [26–30]. In this study, up to four isomers of both glucuronide and sulfate conjugates were determined in urine samples as previously reported [27], although only glucuronide conjugates were found in plasma samples. In fact, resveratrol glucuronides and sulfates are the most frequently reported phase II metabolites [14,31] and, although the main ones described were glucuronides [22–24], it seems that sulfate conjugates may be found in greater amounts, always after administration of higher doses of resveratrol [29,30]. This aspect must be highlighted, since a possible saturation of glucuronosyltransferases has been observed when higher doses of resveratrol were administered, in contrast to sulfation pathway which showed a non-competitive substrate inhibition [41]. In addition, this saturation could entail a shift from glucuronidation to sulfation in resveratrol metabolism [42], which would explain the higher amounts of sulfate metabolites in studies with higher intake of dietary resveratrol [29,30].

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2012.08.001.

Less information is available regarding resveratrol glucosides in humans. They have been previously identified in human LDL after RW intake [26], as well as in urine, plasma and tissues of Wistar rats after oral administration of pure *trans*-piceid [43–45]. In this report, not only were both isomers of resveratrol glucosides identified, but also one glucuronide and two sulfates of piceid in urine, as well as a glucuronide in plasma, were identified for the first time in humans after RW or GE intake through MS/MS experiments (Supplemental Fig. S2). Previously, these metabolites were determined in rats after administration of pure glucoside [44,45].

In this work, DHR and its phase II metabolites were also studied as conjugated metabolites derived from hydrogenation of resveratrol by the colonic microbiota [46], although free DHR were not found in any sample. The identification of two glucuronides and two sulfates of DHR in urine of these volunteers was confirmed through MS/MS experiments (Supplemental Fig. S2). Only few studies have considered the effect of intestinal microbiota on the metabolism of resveratrol. However, some conjugated forms of DHR have been observed only after administration of pure compounds: in human urine after a 25 mg dose of labeled resveratrol [28], in rat urine or plasma after resveratrol [47], piceid [45] or DHR [32] administration, and in pig tissues after resveratrol intake [48].

3.2. Effect of food matrix on the pharmacokinetic parameters and urinary excretion

The influence of food matrices on the form in which resveratrol is delivered must always be considered when interpreting results from clinical or epidemiological studies, since other compounds present in food, such as alcohol, tablet excipients and others, could affect the absorption, distribution and elimination of the metabolites studied. However, there is currently little and

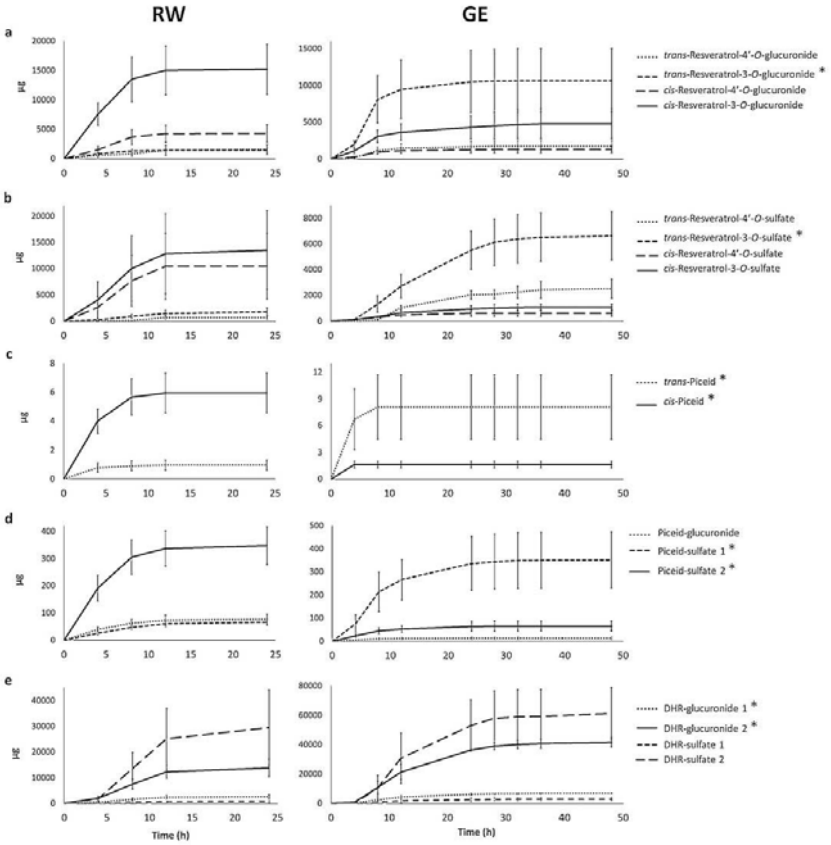

Fig. 2. Accumulated excreted amounts of (a) resveratrol glucuronides, (b) resveratrol sulfates, (c) *trans*- and *cis*-piceid (d) piceid metabolites and (e) DHR conjugates after consumption of RW and GE tablets in seven and three volunteers, respectively. Values are mean ± SE; $^{*}P < 0.05$, comparing RW vs. GE groups.

conflicting information available regarding the bioavailability of resveratrol in humans. Goldberg et al. [22] tested the matrix effect of pure *trans*-resveratrol absorption when dissolved in white wine, grape juice or vegetable homogenate, although no differences were observed. Recently it was reported that absorption of total resveratrol was higher (6-fold) from natural products than from tablets enriched with pure *trans*-resveratrol [25]. However, a previous study obtained better recoveries of resveratrol when pure compounds were administered, compared with those obtained after grape juice consumption [24]. In order to provide more information in this field, this study was focused on resveratrol bioavailability, taking into account pharmacokinetic parameters of the metabolic profile of resveratrol, after administration of resveratrol in different dosage forms: RW and a nutraceutical with resveratrol (GE extract).

Table 2 summarizes the pharmacokinetic parameters, corresponding to the nine resveratrol and piceid metabolites identified in plasma samples after the intake of either RW or GE. Maximum concentrations of resveratrol glucuronides were reached 2–2.5 h after RW consumption. Even more time was needed after GE intervention (Fig. 1) and remain longer in organism (higher MRT_{last} values), with statistically significant differences for *trans*-resveratrol-4′-*O*-glucuronide and *trans*-resveratrol-3-*O*-glucuronide, although similar appearance rates (C_{max}/AUC_{last}) were shown (Table 2). The main resveratrol phase II metabolite found in plasma after RW consumption was *cis*-resveratrol-3-*O*-glucuronide, with C_{max} and AUC_{last} values ~2–6-fold higher than other resveratrol glucuronides, whose C_{max} values and those of the *cis*-4′ isomer were statistically higher than after GE ingestion. This difference could be due to the ~6-fold higher content of *cis*-resveratrol and *cis*-piceid in RW compared with GE. The concentration of glucuronide metabolites in plasma showed two maximum peaks (Fig. 1). The second peak, corresponding to the 8-h sampling point, could suggest that resveratrol undergoes enterohepatic recirculation [29,49]. When urinary excretion of resveratrol phase II metabolites was considered, similar excretion values were obtained for resveratrol glucuronides and sulfates, although some differences were observed between the two interventions. In fact, the most excreted resveratrol phase II metabolites after RW consumption were *cis*-resveratrol-3-*O*-glucuronide, and *cis*-resveratrol-3 and 4′-*O*-sulfates, while *trans*-resveratrol-3-*O*-glucuronide and *trans*-resveratrol-3-*O*-sulfate were the highest after GE consumption (Fig. 2). These results could be explained by the higher *trans*-isomers prevalence in GE composition, comprising 93% of total resveratrol. Moreover and when the kinetics of the accumulated excreted amounts is considered (Fig. 2), a later excretion could be appreciated for resveratrol sulfates. This could suggest that glucuronidation pathway of resveratrol is saturated, but not the sulfation one, as was previously hypothesized [38]. It is

interesting to note that resveratrol aglycone was not found in any sample; in fact, due to its extensive metabolism [49]. Previous studies found free *trans*-resveratrol when higher amounts (>25 mg) were administered [19,22,23,28]. However, when resveratrol is administered through consumption of wine or juices, the free form is either not detectable at all or present in very low concentrations in plasma [49,50].

Resveratrol glucosides were already appeared almost 1 h after both interventions, which are in line with the high absorption rate (C_{max}/AUC_{last}). These both isomers of piceid had the lowest concentrations in plasma, as well as the lowest AUC_{last} values after both administration sources, along with those of its glucuronide conjugate. These values were lower, by far, than those of resveratrol phase II metabolites, although the piceid concentration in RW was higher than its aglycone [51]. Indeed, piceid is absorbed in the enterocyte using the active transporter sodium-dependent glucose transporter 1 [52] and could then be mainly hydrolyzed by cytosolic-β-glucosidase or by the membrane-bound lactase phlorizin hydrolase, resulting in the formation of resveratrol aglycone [53]. Therefore, the small amounts of unmetabolized resveratrol glucosides found in this study could derive from the direct absorption of glucosides in the enterocyte, as has also been observed in rats [43–45] and with other phenolic compounds [54–56]. In this way, glucosilation of the parent resveratrol could be considered as a protection before biotransformation [57]. Despite the low plasma values of resveratrol glucosides, C_{max} and AUC_{last} values for *trans*-piceid were higher after GE consumption, while *cis*-piceid has higher values after RW consumption but without statistical significance. Statistical differences were obtained for urinary excretion, with a *cis*-piceid excretion 3.6-fold higher after RW intake and *trans*-piceid values 8.5-fold higher after GE consumption. Urinary recovery of *trans*-piceid showed the same trend with values of 0.03% after RW intake and 0.43% after GE intake. Unlike foregoing discussion for resveratrol glucuronides, these results in both plasma and urine could not be explained by RW and GE composition, since RW contained higher amount of *trans*-piceid than the GE. Thus, these results could lead the hypothesis that after GE intake, the body could use metabolic pathways to metabolize *trans*-resveratrol (present in higher amounts in GE), and could allow the directly absorption of *trans*-piceid, which would be in the organism for a longer period of time, as shown the 11.7-fold statistically higher MRT_{last} values. In contrast, after RW consumption, where *trans*-piceid predominates, metabolic pathways could hydrolyze it into resveratrol and conjugate it allowing lower direct absorption of *trans*-piceid.

Regarding piceid phase II metabolites, piceid sulfate 2 was the main excreted glucoside after RW consumption and piceid sulfate 1 was mainly excreted after GE ingestion, with statistical differences between both products (Fig. 2). Due to the different composition of RW and GE, and regarding the results discussed, here we could hypothesize that the isomer 1 could be a *trans*-piceid-sulfate, while isomer 2 could be a *cis*-piceid-sulfate. In the same way, the glucuronide of piceid, which demonstrate statistically higher C_{max} and AUC_{last} values and a tendency for higher urinary excretion after RW than after GE consumption, could follow the same trend as *cis*-piceid and be a *cis*-piceid-glucuronide.

According to the intestinal transit time, microbial metabolism of resveratrol in the gut [32] seems to involve more time to reach C_{max} (Table 2 and Fig. 1) than that required for phase II metabolites of resveratrol. It should be note that there was high individual variability in C_{max} and AUC_{last} values for DHR-glucuronides which could be due to the high variability of bacterial species [58]. These results are in line with those obtained in urine, where higher excretion of resveratrol phase II metabolites was achieved in the 0–8 h period, while between 4 and 12 h were needed for DHR metabolites after RW consumption and up to 24 h after GE intake (Fig. 3).

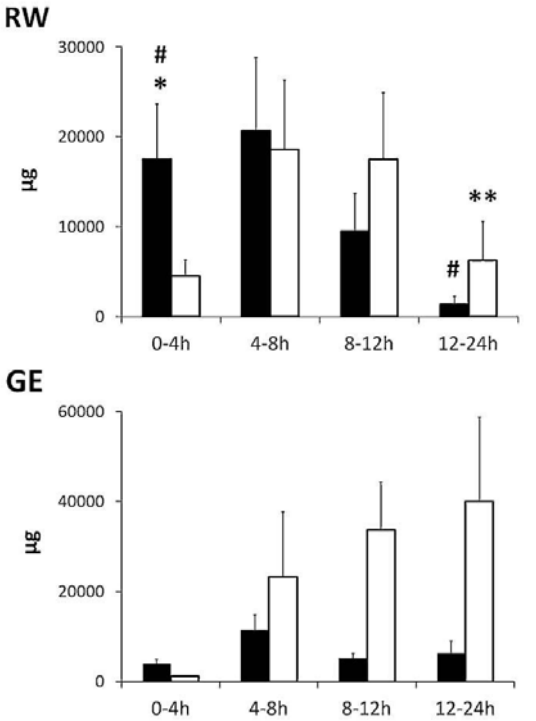

Fig. 3. Sum of urinary resveratrol phase II metabolites (black boxes) and sum of DHR metabolites (white boxes) in each urine collection fraction after RW consumption and GE tablets intake. Values are mean ± SE; $^{*}P < 0.05$, comparing vs. DHR metabolites values in the same intervention. $\#P = 0.053$ and $^{**}P < 0.05$, comparing vs. the same urinary fraction after GE intake.

Despite this late absorption and although preliminary tests could suggest a high excretion after 24 h in the GE intervention, more than 90% of metabolites were excreted within 24 h. MRT_{last} values for DHR metabolites were higher than those of resveratrol phase II metabolites after both interventions (Table 2). Even higher MRT_{last} values for DHR glucuronides were shown when GE was ingested. The higher the MRT values, the longer the compound remains in the body to possibly exert potentially beneficial effects, although the activity of metabolites has been scarcely studied and mainly focused on resveratrol sulfates [16–18], which were less active than the aglycone [18]. Obviously, resveratrol and its glucoside from GE tablets require time to be dissolved in vivo and the compounds from nutraceuticals will stay longer in the gut where microbiota would have more time to metabolize them. This could be the reason why urinary excretion values were higher after GE ingestion than after RW intake, with values 2.1–3.6-fold higher for each microbial metabolite (Fig. 2), with statistical differences only performed for glucuronide conjugates. DHR after hydrolysis was also the highest excreted in the study of Ortuño et al. [25], and Wang et al. described DHR and its sulfate as the main urinary metabolites, along with glucuronide and sulfate conjugates of resveratrol [47]. No previous studies determined the glucuronide or sulfate positions in the DHR although Azorin-Ortuno et al. [59] found only one glucuronide conjugate as DHR-3-*O*-glucuronide in pig tissues after comparing with its synthesized standard. In this study, we would tentatively hypothesize some proposed chemical structures for the identified 2 glucuronide and 2 sulfate isomers of DHR in urine. Since

only 3 or 4′ positions are possible and taking into account that 3-position was the predominant in the excretion of glucuronide and sulfate conjugates of resveratrol after RW and GE, as was also observed in a previous work [59], the isomer 2 of glucuronide and sulfate of DHR (majority) (Fig. 2), would tentatively be the DHR-3-*O*-glucuronide and DHR-3-*O*-sulfate, respectively, while isomer 1 (minor) would tentatively be the DHR-4′-*O*-glucuronide and DHR-4′-*O*-sulfate, respectively.

Despite the differences observed after both interventions, when the sum of all resveratrol metabolites was considered and after taking into account the ingested dose, the total urinary excretion (mean ± SE) was 96.3 ± 32.8 mg (281 ± 101 μmol) after RW and 125.2 ± 17.9 mg (398 ± 65 μmol) after GE, without any statistical differences between them ($P = 0.383$). In fact, one shortcoming of this study design could be that the amount of each resveratrol isomer administered in both sources was different, although it allowed hypothesizing which are the main resveratrol and piceid metabolic pathways and the possible chemical structures of some phase II metabolites, since *trans*-isomers were mainly provided in the GE intervention, while the *cis*-ones in RW intervention. Another drawback is the low number of volunteers in the GE intervention.

4. Conclusions

To our knowledge, this is the first pharmacokinetic study of resveratrol and piceid in humans, considering a large metabolic profile, after an acute intake of moderate RW and a similar amount of total resveratrol as a nutraceutical, in order to study the effect of these different matrices of both common resveratrol and piceid sources. Statistically significant differences between both treatments were obtained for some metabolites, mainly due for the different composition of resveratrol and piceid in both sources. However, DHR conjugates showed higher excretion values after GE intervention, since delayed absorption of tablet compounds could leave resveratrol longer in the gut, where microbiota would have more time to metabolize it into a greater degree. Moreover, in this study, nutraceuticals increased exposition and residence times of *trans*-piceid in the organism, compared to RW. Therefore, nutraceuticals could be a way to take resveratrol in order to bring benefits to human health. However, it should be borne in mind that 15 GE tablets were necessary to reach the ingested resveratrol and piceid levels of a moderate RW consumption in men. Therefore, some other sources with higher resveratrol or piceid amounts in the supplements could be considered, although recent studies have already focused on the resveratrol delivery in order to improve its bioavailability [60,61]. Moreover, additional investigation is needed into tissue distribution and the biological activity that could be exerted by conjugate metabolites, specifically for those metabolites present in plasma after oral intake of resveratrol and those that remain in the organism for a longer period of time.

Conflict of interests

The authors declare no conflict of interest.

Acknowledgments

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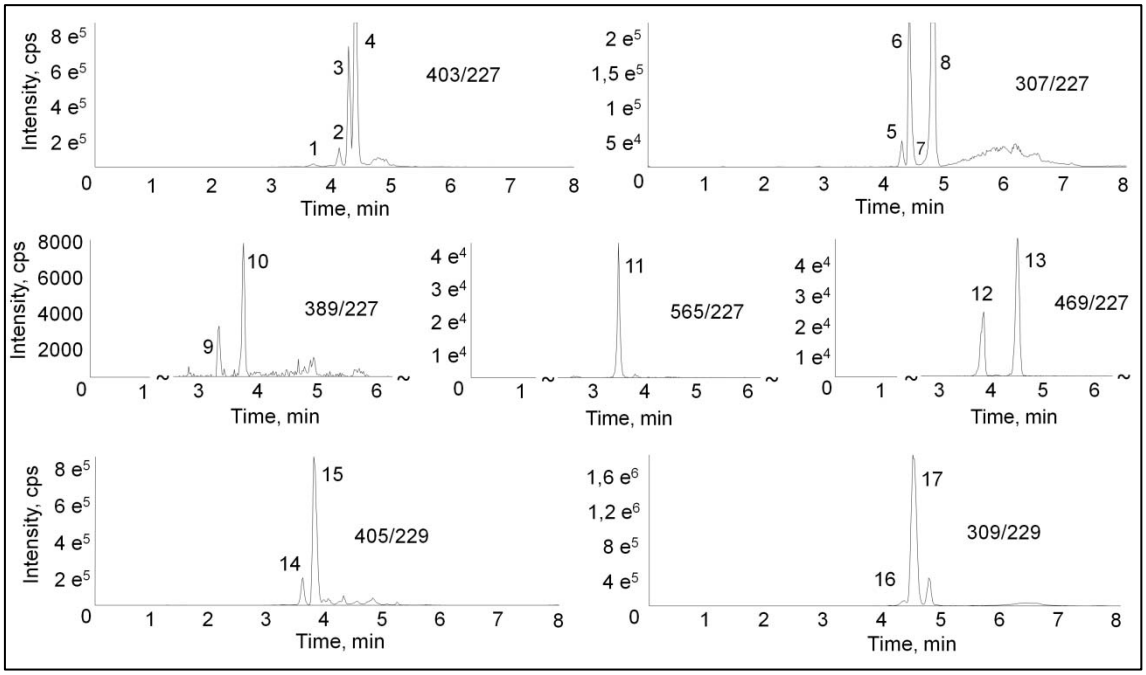
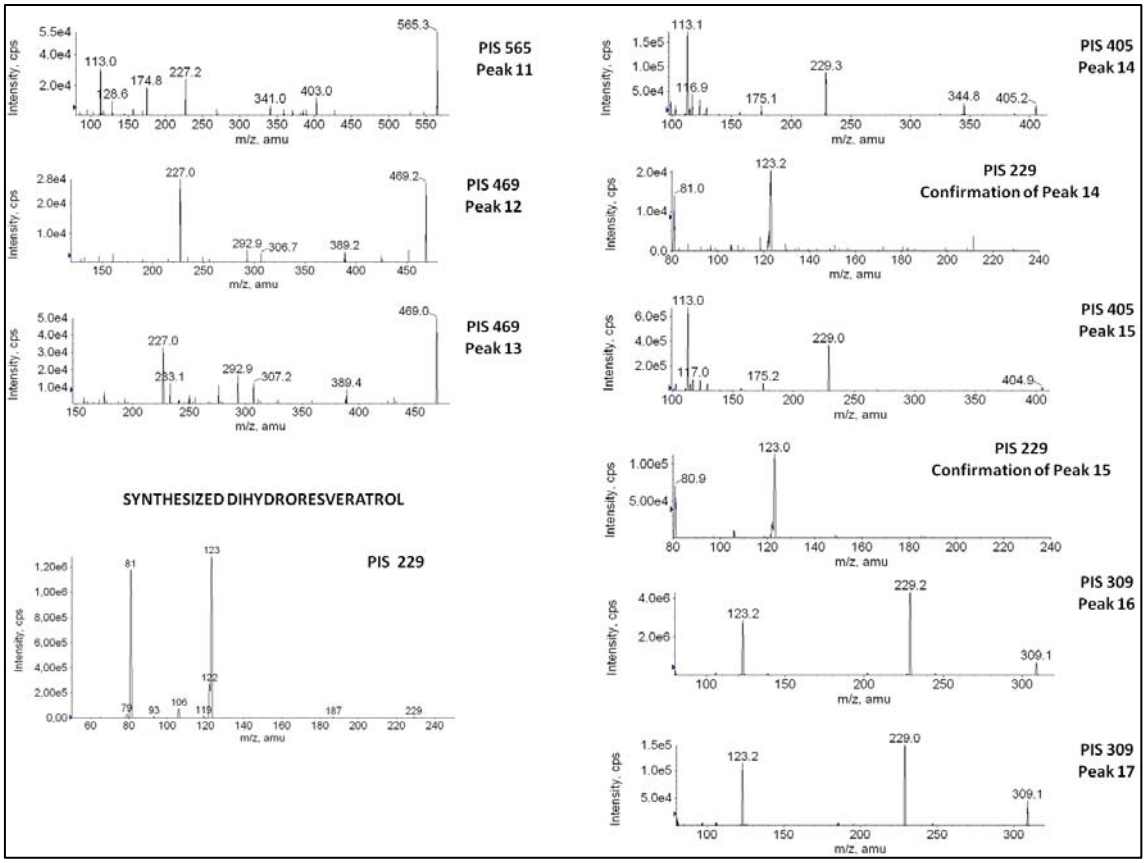
SUPPLEMENTARY DATA

Supplemental Fig. S1. Representative multiple reaction monitoring trace chromatogram of resveratrol glucuronides (403/227), resveratrol sulfates (307/227), resveratrol glucosides (389/227), piceid glucuronide (565/227), piceid sulfates (469/227), dihydroresveratrol glucuronides (405/229) and dihydroresveratrol sulfates (309/229) in urine samples after RW. (1) *trans*-resveratrol-4'-*O*-glucuronide, (2) *trans*-resveratrol-3-*O*-glucuronide, (3) *cis*-resveratrol-4'-*O*-glucuronide, (4) *cis*-resveratrol-3-*O*-glucuronide, (5) *trans*-resveratrol-4'-*O*-sulfate, (6) *trans*-resveratrol-3-*O*-sulfate, (7) *cis*-resveratrol-4'-*O*-sulfate, (8) *cis*-resveratrol-3-*O*-sulfate, (9) *trans*-piceid, (10) *cis*-piceid, (11) piceid-glucuronide, (12, 13) piceid-sulfates 1 and 2, respectively, (14, 15) DHR-glucuronides 1 and 2, respectively and (16, 17) DHR-sulfates 1 and 2, respectively.



Supplemental Fig. S2. Product ion scan (PIS) of piceid glucuronide (peak 11, *m/z* 565), piceid sulfates (peaks 12-13, *m/z* 469), synthesized dihydroresveratrol (DHR) (*m/z* 229), DHR-glucuronides (peaks 14-15, *m/z* 405), with their confirmation through the PIS of *m/z* 229 in CID-MS/MS experiments, and DHR-sulfates (peaks 16-17, *m/z* 309).



ESTUDI 2

ESTUDI DEL PERFIL METABÒLIC DEL RESVERATROL, TANT INTESTINAL COM MICROBIÀ, DESPRÉS D'UN CONSUM REGULAR I MODERAT DE VI NEGRE EN COMPARACIÓ AMB EL MATEIX VI DESALCOHOLITZAT PER UN MÈTODE OPTIMITZAT EN UPLC-MS/MS.

L'estudi del perfil metabòlic del resveratrol després d'un consum regular i moderat de vi negre en comparació amb el mateix vi desalcoholitzat, s'ha presentat, juntament amb la optimització del mètode (Estudi 2), en una de les revistes considerada pel *Science Citation Index*, amb un índex d'impacte de 4,531 i situada en el primer decil de la categoria C*hemistry, Analytical* (6 de 73) i en el primer quartil de la categoria *Biochemical Research Methods* (13 de 72). Aquest estudi ha estat emmarcat en el projecte AGL 2006-14228-C03-02/01 del Pla Nacional (MINECO).

Publicació 2: Maria Rotches-Ribalta, Mireia Urpi-Sarda, Rafa Llorach, Maria Boto-Ordóñez, Olga Jauregui, Gemma Chiva-Blanch, Lluïsa Perez-Garcia, Walter Jaeger, Marisa Guillen, Dolores Corella, Francisco J. Tinahones, Ramon Estruch, Cristina Andres-Lacueva. Gut and microbial resveratrol metabolite profiling after moderate long-term consumption of red wine versus dealcoholized red wine in humans by an optimized ultra-high-preassure liquid chromatogrphy tandem mass spectrometry method. *Journal of Chromatography A*, 2012;1265:105–113.

Resum

El resveratrol presenta una elevada varietat d'activitats biològiques i farmacològiques, les quals han estat observades malgrat la seva baixa biodisponibilitat i la seva ràpida

desaparició de la circulació sanguínia, fruit del seu extens metabolisme, tant en l'intestí com al fetge. Per tal de poder quantificar d'una manera més acurada tot el perfil metabòlic del resveratrol, es va optimitzar un mètode analític amb estàndards purs d'alguns d'aquests metabòlits. Les millores en la metodologia, dirigides principalment a la detecció cromatogràfica dels conjugats disulfats i sulfoglucurònids, van permetre obtenir una millor resolució cromatogràfica de tots els compostos sulfatats, utilitzant una solució tamponada, amb valors de recuperació de tots els compostos del voltant del 99%.

Aquesta metodologia va ser després aplicada a l'estudi d'intervenció presentat anteriorment, en les mostres d'orina del qual es van identificar fins a 21 metabòlits del resveratrol i piceid, incloent-hi els derivats de l'acció microbiana. Es va poder observar que després del consum a llarg termini de vi negre i vi negre desalcoholitzat, l'excreció urinària del metabòlits del resveratrol era significativament superior a la que es va obtenir en condicions basals. Ara bé, no es van observar diferències entre el consum regular de les dues begudes riques en polifenols, indicant que la biodisponibilitat i biotransformació del resveratrol no es veu afectada per la matriu alcohòlica del vi.

Així doncs, s'ha establert un mètode sensible per a la quantificació d'un ampli perfil metabòlic del resveratrol en orina, el qual també podrà ser aplicat a mostres de plasma o de diferents teixits. A més a més, podem dir que aquest perfil metabòlic suposarà un excel·lent marcador per a determinar la biodisponibilitat del resveratrol en estudis clínics i epidemiològics que es duguin a terme amb l'objectiu d'investigar els efectes beneficiosos del consum de productes derivats del raïm o altres aliments que continguin resveratrol. En aquest sentit, aconseguir un valor de concentració precís dels metabòlits de resveratrol en diferents mostres biològiques, gràcies a l'acurada quantificació que permet el fet de disposar d'estàndards, millora la interpretació dels resultats obtinguts i redueix els errors metodològics que ha sofert històricament la quantificació del resveratrol biològic, donada la prèvia inexistència d'estàndards purs.

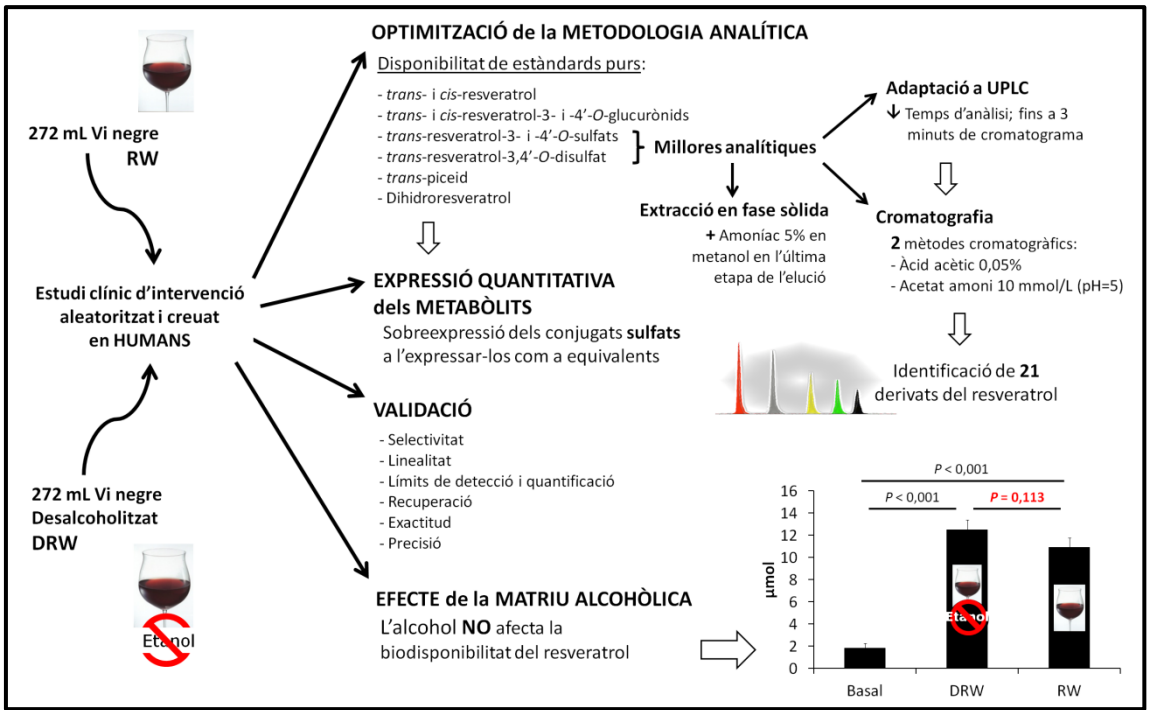


Figura 11. Gràfic resum de l'estudi del perfil metabòlic del resveratrol després d'un consum regular i moderat de vi negre en comparació amb el mateix vi desalcoholitzat per un mètode optimitzat en UPLC-MS/MS (estudi 2).

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Gut and microbial resveratrol metabolite profiling after moderate long-term consumption of red wine *versus* dealcoholized red wine in humans by an optimized ultra-high-pressure liquid chromatography tandem mass spectrometry method

Maria Rotches-Ribalta[a,b], Mireia Urpi-Sarda[a,*], Rafa Llorach[a,b], Maria Boto-Ordoñez[c,d], Olga Jauregui[b,e], Gemma Chiva-Blanch[c,d], Lluïsa Perez-Garcia[f], Walter Jaeger[g], Marisa Guillen[d,h], Dolores Corella[d,h], Francisco J. Tinahones[d,i], Ramon Estruch[c,d], Cristina Andres-Lacueva[a,b]

[a] Nutrition and Food Science Department, XaRTA-INSA, Pharmacy Faculty, University of Barcelona, Av Joan XXIII, s/n, 08028 Barcelona, Spain
[b] Ingenio-CONSOLIDER Program, FUN-C-FOOD CSD2007-063, Ministry of Science and Innovation, Barcelona, Spain
[c] Department of Internal Medicine, Hospital Clinic, Institut d'Investigació Biomèdica August Pi i Sunyer (IDIBAPS), University of Barcelona, Villarroel 170, 08036 Barcelona, Spain
[d] CIBER Fisiopatología de la Obesidad y la Nutrición, Instituto de Salud Carlos III, Madrid, Spain
[e] Scientific and Technological Centers of the University of Barcelona (CCIT-UB), Barcelona, Spain
[f] Pharmacology and Therapeutical Chemistry Department, Pharmacy Faculty and Institute for Nanoscience and Nanotechnology (IN2UB), University of Barcelona, Av Joan XXIII, s/n, Barcelona, Spain
[g] Department of Clinical Pharmacy and Diagnostics, University of Vienna, Vienna, Austria
[h] Department of Preventive Medicine and Public Health, University of Valencia, Valencia, Spain
[i] Endocrinology and Nutrition Service, Virgen de la Victoria Clinical Hospital, Málaga, Spain

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ABSTRACT

Resveratrol exerts a variety of biological and pharmacological activities, which are observed despite its extremely low bioavailability and rapid clearance from the circulation due to extensive sulfation and glucuronidation in the intestine and liver. In order to more accurately quantify all known resveratrol metabolites, a sensitive and optimized analytical assay was developed and validated by pure standards. Methodology improvements aimed to the chromatographic detection of disulfates and sulfoglucuronides, improving resolution of sulfates, by using a buffered solution, with recovery values of resveratrol and its metabolites, even of sulfates, of 99%. The adapted methodology was then applied to a clinical study with high cardiovascular risk subjects, after the moderate consumption of red wine (RW) or dealcoholized red wine (DRW) for 28 days. Up to 21 resveratrol metabolites, including those formed by gut and microbial metabolism, were identified in 24-h urine samples. Interestingly, after long-term consumption of RW and DRW, resveratrol metabolite concentration significantly increased in urine with no differences between the two interventions, indicating that bioavailability and biotransformation of resveratrol is not affected by the alcoholic matrix of wine. In summary, we established a sensitive analytical assay for the quantification of a wide resveratrol metabolic profile in human urine, also regarding gut microbial-derived metabolites, which may also be applied to blood and tissue samples. The resveratrol metabolic pattern might therefore act as an excellent marker for the efficacy of resveratrol in clinical and epidemiological studies for the study of the beneficial effects of grape product consumption. In this sense, having a more precise concentration value of all the resveratrol metabolites in target tissues would finally lead to a better interpretation of the obtained results.

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1. Introduction

Resveratrol (RV) is a stilbene mainly consumed through grape and grape products, such as red wine [1]. Although several studies have already demonstrated the RV ability to protect against several diseases [2], it has aroused some controversia due to its low bioavailability [3], since it is rapidly metabolized after its oral absorption [4]. Indeed, free RV found in plasma samples constituted less than 2% of the total RV consumed [5], and therefore it is mainly the metabolic forms which will reach the target tissues. Thus, more studies are necessary in order to investigate the biological mechanisms for which RV or its metabolites could exert

* Corresponding author. Tel.: +34 93 4024508.
E-mail address: murpi@ub.edu (M. Urpi-Sarda).

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a biological activity. Actually, scarce information is now available regarding the possible benefits of these metabolites [6–9], which mainly focuses on the sulfate conjugates. Prior to studying the possible beneficial effects of RV metabolites, development of highly sensitive and selective techniques is necessary for their identification and quantification in biological samples. Several liquid chromatography–mass spectrometry (LC–MS) techniques have already been validated for the analysis of RV metabolites [10–16], and although it is now widely accepted that RV is mainly metabolized to glucuronide and sulfate conjugates [3], including several isomers of mono and di-conjugates [10–12,17,18], less information is now available regarding piceid metabolism [11,19], as well as those metabolites derived from gut microbial action, represented by dihydroresveratrol (DHR) and its phase II metabolites [19–21]. Standards of these metabolites are rarely available and their quantification in biological samples is made by expressing results as aglycone equivalents [10–12], which is useful as a first approach, but this is open to error, since metabolite ionization in LC-ESI–MS techniques could be different from their aglycones. Several studies attempted to avoid this known technical drawback by measuring RV metabolites indirectly after enzymatic hydrolysis [20,22,23] or with diode-array detection (DAD) [17,24]. However, other drawbacks are shown using these techniques, such as the low efficiency of enzymatic hydrolysis, as well as losing all information of the metabolic profile, and the low sensitivity for DAD. Studies on the RV metabolic profile need more sensitive methodologies only achieved by LC-ESI–MS techniques, which are being used not only for RV metabolites [10–12] but also for other polyphenols [25,26], despite their quantification drawback. Nowadays, some purified standards of RV metabolites, along with the labeled C^{13}-resveratrol are already commercially available, allowing an accurate quantification of these compounds in biological samples; so, data from clinical and epidemiological studies could be more precise. Moreover, other drawbacks in chromatography, bearing in mind the different chemical characteristics of the high number of determined and identified metabolites, need to be minimized. It is known that while aglycone, glucuronided and glucosided metabolites of RV and other polyphenols are well resolved when reverse analytical columns are used, other metabolites, such as sulfate conjugates, show poor chromatographic behavior [21]. Although in our previous methodology [10] we solved this problem with the addition of more apolar mobile phase, such as acetone, the determination of disulfate metabolites was not evaluated. Thus, analytical solutions are required in this aspect.

Therefore, the main aim of this study is to validate a methodology for the analysis of RV metabolites in urine samples, through optimization of solid-phase extraction (SPE) and ultra-performance liquid chromatography (UPLC) analysis, using the recent available standards of glucuronide and sulfate conjugates and gut microbial metabolites. This methodology was applied to a randomized, crossover, controlled clinical trial, where volunteers with cardiovascular risk factors consumed, for one month, a daily moderate dose of RW or the same dose of dealcoholized RW (DRW) in order to investigate a possible alcoholic matrix effect on the bioavailability of a detailed RV metabolic profile, which will be accurately quantified with the corresponding standards.

2. Materials and methods

2.1. Standards and reagents

All samples and standards were handled with no exposure to light. Standards of *trans*-RV (99% purity) and *trans*-3,4′,5-trihydroxystilbene-3-β-D-glucopyranoside (*trans*-piceid) (97% purity) were purchased from Sigma–Aldrich (St. Louis, MO, USA) and taxifolin (>90% purity) from Extrasynthese (Genay, France).

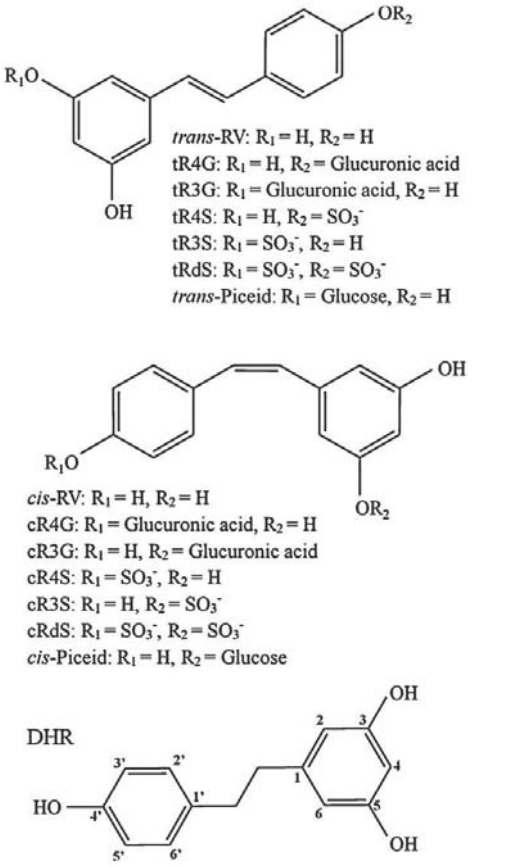

Fig. 1. Chemical structures of RV and its metabolites identified using the available commercial standards.

Standards of *cis*-RV (97% purity), *trans*- and *cis*-resveratrol-3-*O*-glucuronide (tR3G and cR3G, respectively) (98% purity each), *trans*- and *cis*-resveratrol-4′-*O*-glucuronide (tR4G and cR4G, respectively) (98% and 96% purity, respectively), *trans*-resveratrol-3-*O*-sulfate (tR3S) (98% purity) and *trans*-RV-$^{13}C_6$, to be used as internal standard, were acquired from Toronto Research Chemicals Inc. (North York, ON, Canada). *trans*-Resveratrol-4′-*O*-sulfate (tR4S) (>95% purity) and *trans*-resveratrol-3,4′-*O*-disulfate (tRdS) (>91% purity) were obtained as reported previously [27]. DHR aglycone (80% purity) was synthesized following the work by Thakkar et al. [28] (see Section 2.2), using palladium on charcoal and celite, which were purchased from Sigma–Aldrich (St. Louis, MO, USA). Standards were prepared as 80% (v/v) methanol stock solutions.

Methanol, ethyl acetate and acetonitrile (ACN) of LC grade and ammonium acetate (>99%) were purchased from Scharlau Chemie, S.A. (Sentmenat, Spain). LC grade solvents glacial acetic acid, acetone and ammonia (35%) were purchased from Panreac Quimica, S.A.U. (Castellar del Vallès, Spain) and the deuterated dimethyl sulfoxide (DMSO-d_6, 99.96% deuterated) from Euriso-top, SAS (Cedex, France). Ultrapure water (MilliQ) was obtained from Millipore (Bedford, MA, USA) and blank human urine from volunteers after one week of polyphenol-free diet.

Table 1
MRM transitions and optimized parameters of collision energy (CE) and declustering potential (DP).

Analyte	MRM transitions	Identified by	CE	DP
Mobile phase A: 0.05 mL/L acetic acid				
trans-RV	227/185	STD	−30	−55
cis-RV		STD		
tR4G	403/227	STD	−40	−50
tR3G		STD		
cR4G		STD		
cR3G		STD		
trans-Piceid	389/227	STD	−25	−50
cis-Piceid		Isomerized STD		
Pic-G	565/227	PIS		
DHR	229/123	STD	−25	−45
DHR-G	405/229	PIS		
Mobile phase A: ammonium acetate 10 mM				
tR4S	307/227	STD	−30	−50
tR3S		STD		
cR4S		Isomerized STD		
cR3S		Isomerized STD		
tR34dS	387/227	STD	−20	−35
cR34dS		Isomerized STD		
RV-SG	483/307	PIS	−20	−35
Pic-S	469/227	PIS	−25	−50
DHR-S	309/229	PIS	−25	−45
DHR-SG	485/309	PIS		
t-RV-$^{13}C_6$ (IS)	233/191	STD	−25	−55
Taxifolin (IS)	303/285	STD	−25	−50

2.2. Synthesis and structural identification of DHR

For DHR synthesis, 100 mg of *trans*-RV were dissolved in ethanol (120 mL) and hydrogenated at 40 psi in the presence of 10% palladium on charcoal for 24 h. The solution was filtered through celite to remove the catalyst and was evaporated to dryness. DHR structure was confirmed by nuclear magnetic resonance (^{1}H NMR) measurements using a Varian 400-MHz instrument VNMR System (Varian, Palo Alto, CA). Synthesized DHR was dissolved in deuterated DMSO (99.96% deuterated) using dinitrobenzene (1.6 mmol/L) as internal standard. The ^{1}H NMR spectrum was acquired with the following signals numbered according to Fig. 1: δ (ppm) 9.0 (broad band, 3H, −OH), 6.97 (doublet, 2H, J = 8.6 Hz, H-2′,6′), 6.63 (doublet, 2H, J = 8.6 Hz, H-3′,5′), 6.03 (doublet, 2H, J = 4.0 Hz, H-2,6), 6.00 (double doublet, 1H, J = 4.0 Hz, H-4), 2.68–2.56 (multiplet, 4H, −CH_2−CH_2−). Standard purity was calculated following the validated protocol by Malz and Jancke [29], obtaining 80% purity, for quantifying purposes. Only one peak was detected after a full-scan MS experiment (m/z 229) and no peaks of the parent compound RV were detected, since 97% purity related to the starting material was shown.

2.3. Intervention beverages

RW and DRW of the Merlot variety (Penedes appellation) were selected for its large amount of RV and piceid, in comparison to other red varieties [30]. The phenolic composition of both wines was analyzed throughout the study period [31–33] and no significant differences were obtained for any phenolic compound between RW and DRW (Supplementary Table S1).

2.4. Human experimental design

A total of 73 high-risk subjects with high cardiovascular risk, aged ≥55 years, were recruited for a randomized, crossover, controlled clinical trial [34], although in this work, we analyzed available urine from 59 included subjects to study the metabolic profile of RV. Subjects were firstly asked to follow a 15-day run-in period in which they consumed neither grape-derived products nor alcoholic beverages and, after that, they were requested to consume 272 mL of RW (30 g ethanol/day) or DRW every day for 4 weeks, following the same background diet. Twenty-four hour urine samples were collected on the last run-in period day and on the last day after each intervention period. Aliquots were immediately stored at −80 °C until analysis, after noting the corresponding total excreted volume.

This trial was registered in the Current Controlled Trials at the International Standard Randomized Controlled Trial Number Register, at controlled-trials.com, as ISRCTN88720134.

2.5. Sample extraction

RV metabolites were extracted from urine samples by SPE as previously described [10] with slight modifications. Briefly, 1 mL of urine with 100 μL of *trans*-RV-$^{13}C_6$ as internal standard (1.71 μmol/L) was loaded onto a preconditioned Waters Oasis® HLB 96-well SPE plate (30 mg) (Milford, MA, USA). After washing the plate, elution of RV metabolites was achieved with 0.5 mL of 1 mol/L acetic acid in methanol, 2 × 0.5 mL of 1 mol/L acetic acid in ethyl acetate and 0.5 mL of ammonia 5% (v/v) in methanol. The eluate was evaporated to dryness and then reconstituted with a solution of 100 μL of taxifolin (1.64 μmol/L) as secondary internal standard diluted in acidified water (0.5 mL/L acetic acid) with 10% organic mobile phase.

2.6. Chromatography optimization

The mobile phase has a significant influence on the ionization efficiency. Therefore, mobile phases were examined in order to optimize chromatographic conditions for RV metabolites, using the available pure standards (1 mg/L) in MRM mode. These mobile phases included: 0.5 mL/L acetic acid [11], and a buffered solution with ammonium acetate (10 mmol/L) at different pH (3, 4 and 5),

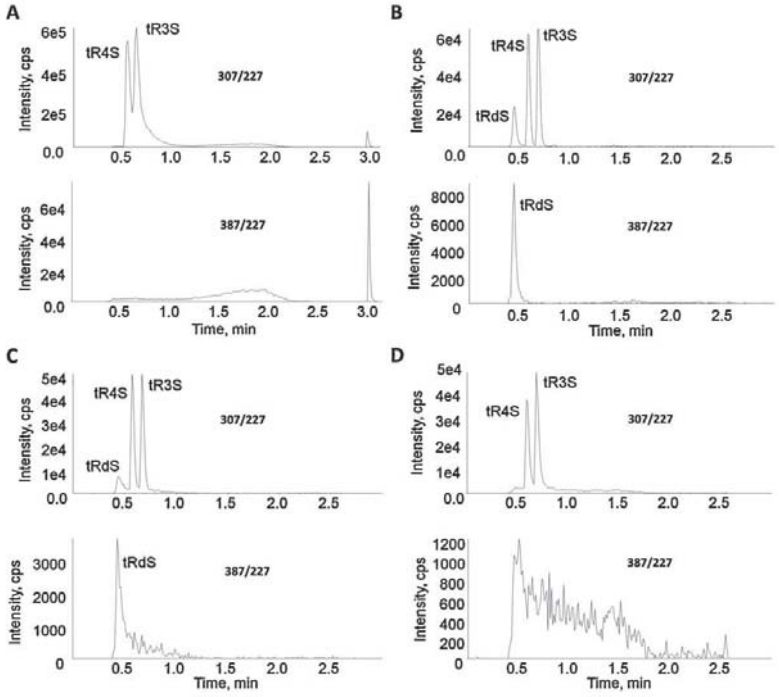

Fig. 2. Representative MRM chromatograms of tR3S (peak 5 – Fig. S1) and tR4S (peak 6 – Fig. S1) (MRM 307/227) and tRdS (peak 9 – Fig. S1) (MRM 387/227) standards, using (A) 0.5 mL/L acetic acid, (B) ammonium acetate 10 mmol/L at pH 5, (C) ammonium acetate 10 mmol/L at pH 4 and (D) ammonium acetate 10 mmol/L at pH 3.

as the aqueous solvent A and acetone:ACN (70:30, v/v) as solvent B in gradient elution (see Section 2.7). Ammonium acetate, along with formate salt, has already been used for the determination of RV-sulfates [13,27,35] as well as for other sulfate metabolites [36,37]. Acetic acid 10% (v/v) was used to adjust the mobile phase at different pH values.

2.7. UPLC–MS/MS analysis

The analysis of RV metabolites in urine samples was carried out by UPLC coupled to tandem mass spectrometry (UPLC–MS/MS) adapted from a previous validated methodology [10]. A Waters Acquity UPLC system (Milford, MA, USA), equipped with a binary solvent manager and a refrigerated autosampler plate, was used to couple an AB Sciex API 3000 triple quadrupole mass spectrometer equipped with a turbo ion spray, ionizing in negative mode. An Acquity UPLC BEH C18 (Milford, MA, USA) analytical column (1.7 μm, 2.1 mm × 5 mm), maintained at 40 °C, was used for chromatographic separation at a flow rate of 1 mL/min, split (1:1) before MS analysis. Injection volume was 5 μL. Two different aqueous mobile phases A were used: 0.5 mL/L acetic acid and then ammonium acetate 10 mmol/L at pH 5. Mobile phase B consisted of acetone:ACN (70:30). A linear gradient profile was applied with the following proportions (v/v) of phase A [*t*(min),%A]: (0,90); (1,70); (2,0); (2.3,0); (2.31,90); (3,90). MS and MS/MS parameters were optimized in infusion experiments for each RV metabolite standard (Table 1) in the corresponding mobile phase A/B (1:1) at a flow rate of 5 μL/min in a mass spectrometer using a model 11 syringe pump (Harvard Apparatus, Holliston, MA), with the general parameters of collision cell exit potential (−15), focusing potential (−200 V), entrance potential (−10 V) and capillary voltage (−3500 V) obtained.

The availability of pure standards of RV phase II metabolites allows their identification by comparing the retention time at the corresponding MRM transitions. Other RV and piceid phase II metabolites, along with the gut microbial ones, were identified by product ion scan (PIS) (Supplementary Figs. S1 and S2) [19]. For quantitative analysis, calibration curves in blank human urine were constructed with available standards subjected to the same SPE procedure as the samples, using the MRM transitions shown in Table 1. When the metabolite standard was not available, concentrations were estimated using the most similar compound standard curve and expressed as their equivalents (Table 3). A commercial standard of cR3G was not available when urine samples were analyzed and, thus, they were quantified using a cR4G standard curve, although afterwards cR3G was validated for this method. After quantification, values under LOQ were considered as the LOD value and values under LOD were considered zero.

2.8. Assay validation

Selectivity, linearity, detection and quantification limits, recovery, accuracy and precision of the available standards were evaluated for the optimized method, according to the Food and Drug Administration (FDA) [38]. The selectivity of the method was assessed by analyzing blank human urine in order to ensure that there were no interferences at the same retention time and MRM transition for the analytes. Five calibration curves in blank human urine of all available RV metabolite standards were performed on three different analysis days for linearity evaluation at

Table 2
Limits of detection (LOD), recovery, accuracy and precision data ($n = 8$) obtained from the UPLC–MS/MS of all available standards in blank human urine for each chromatographic method.

Analyte	LOD (μg/L)	Recovery (mean ± SD, %)	Added Conc. (μg/L)	Calculed Conc. (mean, μg/L)	Accuracy (%)	Precision (RSD, %)
Mobile phase A: 0.5 mL/L acetic acid						
trans-RV	0.48 ± 0.03	105.1 ± 11.5	50.0	54.1	108.18	6.81
			500.0	506.1	101.21	9.30
			1000.0	974.4	97.44	7.68
cis-RV	0.55 ± 0.04	101.6 ± 8.4	51.8	53.5	102.86	9.31
			518.0	482.0	93.04	8.66
			1036.0	960.5	92.71	11.54
tR4G	0.76 ± 0.10	101.2 ± 5.1	50.0	54.9	109.50	0.39
			500.0	546.5	109.30	0.39
			1000.0	958.0	95.80	1.18
tR3G	1.41 ± 0.20	98.7 ± 7.2	51.2	51.4	100.76	4.04
			512.0	473.0	92.38	7.90
			1024.0	970.3	94.76	7.94
cR4G	1.75 ± 0.20	102.0 ± 6.4	51.5	50.3	96.79	8.69
			515.0	482.6	93.72	7.84
			1030.0	988.5	95.98	10.14
cR3G	1.53 ± 0.15	96.1 ± 9.1	50.5	48.6	95.28	6.22
			505.0	497.6	98.53	12.32
			1011.0	1063.6	105.20	12.12
trans-Piceid	0.42 ± 0.05	96.5 ± 5.0	50.0	47.8	95.51	8.63
			500.0	523.6	104.72	9.21
			1000.0	1002.0	100.20	10.57
DHR	0.77 ± 0.03	96.1 ± 5.4	51.6	51.3	98.67	9.13
			516.0	531.7	103.05	5.83
			1032.0	1004.0	97.29	10.32
Mobile phase B: ammonium acetate 10 mM						
tR4S	0.53 ± 0.05	97.4 ± 10.0	50.0	54.4	108.81	8.27
			500.0	523.4	104.68	5.55
			1000.0	954.7	95.47	9.05
tR3S	0.36 ± 0.04	95.6 ± 9.9	50.0	51.5	103.04	6.04
			500.0	546.1	109.23	5.19
			1000.0	981.4	98.14	9.19
tR34dS	0.62 ± 0.09	100.5 ± 6.4	53.3	49.7	93.74	10.08
			533.0	510.1	95.70	6.11
			1065.0	1043.3	97.96	10.97
trans-Piceid	0.85 ± 0.10	–	50.0	49.8	99.53	12.09
			500.0	486.2	97.23	6.06
			1000.0	919.4	91.94	11.03
DHR	1.05 ± 0.13	–	51.6	51.3	98.68	12.42
			516.0	495.0	95.93	7.48
			1032.0	904.9	87.68	9.60

eight concentration points from 1 to 1000 μg/L. Detection limit was defined as the analyte concentration that produced a signal-to-noise ratio of at least 3. The lowest concentration with accepted precision and accuracy criteria for each standard on the calibration curve was the lower limit of quantification (LLOQ). Three different concentrations (low, medium and high) of each available standard were used to evaluate their precision and accuracy ($n = 8$). The latter was obtained as the percentage of the ratio between the mean calculated concentration and the known added concentration and precision as its relative standard deviation. The same concentration levels were used to perform the extraction recovery, for which the standards were spiked to blank human urine before extraction and compared to the standards spiked to extracted blank human urine.

Validation parameters were evaluated for *trans*- and *cis*-RV, the available four glucuronide isomers, *trans*-piceid and DHR using the chromatographic method with 0.5 mL/L acetic acid as mobile phase A, while for sulfate and disulfate conjugates the validation procedure was performed using the mobile phase A consisting of ammonium acetate 10 mmol/L. Standards of *trans*-piceid and DHR were also validated for the ammonium acetate 10 mmol/L mobile phase A, since their calibration curves were used to quantify their respective sulfate and sulfoglucuronide (SG) conjugates.

2.9. Statistical analysis

As urinary RV data were skewed (Kolmogorov and Levene tests) and the natural logarithm of the variable did not normalized the data, comparisons between interventions were performed using the nonparametric Friedman test and the paired Wilcoxon test. IBM SPSS Statistics software, version 20 (Chicago, IL), was used to perform the statistical analysis. Statistical significance was defined as $P \leq 0.05$.

3. Results and discussion

3.1. Method optimization for sulfate metabolites

The availability of purified standards of RV metabolites, and specifically tRdS, has allowed some improvements of the previously validated methodology for the analysis of the RV metabolic profile

Table 3
24-h urinary excretion (mean ± SEM) of individual metabolites at baseline (BAS) and after regular consumption of dealcoholized red wine (DRW) and red wine (RW).

Metabolites	Urinary excretion (nmol/24 h)		
	Baseline	DRW	RW
RV phase II metabolites			
trans-RV	n.d.	n.d.	n.d.
cis-RV	n.d.	n.d.	n.d.
tR4G	85.82 ± 28.36*	838.11 ± 259.78	390.58 ± 145.55
tR3G	89.58 ± 24.70*	192.88 ± 29.34	192.69 ± 29.72
cR4G	53.59 ± 15.97*	487.35 ± 84.54	449.89 ± 60.64
cR3G[a]	292.35 ± 97.22*	2410.23 ± 267.05	2304.85 ± 231.88
tR4S	80.33 ± 61.95*	140.56 ± 77.24	91.08 ± 47.40
tR3S	55.83 ± 25.32*	488.62 ± 69.93	591.88 ± 93.27
cR4S[b]	180.43 ± 62.57*	1044.86 ± 152.15	931.97 ± 143.70
cR3S[c]	83.34 ± 41.40*	891.18 ± 132.27	753.16 ± 132.13
tR34dS	47.63 ± 16.78*	414.45 ± 58.92	418.60 ± 53.37
cR34dS[d]	n.d.	n.d.	n.d.
RV-SG[d]	35.35 ± 19.36*	211.14 ± 30.73	170.17 ± 22.37
Sum of the total RV phase II metabolites	1004.26 ± 252.75*	7119.38 ± 658.16	6294.88 ± 620.00
RV glucosides			
trans-Piceid	0.00 ± 0.00*	2.94 ± 0.53	2.63 ± 0.55
cis-Piceid[e]	2.26 ± 0.92*	14.71 ± 1.78	17.84 ± 2.41
Pic-G[e]	3.80 ± 1.52*	29.64 ± 2.97	31.80 ± 4.55
Pic-S 1[e]	6.91 ± 2.44*	50.46 ± 6.11	54.68 ± 7.06
Pic-S 2[e]	4.39 ± 1.56*	44.86 ± 5.08	39.42 ± 4.01
Sum of the total RV glucosides	17.36 ± 5.87*	142.60 ± 13.23	146.38 ± 13.40
Gut microbial metabolism			
DHR	1.46 ± 1.01*	18.08 ± 2.47	20.27 ± 3.56
DHR-G 1[f]	7.94 ± 3.29*	92.18 ± 8.03	73.16 ± 8.95
DHR-G 2[f]	58.21 ± 22.06*	500.81 ± 43.85	455.99 ± 42.34
DHR-S 1[f]	515.83 ± 166.71*	3286.05 ± 279.89	2799.04 ± 254.89
DHR-S 2[f]	144.81 ± 27.51*	839.04 ± 97.16	681.17 ± 106.40
DHR-SG[f]	49.75 ± 17.43*	333.12 ± 34.09	300.42 ± 34.22
Sum of the total gut microbial metabolism of RV	778.00 ± 222.87*	5069.28 ± 383.77	4330.04 ± 362.33

Changes in excretion values in response to the intervention treatment were determined by Wilcoxon non-parametric test for 2 related samples. n.d., not detected.
[a] Expressed as cR4G equivalents.
[b] Expressed as tR4S equivalents.
[c] Expressed as tR3S equivalents.
[d] Expressed as tRdS equivalents.
[e] Expressed as *trans*-piceid equivalents.
[f] Expressed as DHR equivalents.
* $P < 0.05$, comparing baseline to DRW and RW interventions.

[10,11], including also a reduction of time analysis with UPLC. The poor chromatographic behavior previously reported for RV-sulfates [21] was improved using ammonium acetate (10 mmol/L) as mobile phase. In our previous publication we improved sulfate metabolite resolution using a ternary mobile phase B with acetone [10], but disulfate metabolites were not previously considered. In fact, the use of a buffered solution in chromatography analysis allowed the identification of tRdS, since it was not observed when 0.5 mL/L acetic acid was used (Fig. 2A). Certainly, ammonium acetate improves the separation and enhances peak shapes of sulfate conjugates, avoiding adsorption and tailing previously shown [3]. MRM analysis of metabolites (Fig. 2) supports these improvements, since monosulfate conjugates provided a signal-to-noise ratio around 7-fold higher for tR4S and tR3S, respectively, with ammonium acetate at pH 5 compared with 0.5 mL/L acetic acid (Fig. 2A and B). Regarding pH values, mobile phase at pH = 5 provided signal-to-noise ratios from 9- to 20-fold higher for tRdS and around 7-fold higher for tR4S and tR3S, compared to mobile phases at pH 4 and 3. Moreover, the higher the pH value, the better the resolution of sulfate peak shapes shown (Fig. 2). However, the signal-to-noise ratios for aglycones (*trans*- and *cis*-RV and DHR), as well as their glucoside (*trans*-piceid) and glucuronide conjugates, was up to 4-fold higher with 0.5 mL/L acetic acid mobile phase, compared to the buffered solution. Bearing in mind these differences in chromatography resolution, both aqueous mobile phases were used for sample analysis, since low chromatographic times were achieved with UPLC. Regarding the benefits of ammonia solution for sulfate detection, we added a final stage of the elution process in the SPE with 5% ammonium hydroxide in methanol, obtaining recoveries from 96% to 105% for all studied standards (Table 2).

3.2. Quality parameters of the method

Selectivity of the method was evaluated in blank human urine and no interference peaks were observed at the same retention time of the metabolites. The five calibration curves were determined by weighted ($1/x^2$) least-square regression analysis, and linearity over the concentration range was studied by obtaining correlation coefficients for all analytes higher than 0.994, with accuracy values ranging from 85 to 115%. Detection limit for each standard ranged from 0.36 to 1.75 μg/L (Table 2), and the lower limit of quantification (LLOQ) was 5 μg/L, which evinced the great sensitivity achieved by the analytical method. Results for accuracy and precision, displayed in Table 2, met the acceptance criteria of the FDA [38]. Thus, results obtained with this new adapted methodology will be repetitive and exact at low, medium and high concentrations, since accuracy values were from 88 to 111%, with mean variations in precision up to 12%. Mean recovery values for all

the compounds were 99% (Table 2), with 95% for the RV isotope (*trans*-RV-$^{13}C_6$), which was used as internal standard.

3.3. Identification of metabolite profiling of RV

Up to 15 RV and derivates were unequivocally identified using the available commercial standards, including also *cis*-isomers of RV sulfates and piceid, which were identified by isomerization of the *trans*-isomer standard solutions after 30 min in the sunlight [11] (Table 1). Although *cis*-resveratrol-3,4′-*O*-disulfate (cRdS) was also identified after isomerization of the *trans*-standard, it was not found in any sample after RW or DRW consumption. Also, neither *trans*- nor *cis*-RV were found in urine samples, as was previously reported [1,17], due to their extensive metabolism [39].

The presence of other metabolites, whose standard were not yet available, was also studied through MS/MS experiments. We identified up to 9 RV metabolites using the PIS (Supplementary Figs. S1 and S2): RV-SG, piceid-glucuronide (Pic-G), two piceid-sulfates (Pic-S), two DHR-glucuronides (DHR-G), two DHR-sulfates (DHR-S) and a DHR-SG. Other metabolites such as RV-diglucuronides (MRM 579/403), DHR-diglucuronides (MRM 581/229) and DHR-disulfates (MRM 389/229) were also sought, although any peak could be confirmed in PIS experiments.

Piceid and its phase II metabolites were also previously detected in human samples [11,19] and in rats [15,40,41], indicating that RV glucoside might be absorbed in its intact form, conjugated and finally excreted in urine. Gut microbial metabolism of RV was observed with the presence of DHR and its glucuronide and sulfate conjugates, as already published in some previous works in humans [19–21], in rats [14,15,42] and in pigs [43] after RV or RW administration. However, it should be borne in mind that this is the first work linking all the resveratrol derivates and their metabolites in a unique metabolic profile of up to 21 compounds, considering resveratrol and piceid phase II metabolites and also those obtained by microbial action.

3.4. Quantitative expression of RV metabolites

With the recent commercial availability of RV metabolites, an accurate quantification of these compounds in biological samples could be achieved. In this work, we have been able to quantify 6 RV phase II metabolites with the corresponding standard: tR4G, tR3G, cR4G, tR4S, tR3S and tRdS. phase II metabolites of RV have already been studied, with glucuronide and sulfate forms being the most reported ones [3,39], although many studies measured them indirectly as free aglycone after enzymatic hydrolysis [20–23], or directly expressing results as aglycone equivalents [10–12]. In this work, the effect of quantifying a RV metabolite with its corresponding standard or as aglycone equivalents has been evaluated in the urine samples of this study (Fig. 3). Statistical differences between both quantification methods were obtained for the evaluated metabolites, with higher excretion values using aglycone equivalents, except for tR4G (Fig. 3). However, while only 0.7–1.2-fold higher values were achieved for glucuronide conjugates, amounts of sulfate metabolites were more than 8-fold higher when expressed as aglycone equivalents. Thus, sulfate conjugates seem to be greatly overestimated compared to glucuronides, when quantified as RV equivalents.

Despite all these reported differences, a wide RV metabolic profile of up to 21 compounds was considered, with 8 of those accurately quantified as the real metabolite and 13 expressed as equivalents of the most similar compound (Table 3), since commercial standards are not yet available. It would now be very interesting to have these standards available, in order to finally achieve a more accurate concentration of the whole metabolic profile of RV and its relation to the beneficial effects of grape product consumption. To

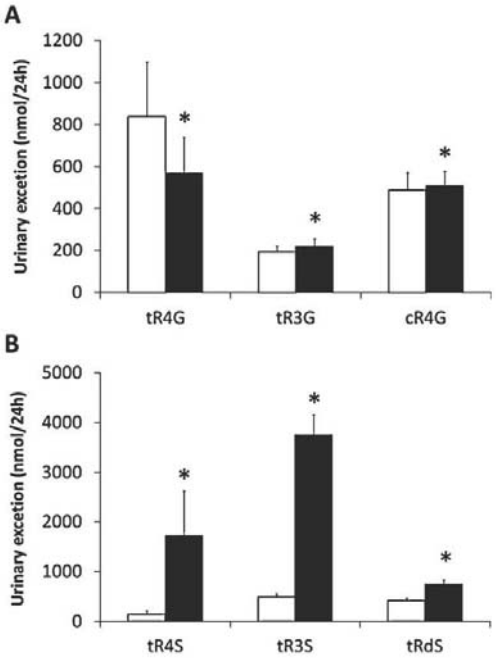

Fig. 3. Mean excreted amounts (and SEM) of RV glucuronides (A) and RV sulfates (B) accurately quantified with their corresponding standard (white boxes) or as RV equivalents (black boxes) after the consumption of DRW. *$P < 0.05$ when comparing both quantification expressions (Wilcoxon test).

our knowledge, few studies have been performed on the possible biological activity of RV metabolites [6–9] and, although the *in vitro* activity of these metabolites has been shown to be less effective than that observed for RV [6,8,9], it is thought that, *in vivo*, enzymes could release the aglycone [7], which exerts its biological activity.

3.5. Effect of alcohol on the bioavailability of RV

Statistical analysis of 24-h urinary excretion of all the RV metabolites showed significant differences between baseline period and both interventions ($P < 0.001$, Friedman test). In fact, these differences in the excreted amounts of all the metabolites studied were significantly higher after a regular consumption of RW and DRW compared with values obtained after the run-in period (Table 3) ($P < 0.05$). Increments on excreted RV metabolites after RW consumption were previously observed [10,44,45] and, indeed, Zamora-Ros et al. described urinary glucuronide and sulfate conjugates of RV as biomarkers of moderate wine intake [44,45].

Interestingly, when the 24-h excretion values of volunteers after 4 weeks of moderate RW and DRW consumption were compared, no differences were obtained for any individual metabolite or for the sum of RV phase II metabolites, RV glucosides and those of gut microbial metabolites (Table 3) ($P > 0.05$). Therefore, excretion of RV metabolites, and thus RV bioavailability, would not be influenced by the alcoholic matrix of wine. Previous reports showed that alcohol from RW could improve polyphenol absorption by increasing their solubility [46], as was reported for quercetin in *in vitro* and *in vivo* studies [22,47]. However, different conclusions are reached in the few *in vivo* human studies focused on the bioavailability of RV related to alcohol matrix effects. In one of the first RV bioavailability studies in humans [22], pure *trans*-RV was

dissolved in three different matrices considering absence/presence of alcohol – white wine, grape juice and vegetable juice – resulting in an equivalent absorption of this polyphenol either in aqueous or alcoholic matrices. These results are in line with those obtained by Ortuño et al. [20], who compared pharmacokinetics of RV after wine or grape juice administration, obtaining similar values for *trans*-RV when samples were hydrolyzed. Moreover, another study concluded that the combined intake of 2000 mg of *trans*-RV with 500 mg of quercetin and 5% of alcohol (100 mL) did not improve RV absorption [48]. The same results were obtained for other polyphenols, such as catechin, malvidin-3-glucoside, caffeic acid and production of 4-O-methylgallic acid when interventions with RW and DRW were compared [49–51]. It is important to note that the above-mentioned studies on RV bioavailability only looked for RV aglycone, as itself or after enzymatic hydrolysis, with their results being in line of those obtained in this study. Thus, the utility of this method to give a global value for the resveratrol metabolism, largely used in clinical and epidemiological studies [34,45,52], might not be discarded, since similar results were obtained not only for the individual compounds but also for the total sum. However, the importance of the study of the large metabolic profile must be highlighted, since different concentration values of RV metabolites could reach target tissues, and thus they could have different capacities to exert their possible biological activity.

4. Conclusions

Although several studies have already demonstrated the RV ability to protect against several diseases, its low bioavailability makes them quite controversial. Thus it is important to have a sensitive and accurate methodology for the resveratrol analysis in biological samples, providing a complete profiling of the metabolism, including those formed by gut and microbial metabolism, in order to elucidate their possible biological activities. In this work, the analysis of RV metabolite profiling has been optimized, specifically for disulfate and sulfate metabolites, improving their chromatographic resolution and their extraction, as well, by using ammonium solutions. The improved methodology using combined mobile phases to consider the different chemical characteristics and analytical behavior of all the compounds constituting the metabolic profile has been validated for 11 RV derivates in a wide concentration range, with the recent availability of pure standards, which also allows an accurate quantification of biological samples. Furthermore, the study of the metabolic profile of RV in humans has been extended to a total of 21 metabolites in order to get the most comprehensive metabolic profile of resveratrol described in the literature with the inclusion of tentative identification of piceid and gut microbial-derived metabolites, which have aroused a great interest in this field, due to the microbiota effect on polyphenols activity. The analysis of all these metabolites has been applied to a clinical study where RV metabolites were excreted in higher proportions after long-term consumption of RW and DRW, with no differences observed between the interventions. Thus, it seems that RV bioavailability would not be influenced by the alcoholic matrix of wine. Having a more precise concentration value of all the RV metabolites in target tissues would finally lead to a better interpretation of the obtained results from clinical and epidemiological studies for the study of the beneficial effects of grape product consumption, since it has been demonstrated that the resveratrol metabolic pattern might act as an excellent marker for the efficacy of resveratrol in such studies. However, not only a global sum of total metabolites could be provided, but also the individual excretion of all the metabolic profile and, thus, it would be achieved higher possibilities to know which compounds would possibly have higher biological activity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2012.09.093.

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SUPPLEMENTARY DATA

Table S1. Phenolic composition of intervention beverages, Red Wine (RW) and dealcoholized RW (DRW), during the study period.

Phenolic compound (mg/L) [a]	**DRW**	**RW**	***P***
trans-RV [b]	2.73 ± 0.23	2.92 ± 0.36	0.352
cis-RV [b]	2.75 ± 0.15	2.79 ± 0.15	0.761
trans-Piceid [b]	10.53 ± 0.96	9.41 ± 1.12	0.160
cis-Piceid [b]	7.08 ± 0.87	7.71 ± 0.34	0.226
Gallic acid [c]	73.17 ± 7.01	68.48 ± 6.40	0.306
Protocatechuic acid [c]	5.85 ± 0.51	5.22 ± 0.62	0.246
Tyrosol [c]	47.81 ± 3.90	43.59 ± 4.73	0.298
Catechin [c]	126.45 ± 13.35	123.51 ± 11.30	0.786
Epicatechin [c]	70.57 ± 8.22	67.86 ± 7.74	0.699
trans-Caftaric [c]	19.21 ± 1.62	18.62 ± 1.44	0.595
trans-Caffeic [c]	12.18 ± 0.92	11.50 ± 0.79	0.246
trans-Coutaric [c]	5.62 ± 0.52	5.21 ± 0.45	0.182
2-S-Glutathionylcaftaric [c]	10.76 ± 1.26	10.30 ± 1.00	0.956
Quercetin-3-glucuronide [c]	11.25 ± 1.42	11.88 ± 1.38	0.770
Quercetin [c]	23.82 ± 2.37	26.66 ± 0.78	0.161
Isorhamnetin [c]	2.96 ± 0.14	3.34 ± 0.27	0.114
Delphinidin-3-glucoside [c]	14.71 ± 1.62	15.25 ± 0.89	0.589
Petunidin-3-glucoside [c]	12.04 ± 1.15	12.29 ± 1.06	0.755
Peonidin-3-glucoside [c]	6.68 ± 0.57	6.78 ± 0.62	0.797
Malvidin-3-glucoside [c]	49.86 ± 4.27	48.83 ± 4.45	0.787
Malvidin-(6-acetyl)-3-glucoside [c]	10.41 ± 1.20	10.97 ± 0.96	0.563
Malvidin-(6-coumaroyl)-3-glucoside [c]	3.54 ± 0.33	4.15 ± 0.27	0.066
Total Phenol (meqAG/L) [d]	2694.92 ± 86.79	2933.35 ± 377.31	0.426

[a] Values are expressed as Mean ± SD (n=5). [b]Analyzed following the work by Romero-Perez, et al. (Romero-Perez *et al*., 1999). [c]Determined as previously described by Ibern-Gomez, et al. (Ibern-Gomez *et al*., 2002). [d]Analyzed by Folin-Ciocalteu methodology (Singleton i Rossi, 1965)

Figure S1. Representative MRM trace chromatogram in urine samples after RW intake using different aqueous mobile phases: **A)** Acetic acid (0.05%): RV glucuronides (403/227): 1, tR4G, 2, tR3G, 3, cR4G, 4, cR3G; RV glucosides (389/227): 11, *trans*-piceid, 12, *cis*-piceid; 13, Pic-G (565/227); 16, DHR (229/123); 17, 18, DHR-G (405/229); and **B)** Ammonium acetate (10 mmol/L at pH 5): RV sulfates (307/227): 5, tR4S, 6, tR3S, 7, cR4S, 8, cR3S; 9, tRdS (387/227); 10, RV-SG (483/307); 14, 15, Pic-S (469/227); 19, 20, DHR-S (309/229) and 21, DHR-SG (485/309).

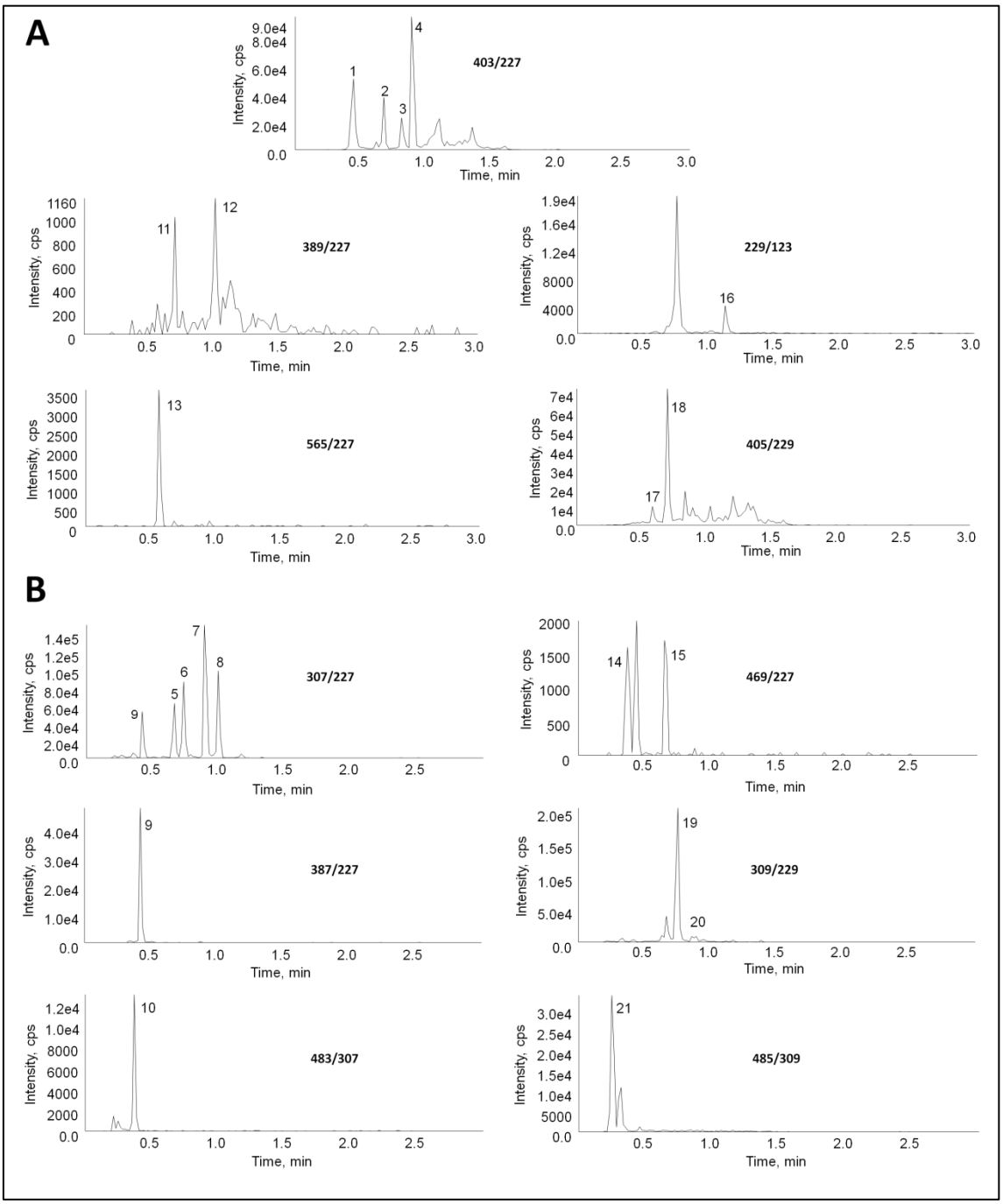


Figure S2. Product ion scan (PIS) of RV metabolites in urine samples after RW intake using the aqueous mobile phase of 0.05% acetic acid: A, Pic-G (peak 13, Figure S1, *m/z* 565) and B, DHR-G (peaks 17-18, Figure S1, *m/z* 405). Typical losses of the glucuronide moiety (-176 u) were shown, as well as *m/z* 175 and *m/z* 113, corresponding to the glucuronic acid moieties.

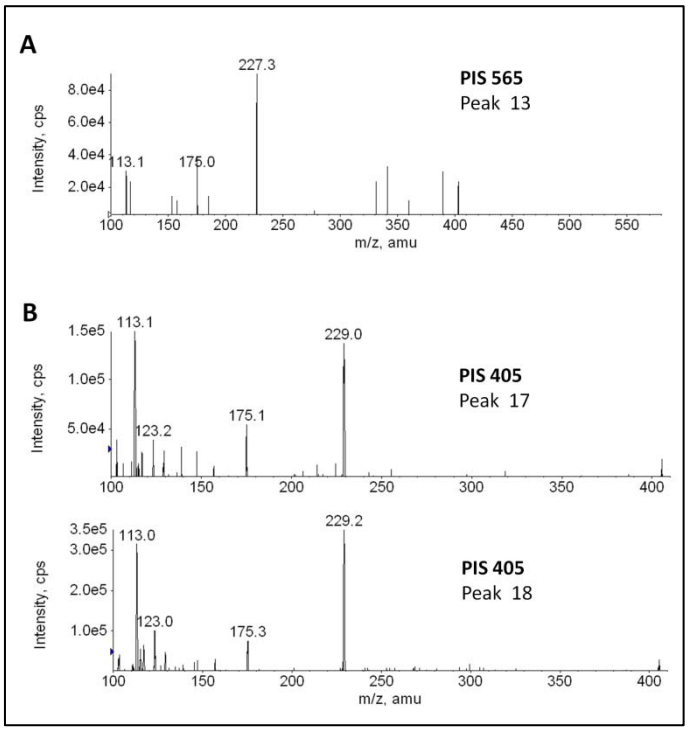
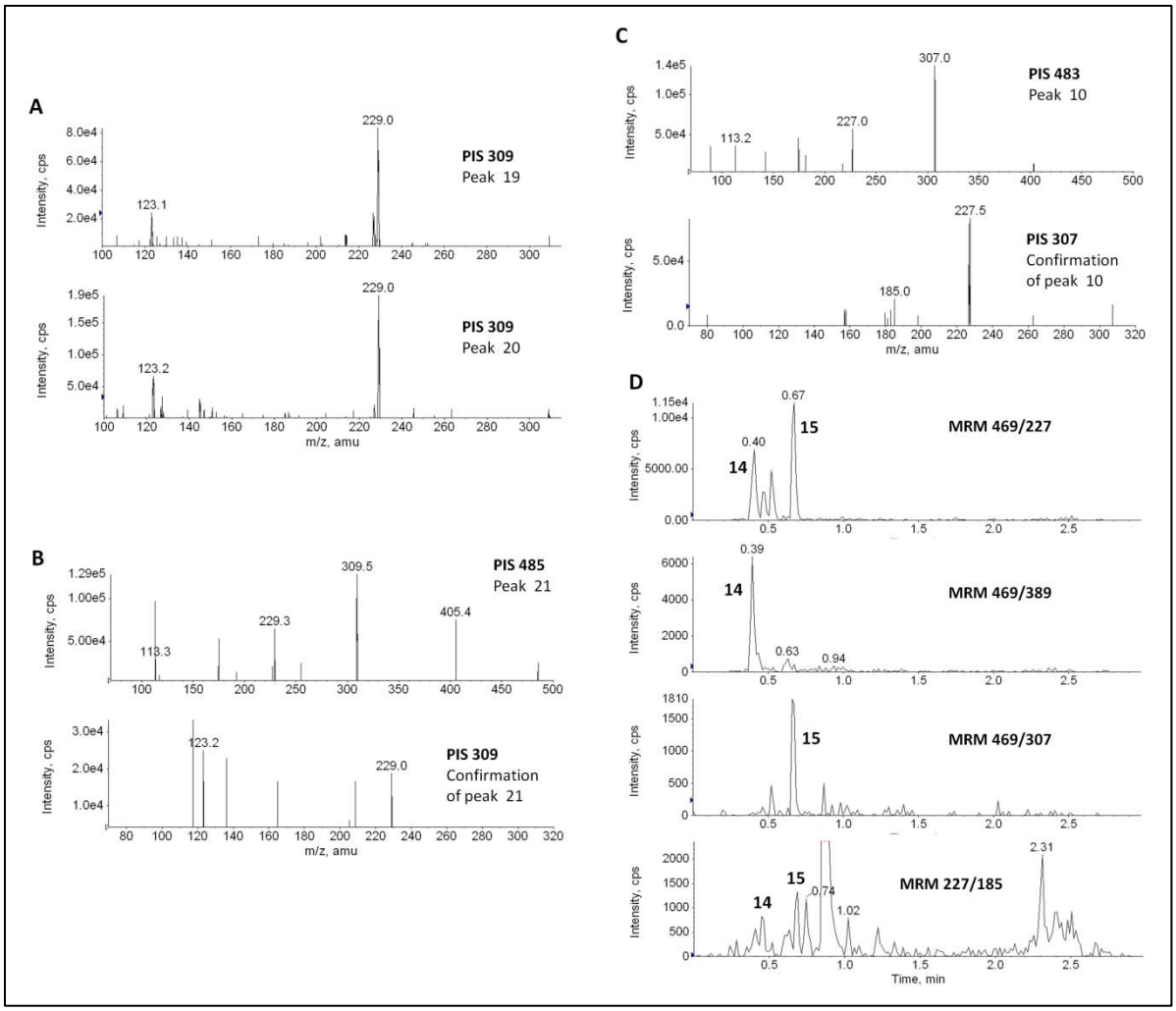


Figure S3. Product ion scan (PIS) of RV metabolites in urine samples after RW intake using the aqueous mobile phase of ammonuim acetate (pH 5), where the typical losses of the sulfate group (-80 u) could be shown: A, DHR-S (peaks 19-20, m/z 309); B, DHR-SG (peak 21, m/z 485) with its confirmation through the PIS of m/z 229 in CID-MS/MS experiment, and C, RV-SG (peak 10, m/z 483) with its confirmation through the PIS of m/z 227 in CID-MS/MS experiment. Pic-S (peaks 14-15, m/z 469) (D) were identified and confirmed by MRM experiments due to the low concentrations in urine samples.



ESTUDI 3

ESTUDI METABOLÒMIC DEL RESVERATROL DESPRÉS DE LA INGESTA PUNTUAL I REGULAR D'UNA BEGUDA FUNCIONAL D'EXTRACTE DE RAÏM EN HOMES I DONES.

Els resultats obtinguts en l'estudi metabolòmic dirigit del resveratrol després de la ingesta puntual i regular d'una beguda funcional d'extracte de raïm en homes i dones (Estudi 3), s'han enviat recentment a una de les revistes considerada pel *Science Citation Index*, *Nutrition*, que actualment es troba en procés de revisió. Aquest estudi ha estat emmarcat en el projecte CENIT-HIGEA (nº FBG306071).

Publicació 3: Maria Rotches-Ribalta, Mireia Urpi-Sarda, Mercè Mercader-Martí, Guillermo Reglero, Cristina Andres-Lacueva. Resveratrol metabolomic fingerprinting in acute and chronic intakes of a functional beverage in men and women. Enviada.

Resum

L'augment de la demanda de productes alimentaris amb compostos fitoquímics que promoguin un benefici per a la salut humana, ha despertat l'interès dels productes funcionals rics en polifenols. Abans de fer declaracions nutricionals o de propietats saludables, però, és necessari tenir un ampli coneixement sobre la biodisponibilitat dels suposats compostos bioactius considerant diferents matrius. En aquest treball es va estudiar la biodisponibilitat del resveratrol, tenint en compte tant els metabòlits intestinals com els obtinguts per acció microbiana, després del consum puntual i regular d'una beguda funcional a base d'extracte de raïm. Amb aquest objectiu es va dur a terme un estudi clínic d'intervenció aleatoritzat, creuat i controlat, que s'ha

explicat anteriorment. Les mostres d'orina obtingudes abans i després dels períodes de tractament van ser analitzades per LC-MS/MS, després d'un tractament amb SPE.

Després de corroborar que la ingesta de la beguda funcional incrementava l'excreció del resveratrol urinari, es van comparar els valors obtinguts després del consum puntual i regular d'aquesta beguda funcional. Es van obtenir valors similars per als metabòlits de fase II del resveratrol entre ambdues ingestes (P=0,62), i es va poder observar una menor excreció dels metabòlits obtinguts per acció microbiana 4 hores després de la ingesta puntual de la beguda funcional en comparació amb el seu consum regular (P<0,001), fet que implica una excreció més tardana d'aquests metabòlits microbians. Amb aquests resultats es va poder concloure que les diferències entre les ingestes puntual i regular del resveratrol són degudes principalment als metabòlits del resveratrol originats per la microbiota colònica. Pel que fa l'efecte del gènere, es van observar diferències en l'estudi agut, amb una major excreció dels metabòlits de resveratrol per part de les dones (P=0,010), donat un major metabolisme cap a la formació de glucurònids (P=0,014).

Amb aquests resultats, es pot concloure que el període d'administració del resveratrol determina el perfil metabòlic qualitatiu d'aquest, ja que els metabòlits provinents de la microbiota colònica seran identificats en estudis on es considerin temps d'excreció llargs i dosificacions sostingudes en el temps. Per primera vegada en humans s'han observat diferències significatives per la variable gènere en el perfil metabòlic del resveratrol excretat, tot i que seran necessaris més estudis per a verificar l'efecte del gènere en la biodisponibilitat d'aquest compost.

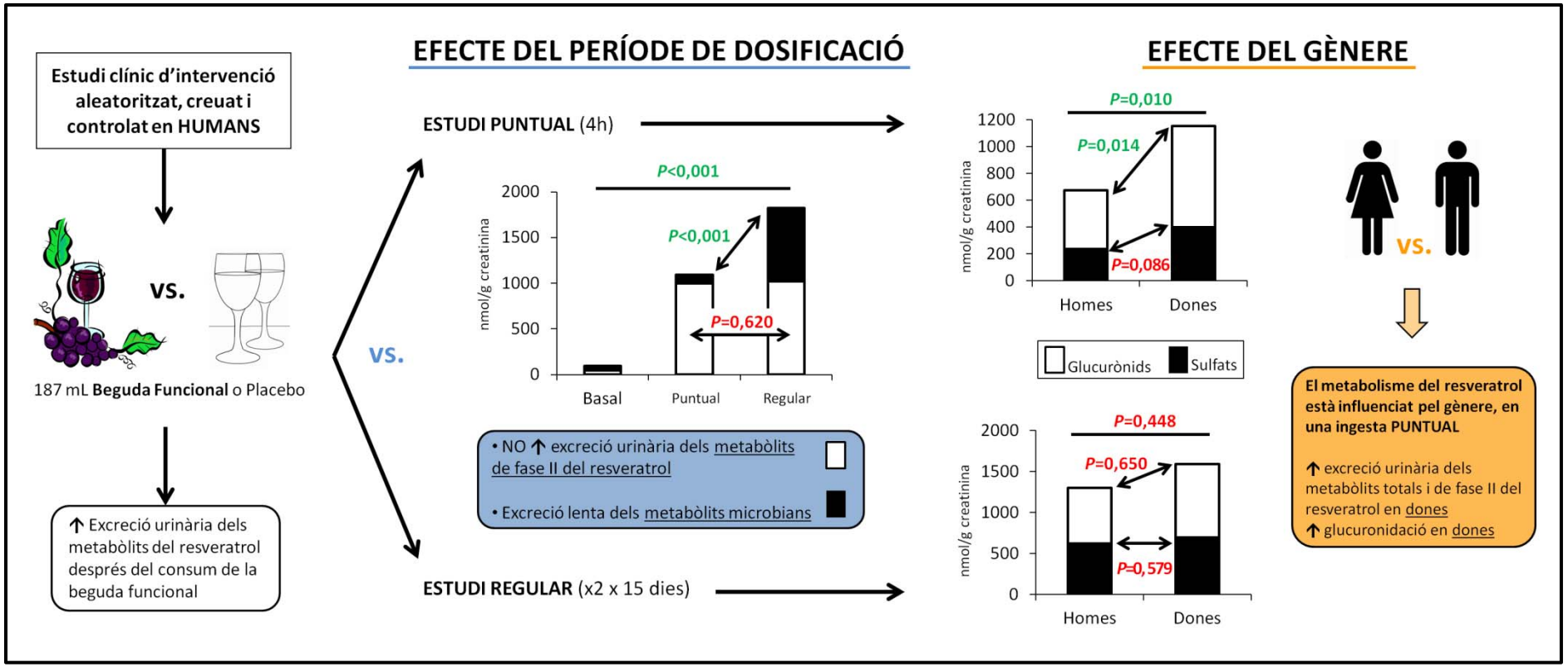


Figura 12. Gràfic resum de l'estudi metabolòmic del resveratrol després de la ingesta puntual i regular d'una beguda funcional d'extracte de raïm en homes i dones (estudi 3).

Resveratrol metabolomic fingerprinting in acute and chronic intakes of a functional beverage in men and women

Maria Rotches-Ribalta, Master[a,b]; Mireia Urpi-Sarda, Ph.D.[a,*]; Mercè Mercader-Martí[c]; Guillermo Reglero, Ph.D.[d]; Cristina Andres-Lacueva, Ph.D.[a,b]

[a]*Nutrition and Food Science Department, XaRTA-INSA. Pharmacy Faculty, University of Barcelona, Av Joan XXIII, s/n, 08028 Barcelona, Spain*

[b]*Ingenio-CONSOLIDER program, FUN-C-FOOD, Spain CSD2007-063. Ministry of Science and Innovation, Barcelona, Spain*

[c]*Miguel Torres, S.A. c/ M. Torres, 6. 08720 Vilafranca del Penedès. Barcelona. Spain*

[d]*Instituto de Investigación en Ciencias de la Alimentación CIAL (CSIC-UAM) C/Nicolás Cabrera 9, Universidad Autónoma de Madrid, 28049 Madrid, Spain*

*Corresponding author: Dr. Mireia Urpi-Sarda; Tel.: +34934024508, Fax: +34934035931, e-mail: murpi@ub.edu

RUNNING TITLE: Acute and chronic resveratrol bioavailability study

KEYWORDS: functional beverage; gender variability; grape extract; microbial metabolism; resveratrol

ABSTRACT

Objective. Before making nutritional claims about functional foods, such as polyphenol-rich beverages, more information is required on their bioavailability. In this work, we aimed to study resveratrol bioavailability, considering its gut and microbial metabolites after both acute and chronic consumption of a functional beverage, with special regard to a possible gender effect.

Research Methods & Procedures. A randomized, crossover, placebo-controlled clinical trial was performed with 26 volunteers. They consumed, after a run-in period of 7 days, 187 mL of a control placebo or a functional beverage for an acute study over 4 h and twice a day over 15 days for the chronic study. Urine samples obtained before and after intervention treatments were analyzed by LC-MS/MS.

Results. Similar values were obtained for resveratrol phase II metabolites after acute and chronic consumption of a functional beverage (P=0.62), although a later excretion was observed for microbial metabolites, since 4 h was not enough to excrete them all (P<0.001). A gender effect was observed in the acute intervention study, with higher excretion of resveratrol metabolites in women (P=0.010), due to a higher glucuronide metabolism of resveratrol (P=0.014).

Conclusions. Differences between acute and chronic resveratrol consumption are due to a later excretion of microbial metabolites. Resveratrol metabolism seems to be influenced by gender in the acute but not in the chronic intervention period, with women excreting more highly than men. Factors affecting bioavailability should take into consideration before making recommendations for regular functional foods consumption.

INTRODUCTION

The numerous health effects reported for resveratrol [1] have aroused interest in its consumption, and thus a lot of nutraceuticals and functional foods containing this compound have been developed [2]. But before making nutritional claims, bioavailability studies are required in order to ensure that sufficient amounts of the compound are available at target tissues after consumption of a reasonable dose.

Nowadays, more information is still needed on resveratrol bioavailability yielding its whole metabolic profile, since previous research has been conducted in a variety of different ways, and thus it is difficult to reach a concise conclusion [3], because a lot of factors could influence this bioavailability, including age, gender, food matrix, etc. In fact, previous studies were mainly conducted after administration of the pure compound with high doses administered [4], which might not reflect usual resveratrol consumption. In addition, most resveratrol bioavailability studies were performed after an acute intake, with limited data after chronic administration [3], and usually in male volunteers due to the estrogenic effect of resveratrol [5]. However, functional foods are developed to be consumed in a regular way in order to bring their benefits to the general population. Thus, more information is needed about the long-term consumption of polyphenol-rich functional foods and the possible gender effect in their bioavailability, which could modulate their beneficial properties [6].

This work is an important step in this direction, since the bioavailability of both gut and microbial metabolites of resveratrol after the consumption of a grape extract functional beverage (FB) was studied aiming to compare the acute and chronic administration of this beverage in a randomized, crossover, placebo-controlled clinical trial. Moreover, gender, as an influencing factor on resveratrol bioavailability, was also studied.

MATERIALS AND METHODS

Standards and reagents. In this study we worked with standards and reagents as previously described [7].

Human experimental design. A total of 31 healthy subjects (aged 25-55 years) were recruited for a randomized, crossover, placebo-controlled clinical trial. Five subjects withdrew before completing the study, and therefore 26 subjects (13 men and 13

women) were included. Participants were instructed to maintain a low polyphenol diet during a run-in period of 7 days and during the intervention periods. For the acute study, subjects consumed 187 mL of functional or control-placebo beverages (FB or CB, respectively) in a randomized order, together with a standard diet (white bread, cheese and yoghurt). After that, they were instructed to follow a 15-day period consuming, twice a day (at lunch and dinner time), 187 mL of the same beverages as in the acute study. The same procedure was repeated after a washout period of 7 days following a crossover experimental design. Twenty-four-hour urine samples were collected the day before the acute intervention study and on the last day of the chronic study. For the acute study, participants collected the urine during the first 4 hours.

The study protocol was approved by the Ethics Committee of the Hospital Universitario La Paz, Madrid.

Intervention beverages. Two different beverages were administered to the subjects included in this study. The FB was made with grape extract obtained by solid/liquid extraction of grape skins with alcohol mixtures at different temperature conditions. The selected extract had the highest antioxidant activity and total polyphenol index (TPI = 116) and CB was prepared with the same macronutrient composition as FB: 10 g/L of carbohydrates (sugars) and energy of 40 kcal/L. The phenolic composition of the FB was 4280 mg/L of hydroxycinnamic acids, 16 mg/L of anthocyanins, 96 mg/L of flavanols, 83 mg/L of hydroxybenzoic acids and 5.7 mg/L of stilbenes [8]. No polyphenolic content was detected for CB.

Sample preparation and analysis. Urine samples provided from the study were analyzed by LC-MS/MS after an SPE procedure as previously described elsewhere [7]. The excretion values were quantified following previous methodology [7], were corrected for urinary creatinine level and were reported as nmol per gram of creatinine. The following total sums of different kinds of metabolites were considered [7]: resveratrol phase II metabolites (RV-PhII), which were calculated as the sum of individual metabolites including *trans*- and *cis*-resveratrol-4'-*O*-glucuronide, *trans*- and *cis*-resveratrol-3-*O*-glucuronide, *trans*- and *cis*-resveratrol-4'-*O*-sulfate, *trans*- and *cis*-resveratrol-3-*O*-sulfate, *trans*-resveratrol-3,4'-*O*-disulfate and resveratrol-sulfoglucuronide, and microbial metabolites of resveratrol (RV-MM) considered as the sum of free dihydroresveratrol, two dihydroresveratrol-glucuronides, two

dihydroresveratrol-sulfates and a dihydroresveratrol-sulfoglucuronide. Total resveratrol metabolites (TRM) were the sum of all individual metabolites [7].

Statistical Analysis. IBM SPSS Statistics software, version 20 (Chicago, IL), was used to perform the statistical analysis. The nonparametric Friedman test, coupled with the paired Wilcoxon test for related samples, was used to compare changes in the excretion values of resveratrol metabolites before and after each intervention treatment since data were skewed (Kolmogorov and Levene tests) and could not be normalized. For the gender-effect evaluation, the Mann-Whitney test was performed. Statistical significance was defined as $P \leq 0.05$.

RESULTS

Changes in the urinary excretion of resveratrol metabolites performed after interventions with both beverages in comparison to baseline conditions are summarized in Table 1. Higher excretion values were obtained after acute and chronic consumption of FB than those observed at baseline and after CB consumption, with no differences between these low polyphenolic intake periods. Individual excretion values of the 26 subjects after FB intake at baseline and after acute and chronic consumption periods are represented in Figure 1. RV-PhII were excreted after 4-h acute intake with similar values after LT intake ($P>0.05$), while longer consumption of the functional beverage is needed for the excretion of RV-MM. For TRM, an intermediate value was observed after acute intake compared to baseline ($P<0.001$) and to the chronic intake ($P=0.002$) of the FB due to the low RV-MM excretion 4 h after FB intervention. The high range of values observed in Figure 1 and Table 1 has proved a high variability between subjects.

When the population was distributed by gender, similar behavior was observed between acute and chronic intakes except for the group of women, whose TRM had similar and not statistically significant values between chronic and acute intakes (Table 2). We observed significant differences between women and men in the urinary excretion of RV-PhII and TRM after 4-h FB intake, with a tendency for RV-MM ($P=0.086$). Otherwise, these differences were not observed in the urinary levels after chronic intervention. Urinary excretion values of glucuronide and sulfate conjugates were split up from the total sums in order to study the gender effect on the resveratrol metabolism

(Table 2). Women excreted statistically higher resveratrol glucuronides and total glucuronide conjugates than men ($P<0.05$) after 4-h FB intake, and nearly significant values were obtained for sulfate conjugates of RV-MM ($P=0.077$).

DISCUSSION

FB intake entails a significant overall increase in the urinary excretion of the whole resveratrol metabolic profile compared to baseline. The effect of the acute and chronic administration of FB was considered for gut and microbial metabolism of resveratrol, obtaining a total excretion for RV-PhII at 4 h, which is maintained during chronic intake, and a later excretion for RV-MM (Figure 1). These results are in line with a recent pharmacokinetic study of the resveratrol metabolic profile, where the maximum excretion for RV-MM is described between 4 and 12 hours after red wine intake [9], although slight urinary excretions were already observed in the first 4 h after ingestion. Previous pharmacokinetic studies have reported rapid absorption and metabolism of resveratrol [10], which was observed with the increased RV-PhII excretion values after acute FB intake. In a recent pharmacokinetic study, tmax values for resveratrol glucuronides were found ~2 h after red wine intake, with mean residence times of up to 4 h [9], explaining why there were no differences between acute and chronic intakes, since, moreover, the maximum excretion values for RV-PhII were reported to be reached in the first 4 h [9]. To our knowledge, no studies have focused on the differences between acute and chronic intakes of products with resveratrol [11], considering both gut and microbial metabolism of resveratrol. In humans, only one study has been carried out after a 200 mg single dose of trans-resveratrol and 3-day multiple doses of 200 mg twice daily, but evaluating only free resveratrol, for which no differences were observed [12]. In our study, where the whole resveratrol metabolic profile was considered, some differences were obtained in both time periods for RV-MM, which have been less studied [4], maybe due to the low excretion values obtained in an acute study.

The bioavailability of resveratrol has been extensively studied in males or in both genders together [10]. However, with the wide physiological differences between men and women, nutrition could influence the health of both genders differently [6]. Regarding the estrogenic effect of resveratrol [5], and as no previous studies have been

conducted, it could be hypothesized that its bioavailability, and thus its biological activity, might be gender-dependent [6]. In fact, women might have more receptors for the absorption of resveratrol and thus it would be more rapidly absorbed and more highly excreted. In this trial, where half of the participants were women, the gender differences in the urinary excretion of resveratrol metabolites were studied, finding, as hypothesized, higher excretion for RV-PhII and TRM in women after acute FB consumption, although no differences were obtained after chronic FB consumption. In this context, interindividual variability must not be discarded (Table 2), nor differences in the menstrual cycle of the women included in the study, which could modify the excretion values of TRM. Thus, further studies are required in this aspect, since to our knowledge few resveratrol bioavailability studies have focused on the gender effect. Only one human study has been conducted in the gender effect after administering 200 mg of trans-resveratrol during 3 days at 8-h intervals, concluding that its kinetic profile was independent of age and gender [12]. However, only free trans-resveratrol in plasma samples was analyzed, although it has been reported to account, in biological samples, for less than 2% of the total resveratrol consumed [13]. Taking into account other polyphenols with estrogenic effect, such as isoflavones [14], plasma concentrations of daidzein were higher in women than in men, although no differences were obtained for equol, its microbial-derived metabolite [15]. However, other studies found higher plasma values of daidzein and its metabolites in male rats after a commercial isoflavone supplement [16]. So, more studies are required in this context in order to elucidate the bioavailability of polyphenols in both genders, with a special regard to those with estrogenic properties. In addition, the sulfation and glucuronidation activity of some polyphenols, such as apigenin, has also been reported to be gender-dependent. It has been suggested that female rats would excrete higher proportions of glucuronide metabolites, while sulfate conjugates were more highly excreted in male rats [17]. In our study, the same behavior for glucuronide conjugates of resveratrol after acute FB intake (Table 2) was observed between men and women, although similar values of resveratrol sulfates were obtained for both genders and a tendency was even observed for the higher excretion of dihydroresveratrol sulfates in women.

CONCLUSIONS

The later microbial metabolism involves differences between acute and chronic resveratrol consumption from a FB. Its metabolism seems to be influenced by gender after the acute intervention, with women excreting more highly than men. Factors affecting bioavailability should be taken into consideration before making recommendations of functional products consumption.

ABBREVIATIONS USED

CB, control beverage; FB, functional beverage; RV-MM, resveratrol microbial metabolites; RV-PhII, resveratrol phase II metabolites; TPI, total polyphenol index.

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The authors' contributions were as follows: CA-L, GR, MM-M: conception and design of the study; MR-R and MU-S: generation, collection, assembly, analysis and/or interpretation of data; MR-R, MU-S, MM-M, GR and CA-L: drafting and revision of the manuscript. All authors read and approved the final version of the manuscript. None of the authors had a conflict of interest.

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Table 1. Changes in urinary excretion as median (IQR) of resveratrol phase II metabolites (RV-PhII), resveratrol microbial metabolites (RV-MM) and total resveratrol metabolites (TRM) after acute and chronic consumption of the control placebo and the functional beverages.

	Control Beverage		**Functional beverage**		
	Acute intake	Chronic intake	Acute intake	Chronic intake	*P*
RV-PhII	2.18 (-16.98 – 56.68)[a]	2.38 (-27.45 – 39.20)[a]	837.83 (581.56 – 1222.71)[b,*]	907.76 (523.10 – 1404.73)[b,*]	< 0.001
RV-MM	1.91 (-28.48 – 55.74)[a]	-22.68 (-53.00 – 22.35)[a]	45.68 (-0.05 – 89.66)[b,*]	596.14 (244.83 – 1100.92)[c,*]	< 0.001
TRM	14.55 (-23.50 – 68.21)[a]	-20.85 (-62.00 – 61.84)[a]	882.70 (628.62 – 1366.93)[b,*]	1684.51 (1080.26 – 2402.73)[c,*]	< 0.001

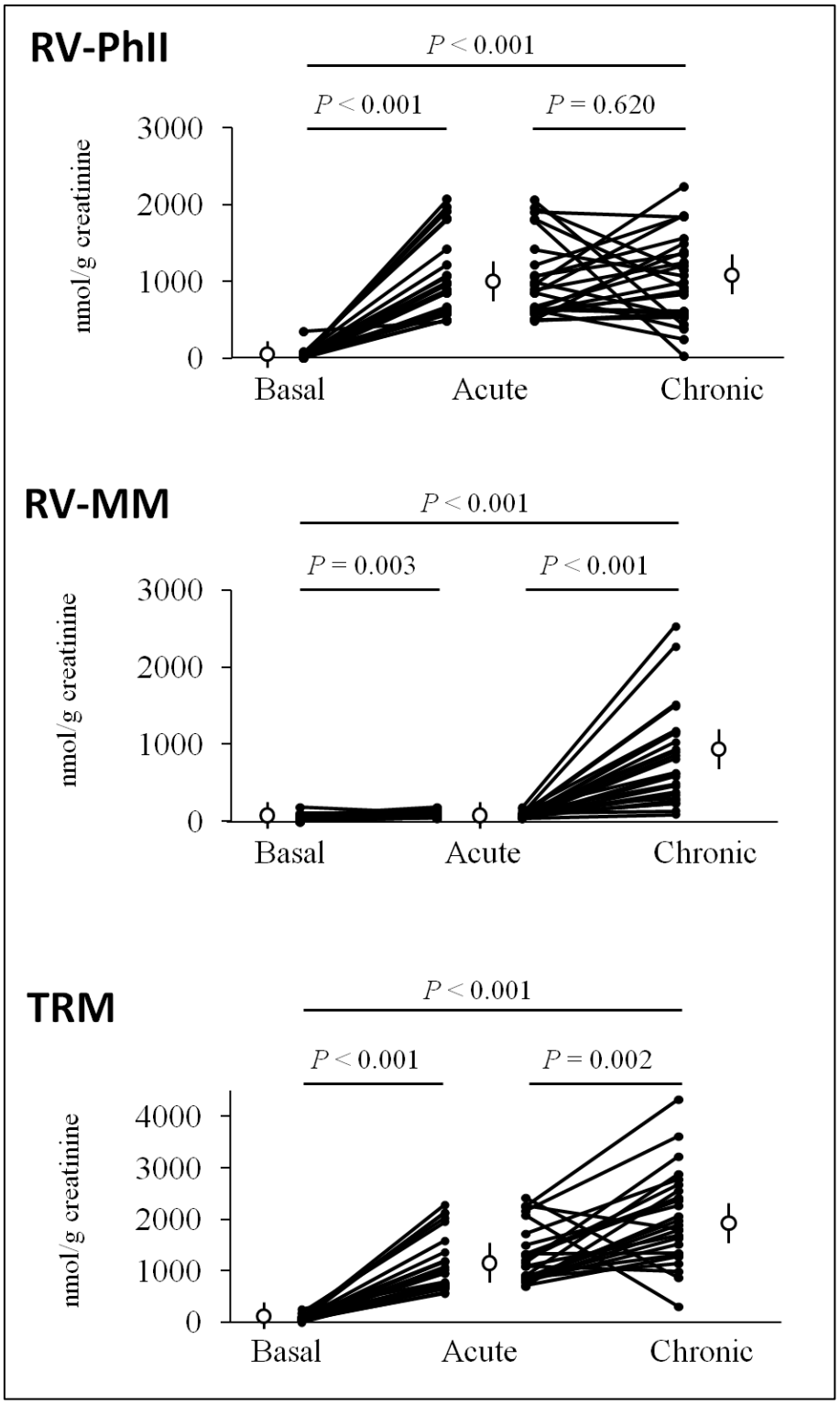
Values in a row with different superscript letters are significantly different ($P<0.05$, Wilcoxon test).
*Values significantly different from baseline ($P<0.05$, Wilcoxon test).

Table 2. Changes in urinary excretion as median (IQR) of resveratrol phase II metabolites (RV-PhII), resveratrol microbial metabolites (RV-MM) and total resveratrol metabolites (TRM), considering its glucuronide and sulfate conjugates, for men and women after acute and chronic consumption of the functional beverage.

	Acute intake		Chronic intake	
	Men	**Women**	**Men**	**Women**
RV-PhII	618.79 (555.36 – 837.83)	1176.3 (741.76 – 1804.75)*	837.26 (556.24 – 1283.46)	1014.7 (316.78 – 1810.1)
Glucuronides	405.62 (341.85 – 460.69)	705.99 (423.01 – 1343.2)*	580.83 (326.61 – 747.37)	780.55 (68.93 – 1214.2)
Sulfates	230.23 (134.05 – 319.04)	327.21 (229.40 – 443.16)	231.36 (159.94 – 307.28)	269.79 (141.27 – 404.05)
RV-MM	20.42 (-3.70 – 49.69)	79.37 (19.47 – 96.73)	581.78 (341.97 – 915.55)†	610.50 (221.39 – 1331.5)†
Glucuronides	0.00 (0.00 – 17.05)	31.22 (0.00 – 35.50)	91.05 (60.46 – 148.42)†	81.73 (24.01 – 277.16)†
Sulfates	16.02 (-6.64 – 28.08)	43.63 (9.00 – 54.71)	470.37 (253.52 – 687.04)†	416.85 (131.63 – 1167.2)†
TRM	672.30 (574.98 – 882.70)	1317.89 (839.36 – 1956.9)*	1494.8 (1113.8 – 2041.7)†	2086.5 (794.4 – 2801.2)
Glucuronides	436.04 (356.58–472.86)	753.52 (459.26–1424.7)*	679.26 (416.50 – 899.79)†	895.50 (154.17 – 1524.3)
Sulfates	236.44 (149.21–351.43)	398.32 (241.41–504.81)	619.41 (544.41 – 1031.8)†	694.81 (503.84 – 1526.1)†

*Values significantly different in comparison to the respective value for men (P<0.05, Mann-Whitney test).
†Values significantly different in comparison to the respective value after acute intake (P<0.05, Wilcoxon test).

Figure 1. Individual and mean values (± SEM) of urinary excretion of resveratrol phase II metabolites (RV-PhII), microbial metabolites (RV-MM) and total resveratrol metabolites (TRM) at baseline and after acute and chronic administration of the functional beverage for the 26 subjects. Statistical significance (P<0.05) performed by a Wilcoxon test.



DISCUSSIÓ
GENERAL

DISCUSSIÓ GENERAL

L'estudi qualitatiu i quantitatiu del perfil metabòlic del resveratrol, administrat a dosis dietètiques, requereix tècniques analítiques suficientment sensibles i optimitzades a les característiques químiques de tot el perfil, donat que els conjugats sulfats del resveratrol, a diferència de l'aglicona, els seus glucòsids o els metabòlits glucuronidats, presenten una baixa resolució cromatogràfica que dificulta la seva caracterització i quantificació (Walle, 2011). L'ús d'una fase mòbil ternària amb acetona havia resolt aquest inconvenient pels metabòlits monosulfats (Urpi-Sarda *et al*., 2007), però la detecció dels disulfats i sulfoglucurònids encara tenia marge de millora cromatogràfica. La recent disponibilitat d'estàndards purs de certs conjugats del resveratrol (gràcies a la síntesi química o recent disponibilitat comercial) ha permès el desenvolupament d'una metodologia millorada de l'anàlisi dels metabòlits del resveratrol (Rotches-Ribalta *et al*., 2012b), que ha estat validada segons els paràmetres de qualitat establerts per la FDA, com ara l'exactitud, precisió i recuperació. Alhora, la metodologia proporciona una clara millora cromatogràfica en relació als conjugats sulfats, podent identificar els conjugats disulfats del resveratrol com ara el *trans* i *cis*-resveratrol-3,4'-*O*-disulfat i els conjugats sulfoglucurònids de resveratrol. També inclou la identificació del dihidroresveratrol i els seus conjugats glucuronids, sulfats i sulfoglucurònids com a metabòlits del resveratrol obtinguts per acció de la microbiota intestinal, els quals són d'especial interès per als estudis clínics a llarg termini, donada la seva formació més tardana (Rotches-Ribalta *et al*., 2012a). Amb aquestes millores, s'ha ampliat el perfil metabòlic del resveratrol fins a 21 compostos: els glucurònids i sulfats de resveratrol, el corresponent sulfoglucurònid i disulfat, els glucòsids de resveratrol i els seus metabòlits de fase II, i els derivats del metabolisme microbià (dihidroresveratrol i conjugats). Cal destacar que cap dels isòmers de l'aglicona (*trans*- i

cis-resveratrol) ha sigut detectat en les mostres analitzades en aquests estudis. De fet, quan el resveratrol és administrat a través dels aliments, on es troba en baixes quantitats, aquest és extensament metabolitzat i, per tant, l'aglicona lliure es troba en quantitats molt baixes, per sota del límit de detecció, en mostres biològiques (Wenzel i Somoza, 2005).

La metodologia presentada aporta el perfil metabòlic de resveratrol més complet descrit en la literatura; afrontant i resolent les diferents singularitats que presenta cada grup de metabòlits del resveratrol. Anteriorment, els estudis que se centraven en els derivats de piceid no estudiaven el metabolisme microbià, i viceversa (Wang *et al*., 2011; Azorin-Ortuno *et al*., 2011; Wang *et al*., 2005). A més a més, els resultats de l'estudi farmacocinètic (Rotches-Ribalta *et al*., 2012a) han permès proposar les possibles estructures químiques dels metabòlits de fase II del piceid i el dihidroresveratrol, dels quals no es disposa estàndard. Per als metabòlits de fase II del piceid s'ha pogut discernir sobre la seva isomerització *trans*- i *cis*-, donada la diferent proporció de cada un d'aquests isòmers en els productes administrats en aquest estudi; essent les formes *trans*- majoritàries després del consum dels comprimits i els isòmers *cis*- després de la ingesta de vi. Així doncs, s'ha identificat al *cis*-piceid-glucurònid i el *trans*- i *cis*-piceid-sulfat. Pel què fa als metabòlits microbians, cal dir que la hidrogenació del doble enllaç de la molècula del resveratrol implica la pèrdua de la capacitat de isomerització *trans*- i *cis*- en el dihidroresveratrol i els seus conjugats. Les estructures d'aquests metabòlits han pogut ser identificades en funció de la seva posició en la molècula de dihidroresveratrol, ja que s'ha observat que la posició -3- és predominant en l'excreció dels conjugats del resveratrol en front a la -4'-, com també han descrit altres estudis (Azorin-Ortuno, *et al*., 2010). Traslladant aquesta observació, els isòmers majoritaris dels conjugats de dihidroresveratrol serien dihidroresveratrol-3-*O*-glucurònid i dihidroresveratrol-3-*O*-sulfat, respectivament; mentre que els isòmers minoritaris serien dihidroresveratrol-4'-*O*-glucurònid i dihidroresveratrol-4'-*O*-sulfat.

La disponibilitat d'estàndards purs ha permès, també, quantificar, amb el corresponent estàndard pur, fins a 8 dels compostos determinats en l'ampli perfil metabòlic. La resta dels metabòlits, dels quals no es disposa estàndard, han estat expressats com a equivalents del compost químicament més similar. Aquesta última forma de

quantificació, expressant-ho com a equivalents d'aglicona, és útil com a primera aproximació, però ha generat certa controvèrsia, donat que la ionització dels compostos en LC-ESI-MS/MS no és igual per a tots ells. Molts estudis eviten aquesta qüestió determinant les formes conjugades de manera indirecta després d'un procés d'hidròlisi enzimàtica (Ortuño *et al*., 2010; Goldberg *et al*., 2003; Soleas *et al*., 2001), o bé utilitzant detectors de llum UV/vis (Azorin-Ortuno *et al*., 2010; Burkon i Somoza, 2008). Tanmateix, aquestes tècniques també tenen els seus inconvenients, com la baixa eficiència del procés d'hidròlisi enzimàtica i la pèrdua del coneixement del perfil metabòlic o la baixa sensitivitat de la detecció per DAD. L'estudi sobre el perfil metabòlic del resveratrol administrat a dosis dietètiques requereix tècniques d'elevada sensibilitat, donades les baixes concentracions assolides en mostres biològiques. Aquesta sensibilitat analítica requerida s'aconsegueix amb la LC-ESI-MS/MS, que ha estat àmpliament utilitzada, malgrat la problemàtica plantejada per a la quantificació (Kapetanovic *et al*., 2010; Urpi-Sarda *et al*., 2007; Urpi-Sarda *et al*., 2005). Per tal de discernir sobre aquesta controvèrsia, en l'estudi 2 s'ha avaluat l'efecte de la quantificació dels metabòlits de resveratrol com a tal o bé expressats com a equivalents de l'aglicona. La concentració urinària de 5 dels 6 metabòlits de fase II del resveratrol avaluats s'ha vist sobreestimada quan es quantifica com a equivalent de l'aglicona, essent molt més acusat per el cas dels sulfats, en comparació als glucurònids.

Considerant el perfil metabòlic del resveratrol de manera quantitativa cal destacar les diferències interindividuals que s'han observat entre els subjectes que han participat en cadascun dels estudis clínics i que podrien ser deguts a possibles polimorfismes dels enzims intestinals (Manach *et al*., 2005). L'estudi 1 (Rotches-Ribalta *et al*., 2012a) fa èmfasi en aquest aspecte després d'observar una gran variabilitat en les concentracions plasmàtiques i urinàries dels metabòlits microbians i que pot ser atribuïda a l'elevada variabilitat descrita en la microbiota humana (Bode *et al*., 2013; Blaut i Clavel, 2007). L'estudi 3 es focalitza també en aquest aspecte comparant les excrecions urinàries del perfil metabòlic entre homes i dones, per tal d'avaluar un possible efecte del gènere. Fins ara, tots els estudis sobre la biodisponibilitat del resveratrol s'han dut terme en homes, ja que l'efecte estrogènic que ha demostrat el

resveratrol (Gehm *et al*., 1997) podria modificar els resultats d'estudis que contemplin les dones. Els aliments funcionals es desenvolupen amb la finalitat de ser utilitzats i d'aportar els seus beneficis a la població en general i, per tant, és d'interès conèixer si el gènere pot afectar la seva biodisponibilitat. Aquest aspecte s'estudia en el tercer treball, els resultats del qual han mostrat una major excreció dels metabòlits de fase II del resveratrol, principalment glucurònids, en dones 4 hores després de la ingesta d'una beguda funcional a base d'extracte de raïm. Les propietats estrogèniques del resveratrol (Gehm *et al*., 1997) podrien explicar que les dones tinguessin més receptors per a l'absorció d'aquest, o amb una major afinitat, que l'absorbirien més ràpidament, essent excretat en major proporció que els homes en un temps de curta durada. El consum regular durant 15 dies d'aquesta beguda, però, igualaria estadísticament l'excreció urinària dels metabòlits de resveratrol entre homes i dones. Aquest fet s'explicaria per l'elevada variabilitat observada en les dones, que podria ser atribuïble a les diferències en el seu cicle menstrual.

El coneixement de la biodisponibilitat i el metabolisme del resveratrol al llarg del temps s'ha dut a terme en l'estudi farmacocinètic (estudi 1) (Rotches-Ribalta *et al*., 2012a). Les formes intactes dels glucòsids de resveratrol s'han observat en plasma gairebé una hora després de les intervencions. És interessant remarcar que,; malgrat que el piceid es troba en l'aliment en major proporció (Romero-Perez *et al*., 1999), les concentracions de *trans*- i *cis*-piceid, juntament amb els seus conjugats, han esdevingut realment baixes (<4 ng/mL), comparades amb la de la resta de metabòlits. Sembla ser que en el procés d'absorció a l'enteròcit, el piceid és principalment hidrolitzat a resveratrol, el qual arribarà al corrent sanguini. Tot i això, petites proporcions del glucòsid es podrien absorbir directament, tal com també han demostrat altres estudis (Wang *et al*., 2011; Zhou *et al*., 2007; Lv *et al*., 2006). La formació dels diferents metabòlits de fase II ha requerit més temps per aconseguir les concentracions màximes plasmàtiques, que han esdevingut de fins a 130 ng/mL per al *cis*-resveratrol-3-*O*-glucurònid després del consum de vi. També s'han vist augmentats els temps de vida mitja, que s'han situat cap a les 4 hores després del consum de vi i de més de 5 hores després de la ingesta dels comprimits. Els metabòlits obtinguts per acció microbiana han assolit uns temps màxims de concentració en plasma encara

superior (fins a 8 hores després del consum de comprimits), que s'ha plasmat en una excreció urinària més tardana. La formació més lenta dels metabòlits microbians s'ha pogut corroborar en les dades obtingudes a l'estudi 3, ja que les 4 hores posteriors a la ingesta puntual no han estat suficients per a excretar els metabòlits de la microbiota en la seva totalitat, mentre sí que ho han aconseguit els metabòlits de fase II del resveratrol. Amb aquests resultats cal destacar, doncs, la importància de disposar de mostres urinàries obtingudes durant un període de temps superior al temps de vida mitja dels compostos a analitzar, és a dir, superior a 8 hores si es vol considerar tot el perfil metabòlic.

L'estudi cinètic també ha demostrat una excreció més lenta per als sulfats de resveratrol, fet que ha suggerit un canvi en el metabolisme del resveratrol que passaria de la glucuronidació a la sulfatació de l'aglicona (Kapetanovic *et al*., 2010). Això, podria explicar perquè aquests conjugats són presents en majors quantitats en estudis on s'administren dosis extremadament elevades de l'aglicona (Burkon i Somoza, 2008; Boocock *et al*., 2007a). En canvi, quan es consumeixen dosis moderades de resveratrol només s'han descrit conjugats glucurònids en mostres de plasma humanes (Vitaglione *et al*., 2005), tal com s'observa en els resultats corresponents a l'estudi 1. De fet, anteriorment ja es va descriure una possible saturació dels enzims que duen a terme la glucuronidació quan el substrat es troba en elevades quantitats (fins a 20 vegades superior a una dosi moderada), mentre que la via de la sulfatació no mostra cap tipus d'inhibició competitiva pel substrat (Andres-Lacueva *et al*., 2012; Maier-Salamon *et al*., 2006).

La influència de la matriu alimentària en el metabolisme del resveratrol és un factor important a tenir en compte ja que diferents compostos presents en aquesta matriu i que acompanyen al resveratrol, com l'alcohol, excipients, altres components de l'aliment o de la presentació dietètica, entre d'altres, podrien modificar-ne la biodisponibilitat. Estudis previs han suggerit que l'alcohol del vi negre pugui millorar l'absorció dels polifenols augmentant-ne la seva biodisponibilitat (Serafini *et al*., 1997), tal com han descrit estudis *in vitro* i *in vivo* per a la quercetina (Dragoni *et al*., 2006; Goldberg *et al*., 2003). Tanmateix, l'evidència *in vivo* per a altres polifenols, com la catequina, malvidina-3-glucòsid, l'àcid cafeic o la formació d'àcid 4-*O*-metilgàlic, ha

demostrat una biodisponibilitat similar després del consum de vi negre o vi negre desalcoholitzat (Forester i Waterhouse, 2009; Caccetta *et al*., 2000), tot i que s'ha observat una tendència per a la catequina urinària amb un augment d'un 20% després del consum de vi negre (Forester i Waterhouse, 2009). Per al cas concret del resveratrol, es van obtenir valors farmacocinètics similars després del consum de vi i de most (Ortuño *et al*., 2010). L'alcohol tampoc va millorar la biodisponibilitat del resveratrol en un altre estudi en el que es va administrar dissolt en vi blanc, most o homogeneïtzat de verdures (Goldberg *et al*., 2003), ni en l'estudi de la Porte i col·laboradors, en el que es va administrar conjuntament amb la quercetina (la Porte *et al*., 2010). Els resultats de l'estudi 2 aporten més evidència en aquest sentit, demostrant que l'alcohol del vi no afecta la biodisponibilitat del resveratrol (Rotches-Ribalta *et al*., 2012b), de manera que un vi desalcoholitzat podria ser una manera de consumir un producte, que sí podria ser considerat funcional i que, si els estudis científics finalment ho corroboren, podria aportar un benefici addicional a la salut humana.

Pel què fa a l'efecte de la matriu que constitueix el vi i el producte nutracèutic administrats en el primer disseny d'estudi (Rotches-Ribalta *et al*., 2012a), cal destacar que no s'han obtingut diferències significatives globals, considerant la suma total dels metabòlits excretats, entre aquests dos tipus de productes. Tenint en compte als metabòlits de forma individual s'ha observat un augment dels metabòlits microbians després del consum dels comprimits. De fet, aquesta forma de presentació requereix més temps per a dissoldre's *in vivo,* deixant als components d'aquest més temps en l'intestí on la microbiota els metabolitzarà en major proporció. Així doncs, es podria dir que els productes nutracèutics a base d'extracte de raïm podrien ser una font alimentaria de resveratrol, però cal tenir en compte que, en aquest estudi, s'han administrat 15 comprimits per tal d'igualar la quantitat del resveratrol ingerida en una dosi moderada de vi negre en homes (3 copes). Per la qual cosa, la quantitat de resveratrol en aquests comprimits és molt baixa respecte a la quantitat de resveratrol en una copa de vi. És per això que alguns estudis es focalitzen en obtenir productes naturals amb quantitats superiors de resveratrol i piceid o bé, en millorar la forma d'alliberació d'aquests per tal d'augmentar la seva biodisponibilitat facilitant la seva

posterior activitat biològica. De totes maneres, encara es necessita més informació pel què fa a l'efecte que puguin aportar els diferents metabòlits per al benefici de la salut humana.

Amb els resultats derivats dels tres estudis presentats i, comptant amb l'àmplia literatura prèvia sobre la biodisponibilitat i el metabolisme del resveratrol recollida a la part introductòria, es pot afirmar que cada vegada es disposa de més informació en aquest context, però encara cal més evidència científica per tal de clarificar els efectes beneficiosos del consum de resveratrol i de productes derivats del raïm. Estudis com els que recull la present Tesi Doctoral, en humans i després del consum de dosis moderades de resveratrol en aliments o productes funcionals, són els que recomana el comitè organitzador del "*2nd International Scientific Conference on Resveratrol and Health*" (http://www.resveratrol2012.eu/) amb l'objectiu d'aportar, finalment, l'evidència científica necessària per a poder fer recomanacions per al consum d'aquesta molècula.

CONCLUSIONS

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Els resultats obtinguts en els diferents treballs que constitueixen la present Tesi Doctoral deriven en les següents conclusions:

- S'ha desenvolupat i validat una nova metodologia per a l'anàlisi del perfil metabòlic del resveratrol en mostres biològiques per espectrometria de masses. Els canvis implementats han suposat una millora dels paràmetres de validació i una major resolució cromatogràfica pels metabòlits sulfats i disulfats. Això ha permès la identificació conjunta del perfil metabòlic del resveratrol més complet descrit en la literatura científica, constituït per 21 compostos que engloben als metabòlits de fase II del resveratrol, del piceid i dels derivats de l'acció de la microbiota intestinal.

- La ràtio de quantificació dels metabòlits conjugats del resveratrol expressats com a equivalents de l'aglicona, respecte a la quantificació amb el corresponent estàndard pur, analitzats per espectrometria de masses, ha estat 8 vegades superior en el cas dels metabòlits sulfatats de resveratrol i d'entre 0,7 i 1,2 en el cas dels metabòlits glucuronidats.

- L'estudi cinètic ha mostrat que el piceid, en la seva forma intacta, té un temps de màxima concentració (t_{max}) en plasma de 1 hora després del consum de vi negre o comprimits d'extracte de raïm, mentre que els metabòlits de fase II del resveratrol assoleixen les màximes concentracions entre les 2 i 7h després del consum d'aquests productes. S'ha observat una formació màxima més lenta dels metabòlits derivats de l'acció microbiana (t_{max} = 8h), per als quals s'ha descrit temps mitjos de residencia de fins a 12h.

- S'han observat diferencies estadísticament significatives per alguns metabòlits quan es compara el consum puntual de vi negre i comprimits d'extracte de raïm. Les concentracions màximes en plasma (C_{max}) pels *cis*-resveratrol-3 i 4-*O*-glucurònids van ser superiors després del consum de vi negre i les concentracions urinàries pels *trans*-resveratrol-3-*O*-glucurònid i *trans*-resveratrol-3-*O*-sulfat van ser superiors després del consum de comprimits d'extracte de raïm. Aquestes diferències poden ser degudes a la diversa proporció dels isòmers *trans* i *cis* del resveratrol i piceid de les formes administrades.

- L'excreció urinària global del perfil metabòlic del resveratrol no es veu afectada per la matriu que constitueixen els comprimits d'extracte de raïm en comparació amb la del vi negre. Tot i això, s'ha observat un augment significatiu, fins a 3 vegades superior, en l'excreció urinària dels metabòlits microbians entre les 12 i 24h després del consum dels comprimits d'extracte de raïm, la formulació del qual sembla que podria retardar l'absorció del resveratrol, augmentant el temps de permanència al colon que afavoriria el metabolisme de la microbiota.

- L'alcohol no afecta la biodisponibilitat i el metabolisme del resveratrol considerant el perfil metabòlic excretat.

- La variabilitat interindividual observada en els resultats del perfil metabòlic del resveratrol obtingut després del consum de vi, de productes funcionals i nutracèutics és un factor important a considerar a l'hora de dur a terme estudis de biodisponibilitat.

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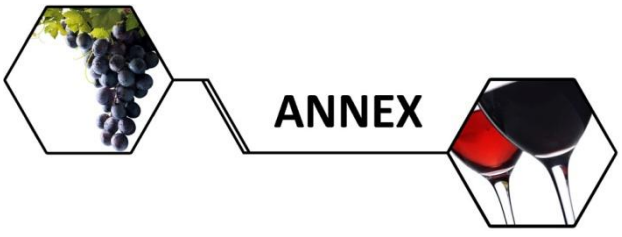
ANNEX

ANNEX

ALTRES PUBLICACIONS EN REVISTES

Publicació I: Chiva-Blanch G, Urpi-Sarda M, Rotchés-Ribalta M, Zamora-Ros R, Llorach R, Lamuela-Raventós RM, Estruch R, Andrés-Lacueva C. Determination of resveratrol and piceid in beer matrices by solid-phase extraction and liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A*, 2011; 1218(5):698-705.

La quantitat de resveratrol i piceid presents en diferents tipus de cerveses s'ha determinat en aquest treball mitjançant un nou mètode per SPE i LC-MS/MS, validat en matriu de cervesa. El desenvolupament d'un nou mètode per a l'anàlisi de resveratrol en aliments, va permetre conèixer millor a aquest compost, de cara a la validació de la metodologia optimitzada en l'estudi 2.

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Determination of resveratrol and piceid in beer matrices by solid-phase extraction and liquid chromatography–tandem mass spectrometry

Gemma Chiva-Blanch[a,b], Mireia Urpi-Sarda[a,c], Maria Rotchés-Ribalta[a,c], Raul Zamora-Ros[a,1], Rafael Llorach[a,c], Rosa Maria Lamuela-Raventós[a,d], Ramon Estruch[b,d], Cristina Andrés-Lacueva[a,c,*]

[a] Nutrition and Food Science Department, XaRTA, INSA, Pharmacy Faculty, University of Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain
[b] Department of Internal Medicine, Hospital Clinic, Institut d'Investigació Biomèdica August Pi i Sunyer (IDIBAPS), University of Barcelona, Villarroel 170, 08036 Barcelona, Spain
[c] INGENIO-CONSOLIDER Program, Fun-c-food CSD2007-063, Ministry of Science and Innovation, Barcelona, Spain
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ABSTRACT

Beer is one of the most commonly consumed undistilled alcoholic beverages in many countries. In recent studies, the stilbenes resveratrol and piceid have been found in some hop varieties which are used in the production of beer. Therefore, they could be transferred to beer. The aim of the present work was to validate a method to study the potential content of *trans*- and *cis*-resveratrol and piceid in 110 commercial beers from around the world. The resveratrol and piceid contents of 110 beers were analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) after a solid-phase extraction (SPE) using optimized and validated procedures for the beer matrix. The beer matrix effect was also studied. Stilbenes were found in quantifiable amounts in 92 beers, while concentrations below the limit of quantification (LOQ) were found in 18 beers. Resveratrol was found in the range of 1.34–77.0 μg/L in 79% of the beers analyzed, and piceid was found in the range of 1.80–27.3 μg/L in only 33% of them. The mean of total resveratrol in all the beers was 14.7 ± 20.5 μg/L. The content of resveratrol has been compared with other resveratrol containing foods. A serving of beer contains similar amounts of stilbenes as berries, less than chocolate and grape products but more than pistachios, peanuts or tomatoes. Overall, beer is one of the products with the lowest levels of total resveratrol (μg/L), and despite its high consumption it should not be considered as a representative source of resveratrol.

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1. Introduction

Resveratrol (3,5,4′-trihydroxystilbene) is a phenolic *phytoalexin* with potential preventive activity in several human diseases [1–6]. The described health effects depend on the ingested amount and bioavailability of these compounds. The presence of *trans*-resveratrol, *trans*-piceid (the resveratrol glucoside) and their respective *cis*-isomers in the human diet is limited. The major sources of resveratrol include grapes and grape products such as wines and grape juice [7]. Although it has been found in other foods such as peanuts, pistachios and some berries, their total resveratrol levels are from 10 to 100-times less than those in grape products [7]. Recently, it has also been found at low levels in the skin of some kinds of tomatoes [8] and in chocolate products [9].

Beer is one of the most commonly consumed undistilled alcoholic beverages in many countries. It is a complex mixture of bioactive substances including carbohydrates, amino acids, minerals, vitamins and phenolic compounds [10]. The majority of phenolic compounds in beer are non-tannic and non-flavonoid compounds (98% of total phenolic compounds), such as phenolic acids [11,12]. Other minor phenols found in beer are flavonols, catechins, procyanidins, tannins and chalcones [13,14]. The content of polyphenols in beer is largely influenced by the genetic factors of its raw materials and therefore by the environmental conditions in which they grow, and also by technological brewing factors [13,15]. Hops are used in the brewing industry to add flavor and bitterness to beer [16]. Although it has been observed that the nature of the harvest year can have a strong influence [17–19], *trans*- and *cis*-piceid have been found in different hop cultivars and in hop pellets in concentrations ranging from 0.5 to 11.7 mg/kg, up to 2 mg/kg of *trans*-resveratrol [20], and *cis*-resveratrol has been found up to

Abbreviations: SPE, solid-phase extraction; LC–ESI-MS/MS, liquid chromatography–electrospray ionization-tandem mass spectrometry; MRM, multiple reaction monitoring; LOD, limit of detection; LOQ, limit of quantification; MF, matrix factor; PE, process efficiency.

* Corresponding author at: Nutrition and Food Science Department, XaRTA, INSA, Pharmacy Faculty, University of Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain. Tel.: +34 93 4034840; fax: +34 93 4035931.
E-mail address: candres@ub.edu (C. Andrés-Lacueva).
[1] Present address: Unit of Epidemiology, Catalan Institute of Oncology, Institute of Biomedical Research of Bellvitge, L'Hospitalet de Llobregat, Spain.

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0.3 mg/kg in hop pellets [21] and up to 1.2 mg/kg in hop cones [22]. Moreover, about 20–30% of beer polyphenols originate from hops, and 70–80% from malt [10,20], although hops are added in 100-times lesser amounts than malt [23,24]. Therefore, a low content of stilbenes from the hops could be expected to be found in the final beer product [18]. Recently, low amounts of *trans*-resveratrol and *trans*-piceid (5 and 15 μg/L, respectively) have been found in four and five regular beers, respectively [25]. Other authors, after analyzing only two beers by high performance liquid chromatography (HPLC-UV), with detection at 280 nm, found up to 200-times higher concentrations of resveratrol (ranging between 0.3 mg/L and 3 mg/L) [26], however neither the *trans*- or *cis*-forms nor the piceid contents were specified. Therefore, very sensitive, selective and validated analytical methods are necessary to strengthen scientific evidence of the presence of *trans*- and *cis*-resveratrol and piceid in beers. Added to this, when performing MS analysis of food components, a large matrix effect can be observed, which leads to a diminution in the signal intensity of the analytes and the sensitivity of the method. The matrix effect during the validation of analytical methods may be best examined by comparing the response of an analyte at any given concentration spiked into the target matrix, to the response of the same analyte present in the "neat" mobile phase [27–29].

The aim of this study was to validate an analytical method for beer matrix and to study the content of *trans*- and *cis*-resveratrol and piceid in 110 beers from around the world, including alcohol-free, lager, ale, weissbier, stout, and abbey beers, and compare them with other dietary sources of resveratrol, like red wine or grape products and other foods with stilbenes.

2. Materials and methods

2.1. Standards and reagents

All samples and standards were handled avoiding exposure to light. Standards of *trans*-resveratrol (99% purity), *trans*-3,4′,5-trihydroxystilbene-3-β-D-glucopyranoside (*trans*-piceid) (97% purity) and ethyl gallate were purchased from Sigma–Aldrich–Fluka (St. Louis, MO), *cis*-resveratrol (97% purity) from Toronto Research Chemicals Inc (Toronto, ON, Canada), and taxifolin (>90% purity) from Extrasynthèse (Genay, France). Methanol, acetone, glacial acetic acid, ethyl acetate and acetonitrile of HPLC grade were purchased from Scharlab (Barcelona, Spain). Ultrapure water (Milli-Q) was obtained from Millipore system.

2.2. Samples

A total of 110 international commercial beers were analyzed (Table 1): 52 lagers, 20 ales, 15 abbey beers, 11 weissbiers, 7 stouts and 5 alcohol-free beers. The alcohol content ranged between less than 0.05 and 14% (v/v). All beers were purchased from local commercial markets. Some of the beers selected in this study are the most widely consumed in Spain (19%), while the others are a variety of beers consumed in Europe (66%) and worldwide (15%).

2.3. Sample preparation

Prior to the analysis, all beers were sonicated for 4 min for degasification. All experiments were performed on ice, avoiding light exposure, and all reagents were maintained in an N_2 atmosphere to avoid the oxidation of phenolic compounds. All beers were analyzed immediately after being opened.

2.4. Quality parameters of the method

To obtain the maximum detectivity and sensitivity in the analysis of resveratrol in beer by liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS), sample extraction was optimized, and the quality of the method and the matrix effect were evaluated. Optimization of sample extraction was carried out through the analysis of different parameters, including beer volume and pre-cleaning of samples as recommended by Jerkovic et al. [25]. Different volumes of samples (5 and 1 mL) were considered for loading onto HLB® cartridges (30 mg; 30 μm particle size and 80 Å pore size) (Waters). The pre-cleaning of beers before the solid-phase extraction (SPE) consisted of cleaning beers with toluene (1:1, v/v) followed by a double extraction with cyclohexane (1:1, v/v) as described previously by Jerkovic et al. [25].

After the optimization of the sample extraction, the method was evaluated for selectivity, detectivity, sensitivity, linearity, recovery, accuracy and precision, according to the Food and Drug Administration (FDA) acceptance criteria [30]. Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. This was assessed by analyzing blank beer samples. A blank beer sample was obtained after applying the SPE analysis procedure to 1 mL of blank beer (beer number 57, Table 1). The selected blank beer for the evaluation of the method was a lager beer because it is the most widely consumed kind of beer worldwide and more common on the market. The detectivity of the method was evaluated by determining the limit of detection (LOD) and the limit of quantification (LOQ). The LOD was determined as the concentration of analytes with a signal-to-noise ratio of at least 3 and the LOQ was the lowest standard with a signal-to-noise ratio of at least 10. Sensitivity was expressed as the slope of the analytical curve. Linearity was evaluated by spiking blank beer matrix with known concentrations of analytes at 6 concentration levels (4–100 μg/L for *trans*-resveratrol and 2–50 μg/L for *cis*-resveratrol and *trans*-piceid). Recovery was calculated as the ratio of the mean peak area of the analytes spiked before extraction to the mean peak area of the analytes spiked post-extraction multiplied by 100 in a six-point calibration curve of beer matrix ($n = 3$).

The precision and accuracy of the method were evaluated using three different concentrations [low (near the LOQ), medium and high] within the linear range of the calibration curve of the analytes in the beer matrix ($n = 8$). Accuracy and precision were calculated as the percentage and relative standard deviation, respectively, of the ratio of the mean calculated concentration and the true value of the known added concentration in blank beer samples ($n = 8$) for each concentration.

The matrix effect of the beers was also evaluated by calculating the matrix factor (MF or suppression coefficient), process efficiency (PE) [28] and the variations in the sensitivity of the method [29]. The MF was expressed as the ratio of the mean peak area of the analytes spiked after the SPE procedure in blank beer matrix to the mean peak area of the same analyte standards in aqueous matrix without SPE procedure multiplied by 100. PE was calculated as the ratio of the mean peak area of the analytes spiked before the SPE procedure to the mean peak area of the same analytes standards in aqueous matrix without SPE procedure multiplied by 100. Differences in the sensitivity of the method within the two matrices (beer and water) were expressed as the ratio of the slope of the analytical curve in beer matrix (spiked after the SPE procedure) to the slope of the analytical curve in aqueous matrix without SPE procedure multiplied by 100.

Short and long term stabilities of standards were previously evaluated by our group [31].

Table 1
Type, alcoholic degree (A.D. (%, v/v)), manufacturing country and concentrations of *trans*- and *cis*-resveratrol (μg/L) and *trans*- and *cis*-piceid (μg/L) of the 110 analyzed beers.

ID	Type	A.D. (%, v/v)	Country	*trans*-Resveratrol (μg/L)	*cis*-Resveratrol (μg/L)	*trans*-Piceid (μg/L)	*cis*-Piceid (μg/L)[a]
1	Lager	4.4	Czech Republic	n.d.	4.39	n.q.	6.48
2	Lager	5	Czech Republic	n.d.	n.q.	n.q.	n.q.
3	Lager	4.5	Mexico	n.d.	n.q.	n.d.	n.d.
4	Stout	8	Ireland	11.94	10.86	n.q.	n.d.
5	Stout	5	Holland	7.79	16.12	n.d.	n.q.
6	Stout	5.8	Germany	n.d.	4.84	n.q.	4.53
7	Lager	4.8	Brazil	n.q.	n.q.	n.q.	n.q.
8	Ale	8.4	Belgium	n.q.	n.q.	n.q.	n.q.
9	Lager	4.9	Germany	n.d.	3.45	n.q.	1.80
10	Alcohol-free	<0.05	Italy	4.09	4.74	n.q.	n.q.
11	Lager	4.7	Mexico	11.94	n.d.	n.q.	n.d.
12	Lager	4.8	Germany	n.d.	2.81	n.q.	n.q.
13	Lager	5	Holland	n.q.	n.d.	n.q.	n.q.
14	Stout	4.2	Ireland	10.56	n.d.	n.d.	n.q.
15	Alcohol-free	<0.05	Germany	5.67	n.d.	n.q.	n.q.
16	Alcohol-free	<0.05	Germany	n.d.	n.d.	n.d.	n.d.
17	Lager	<0.5	Germany	n.d.	5.28	n.q.	n.q.
18	Stout	5	Italy	30.86	7.13	n.q.	n.q.
19	Lager	5.1	Italy	n.d.	4.59	n.q.	n.q.
20	Lager	4.7	Italy	4.53	2.29	n.q.	n.q.
21	Lager	5.6	Poland	n.q.	n.q.	n.q.	n.q.
22	Lager	4.8	Germany	4.73	n.d.	n.q.	n.q.
23	Lager	4.6	Italy	7.36	2.58	n.q.	n.q.
24	Ale	4.5	Italy	32.69	17.83	n.d.	2.62
25	Ale	6.5	Italy	32.72	n.q.	n.d.	n.q.
26	Abbey beer	10.5	Belgium	23.18	n.d.	3.16	9.73
27	Lager	4.5	Mexico	n.d.	n.q.	n.q.	n.d.
28	Lager	3.5	Spain	n.q.	n.q.	n.q.	2.85
29	Lager	6.2	Spain	n.d.	1.91	n.q.	n.q.
30	Stout	4.5	England	11.61	3.43	n.q.	3.31
31	Weissbier	5.4	Germany	10.68	2.87	n.d.	n.d.
32	Weissbier	5.5	Germany	n.q.	n.q.	n.d.	n.d.
33	Abbey beer	10	Holland	n.q.	2.92	n.q.	n.q.
34	Ale	8.5	Belgium	n.q.	n.q.	n.d.	n.d.
35	Weissbier	5.3	Germany	n.d.	1.99	n.d.	n.q.
36	Weissbier	8.5	Belgium	n.d.	1.77	n.q.	n.q.
37	Ale	4.7	England	n.q.	1.72	n.q.	n.q.
38	Ale	4.5	England	12.47	n.q.	n.q.	3.51
39	Lager	5.1	Uruguay	n.d.	2.21	n.q.	n.q.
40	Lager	14	Austria	10.22	n.d.	n.d.	2.06
41	Lager	4.7	USA	8.56	n.q.	n.q.	n.q.
42	Abbey beer	8.2	Belgium	9.60	1.34	n.d.	n.q.
43	Stout	4.3	Spain	10.36	2.32	n.d.	n.q.
44	Lager	4.9	Argentina	4.42	1.82	n.d.	n.q.
45	Ale	4.6	Spain	17.78	2.68	n.d.	n.q.
46	Lager	4.8	Italy	n.d.	n.q.	n.q.	n.q.
47	Weissbier	12	France	6.25	6.07	n.q.	4.91
48	Alcohol-free	<0.05	Spain	4.60	n.q.	n.d.	3.13
49	Lager	4.5	Mexico	n.d.	n.q.	n.q.	n.q.
50	Lager	5.3	Mexico	9.66	2.56	n.d.	n.q.
51	Weissbier	5.2	Germany	13.93	n.d.	n.q.	n.q.
52	Lager	5	England	n.q.	n.d.	n.d.	n.q.
53	Lager	4	Colombia	n.d.	1.49	n.q.	n.q.
54	Ale	10	Belgium	6.12	n.q.	n.q.	n.q.
55	Abbey beer	7.3	Belgium	n.d.	3.23	n.q.	2.35
56	Lager	5	UK/Japan	n.d.	n.d.	n.q.	n.q.
57	Lager	4.8	UK/India	n.d.	n.d.	n.d.	n.q.
58	Lager	5	UK/India	n.d.	5.38	n.q.	2.56
59	Lager	5	Germany	n.q.	4.43	n.d.	n.q.
60	Weissbier	8.5	Belgium	18.00	2.70	n.q.	n.q.
61	Lager	5.2	Germany	n.d.	2.04	n.d.	n.q.
62	Weissbier	9	Belgium	14.88	5.74	3.08	24.24
63	Abbey beer	6.5	Holland	6.42	3.41	n.q.	2.15
64	Abbey beer	8	Holland	18.15	8.09	n.q.	5.38
65	Weissbier	5	Germany	n.d.	n.d.	n.d.	n.q.
66	Weissbier	8	Belgium	27.46	5.84	n.q.	n.q.
67	Abbey beer	6.6	Belgium	n.d.	n.q.	n.q.	n.d.
68	Abbey beer	5.5	Holland	n.d.	2.54	n.q.	3.07
69	Lager	4	Colombia	18.97	6.17	n.q.	n.q.
70	Abbey beer	7	Holland	n.d.	5.52	n.q.	n.q.
71	Weissbier	4.9	Belgium	38.74	8.70	n.d.	n.q.
72	Abbey beer	8.2	Belgium	10.06	5.86	n.q.	n.d.
73	Ale	8	Belgium	n.d.	n.d.	1.80	2.68
74	Ale	5.2	England	19.96	3.02	n.q.	4.83
75	Ale	5.1	Spain	33.33	8.33	n.d.	n.d.
76	Lager	3.5	Spain	42.19	5.91	n.d.	1.81

Table 1 (*Continued*)

ID	Type	A.D. (%, v/v)	Country	*trans*-Resveratrol (μg/L)	*cis*-Resveratrol (μg/L)	*trans*-Piceid (μg/L)	*cis*-Piceid (μg/L)[a]
77	Ale	7	Spain	26.21	5.86	n.d.	n.q.
78	Ale	5.4	Spain	32.47	5.66	n.d.	n.q.
79	Lager	4.7	Jamaica	6.65	n.q.	n.q.	n.q.
80	Lager	4.8	Germany	n.q.	n.q.	n.q.	1.89
81	Lager	5	Belgium/Australia	6.00	3.45	n.q.	n.q.
82	Lager	5	Holland	n.d.	n.q.	n.q.	n.q.
83	Ale	3.8	Spain	26.91	22.65	n.d.	n.q.
84	Ale	5.4	England	10.00	1.68	n.d.	3.69
85	Lager	6.4	Spain	13.82	7.40	n.d.	6.55
86	Lager	4.8	Italy	n.d.	2.00	n.d.	n.q.
87	Lager	5.2	Lithuania	n.q.	1.46	n.q.	1.80
88	Lager	4.8	Germany	n.d.	5.21	n.q.	n.q.
89	Ale	5.5	Spain	11.26	10.77	n.d.	n.q.
90	Ale	4.5	England	22.01	n.q.	n.q.	4.47
91	Lager	6.5	Spain	26.82	3.65	n.q.	1.84
92	Lager	4.9	Germany	n.q.	2.88	n.q.	n.q.
93	Alcohol-free	<0.05	Spain	6.03	1.34	n.q.	n.q.
94	Lager	5	Germany/Russia	4.63	3.68	n.q.	2.81
95	Lager	4.7	Italy	n.q.	2.61	n.q.	2.46
96	Abbey beer	6.5	Spain	n.d.	8.95	n.q.	4.77
97	Lager	7.2	Spain	n.d.	2.64	n.q.	n.q.
98	Lager	5.4	Spain	5.39	3.26	n.q.	3.77
99	Abbey beer	7	Belgium	54.52	n.q.	n.q.	4.54
100	Lager	4.5	Spain	n.d.	2.02	n.q.	n.d.
101	Lager	7.2	Spain	4.65	n.q.	n.d.	n.q.
102	Lager	4.8	Spain	n.d.	4.23	n.d.	n.q.
103	Lager	4.8	Spain	n.d.	3.04	n.d.	n.d.
104	Abbey beer	7.5	Belgium	66.74	10.31	n.d.	4.17
105	Abbey beer	9.2	Belgium	7.29	n.d.	n.q.	8.15
106	Ale	11	Belgium	13.97	3.98	2.30	6.66
107	Abbey beer	9.5	Belgium	22.61	1.37	9.31	16.88
108	Ale	8.5	Belgium	23.77	n.q.	1.99	4.47
109	Ale	10.5	Belgium	12.14	n.q.	n.q.	3.65
110	Lager	5.4	Spain	n.d.	n.q.	n.d.	1.80

n.d.: not detected; n.q.: not quantified.

[a] Expressed as *trans*-piceid equivalents.

2.5. Determination of resveratrol and piceid in beer by LC–MS/MS

Resveratrol and piceid analyses were carried out by LC–ESI-MS/MS after an SPE, based on the Urpi-Sarda et al. [31,32] method and optimized for beer samples.

2.5.1. Extraction procedure

Samples (1 mL of each presonicated beer, diluted with ultrapure water to reduce the alcohol percentage to below 5%) with the internal standard (ethyl gallate) all maintained on ice, were loaded onto a Waters Oasis® HLB® 96-well plate that had been preconditioned with 1 mL of methanol and equilibrated with 1 mL of 2 mol/L acetic acid in water. Samples were washed with 1 mL of 2 mol/L acetic acid in water and 1 mL of 2 mol/L acetic acid in water/methanol (85/15, v/v). Elution was achieved with 0.5 mL of 1 mol/L acetic acid in methanol and 2 × 0.75 mL of 1 mol/L acetic acid in ethyl acetate. The eluate was evaporated to dryness under a gentle stream of N_2. The residue was reconstituted with 100 μL of initial mobile phase, with 1.64 μmol/L of taxifolin as an additional external standard. Ethyl gallate was used as the internal standard (mean recovery: 99%, CV = 10%) and taxifolin was used as an additional external standard to assess the performance of the mass spectrometer. Both compounds are absent in beers.

2.5.2. LC–MS/MS analyses

LC analyses were performed using an Agilent 1100 system equipped with a quaternary pump and a refrigerated plate autosampler (Waldbronn, Germany). An Applied Biosystems API 3000 triple quadrupole mass spectrometer, equipped with a turbo ion spray source ionizing in the negative mode, was used to obtain the mass spectrometry data.

A Phenomenex Luna C_{18} column, 50 mm × 2.0 mm i.d., 5 μm (Torrance, CA) maintained at 40 °C was used for chromatographic separation. The injection volume was 15 μL, and the flow rate was 500 μL/min. Gradient elution was carried out with 0.5 mL/L acetic acid as mobile phase A and 700 mL/L acetone, 300 mL/L acetonitrile with 0.4 mL/L acetic acid as mobile phase B. A non-linear gradient profile was applied as follows: 0–0.5 min, 10–15%B; 0.5–5 min, 15–100%B and 5–5.6 min, 100%B. The column was re-equilibrated for 6 min, to return to 10%B.

The MS and MS/MS parameters were as previously described [31]. Briefly, the following parameters were used: capillary voltage–3500 V, nebulizer gas (N_2) 10 (arbitrary units), curtain gas (N_2) 12 (arbitrary units), collision gas (N_2) 6 (arbitrary units), focusing potential −200 V, entrance potential −10 V, declustering potential −50 V, drying gas (N_2) heated to 400 °C and introduced at a flow rate of 6000 cm^3/min. The collision energy was −25 V for resveratrol, piceid and taxifolin, and −30 V for ethyl gallate.

2.5.3. Quantification of analytes

For the quantification of *trans*- and *cis*-resveratrol and *trans*- and *cis*-piceid in beer samples, the multiple reaction monitoring (MRM) mode was used with a dwell time of 300 ms, monitoring four transitions for each analysis: *trans*- and *cis*-resveratrol (227/185), *trans*- and *cis*-piceid (389/227), ethyl gallate (197/169) and taxifolin (303/285). *trans*-Resveratrol was quantified using a six-point calibration curve determined by weighted ($1/x^2$) linear regression between 4 and 100 μg/L in the beer matrix and *cis*-resveratrol and *trans*-piceid between 2 and 50 μg/L in the beer matrix ($n = 3$ for each calibration curve). Ethyl gallate was used for quantification purposes. *cis*-Piceid was expressed as *trans*-piceid equivalents as no commercial standard was available. To identify *cis*-piceid, *trans*-

Table 2
LOD, LOQ and between-day ($n = 8$) precision and accuracy data obtained from the LC–MS/MS of *trans*-resveratrol, *cis*-resveratrol and *trans*-piceid in the beer matrix after solid-phase extraction.

Analyte	LOD (μg/L)	LOQ (μg/L)	Added concentration (μg/L)	Calculated concentration[a] (mean, μg/L)	Accuracy (%)	Precision (RSD[b], %)
trans-Resveratrol	1.10	3.68	4	4.08	102	2.5
			50	48.95	98	8.5
			100	99.43	99	1.0
cis-Resveratrol	0.41	1.34	2	2.11	105	13.6
			10	10.64	106	6.1
			50	49.9	100	8.7
trans-Piceid	0.54	1.80	2	1.85	92	15.1
			10	9.86	99	8.2
			50	49.93	100	0.8

[a] Values are the mean of $n = 8$.
[b] Relative standard deviation.

piceid isomerated by light exposure for 10 min on ice was used [33].

3. Results and discussion

3.1. Quality parameters of the method

Optimization of the sample extraction procedure and evaluation of the methodology were performed.

3.1.1. Sample extraction optimization

The main objective of the sample extraction optimization was to reach the highest detectivity and sensitivity while minimizing the matrix effect in LC–MS/MS. In the first step, we compared the recovery of resveratrol and piceid from the spiked beer matrix (1 μg/mL of final concentration) using different sample volumes (5 and 1 mL) with or without the pre-cleaning procedure ($n = 4$). The recovery values of *trans*-resveratrol, *cis*-resveratrol and *trans*-piceid from the pre-cleaned samples increased by 86%, 157% and 112% respectively, compared to the non-pre-cleaned ones when 5 mL were used, and this is in accordance with Jerkovic et al. [25]. However, when we compared 1 mL of a sample with and without the pre-cleaning procedure, no differences were observed for the recovery of the compounds. These results suggested a higher matrix effect when a higher volume was considered, affecting the analyte ionization and obtaining best signal-to-noise ratio for the analytes extracted with lesser volumes. When we compared different volumes of pre-cleaned samples (1 and 5 mL), the recovery values for *trans*-resveratrol, *cis*-resveratrol and *trans*-piceid after loading 1 mL were 2, 2.5 and 1.3-fold higher, respectively, than when 5 mL were considered. Again, the volume of the sample influenced the matrix effect and the resveratrol ionization. Therefore, as no differences were observed loading 1 mL of sample with and without pre-cleaning, and the recovery was higher than after loading 5 mL of pre-cleaned sample, 1 mL of beer without the pre-cleaning procedure was the selected volume used for the total resveratrol determination in the commercial beers and for the standard calibration curves.

3.1.2. Evaluation of the method

The method met the criteria of selectivity because no endogenous peaks were observed at the same retention time as the analytes in the blank beer samples. The LOD and LOQ of analytes are shown in Table 2, and sensitivity was 3.62×10^{-5}, 2.60×10^{-4} and 9.47×10^{-5} cpm L/μg for *trans*-resveratrol, *cis*-resveratrol and *trans*-piceid, respectively. The 6-point calibration concentrations ($n = 3$) in blank beer matrix determined by weighted ($1/x^2$) least-square regression analysis showed correlation coefficients for all analytes > 0.99. The calibration curves were linear over the concentration range studied. Recovery was evaluated comparing a 6-point calibration curve of analytes with and without the SPE procedure in the beer matrix ($n = 3$). *cis*-Resveratrol, *trans*-resveratrol and *trans*-piceid showed recovery values of 99%, 90% and 102%, respectively, after SPE in the beer matrix. The recovery for the internal standard ethyl gallate was also evaluated at the concentration used in the analysis (50 μg/L) ($n = 8$). Ethyl gallate showed a recovery of 99%. The precision and accuracy of the analytes in the beer matrix after

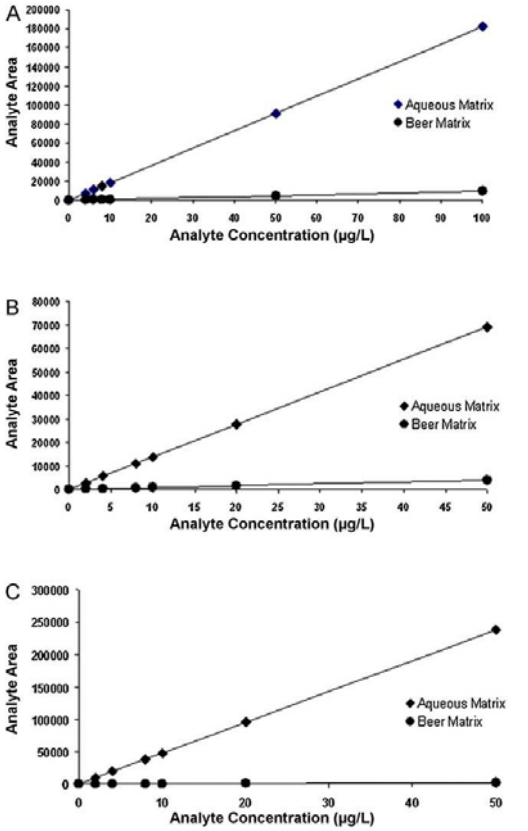

Fig. 1. Matrix effect of beer for: (A) *trans*-resveratrol; (B) *cis*-resveratrol; and (C) *trans*-piceid.

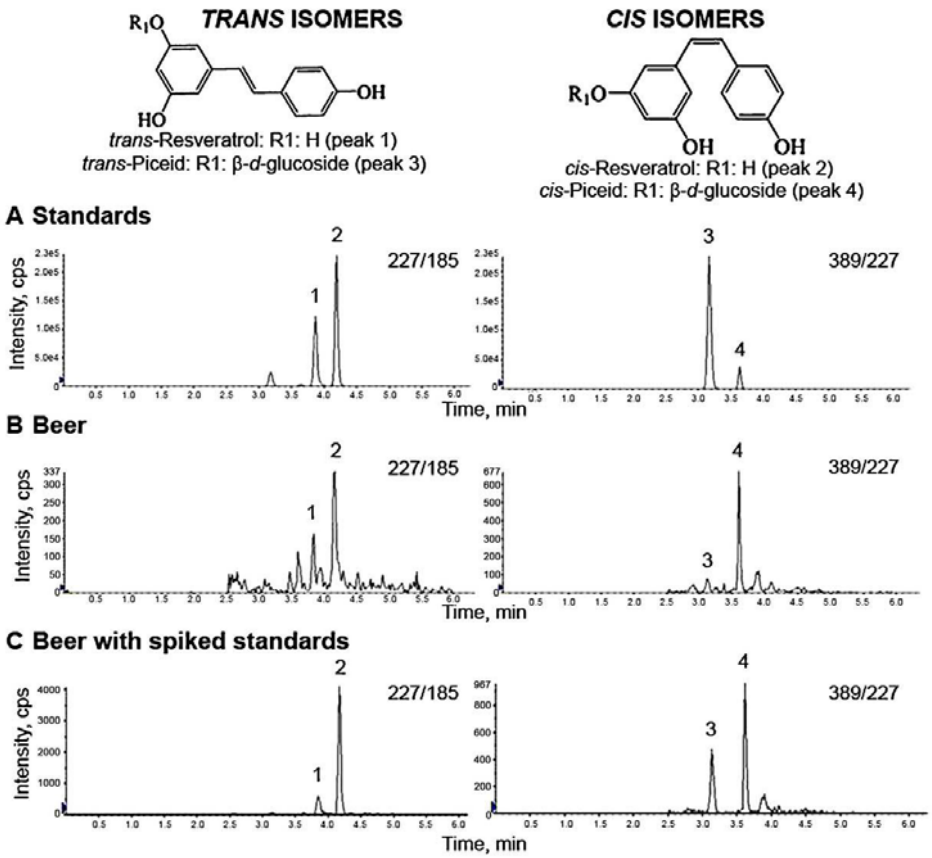

Fig. 2. Chemical structures of resveratrol and piceid in their *trans*- and *cis*-forms and multiple reaction monitoring (MRM) chromatograms for: (A) standards of *trans*-resveratrol (1), *cis*-resveratrol (2), *trans*-piceid (3) and *cis*-piceid (4); (B) beer 62 and (C) beer 62 spiked with standards.

SPE met the acceptance criteria of the FDA [30] and are shown in Table 2.

3.1.3. Evaluation of the matrix effect

To assess the strength of the matrix effect, 6-point calibration curves of analytes ($n = 3$) in the beer matrix and in an aqueous matrix (pure solvent) without the SPE procedure were compared. Briefly, blank beer was prepared with the full extraction procedure and standards were added *a posteriori* to this matrix to compare the differences of peak signal intensity of the analytes due to the matrix effect to calculate the MF, and *a priori* to calculate the PE. The MF for *trans*-resveratrol, *cis*-resveratrol and *trans*-piceid were 5.2%, 5.8% and 1.1% respectively. These matrix factors highlight a great suppression of the ionization of the analytes due to the matrix effect [28,29]. The PE of the method considers the MF and the recovery. The PE was 12.6%, 28.2% and 3.25% for *trans*-resveratrol, *cis*-resveratrol and *trans*-piceid, respectively. Taking into account that the recovery is >90% for all the analytes, the low PE value is attributable to the high MF. As shown in Fig. 1, standard curves in the beer matrix showed a decrease of the sensitivity of 95%, 94% and 99% for *trans*-resveratrol, *cis*-resveratrol and *trans*-piceid, respectively, when compared to the aqueous matrix. This great loss in the peak intensity signal highlights that calibration curves in an adequate matrix, in this case the beer matrix, are needed to avoid an underestimation of the analyte concentration in beer samples. This enormous matrix effect may also explain the differences in the enhancement of the peak intensity when loading 1 or 5 mL of pre-cleaned beer samples compared to non-pre-cleaned samples. To our knowledge, this is the first time that matrix effect of beer in the resveratrol analysis by LC–MS/MS has been highlighted and calculated.

The matrix effect (MF) for the internal standard ethyl gallate was also evaluated at the concentration used in the analysis (50 μg/L) ($n = 8$). Ethyl gallate in the beer matrix spiked after the SPE procedure showed a decrease of the peak intensity of 85% compared with the aqueous matrix without SPE procedure.

3.2. Determination of resveratrol and piceid in beers

The 110 commercial beers were quantified using MRM transitions of 227/185 for *trans*- and *cis*-resveratrol and 389/227 for *trans*- and *cis*-piceid in LC–MS/MS. In this study, *trans*- and *cis*-piceid showed a retention time of 3.15 and 3.62 min, respectively, and *trans*- and *cis*-resveratrol showed a retention time of 3.85 and 4.18 min, respectively (Fig. 2A). The confirmation of resveratrol and piceid in beer samples was based on their retention time and ion fragmentation in the MS/MS mode as compared with those of commercially available standards. Finally, to verify the identity of the

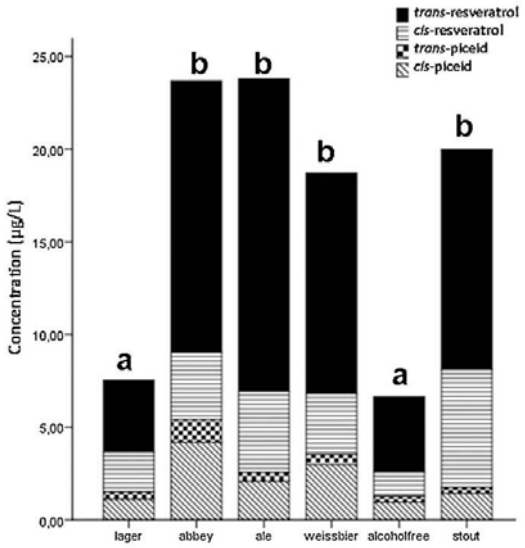

Fig. 3. Mean concentration (µg/L) of *trans*- and *cis*-resveratrol and piceid contents in different beer varieties. Bars with different letters are significantly different in total resveratrol content ($p < 0.05$).

peaks, beer samples (Fig. 2B) and spiked beer samples (Fig. 2C) were injected and compared, confirming the presence of *trans*- and *cis*-resveratrol and *trans*- and *cis*-piceid in beers.

Of the 110 analyzed beers, 79% of them contained free resveratrol (mainly in the *trans*-form), while only 33% of the beers analyzed contained piceid in quantifiable amounts. Table 1 shows that 59 beers contained *trans*-resveratrol between 3.68 and 66.74 µg/L, 69 beers contained *cis*-resveratrol in a range between 1.34 and 22.65 µg/L, 6 beers contained *trans*-piceid between 1.8 and 9.31 µg/L, and 38 beers contained *cis*-piceid between 1.80 and 24.24 µg/L. The beer with the greatest amount of stilbenes (beer number 104) contained 66.74 µg/L, 10.31 µg/L and 4.17 µg/L of *trans*- and *cis*-resveratrol and *cis*-piceid, respectively.

Jerkovic et al. [17,18,21,22] found *trans*-resveratrol (up to 1 mg/kg) and *cis*-resveratrol (up to 1.2 mg/kg) in significantly lower quantities than *cis*- and *trans*-piceid in hops (2–6 mg/kg and 4–9 mg/kg, respectively). In our analyses, resveratrol (mainly in its *trans*-form) has been found to be the most abundant stilbene in beer. This can be attributable to the fact that resveratrol (in the *trans*- and *cis*-form) can be partially regenerated by its glucoside, although piceid in beer remains more stable during the brewing process than resveratrol [26]. It could also be possible that hydrolysis of glycosides by yeast or bacterial β-glucosidase activity through beer fermentation may lead to piceid hydrolysis yielding free resveratrol [17,18]. In addition, an isomerase activity on phenols by the yeast during fermentation has been described previously by Jeandet et al. [34] and other authors [35]. As well as these factors, the amount of resveratrol and piceid extraction from hops to beer may depend on the commercial form of the hops [21,36], as well as on hop freshness [22]. These factors can explain the differences in the *trans*- and *cis*-amounts of resveratrol and piceid in hops and beers.

The mean concentration of total resveratrol distributed in *trans*- and *cis*-resveratrol and piceid in different kinds of beers is shown in Fig. 3. Abbey beers, ale, weissbier and stout beers contained significantly higher amounts of total resveratrol than lager and alcohol-free beers ($p < 0.05$, Mann–Whitney test). These differences in stilbene concentrations could be due mainly to the different hop varieties used, as well as maceration, fermentation and the hopping rate in the boiling kettle during the brewing process [18].

The mean of piceid and resveratrol in their *trans*- and *cis*-forms, as well as the total resveratrol expressed per serving and per liter or kg in beers and in other resveratrol-containing foods is shown in Table 3. On an equal volume basis, beer had ~580-fold lower levels of total resveratrol than red wine [7], ~60-fold lower levels than grape juice and ~50-fold lower levels than white wine [7]. This means that ~260 L of beer contains the equivalent amount of total resveratrol found in one glass of wine (150 mL). Nevertheless, because of their alcoholic content, they should be consumed in moderation.

In conclusion, a method to analyze resveratrol in beer matrix has been developed and evaluated and matrix effect of beer was determined. Total resveratrol was found in a range of 1.99–81.22 µg/L in 92 of the 110 commercial beers studied. *trans*-Resveratrol was the stilbene found in the highest levels and in the largest number of beers. Overall, beer contains only low levels of total resveratrol (µg/L), and despite its high consumption, it is not a representative source of dietary resveratrol.

3.3. Safety

We followed the general guidelines for working with organic solvents and acids. Universal precautions for the handling of chemicals were applied.

Table 3
Average of total resveratrol content (µg) per serving and per liter or kilogram of different food items.

Food	*trans*-Resveratrol (µg/L or µg/kg)	*cis*-Resveratrol (µg/L or µg/kg)	*trans*-Piceid (µg/L or µg/kg)	*cis*-Piceid (µg/L or µg/kg)	Total resveratrol (µg/L or kg)	Serving (g or mL)	Total resveratrol (µg/serving)	Reference
Red wine	1810	440	4950	1270	8470	150	1270	[7,9]
White wine	100	160	260	220	740	150	111	[7]
Grapes, not specified	1560	–	670	–	2230	100	223	[7]
Grape juice	100	tr	360	430	890	250	222	[7]
Peanuts, toasted	60	–	–	–	60	30	1.8	[7,9]
Pistachios, toasted	70	–	–	–	70	30	2.1	[7]
Berries, not specified	80	–	–	–	80	50	4.0	[7]
Tomatoes[a]	11	1.7	0.1	0.2	12.6	100	1.3	[8]
Chocolate, not specified[b]	692	–	2633	–	3325	10–40	88	[9]
Beer, not specified[c]	9	3.1	0.5	1.9	14.7	330	4.8	This work

tr, traces.
[a] Calculated from skin tomato values (5% of dry weight and 92% humidity) from MicroTom, Beafsteak, UglyRipe, Heirloom and PlumTom varieties [8].
[b] Chocolate included: cocoa powder, unsweetened, semi-sweet, dark and milk chocolate, and chocolate syrup [9]. Serving depends on chocolate product.
[c] Values <LOQ (traces) considered as the LOD value.

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Aquest treball presenta els efectes diferencials dels polifenols i alcohol del vi negre sobre l'expressió de molècules d'adhesió i citoquines inflamatòries relacionades amb l'aterosclerosi. És una de les publicacions amb els resultats de la part clínica obtinguts de l'estudi clínic d'intervenció presentat a la publicació 2. De fet, les dades d'excreció urinària dels metabòlits de fase II del resveratrol presentades en la publicació resultant de l'estudi 2 van servir com a mesura de compliment de la intervenció per a el treball presentat en aquesta publicació.

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Differential effects of polyphenols and alcohol of red wine on the expression of adhesion molecules and inflammatory cytokines related to atherosclerosis: a randomized clinical trial[1–3]

Gemma Chiva-Blanch, Mireia Urpi-Sarda, Rafael Llorach, Maria Rotches-Ribalta, Marisa Guillén, Rosa Casas, Sara Arranz, Palmira Valderas-Martinez, Olga Portoles, Dolores Corella, Francisco Tinahones, Rosa M Lamuela-Raventos, Cristina Andres-Lacueva, and Ramon Estruch

ABSTRACT

Background: Few clinical studies have focused on the alcohol-independent cardiovascular effects of the phenolic compounds of red wine (RW).

Objective: We aimed to evaluate the effects of ethanol and phenolic compounds of RW on the expression of inflammatory biomarkers related to atherosclerosis in subjects at high risk of cardiovascular disease.

Design: Sixty-seven high-risk, male volunteers were included in a randomized, crossover consumption trial. After a washout period, all subjects received RW (30 g alcohol/d), the equivalent amount of dealcoholized red wine (DRW), or gin (30 g alcohol/d) for 4 wk. Before and after each intervention period, 7 cellular and 18 serum inflammatory biomarkers were evaluated.

Results: Alcohol increased IL-10 and decreased macrophage-derived chemokine concentrations, whereas the phenolic compounds of RW decreased serum concentrations of intercellular adhesion molecule-1, E-selectin, and IL-6 and inhibited the expression of lymphocyte function-associated antigen 1 in T lymphocytes and macrophage-1 receptor, Sialil-Lewis X, and C-C chemokine receptor type 2 expression in monocytes. Both ethanol and phenolic compounds of RW downregulated serum concentrations of CD40 antigen, CD40 ligand, IL-16, monocyte chemotactic protein-1, and vascular cell adhesion molecule-1.

Conclusion: The results suggest that the phenolic content of RW may modulate leukocyte adhesion molecules, whereas both ethanol and polyphenols of RW may modulate soluble inflammatory mediators in high-risk patients. The trial was registered in the International Standard Randomized Controlled Trial Number Register at http://www.isrctn.org/ as ISRCTN88720134. *Am J Clin Nutr* 2012;95:326–34.

INTRODUCTION

CAD[4] is generally due to atherosclerosis and is the leading cause of morbimorbidity in developed countries. Atherogenesis is a multifactorial process that involves a combination of environmental, genetic, and metabolic components that act synergistically to induce oxidative stress and a chronic inflammatory state. Thus, atherosclerosis is considered a low-grade inflammatory disease in which the cell and endothelial expression of adhesion molecules and chemokines participate in the recruitment of circulating leukocytes to the vascular endothelium and further migration into subendothelial spaces (1), which leads to the formation of atherosclerotic lesions (2). In this process, chemokines, the secreted proteins that recruit specific cell types to inflammatory sites, have emerged as major contributors to vascular inflammation. Therefore, the study of cir-

[1] From the Department of Internal Medicine, Hospital Clínic, Institut d'Investigació Biomèdica August Pi i Sunyer (GC-B, MU-S, RC, SA, PV-M, and RE) and the Nutrition and Food Science Department, Xarxa de Referència de Teconolgia dels Aliments de la Generalitat de Catalunya, Instituto Nacional de Salud, Pharmacy Faculty (RL, MR-R, RML-R, and CA-L), University of Barcelona, Barcelona, Spain; the Centro de Investigación Biomédica en Red de Fisiopatologia de la Obesidad y la Nutrición, Instituto de Salud Carlos III, Madrid, Spain (GC-B, MU-S, RC, SA, PV-M, DC, FT, RML-R, and RE); the INGENIO-CONSOLIDER Program, Fun-c-food CSD2007-06, Barcelona, Spain (RL, MR-R, and CA-L); the Department of Preventive and Legal Medicine, School of Medicine, University of Valencia, Valencia, Spain (MG, OP, and DC); and the Endocrinology Department, Biomedical Research Laboratory, Hospital Virgen de la Victoria, Málaga, Spain (FT).

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[3] Address correspondence to R Estruch, Department of Internal Medicine, Hospital Clinic, Villarroel 170, 08036 Barcelona, Spain. E-mail: restruch@clinic.ub.es.

[4] Abbreviations used: CAD, coronary artery disease; CCR2, C-C chemokine receptor type 2; CD40a, CD40 antigen; CD40L, CD40 ligand; CRP, C-reactive protein; DRW, dealcoholized red wine; ICAM-1, intercellular adhesion molecule 1; LFA-1, lymphocyte function-associated antigen 1; Mac-1, macrophage-1 receptor; MCP, monocyte chemotactic protein; MDC, macrophage-derived chemokine; MIP-1α, macrophage inflammatory protein 1α; PBMC, peripheral blood mononuclear cell; RW, red wine; SLe^x, Sialil-Lewis X; VCAM-1, vascular cell adhesion molecule 1; VLA-4, very late activation antigen 4.

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culating chemokines may provide a greater understanding of the underlying pathophysiology of this disease (3).

Several epidemiologic studies have reported beneficial effects of moderate alcohol consumption on cardiovascular disease (4, 5). Other studies that differentiated the actions of different alcoholic beverages have observed that moderate consumption of RW has greater effects on lowering risk of CAD than do other beverages (6–9). RW contains alcohol and nonalcoholic compounds, mainly polyphenols such as anthocyanosides, catechins, proanthocyanidins, stilbenes, and other phenolic compounds (6). The cardiovascular effects of RW phenolic compounds have been well studied in vitro (10–12) and in animal models (13–16), but few human clinical trials have focused on the alcohol-independent effects of phenolic compounds of RW (17–20). Therefore, the differential cardiovascular effects of both components in RW are not well known.

In contrast, previous clinical trials in healthy men (21, 22) and women (23) have shown a reduction in circulating markers of inflammation and monocyte adhesion to endothelial cells after the daily intake of 20–30 g alcohol as RW, which potentially modulates atherosclerosis development. However, it is unknown whether these effects can be extrapolated to high–cardiovascular risk populations, and whether these effects are attributed to alcohol, polyphenols of RW, or the synergistic effect of both.

Therefore, we embarked on a randomized, crossover, controlled clinical trial to evaluate and compare the effects of moderate consumption of 30 g alcohol/d of gin (a nonpolyphenolic alcoholic beverage), RW (a high-polyphenolic alcoholic beverage), and the same amount of DRW (a high-polyphenolic nonalcoholic beverage) on the expression of soluble and leukocyte adhesion molecules as well as proinflammatory cytokines related to the early stages of atherosclerosis in subjects at high risk of CAD in whom diet and exercise were carefully monitored.

SUBJECTS AND METHODS

Subjects

A total of 73 high-risk subjects aged between 55 and 75 y were recruited for the study in the outpatient clinic of the Internal Medicine Department at Hospital Clínic of Barcelona. Subjects included in the trial were moderate alcohol consumers (1–3 drinks/d) and had diabetes or ≥3 of the following cardiovascular disease risk factors: tobacco smoking, hypertension, plasma LDL cholesterol concentrations ≥160 mg/dL, plasma HDL cholesterol concentrations ≤35 mg/dL, overweight or obesity [BMI (in kg/m^2) ≥25], and/or family history of premature CAD. Exclusion criteria included documented CAD, stroke, or peripheral vascular disease, HIV infection, alcoholic liver disease, malnutrition, and neoplastic or acute infectious diseases. The Institutional Review Board of the hospital approved the study protocol, and all participants gave written consent before participation in the study.

Study design

The study was an open, randomized, crossover, and controlled clinical trial, which included 3 4-wk periods. After a run-in period of 2 wk in which subjects were asked not to consume any alcoholic beverage, they received gin (100 mL; 30 g ethanol/d), RW (272 mL; 30 g ethanol/d), and the same amount of polyphenols as RW in the form of DRW (272 mL) according to a computer-generated random-number table. None of the subjects consumed multivitamin or vitamin E supplements or antiinflammatory drugs (steroids, nonsteroidal antiinflammatory drugs, or aspirin >100 mg/d). The RW and DRW were from the Penedès appellation and elaborated with the Merlot grape variety. The phenolic composition of the 3 beverages used in the study is detailed in **Table 1**. The total phenolic content of the 3 beverages was determined by using the Folin-Ciocalteu method (24), the phenolic profile of RW and DRW was determined by using HPLC–diode-array detection as described previously (25), and resveratrol and piceid contents were determined by using HPLC–diode-array detection as described by Romero-Pérez et al (26). No significant differences were observed between phenolic compositions of RW and DRW (Table 1).

Diet and exercise monitoring

Subjects were asked to exclude alcoholic beverages 15-d before the first intervention (run-in period) and during the study. Subjects were also asked not to change their dietary pattern during the study. Natural foods rich in antioxidants, especially fruit and vegetables, were especially monitored so that individual diets had similar antioxidant contents throughout the study. Participants were not blinded to the type of drink they ingested. At the beginning of the study and after each intervention period, a medical record and Minnesota Leisure Time Physical Activity Questionnaire validated in Spain (27) were performed, and a 7-d food record questionnaire (5 weekdays and 2 weekend days), which was also validated in our population (28), was used to assess nutrient intake and to monitor adherence to the study protocol. This information was converted into dietary data by using the Food Processor Nutrition and Fitness Software (*esha* Research). Nutritional variables analyzed included total energy, total protein, carbohydrates, dietary fiber, sugars, total lipids, saturated fatty acids, MUFAs, PUFAs, cholesterol, vitamins C, A, and E, folic acid, and total polyphenols. Subjects were asked to maintain their lifestyle habits and to report any illness or abnormality presented during the study period. At the end of each study period, a clinician assessed any adverse effects from the interventions by administering a checklist of symptoms, including bloating, fullness, or indigestion, altered bowel habits, dizziness, and other symptoms that were possibly associated with the interventions.

Methods

Clinical and laboratory measures

Anthropometric measures were performed with standardized methods (29). Fasting blood and 24-h urine samples were collected at baseline (the last day of the washout period) and the day after the last day of each intervention (RW, DRW, and gin). Serum, EDTA plasma, and urine samples were stored at −80°C until assayed. Clinical investigators and laboratory technicians were blinded to the interventions. To ensure that ethanol consumption did not cause side effects, plasma aminotransferases (aspartate aminotransferase and alanine aminotransferase), γ-glutamyl transpeptidase, albumin, vitamin B-12, and serum and intraerythrocytary folic acid concentrations were measured.

TABLE 1
Phenolic composition of beverages used in the study: RW, DRW, and gin[1]

	RW	DRW	Gin	P^2
Alcohol (%)	14.2	0.42	38	
Phenolic compounds[3]				
Total phenols (mEqGA/L)	2933.35 ± 377.31	2694.92 ± 86.79	ND	0.426
Gallic acid (mg/L)	68.48 ± 6.40	73.17 ± 7.01	ND	0.306
Protocatechuic acid (mg/L)	5.22 ± 0.62	5.85 ± 0.51	ND	0.246
Tyrosol (mg/L)	43.59 ± 4.73	47.81 ± 3.90	ND	0.298
Catechin (mg/L)	123.51 ± 11.30	126.45 ± 13.35	ND	0.786
Epicatechin (mg/L)	67.86 ± 7.74	70.57 ± 8.22	ND	0.699
*t*rans-Caftaric (mg/L)	18.62 ± 1.45	19.21 ± 1.62	ND	0.595
trans-Caffeic (mg/L)	11.50 ± 0.79	12.18 ± 0.92	ND	0.246
trans-Coutaric (mg/L)	5.21 ± 0.45	5.62 ± 0.52	ND	0.182
2-S-glutationylcaftaric (mg/L)	10.30 ± 1.00	10.76 ± 1.26	ND	0.956
Quercetin-3-glucuronide (mg/L)	11.88 ± 1.38	11.25 ± 1.42	ND	0.770
Quercetin (mg/L)	26.66 ± 0.78	23.82 ± 2.37	ND	0.161
Isorhamnetin (mg/L)	3.34 ± 0.27	2.96 ± 0.14	ND	0.114
Delphinidin-3-glucoside (mg/L)	15.25 ± 0.89	14.71 ± 1.62	ND	0.589
Petunidin-3-glucoside (mg/L)	12.29 ± 1.06	12.04 ± 1.15	ND	0.755
Peonidin-3-glucoside (mg/L)	6.78 ± 0.62	6.68 ± 0.57	ND	0.797
Malvidin-3-glucoside (mg/L)	48.83 ± 4.45	49.86 ± 4.27	ND	0.787
Malvidin-(6-acetyl)-3-glucoside (mg/L)	10.97 ± 0.96	10.41 ± 1.20	ND	0.563
Malvidin-(6-coumaroyl)-3-glucoside (mg/L)	4.15 ± 0.27	3.54 ± 0.33	ND	0.066
Total resveratrol (*trans* and *cis*) (mg/L)	5.26 ± 0.83	5.01 ± 0.86	ND	0.566
trans-Resveratrol (mg/L)	2.92 ± 0.36	2.73 ± 0.23	ND	0.352
cis-Resveratrol (mg/L)	2.79 ± 0.15	2.75 ± 0.15	ND	0.761
Total Piceid (*trans* and *cis*) (mg/L)	18.43 ± 1.57	17.98 ± 3.21	ND	0.785
trans-Piceid (mg/L)	9.41 ± 1.12	10.53 ± 0.96	ND	0.160
cis-Piceid (mg/L)	7.71 ± 0.34	7.08 ± 0.87	ND	0.226
Total stilbenes (mg/L)	23.61 ± 1.61	24.53 ± 1.49	ND	0.408

[1] DRW, dealcoholized red wine; mEqGA/L, mEq Gallic acid/L; ND, not detected; RW, red wine.
[2] *P* values are for comparison between RW and DRW polyphenols (Student's *t* test for independent samples).
[3] Mean ± SD (n = 2) (all such values).

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Resveratrol conjugates derived from phase II and microbial metabolism were measured in 24-h urine samples by using the validated methodology described by Urpi-Sarda et al (30), which was adapted to this study, as a biochemical marker of RW- and DRW-intervention compliance. Ethylglucuronide was measured in 24-h urine samples by using liquid chromatography as a biomarker of alcohol intake. High-performance liquid chromatography was performed on an LC Agilent series 1200 (Agilent Technologies) coupled with a hybrid quadrupole time-of-flight mass spectrometer QSTAR Elite (Applied Biosystems/MDS Sciex).

The following serum-soluble adhesion molecules and cytokines and other regulator molecules of adhesion and inflammation processes were quantified by customized human multianalyte profiling (Human MAP; Rules Based Medicine Inc): CD40a, CD40L, CRP, E-selectin, ICAM-1, IL-1α, -10, -16, -18, and -6, MCP-1, -2, and -3, MDC, MIP-1α, myeloid progenitor inhibitory factor 1, tumor necrosis factor-α, and VCAM-1.

PBMC immunophenotyping

PBMCs were isolated from whole blood by Ficoll-Hypaque (Pharmacia) density gradient. The expression of adhesion molecules on the surface of PBMCs was analyzed via double direct immunofluorescence with the use of commercial monoclonal antibodies by following the manufacturer's instructions. The adhesion molecules analyzed were as follows: VLA-4 (CD49-d; Cytogmos), LFA-1 (CD11a; (Bender MedSystems), Mac-1 (CD11b/CD18; Bender MedSystems), SLe^x (CD15s; Beckman Coulter), CD40 (Caltag Laboratories), CD36 (Beckman Coulter), and CCR2 (R&D Systems). Monocytes were identified and selected with the CD14 monoclonal antibody (Caltag Laboratories), and T lymphocytes were identified and selected with the CD2 monoclonal antibody (Caltag Laboratories). Cell counting (5000 events for T lymphocytes and 2000 events for monocytes) and fluorescence analysis were performed in a FACSCalibur Flow Cytometer (Becton Dickinson) with the use of CellQuest software (version 3.3; BD Biosciences).

Statistical analysis

Statistical analysis was performed with SAS Statistical Analysis Systems software (version 9.2; SAS Institute Inc). Descriptive statistics (means ± SDs) were used for baseline characteristics of participants. Values with a skewed distribution (ICAM-1 and MCP-1) were transformed to their natural logarithm for analyses. ANCOVA with baseline value as the covariate was used to compare changes in outcome variables in response to intervention treatments. One-factor ANOVA for repeated measures was used to compare changes in outcome variables in response between intervention treatments and baseline. To exclude the presence of a carryover effect for the 3 periods, the interaction between treatment (RW, DRW, and gin) and sequence of treatment was analyzed in the repeated-measures ANCOVA analyses. Within- and between-group differences are expressed as means and 95% CIs. $P < 0.05$was considered to be significant.

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RESULTS

Baseline characteristics, intervention compliance, diet, exercise monitoring, and side effects

Of the 73 subjects included, 6 subjects withdrew before completing the 3 phases of the study because of physical illness ($n = 2$), journeys ($n = 2$), or not being able to drink DRW ($n = 2$). Therefore, 67 subjects were included in the study. Baseline characteristics of the 67 participants are shown in **Table 2**. Most participants were overweight or obese (~91%), more than one-half of the population had hypertension (~57%), more than three-quarters of subjects had a family history of cardiovascular disease (~78%), and more than one-fifth of subjects had dyslipemia (~24%), had type-2 diabetes (~22%), or were smokers (~24%). Biochemical safety analytes (serum and intraerythrocytary folic acid, vitamin B-12, albumin, aspartate aminotransferase, alanine aminotransferase, and γ-glutamyl transpeptidase) remained within the normal range throughout the study. None of the subjects reported adverse effects related to the interventions.

Protocol adherence was optimum in all subjects, and complete agreement was observed between the reports of participants and the number of empty bottles returned. All the subjects complained about the taste and texture of the DRW. As a measure of intervention compliance, a sum of total resveratrol metabolites, which is a marker of RW and DRW consumption (31), was determined in 24-h urine samples collected the last day of the run-in period and the last day of each intervention. After consumption of RW and DRW, 24-h urinary excretion of total resveratrol metabolites increased in relation to baseline amounts from 1.24 μmol (95% CI: 0.91, 1.65 μmol) to 4.69 μmol (95% CI: 3.86, 5.53 μmol) and 8.33 μmol (95% CI: 6.86, 10.19 μmol), respectively ($P < 0.001$, both). Resveratrol metabolites concentrations were statistically higher after the DRW compared with RW intervention ($P = 0.002$) and were also statistically higher after RW and DRW interventions than those obtained after gin consumption [0.76 μmol (95% CI: 0.48, 1.11 μmol); $P < 0.0001$]. After the gin intervention, the concentration of resveratrol metabolites did not change significantly compared with that at baseline ($P = 0.832$). Ethylglucuronide was used as a biomarker of alcohol consumption. Urinary ethylglucuronide concentrations increased significantly after the RW and gin periods compared with those observed at baseline, with increases of 342% (95% CI: 245%, 773%) and 256% (95% CI: 179%, 599%), respectively ($P < 0.001$, both). Moreover, concentrations after the RW and gin interventions were also higher than those obtained after the DRW intervention: 634% (95% CI: 468%, 1424%) and 491% (95% CI: 359%, 1121%), respectively ($P < 0.001$, both). No significant differences were observed between DRW and baseline periods [66% (95% CI: 64%, 75%); $P = 1.000$] and between RW and gin interventions [24% (95% CI: 24–25%); $P = 1.000$]. According to these results, compliance with the 3 interventions was excellent.

Dietary intake data for the 3 intervention periods are shown in **Table 3**. No significant differences were observed in nutrient intake at the beginning of the study and after each intervention. No significant differences were observed in the daily intake of antioxidants or fat before and after each intervention period or in the daily average energy expended in physical activity during the period of intake of RW, DRW, and gin. In addition, no changes were reported in drug intake (Table 2) in any subjects throughout the study. No effect of the treatment from the previous time period on the response in the current time period was observed ($P > 0.05$, all), and thus, no carryover effect was observed for any variables.

TABLE 2
Baseline characteristics of 67 subjects[1]

	Values
Age (y)	60 ± 8[2]
Current smokers [*n* (%)]	16 (23.9)
Sedentarism [*n* (%)]	40 (59.7)
Family history of premature CAD [*n* (%)]	52 (77.6)
BMI (kg/m^2)	29.6 ± 3.9
BMI ≥25 kg/m^2 [*n* (%)]	61 (91.0)
WHR	0.975 ± 0.045
Type 2 diabetes [*n* (%)]	15 (22.4)
Hypertension [*n* (%)]	38 (56.7)
Dyslipemia [*n* (%)]	16 (23.9)
Medications [*n* (%)]	
ACE inhibitors	28 (41.8)
Diuretics	5 (7.5)
Statins	22 (32.8)
Oral hypoglycemic drugs	14 (20.9)
Aspirin or antiplatelet drugs	15 (22.4)
Systolic blood pressure (mm Hg)	142 ± 18
Diastolic blood pressure (mm Hg)	81 ± 8
Heart rate (beats/min)	69 ± 10
Glucose (mg/dL)	111 ± 34
Triglycerides (mg/dL)	128 ± 60
Total cholesterol (mg/dL)	204 ± 33
LDL cholesterol (mg/dL)	133 ± 32
HDL cholesterol (mg/dL)	43 ± 7
LDL cholesterol:HDL cholesterol ratio	3.08 ± 0.10
Folic acid (serum) (ng/mL)	9.8 ± 4.0
Intraerythrocytary folic acid (ng/mL)	386 ± 98
Vitamin B-12 (pg/mL)	406 ± 163
Albumin (mg/mL)	45.4 ± 2.8
ASAT (UI/L)	25.8 ± 9.5
ALAT (UI/L)	29.2 ± 11.7
GGT (UI/L)	30.7 ± 12.6

[1] ACE, angiotensin-converting enzyme; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; CAD, coronary artery disease; GGT, γ-glutamyl transpeptidase; WHR, waist-to-hip ratio.
[2] Mean ± SD (all such values).

Changes in circulating inflammatory markers

Changes in circulating inflammatory markers are shown in **Table 4.** Serum E-selectin concentrations were lower after the DRW intervention than after the RW and gin interventions [$P = 0.009$ and 0.016, respectively (Bonferroni post hoc test)]. Serum ICAM-1 and IL-6 concentrations were significantly lower after the RW and DRW interventions than after the gin intervention ($P = 0.014$, 0.016, 0.026, and 0.023 for ICAM-1 and IL-6, respectively). The IL-10 concentration was shown to be significantly higher and MDC was shown to be significantly lower after the RW and gin interventions than after the DRW intervention ($P = 0.046$, 0.023, 0.003, and 0.007, for IL-10 and MDC, respectively). Serum concentrations of CD40a and CD40L decreased significantly after the 3 interventions (RW, DRW, and gin) compared with the baseline situation ($P = 0.008$, 0.043, 0.001, 0.001, 0.019, and 0.015 for CD40a and CD40L, respectively) as

TABLE 3
Daily energy and dietary intakes in 67 subjects at baseline and after 3 interventions[1]

	Baseline	RW intervention	DRW intervention	Gin intervention	*P*
Energy (kcal/d)	1816 ± 457	1782 ± 325	1862 ± 320	1887 ± 336	0.109
Total protein (g/d)	90.39 ± 20.60	89.06 ± 18.35	94.29 ± 17.56	95.04 ± 18.73	0.133
Carbohydrates (g/d)	193 ± 51	201 ± 44	193 ± 38	206 ± 40	0.111
Dietary fiber (g/d)	19.67 ± 9.83	20.63 ± 8.08	19.88 ± 6.82	22.05 ± 10.01	0.254
Sugars (g/d)	62.89 ± 23.55	66.74 ± 25.59	67.33 ± 20.08	70.11 ± 20.84	0.489
Total lipids (g/d)	74.87 ± 24.91	72.72 ± 16.27	78.55 ± 20.41	79.86 ± 21.89	0.194
SFA (g/d)	18.65 ± 8.66	18.40 ± 7.06	18.90 ± 6.15	19.01 ± 5.48	0.804
MUFA (g/d)	36.85 ± 12.06	35.84 ± 8.23	37.97 ± 10.18	38.31 ± 9.60	0.522
PUFA (g/d)	10.31 ± 3.89	10.65 ± 4.21	11.94 ± 4.42	10.71 ± 3.29	0.644
Cholesterol (mg/d)	346 ± 142	355 ± 125	342 ± 103	360 ± 162	0.710
Vitamin C (mg/d)	112 ± 75	125 ± 83	121 ± 68	133 ± 91	0.534
Vitamin A (μg retinol equivalent/d)	640 ± 360	688 ± 340	729 ± 343	791 ± 481	0.345
Vitamin E (mg/d)	9.49 ± 3.88	9.65 ± 3.22	9.47 ± 3.34	10.29 ± 4.65	0.153
Folic acid (μg/d)	426 ± 198	454 ± 155	443 ± 143	498 ± 226	0.181
Total polyphenols (mg/d)	340 ± 185	318 ± 142	311 ± 146	327 ± 170	0.555

[1] All values are means ± SDs. Energy, nutrient, and total polyphenol contributions from interventions were excluded. Changes in outcome variables were determined by using repeated-measures ANCOVA with the baseline value as the covariate. No changes were observed between baseline and interventions determined by repeated-measures ANOVA ($P > 0.05$, all). DRW, dealcoholized red wine; RW, red wine.

did IL-16 ($P = 0.013$, 0.005, and 0.008 for RW, DRW, and gin, respectively), MCP-1 ($P = 0.016$, 0.009, and 0.007 for RW, DRW, and gin, respectively) and VCAM-1 ($P = 0.002$, 0.003, and 0.027 for RW, DRW, and gin, respectively) concentrations. Serum ICAM-1 and IL-6 concentrations decreased significantly after the RW and DRW interventions compared with those at baseline ($P = 0.005$, 0.003, 0.019, and 0.009 for ICAM-1 and IL-6, respectively), and IL-10 concentrations increased and MDC concentrations decreased after the RW and gin interventions compared with those at baseline ($P = 0.013$, 0.023, 0.008, and 0.013 for IL-10 and MDC, respectively). Otherwise, serum concentrations of the other molecules evaluated were shown to remain practically unaltered between baseline and after the 3 interventions.

Expression of cell adhesion molecules on leukocyte cell surfaces

Changes in the expression of cell adhesion molecules on leukocyte cell surfaces are shown in **Table 5**. LFA-1 expression on T-lymphocyte membrane was significantly higher after the gin intervention than after the RW and DRW interventions [$P =$

TABLE 4
Expression of soluble adhesion molecules and cytokines in 67 subjects at baseline and after 3 interventions[1]

	Baseline	RW intervention	DRW intervention	Gin intervention	*P*
CD40a (ng/mL)	0.865 ± 0.225	0.805 ± 0.195[2]	0.818 ± 0.201[2]	0.799 ± 0.202[2]	0.734
CD40L (ng/mL)	2.15 ± 1.16	1.62 ± 1.23[2]	1.81 ± 1.24[2]	1.79 ± 1.24[2]	0.477
CRP (μg/mL)	2.18 ± 0.31	2.17 ± 0.33	1.76 ± 0.20	2.15 ± 0.28	0.210
E-selectin (ng/mL)	9.38 ± 4.30	8.78 ± 3.25[a]	8.33 ± 3.17[b]	8.91 ± 3.93[a]	0.008
ICAM-1 (ng/mL)	91.41 ± 28.63	81.33 ± 25.98[a,2]	79.31 ± 21.46[a,2]	87.82 ± 20.39[b]	0.029
IL-1α (pg/mL)	4.46 ± 2.23	4.24 ± 2.23	4.41 ± 2.58	4.82 ± 2.77	0.188
IL-10 (pg/mL)	6.44 ± 2.63	7.68 ± 1.96[a,2]	6.69 ± 2.63[b]	7.89 ± 2.38[a,2]	0.043
IL-16 (pg/mL)	478 ± 142	450 ± 143[2]	428 ± 168[2]	436 ± 147[2]	0.582
IL-18 (pg/mL)	399 ± 123	384 ± 150	384 ± 129	383 ± 127	0.985
IL-6 (pg/mL)	3.67 ± 1.15	2.76 ± 1.06[a,2]	2.44 ± 1.29 [a,2]	3.70 ± 1.14[b]	0.039
MCP-1 (pg/mL)	488 ± 205	455 ± 201[2]	454 ± 196[2]	446 ± 219[2]	0.817
MCP-2 (pg/mL)	43.67 ± 12.57	43.67 ± 10.37	43.17 ± 12.19	43.02 ± 11.60	0.724
MCP-3 (pg/mL)	60.64 ± 20.03	60.72 ± 17.50	59.10 ± 17.69	59.10 ± 17.11	0.546
MDC (pg/mL)	450 ± 104	416 ± 115[a,2]	446 ± 120[b]	418 ± 104[a,2]	0.009
MIP-1α (pg/mL)	141 ± 30	138 ± 30	140 ± 31	142 ± 29	0.248
MPIF-1 (ng/mL)	1.41 ± 0.29	1.41 ± 0.27	1.43 ± 0.31	1.39 ± 0.31	0.274
TNF-α (pg/mL)	7.60 ± 3.79	8.09 ± 5.59	7.034 ± 3.96	7.64 ± 5.95	0.121
VCAM-1 (ng/mL)	608 ± 114	572 ± 112[2]	572 ± 113[2]	576 ± 112[2]	0.952

[1] All values are means ± SDs. Changes in outcome variables in response to the intervention treatment were determined by repeated-measures ANCOVA with the baseline value as the covariate. Values in a row with different superscript letters are significantly different, $P < 0.05$ (Bonferroni post hoc test). CD40a, CD40 antigen; CD40L, CD40 ligand; CRP, C-reactive protein; DRW, dealcoholized red wine; ICAM-1, intercellular adhesion molecule 1; MCP, monocyte chemotactic protein; MDC, macrophage-derived chemokine; MIP-1α, macrophage inflammatory protein 1α; MPIF-1, myeloid progenitor inhibitory factor 1; RW, red wine; VCAM-1, vascular cell adhesion molecule 1.
[2] Significantly different from baseline, $P < 0.05$ (repeated-measures ANOVA and Bonferroni post hoc test).

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TABLE 5
Expression of adhesion molecules on the surface of T lymphocytes and monocytes in 67 subjects at baseline and after 3 interventions[1]

	Baseline	RW intervention	DRW intervention	Gin intervention	*P*
T lymphocytes					
LFA-1 (MFI)	66.03 ± 15.72	65.95 ± 17.51[a]	66.98 ± 13.61[a]	75.92 ± 12.98[b]	0.003
Mac-1 (MFI)	43.44 ± 13.79	39.73 ± 10.90	37.80 ± 14.29	40.76 ± 15.79	0.271
VLA-4 (MFI)	36.67 ± 8.99	36.96 ± 8.34	37.27 ± 7.73	37.08 ± 7.97	0.775
SLe^x (MFI)	77.30 ± 14.06	68.46 ± 13.62[2]	68.94 ± 14.21[2]	73.28 ± 14.31	0.268
CD40 (MFI)	38.44 ± 12.73	37.36 ± 13.84	38.72 ± 11.39	38.37 ± 12.12	0.585
Monocytes					
LFA-1 (MFI)	30.59 ± 8.41	31.50 ± 9.29	31.01 ± 8.90	31.66 ± 9.19	0.781
Mac-1 (MFI)	30.05 ± 12.78	26.32 ± 9.60[a,b,2]	25.46 ± 6.41[a,2]	28.83 ± 11.48[b]	0.044
VLA-4 (MFI)	23.16 ± 8.07	22.74 ± 6.22	23.03 ± 9.57	22.69 ± 6.25	0.909
SLe^x (MFI)	40.12 ± 10.95	36.90 ± 10.40[2]	36.93 ± 11.05[2]	37.39 ± 10.53	0.861
CD40 (MFI)	23.83 ± 6.94	24.22 ± 5.23	23.67 ± 5.49	23.53 ± 5.21	0.266
CD36 (MFI)	19.89 ± 7.67	20.73 ± 7.90	20.50 ± 8.23	20.79 ± 7.74	0.279
CCR2 (MFI)	169.53 ± 36.78	116.94 ± 41.04[a,2]	109.52 ± 38.81[a,2]	168.83 ± 35.10[b]	0.009

[1] All values are means ± SDs. Changes in outcome variables in response to the intervention treatment were determined by repeated-measures ANCOVA with the baseline value as the covariate. Values in a row with different superscript letters are significantly different, $P < 0.05$ (Bonferroni post hoc test). CCR2, C-C chemokine receptor type 2; DRW, dealcoholized red wine; LFA-1, lymphocyte function-associated antigen 1 (CD11a); Mac-1, macrophage-1 receptor; (CD11b/CD18); MFI: mean fluorescence intensity; RW, red wine; SLe^x, Sialil-Lewis X, CD15s; VLA-4, very late activation antigen 4 (CD49-d).
[2] Significantly different from baseline, $P < 0.05$ (repeated-measures ANOVA and Bonferroni post hoc test).

0.003 and 0.005, respectively (Bonferroni post hoc test)], Mac-1 monocyte expression was lower after the DRW intervention than after the gin period ($P = 0.014$), and CCR2 monocyte fluorescence intensity was also lower after the RW and DRW interventions than after the gin intervention ($P = 0.019$ and <0.0001, respectively). In addition, compared with the baseline situation, we observed that SLe^x lymphocyte and monocyte expression decreased significantly after the RW and DRW interventions ($P = 0.006$, 0.012, 0.027, and 0.037 for lymphocyte and monocyte SLe^x, respectively) as did the Mac-1 ($P = 0.018$ and 0.003, respectively) and CCR2 monocyte fluorescence intensity ($P = 0.024$ and 0.006, respectively). The expression of VLA-4, Mac-1, and CD40 on the lymphocyte surface remained practically unaltered. The expression of LFA-1, VLA-4, CD40, and CD36 on monocyte cell membrane were not altered.

DISCUSSION

Atherosclerosis is a low-grade inflammatory disease characterized by local inflammation in the vessel wall, as well as a systemic immune response (32), in which recruitment and migration of leukocytes through cell adhesion molecules into the arterial wall is a crucial step in early atherogenesis (33). We measured several chemokines and adhesion molecules implicated in the onset and progression of the atherosclerotic process after a 1-mo intervention with RW (constituted mainly by alcohol plus polyphenols), DRW (polyphenols), and gin (alcohol) in high-risk subjects. A downregulation of serum concentrations of CD40a, CD40L, IL-16, MCP-1, VCAM-1, and E-selectin was observed after the RW, DRW, and gin interventions. Therefore, these effects could be attributed to both ethanol and the phenolic compounds of RW but in a nonadditive manner. However, an additive effect of ethanol and RW polyphenols was suggested in the case of CD40L, albeit without achieving significance. RW polyphenols also exert a protective effect on atherosclerosis because the RW and DRW interventions decreased ICAM-1 and IL-6 serum concentrations and inhibited the expression of LFA-1 and SLe^x in the T-lymphocyte surface and Mac-1, SLe^x, and CCR2 expression in monocytes. Finally, ethanol by itself exerts a dual protective effect by increasing IL-10 and decreasing MDC concentrations.

To our knowledge, no previous consumption trials have compared the antiinflammatory effects of DRW with those of RW and gin in humans. However, our results agree with those obtained in experimental studies such as the study by Norata et al (34) in which MCP-1, MIP-1α and-1β, and IL-6 decreased in the arterial wall of apolipoprotein $E^{-/-}$ mice after oral administration of a mixture of polyphenols. Similarly, in the human umbilical vein endothelial cells cell line, Cullen et al (35) showed that ethanol inhibited the endothelial production of MCP-1, and Carluccio et al (36) observed that resveratrol inhibits the synthesis of VCAM. Lastly, the incubation platelets from healthy humans with extracts of grape seed and skin inhibited the release of soluble CD40L (37).

We observed increased antiinflammatory IL-10 concentrations only after the gin intervention. Similarly, Mandrekar et al (38) showed an increase of IL-10 in human monocytes after acute alcohol intake. We also showed a diminution of MDC after the RW and gin interventions. To our knowledge, this is the first time that MDC and IL-16 expression has been studied after moderate RW or ethanol consumption.

More interestingly, the polyphenols of RW after the RW and DRW interventions decreased plasma concentrations of ICAM-1 and IL-6 and inhibited the expression of LFA-1 and SLe^x in the T-lymphocyte surface and that of Mac-1, SLe^x, and CCR2 on monocytes. These results agree with in vitro or animal models that analyzed the effect of polyphenols of RW, mainly resveratrol, in the regulation of these molecules. Resveratrol reduced ICAM-1 expression in human umbilical vein endothelial cells (39) and IL-6 in vascular smooth-muscle cells (40). The preincubation of polymorphonuclear leukocytes with *trans*-resveratrol resulted in a concentration-dependent inhibition of fMLP (formyl methionyl leucyl phenylalamine)-induced Mac-1 expression (41). Norata et al also showed a decrease in CCR2 expression in the arterial

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wall of apolipoprotein $E^{-/-}$ mice after oral administration of resveratrol (34), and Cullen et al (42) showed that CCR2 expression was inhibited in a time- and dose-dependent manner by resveratrol in THP-1 monocytes.

Only a few clinical trials have analyzed the changes in the cellular expression of adhesion molecules (21, 23) or in the inflammatory biomarkers related to atherosclerosis (43) after 1-mo of moderate RW consumption in healthy humans. Therefore, we wondered whether the results observed may be extrapolated to a high–cardiovascular risk population. In a previous study in which 30 g ethanol/d in the form of gin and RW was administered to healthy men, significant reductions of VCAM-1 (16.5%), ICAM-1 (9.2%), and IL-1α (20.3%) concentrations were shown after RW consumption (21). In addition, a reduction of 13.9% in VLA-4 lymphocyte expression and LFA-1 (27.1%), Mac-1 (26.7%), VLA-4 (32.5%), and MCP-1 (45.7%) monocyte expression was also observed after RW consumption. However, no significant effects were shown after the gin intervention, except for a decrease in IL-1α concentrations (22.6%). No significant effects were detected in TNF-α, transforming growth factor β1, IL-6, ICAM-1, and VCAM-1 in another human trial performed by Djurovic et al (43) that included healthy subjects (men and women) who consumed 15 g alcohol/d of RW during 3 wk. Differences in the cardiovascular effects observed between the 2 studies (21, 43) may have been attributed to the different amount of alcohol administered (15 compared with 30 g ethanol/d). In the current study, in which 30 g ethanol/d was administered, reductions of ~6%, 11%, and ~12% of VCAM-1 and ICAM-1 concentrations and Mac-1 monocyte expression, respectively, were observed after RW consumption. Nevertheless, in difference from the aforementioned studies, we also observed a significant decrease of ~6% and ~7% in E-selectin and MCP-1 concentrations, respectively, as well as an increase of 15% in LFA-1 lymphocyte expression after the gin period. Thus, the effects of moderate RW consumption observed in healthy people could not be strictly extrapolated to the high–cardiovascular risk population. The fact that more than one-half of the high-risk subjects consumed drugs with known antiinflammatory effects (mainly statins and aspirin) (44) may explain, in part, the differences observed. However, ethanol (gin) exerted some antiinflammatory effect in high-risk subjects, which were actions that were not previously observed in healthy subjects.

One of the main limitations of our study is that there were no washout periods between interventions. Washout periods between interventions would have extended the study 6 wk more, which would have made it difficult to ensure compliance and subject withdrawal more likely. However, because previous studies have shown that changes in cellular and soluble adhesion molecules were already observed after 2 wk of intervention (21, 45), and no carryover effect was observed, the absence of a washout period would probably not have changed the results.

Although the study was far too short to deal with atherosclerosis itself and morbid mortality due to cardiovascular disease, RW polyphenols may provide additional benefits because of its antiinflammatory effects, namely the decreased expression of adhesion molecules related to early stages of atherosclerosis. Thus, another limitation of our study was that our results can only lead to limited conclusions that link the findings to a reduction in atherosclerosis because the biomarkers used in our study are not commonly examined in the clinical setting (46). Moderate alcohol consumption (independent of the type of beverage) is associated with lower risk of cardiovascular disease mortality and endpoints for CAD (47). Because most alcoholic beverages contain alcohol and polyphenols (except for gin and vodka among other alcoholic beverages), it is very difficult to exclude the effects of ethanol from other compounds of alcoholic beverages. Few studies have shown an inverse association between moderate RW consumption and risk of stroke in men (48). Therefore, we cannot conclude that the changes observed were of a potentially clinical relevant magnitude, and thus, additional studies are required regarding the clinical implication of the modulation of these biomarkers (and the role of each compound of alcoholic beverages) on the beneficial effects of alcoholic beverages on the cardiovascular system.

In conclusion, to our knowledge, this is the first randomized, controlled, clinical trial that studied the effects of RW and DRW separately on the expression of adhesion molecules and inflammatory cytokines related to early stages of atherosclerosis and provided information on the separate role of ethanol and phenolic compounds of RW in low-grade inflammation in the arterial wall and endothelial dysfunction. Our results suggest that both ethanol and nonalcoholic compounds contribute to antiinflammatory effects of RW. The phenolic content of RW may modulate leukocyte adhesion molecules, whereas both ethanol and polyphenols of RW may modulate soluble inflammatory mediators in patients at high risk of cardiovascular disease. These positive changes in the inflammatory profile in high–cardiovascular risk patients could contribute to the benefits of moderate wine consumption against early stages and the progression of atherosclerosis.

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Erratum

Chiva-Blanch G, Urpi-Sarda M, Llorach R, Rotches-Ribalta M, Guillén M, Casas R, Arranz S, Valderas-Martinez P, Portoles O, Corella D, et al. Differential effects of polyphenols and alcohol of red wine on the expression of adhesion molecules and inflammatory cytokines related to atherosclerosis: a randomized clinical trial. Am J Clin Nutr 2012;95:326–34.

An error was identified in the 24-h urinary excretion of total resveratrol metabolites, which was incorrect at baseline and after 3 interventions. The statistical analysis between interventions has now been updated. The aim of this measure was to provide intervention compliance through the calculation of the biomarker of wine consumption. This has been previously defined as the sum of 7 phase II metabolites of resveratrol specified in reference 31. These metabolites are as follows: *trans*- and *cis*-resveratrol-3-*O*-glucuronide, *cis*-resveratrol-4′-*O*-glucuronide, *trans*- and *cis*-resveratrol-4′-*O*-sulfate, and *trans*- and *cis*-resveratrol-3-*O*-sulfate.

On page 328, the first sentence of the left-hand column of the Methods section should read as follows: "Resveratrol conjugates derived from phase II metabolism were measured in 24-h urine samples by using the validated methodology described by Urpi-Sarda et al (30) quantitatively adapted due to the commercial and available standards. *trans*- and *cis*-Resveratrol-3-*O*-glucuronide (98% purity each), *cis*-resveratrol-4′-*O*-glucuronide (96% purity), and *trans*-resveratrol-3-*O*-sulfate (98% purity) were purchased from Toronto Research Chemicals Inc. *trans*- and *cis*-Resveratrol-4′-*O*-sulfate and *cis*-resveratrol-3-*O*-sulfate were quantified using the *trans*-resveratrol-3-*O*-sulfate calibration curve."

Incorrect values were given on page 329 in the right-hand column of the Results section. The second sentence of the first paragraph should read as follows: "After consumption of RW and DRW, 24-h urinary excretion of total resveratrol metabolites increased in relation to baseline amounts from 0.94 μmol (95% CI: 0.43, 1.46 μmol) to 6.04 μmol (95% CI: 4.76, 7.31 μmol) and 6.28 μmol (95% CI: 5.10, 7.46 μmol), respectively ($P < 0.001$, both). Resveratrol metabolite concentrations were not statistically different after DRW and RW interventions ($P = 1.00$) and were statistically higher after RW and DRW interventions than after gin consumption [0.51 μmol (95% CI: 0.08, 0.94 μmol); $P < 0.001$]. After the gin intervention, the concentration of resveratrol metabolites did not change significantly compared with that at baseline ($P = 1.00$)." Total resveratrol metabolites have been used only as a measure of compliance in this report and were not used in any of the calculations, did not affect the results or discussion of the article, and therefore have no implications for the main results or their interpretation.

doi: 10.3945/ajcn.112.038810.

Erratum

Vrieling A, Kampman E. The role of body mass index, physical activity, and diet in colorectal cancer recurrence and survival: a review of the literature. Am J Clin Nutr 2010;92:471–90.

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Publicació III: Andres-Lacueva C, Macarulla MT, Rotches-Ribalta M, Boto-Ordóñez M, Urpi-Sarda M, Rodríguez VM, Portillo MP. Distribution of resveratrol metabolites in liver, adipose tissue, and skeletal muscle in rats fed different doses of this polyphenol. *Journal of Agricultural and Food Chemistry*, 2012; 60(19):4833-4840.

En aquest treball s'estudia la distribució dels metabòlits de resveratrol en fetge, teixit adipós i múscul esquelètic de rates alimentades amb diferents dosis d'aquest polifenol. El nou mètode optimitzat i validat en la publicació corresponent a l'estudi 2 de la present Tesi Doctoral es va aplicar també a aquest estudi, després d'una homogeneïtzació-extracció del resveratrol dels teixits amb metanol acidulat.

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Distribution of Resveratrol Metabolites in Liver, Adipose Tissue, and Skeletal Muscle in Rats Fed Different Doses of This Polyphenol

Cristina Andres-Lacueva,[†,‡] M. Teresa Macarulla,[§] Maria Rotches-Ribalta,[†,‡] María Boto-Ordóñez,[†,∥] Mireia Urpi-Sarda,[∥] Víctor M. Rodríguez,[§] and María P. Portillo*[,§]

[†]Department of Nutrition and Food Science, XaRTA, INSA, Faculty of Pharmacy, University of Barcelona, Barcelona, Spain

[‡]INGENIO−CONSOLIDER Program, Fun-C-food (CSD2007-063), Ministry of Science and Innovation, Barcelona, Spain

[§]Department of Nutrition and Food Science, Faculty of Pharmacy, RETIC RD06/0045/0003, Instituto de Salud Carlos III, University the Basque Country (UPV/EHU), Vitoria, Spain

[∥]CIBER: Fisiopatología de la Obesidad y la Nutrición (CIBEROBN), Barcelona, Spain

ABSTRACT: This study aimed to characterize resveratrol metabolite profiles in liver, skeletal muscle, and adipose tissue in rats treated for 6 weeks with 6, 30, or 60 mg of *trans*-resveratrol/kg body weight/d. Resveratrol metabolites were quantified by liquid chromatography−tandem mass spectrometry. The greatest number of metabolites was found in liver followed by adipose tissue. A great number of metabolites in muscle was below the limit of detection. The amounts of sulfate conjugates tended to increase when resveratrol dosage was enhanced, while the glucuronide ones increased only between 6 and 30 mg/kg/d. Microbiota metabolites were detected in higher amounts than resveratrol conjugates in liver, while the opposite occurred in adipose tissue and muscle. So, the largest amounts of resveratrol metabolites were found in liver, intermediate amounts in adipose tissue, and the lowest amounts in muscle. Sulfate conjugates, but not glucuronides, showed a dose−response pattern. Microbiota metabolites were predominant in liver.

KEYWORDS: *resveratrol, glucuronide metabolites, sulfate metabolites, dihydroresveratrol, liver, adipose tissue, skeletal muscle*

INTRODUCTION

Resveratrol (*trans*-3,5,4′-trihydroxystilbene) is a phytoalexin polyphenolic compound occurring naturally in various plants, including grapes, berries, and peanuts, in response to stress or as a defense mechanism against fungal, viral, bacterial infections and damage from exposure to ultraviolet radiation.[1]

Resveratrol has been reported to have several beneficial effects on health.[2] However, the efficacy of orally administered resveratrol depends on its absorption, metabolism, and tissue distribution, since it is quickly absorbed in the intestine, via simple intestinal transepithelial diffusion at a high rate (77−80%).[3,4] Active transport might occur as well, but this is only likely for resveratrol metabolites.[5] Most resveratrol undergoes rapid and extensive metabolism in enterocytes, before entering into the bloodstream, resulting in up to a 20-fold higher concentration of conjugates, and less than 1% resveratrol. Furthermore, it undergoes rapid first-pass metabolism in the liver.[6] Consequently, resveratrol bioavailability is very low and only a small proportion reaches plasma.[7] Besides this intestinal and hepatic metabolism, it has been proposed that bacterial metabolism should also be taken into account.[4] Several approaches that may increase the availability of resveratrol are under evaluation, such as dose escalation studies,[8] as well as repeated or long-term dosing, which might result in saturation of metabolism, leading to higher plasma and tissue levels of resveratrol.[4]

In vitro studies have suggested that concentrations in the range of 5−50 μM are needed to find significant changes in cells.[9−14] Taking into account the low bioavailability of orally administered resveratrol, the doses usually administered in in vivo studies are unlikely to furnish resveratrol levels sufficiently compatible with those that modulate biological actions in vitro.[15,16] A great number of biovailability studies have shown this.[6−8,17] Nevertheless, beneficial effects of resveratrol under in vivo conditions have been reported. This may be due to the fact that some resveratrol metabolites show biological activities[18] or due to the potential conversion of resveratrol metabolites back to resveratrol in target organs.[19]

Thus, the cause−effect relationship between resveratrol concentration in the systemic bloodstream and its reported biological effects is still a conundrum.[15] Consequently, it is essential (a) to establish whether resveratrol metabolites can conceivably contribute to, or account for, different effects of resveratrol in vivo and (b) to quantify resveratrol metabolites in vivo. Localization of tissue uptake, which determines target tissues, and the target/dose relationship are important to understand the positive health effects described for resveratrol.[20]

In this context, the present study aimed to characterize the resveratrol metabolite profile in liver, skeletal muscle, and adipose tissue in rats fed on obesogenic diets supplemented with resveratrol. Different doses of this polyphenol were used for supplementation in order to assess potential saturation of metabolic pathways responsible for transformation of resveratrol into metabolites. These tissues were chosen because they

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play a crucial role in the recently described body-fat-lowering effect of resveratrol. An interesting aspect of this study is that very few data have been published concerning metabolites found in two of these tissues, adipose tissue and skeletal muscle, as well as tissue distribution after a long-term treatment with resveratrol.

MATERIAL AND METHODS

Animals and Experimental Design. The experiment was conducted with 24 male Sprague−Dawley rats with an initial body weight of 180 ± 2 g purchased from Harlan Ibérica (Barcelona, Spain) and took place in accordance with the institution's guide for the care and use of laboratory animals (Reference protocol approval CUEID CEBA/30/2010). Animals from this cohort were previously used to study the potential body-fat-lowering effect of resveratrol.[21]

The animals were individually housed in polycarbonate metabolic cages (Techniplast Gazzada, Guguggiate, Italy) and placed in an air-conditioned room (22 ± 2 °C) with a 12-h day−night rhythm (light on at 21:00). After a 6-day adaptation period, the animals were fed on a commercial obesogenic diet supplied by Harlan Iberica (ref TD.06415) for 6 weeks. All animals had free access to food and water.

Before starting our animal studies with resveratrol, we analyzed the stability of this polyphenol when added into the diet in a reservoir protected from light. We found that resveratrol degraded almost completely over the feeding time.[21] Therefore, we concluded that mixing it in the diet, the most common method used to test the biological effects of a functional molecule intake, was not a suitable system. In a great number of studies performed by our group we have observed that the rats started eating the diet immediately when it was replaced every day at the beginning of the dark period. Taking advantage of this situation, we added resveratrol by using an ethanol solution (30 mg/mL) on the surface of the food reservoir. The amount of resveratrol solution added to the diet was adjusted on a daily basis according to the animal weight to reach the following doses: 6 mg resveratrol/kg body weight/d in RSV1 group, 30 mg resveratrol/kg body weight/d in RSV2 group, and 60 mg resveratrol/kg body weight/d in RSV3 group. These doses are equivalent to 68, 340, and 680 mg in a 70-kg person.[22] In order to avoid differences in the amount of ethanol received by each animal, ethanol was completed to reach 2 mL/kg body weight/d. Because a very small volume was added (0.36−0.69 mL for body weights ranging from 180 g, the initial body weight, to 345 g, the final body weight), the rats ate all the resveratrol provided in the first minutes of feeding period without any degradation. *trans*-Resveratrol (95% purity) was provided by Monteloeder (Elche, Spain) and added to the diet as explained above for 6 weeks.

Tissue Removal. At the end of the experimental period rats were fasted 12 h and sacrificed under anesthesia (chloral hydrate) by cardiac exsanguination. Subcutaneous white adipose tissue, gastrocnemious muscle, and liver were dissected, weighed, immediately frozen, and stored at −80 °C until analysis. Tissue removal was performed 24 h after resveratrol administration.

Standards and Reagents. All samples and standards were handled with no exposure to light. *trans*-Resveratrol (99% purity) and ethyl gallate, as internal standard (96% purity), were purchased from Sigma-Aldrich (St. Louis, MO). The external standard taxifolin (>90% purity) was purchased from Extrasynthese (Genay, France). Standards of *cis*-resveratrol (97% purity) and *trans*-resveratrol metabolites, i.e., *trans*-resveratrol-3-*O*-glucuronide (98% purity), *trans*-resveratrol-4′-*O*-glucuronide (98% purity) and *trans*-resveratrol-3-*O*-sulfate (98% purity), were acquired from Toronto Research Chemicals Inc. (North York, Canada). *trans*-Resveratrol-4′-*O*-sulfate and *trans*-resveratrol-3,4′-disulfate were obtained as reported previously for identification purposes.[23] Dihydroresveratrol aglycon was synthesized following the work by Thakkar et al.[24] Standards were prepared as stock solutions in 80% (v/v) methanol.

Liquid chromatography (LC) grade solvents methanol, ethyl acetate, acetonitrile, and ammonium acetate (>99%) were purchased from Scharlau Chemie, S.A. (Sentmenat, Spain). LC grade solvents glacial acetic acid, acetone, and ammonia (35%) were purchased from Panreac Química, SAU (Castellar del Vallès, Spain). Deuterated dimethyl sulfoxide (DMSO-d_6, 99.96% deuterated) was purchased from Euriso-top, SAS (Saint-Aubin Cedex, France). Ultrapure water (Milli-Q) was obtained from Millipore (Bedford, MA).

Structural Identification of Dihydroresveratrol. Dihydroresveratrol structure was confirmed by nuclear magnetic resonance (^{1}H NMR) measurements using a Varian 400-MHz instrument VNMR System (Varian, Palo Alto, CA). Synthesized dihydroresveratrol was dissolved in deuterated DMSO-d_6 using dinitrobenzene (1.6 mmol/L) as internal standard. ^{1}H NMR spectra were acquired with the following data: δ (ppm) 9.0 (br, 3H, −OH), 6.97 (d, 2H, J = 8.6 Hz, H-2′,6′), 6.63 (d, 2H, J = 8.6 Hz, H-3′,5′), 6.03 (d, 2H, J = 4.0 Hz, H-2,6), 6.00 (dd, 1H, J = 4.0 Hz, H-4), 2.68−2.56 (m, 4H, $-CH_2CH_2-$). Standard purity was calculated following the protocol validated by Malz and Jancke,[25] obtaining 80% purity. Only one peak was detected after a full scan MS experiment, and no peaks of the parent compound resveratrol (m/z 227) were detected.

Sample Preparation and Determination of Resveratrol Metabolites in Tissues. Analysis was carried out by LC−MS/MS after a solid-phase extraction (SPE), as described previously by Urpi-Sarda et al.[26] and Andres-Lacueva et al.[27] with some modifications. Briefly, ∼100 mg of frozen samples was extracted three times with 1 mL of a solution of 1.5 M formic acid with 5% of methanol using a mixer mill (Retsch MM 400, Qiagen, Hilden, Germany) at 30 Hz for 0.5 min and centrifuged each time at 14 000 rpm. After this homogenization, the tissue structure was broken and metabolites linked to protein were released. Pooled supernatant fractions with the internal standard were loaded onto a preconditioned Waters Oasis HLB 96-well plate (30 mg). Acetic acid 2 M in water (1 mL) and in water/methanol (85/15 v/v) (1 mL) were used to wash the plate. Elution was achieved with 0.5 mL of 1 M acetic acid in methanol, 1.5 mL of 1 M acetic acid in ethyl acetate, and 0.5 mL of methanol with 5% (v/v) ammonia. The eluate was evaporated to dryness. The residue was reconstituted with taxifolin dissolved in mobile phase as an additional external standard.

Liquid chromatography analyses were performed using an ACQUITY UPLC (Waters, Milford, MA) and a triple quadrupole mass spectrometer (API 3000) from Applied Biosystems (PE Sciex, Concord, Canada), equipped with a Turbo IonSpray source operated in the negative-ion mode. An ACQUITY UPLC BEH C_{18} column, 50 × 2.1 mm i.d., 1.7 μm, was used for chromatographic separation with mobile phase A (0.05% acetic acid in water, for glucuronide metabolites, or 10 mM ammonium acetate solution for sulfate metabolites) and mobile phase B (acetone:acetonitrile, 70:30). The linear gradient for the determination of resveratrol metabolites at a flow rate of 1 mL/min was [t (min), %B] (0, 10), (1, 30), (2, 100), (2.3, 100), (2.31, 10), (3, 10)]. In each case the sample volume injected was 5 μL. MS/MS parameters used were as follows: capillary voltage, −3500 V; focusing potential, −200 V; entrance potential, −10 V; nebulizer gas, 12 (arbitrary units), curtain gas, 12 (arbitrary units), collision gas, 6 (arbitrary units), auxiliary gas temperature, 400 °C; auxiliary gas flow rate, 7000 cm^3/min.

For quantification of resveratrol metabolites in tissue samples, the multiple reaction monitoring (MRM) mode was used with the following transitions: 227/185 for resveratrol; 403/227 and 307/227 for glucuronide and sulfate conjugates of resveratrol, respectively; 579/403 for resveratrol diglucuronides; 387/227 for resveratrol disulfates; 389/227 for resveratrol glucosides; 229/123 for dihydroresveratrol; 405/229 and 309/229 for glucuronide and sulfate conjugates of dihydroresveratrol and 197/169 and 303/285 for ethylgallate and taxifolin, respectively. Control tissues were used to prepare calibration curves since no resveratrol or its metabolites were detected. Calibration curves were prepared, in the range of expected concentrations, by supplementation with known concentrations of available standards. All of them were quantified using a six-point calibration curve between the limit of quantification (LOQ) and 10 μg/g determined by weighted ($1/x^2$) linear regression. Dihydroresveratrol metabolites and *cis*-resveratrol-3-*O*-sulfate were quantified using the calibration curve of dihydroresveratrol and *trans*-resveratrol-3-*O*-

Table 1. Limits of Detection (LOD) and Limits of Quantification (LOQ) for Each Tissue and Mean Recovery Efficiency from the Three Tissues[a]

	LOD (nmol/g tissue)			LOQ (nmol/g tissue)			
analyte	liver	adipose tissue	skeletal muscle	liver	adipose tissue	skeletal muscle	recovery (%)
trans-resveratrol	0.21 ± 0.01	0.07 ± 0.02	0.27 ± 0.02	0.70 ± 0.02	0.23 ± 0.05	0.90 ± 0.05	105.1 ± 11.5
cis-resveratrol	0.22 ± 0.02	0.14 ± 0.03	0.29 ± 0.02	0.73 ± 0.05	0.46 ± 0.08	1.31 ± 0.07	101.6 ± 8.4
trans- resveratrol-4′-*O*-glucuronide	0.11 ± 0.03	0.20 ± 0.04	0.29 ± 0.03	0.35 ± 0.09	0.66 ± 0.09	0.82 ± 0.94	101.2 ± 5.1
trans- resveratrol-3-*O*-glucuronide	0.09 ± 0.02	0.15 ± 0.04	0.21 ± 0.06	0.31 ± 0.06	0.51 ± 0.07	0.89 ± 0.07	98.7 ± 7.2
trans- resveratrol-3-*O*-sulfate	0.30 ± 0.00	0.06 ± 0.00	0.18 ± 0.04	1.00 ± 0.01	0.19 ± 0.01	0.61 ± 0.08	97.4 ± 10.0
dihydroresveratrol	0.06 ± 0.00	0.05 ± 0.00	0.05 ± 0.01	0.21 ± 0.00	0.15 ± 0.01	0.15 ± 0.02	96.1 ± 5.4

[a]Values are means ± SD.

Table 2. Resveratrol Metabolite Profile (nmol/g tissue) in Liver and Adipose Tissue from Rats Receiving 30 mg of Resveratrol/kg Body Weight/d for 6 Weeks

	liver			adipose tissue		
analyte	mean ± SEM[c]	range	*n*	mean ± SEM[c]	range	*n*
cis-resveratrol	nq	nd−nq	–	nd	–	–
trans-resveratrol-3-*O*-glucuronide	8.66 ± 3.24	nd−28.79	7/8	1.31 ± 0.43	nd−3.24	6/8
trans-resveratrol-4′-*O*-sulfate[a]	1.07 ± 0.27	nq−2.64	7/8	nq	nd−nq	–
trans-resveratrol-3-*O*-sulfate	nq	nd−nq	–	0.24 ± 0.06	nd−0.41	6/8
cis-resveratrol-3-*O*-sulfate[a]	nq	nd−nq	–	nq	nd−0.41	4/8
trans-resveratrol-3,4′-disulfate[a]	nq	nd−nq	–	nq	nd−nq	–
dihydroresveratrol	nq	nd−0.42	2/8	nd	–	–
dihydroresveratrol glucuronide[b]	5.50 ± 1.45	1.77−14.86	8/8	nq	nd−0.18	2/8
dihydroresveratrol sulfate[b]	16.44 ± 4.27	2.90−41.35	8/8	0.32 ± 0.08	nd−0.65	6/8

[a]Quantified as *trans*-resveratrol-3-*O*-sulfate equivalents. [b]Quantified as dihydroresveratrol equivalents. [c]Values <LOQ considered as the LOD value. *n* is the number of rats in which the metabolite was quantified/total rats measured. nd and nq indicate not detected and not quantifiable, respectively (below the limit of detection or quantification, respectively; see Table 1).

Table 3. Resveratrol Metabolite Profile (nmol/g tissue) in Liver and Adipose Tissue from Rats Receiving 60 mg of Resveratrol/kg Body Weight/d for 6 Weeks

	liver			adipose tissue		
analyte	mean ± SEM[c]	range	*n*	mean ± SEM[c]	range	*n*
cis-resveratrol	nq	nd−nq	–	nq	nd−nq	–
trans-resveratrol-3-*O*-glucuronide	10.54 ± 3.06	2.26−25.36	8/8	1.08 ± 0.29	nq−2.33	6/8
trans-resveratrol-4′-*O*-sulfate[a]	3.01 ± 1.14	nq−8.55	6/8	0.26 ± 0.06	nq−0.65	6/8
trans-resveratrol-3-*O*-sulfate	nq	nd−nq	–	0.19 ± 0.06	nd−0.39	5/8
cis-resveratrol-3-*O*-sulfate[a]	nq	nd−nq	–	0.19 ± 0.06	nd−0.32	5/8
trans-resveratrol-3,4′-disulfate[a]	nq	nd−2.39	2/8	nq	nd−nq	–
dihydroresveratrol	0.75 ± 0.20	nd−1.42	7/8	nd	–	–
dihydroresveratrol glucuronide[b]	9.55 ± 2.45	2.65−18.96	8/8	nq	nd−0.30	4/8
dihydroresveratrol sulfate[b]	74.17 ± 23.39	13.00−186.68	8/8	1.02 ± 0.37	0.34−3.65	8/8

[a]Quantified as *trans*-resveratrol-3-*O*-sulfate equivalents. [b]Quantified as dihydroresveratrol equivalents. [c]Values <LOQ considered as the LOD value. *n* is the number of rats in which the metabolite was quantified/total rats measured. nd and nq indicate not detected and not quantifiable, respectively (below the limit of detection or quantification, respectively, see Table 1).

sulfate, respectively, and expressed as their equivalents, as was done previously,[8,26] since no standards are available.

The mean recovery efficiency of the available standards from the different samples was 99.2 ± 11.5%, ranging from 95.6 ± 9.9% (in the case of *trans*-resveratrol-4′-*O*-sulfate) to 105.1 ± 11.5% (in the case of *trans*-resveratrol) (Table 1). Calibration curves were linear over the concentration range with correlation coefficients for all the analytes >0.99. The detection limit (LOD) was defined as the concentration of analyte that produced a signal-to-noise ratio of at least 3, and the limit of quantification (LOQ) was the lowest standard with a signal-to-noise ratio of at least 10. The LOD and LOQ for each standard and evaluated tissue are shown in Table 1. The method had accuracy values (mean ± SD) of 97.25 ± 3.97%, 100.18 ± 3.05%, and 97.69 ± 5.49% for liver, adipose tissue, and skeletal muscle, respectively, and precision values ranged from 3.26 to 13.65% for liver, from 2.65 to 12.48% for adipose tissue, and from 3.22 to 12.85% for skeletal muscle, which met the acceptance criteria of the FDA.[28]

Statistical Analysis. Results are presented as mean ± SEM. Statistical analysis was performed using SPSS 17.0 (SPSS, Chicago, IL). Data were analyzed by one-way ANOVA followed by Newman Keuls post hoc test. Significance was assessed at the $P < 0.05$ level.

RESULTS

The profile of resveratrol metabolites in liver and adipose tissues is detailed in Tables 2 and 3. These metabolites included five phase II metabolites of resveratrol, *cis*-resveratrol, and dihydroresveratrol and its glucurono- and sulfo-conjugates (as microbial metabolites).

When the lowest dose of resveratrol was administered to rats (RSV1 group), two microbial metabolites of resveratrol (dihydroresveratrol conjugates) were found in quantifiable amounts in liver of all eight rats (mean ± SEM): dihydroresveratrol glucuronide (1.48 ± 0.32 nmol/g) and dihydroresveratrol sulfate (2.23 ± 0.32 nmol/g). Otherwise, two metabolites were found in adipose tissue of three rats (range): *trans*-resveratrol-3-*O*-sulfate [not detected (nd)−0.31 nmol/g] and *cis*-resveratrol-3-*O*-sulfate (nd−0.26 nmol/g). Any metabolite has been detected in skeletal muscle.

When higher doses were administered to rats (RSV2 and RSV3 groups) (Tables 2 and 3), the number of quantifiable compounds increased up to four in liver and three in adipose tissue in the RSV2 treatment and five metabolites in liver and adipose tissue in RSV3 treatment. The metabolite mainly detected in liver for all the treatment groups was dihydroresveratrol sulfate, a microbial metabolite, followed by *trans*-resveratrol-3-*O*-glucuronide and dihydroresveratrol glucuronide. Thus, it seems that microbial metabolites predominated in liver. *trans*-Resveratrol-3-*O*-glucuronide was quantified in only one rat in skeletal muscle after RSV2 and RSV3 treatments: 1.02 and 0.76 nmol/g, respectively. Dihydroresveratrol sulfate was quantified in skeletal muscle of two rats after RSV2 treatment (range: nd−0.31 nmol/g) and in six rats after RSV3 treatment [mean ± SEM (range): 0.30 ± 0.10 nmol/g (nd−0.86 nmol/g)]. *trans*-Resveratrol, *trans*-resveratrol-4′-*O*-glucuronide, and resveratrol diglucuronide were not detected in any tissue.

The highest concentrations of resveratrol metabolites were found in liver, followed by adipose tissue and skeletal muscle (Figure 1). A dose−response pattern was found in liver when total resveratrol metabolites were quantified. By contrast, in adipose tissue this pattern was only observed when RSV1 was compared with RSV2 and RSV3 groups (Figure 1). In skeletal muscle no resveratrol metabolites were detected in the RSV1 group, and no significant differences between groups RSV2 and RSV3 were observed (Figure 1).

Total glucuronide and sulfate metabolites in the three tissues are shown in Figure 1. When the distribution between these two kinds of metabolites was analyzed, different patterns of response were also found among tissues. In liver and adipose tissue, animals from the RSV2 group showed significantly higher amounts of glucuronide metabolites than animals from RSV1. Similar amounts of these metabolites were shown in the three tissues of animals from the RSV2 and RSV3 groups, since no statistical differences were obtained between them. A dose−response pattern in all the evaluated tissues was observed for sulfate conjugates. This pattern was also observed when metabolites derived from microbiota were considered (dihydroresveratrol and its conjugates), in the three tissues. Larger amounts of these metabolites than of resveratrol conjugates were detected in liver while the opposite was true in adipose tissue and skeletal muscle (Figure 2).

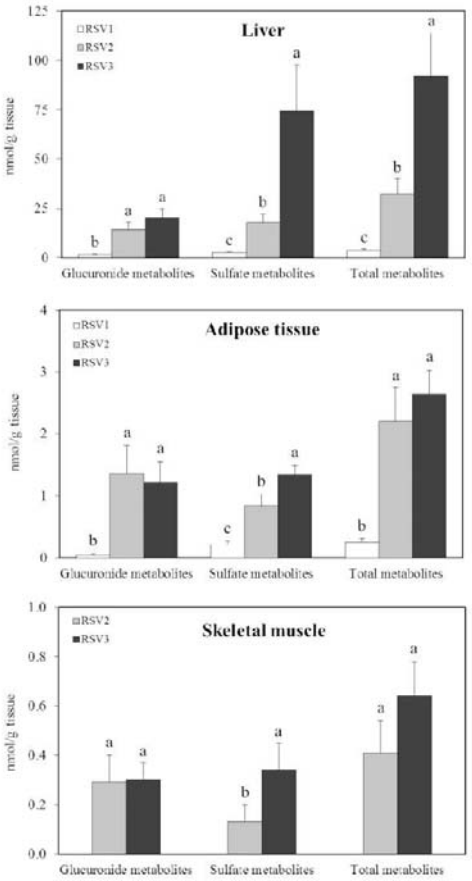

Figure 1. Glucuronide, sulfate, and total analyzed resveratrol metabolites in liver, adipose tissue, and skeletal muscle from rats receiving 6 mg/kg/d (RSV1), 30 mg/kg/d (RSV2), or 60 mg/kg/d (RSV3) of resveratrol for 6 weeks. Results are expressed as mean ± SEM. Statistics shows comparisons among the three dose groups for each metabolite type. Bars not sharing a common letter are significantly different ($P < 0.05$). No detectable amounts of resveratrol metabolites were found in the RSV1 group for the skeletal muscle. Data concerning total metabolites in adipose tissue from rats treated with 30 mg/kg/d have been previously presented.[43] (Values <LOQ considered as the LOD value.)

DISCUSSION

Resveratrol is a polyphenol with a very low availability. Due to its extensive metabolism, free resveratrol reaches very low concentrations in plasma and tissues.[6] Despite that, a great number of published studies have reported beneficial effects on health in animal experimental models. It can be hypothesized that resveratrol metabolites may be active per se, or that they provide a pool for local or systemic regeneration of resveratrol in vivo.[29] Due to these facts, it is essential to quantify resveratrol metabolites in tissues in in vivo studies. Nevertheless, tissue distribution of resveratrol and its metabolites has rarely been assessed compared with the number of papers that refer to the plasmatic concentrations of this polyphenol so far.

It is important to point out that our experimental design was not specifically planned to carry out a pharmacokinetic study. In fact, our first interest was to analyze the effects of resveratrol on body fat accumulation and serum parameters, and thus, rats were fed on an obesogenic diet.[21] We observed that the lowest dose (6 mg/kg/d) was without effect, the intermediate dose (30 mg/kg/d) induced a significant reduction in body fat, and the highest dose (60 mg/kg/d) did not improve the effect of 30 mg/kg/d. In the light of these results, we considered it very interesting to determine the amount of resveratrol and resveratrol metabolites found in target tissues involved in the

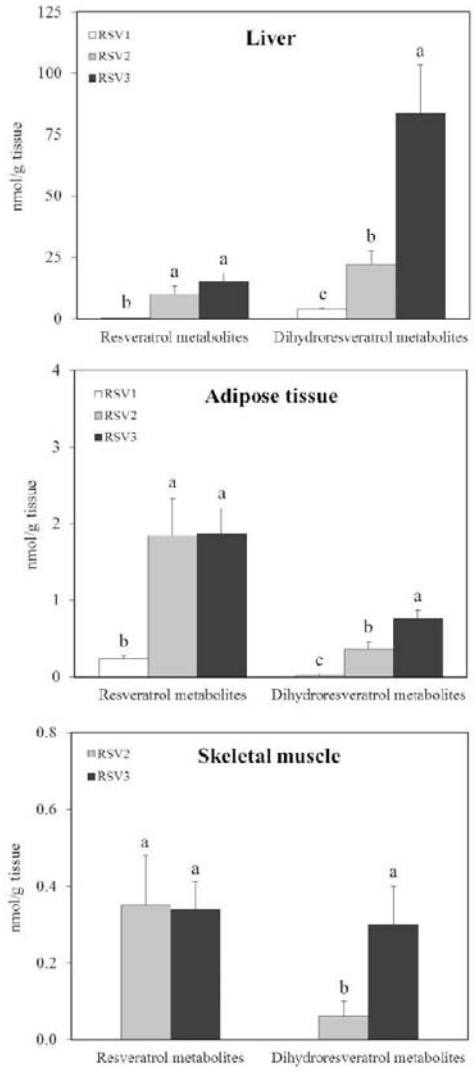

Figure 2. Resveratrol (glucuronide and sulfate conjugates) and dihydroresveratrol (free and its glucurono- and sulfoconjugates; microbial metabolism) metabolites in liver, adipose tissue, and skeletal muscle from rats receiving 6 mg/kg/d (RSV1), 30 mg/kg/d (RSV2), or 60 mg/kg/d (RSV3) of resveratrol for 6 weeks. Results are expressed as mean ± SEM. Statistics shows comparisons among the three dose groups for each metabolite type. Bars not sharing a common letter are significantly different ($P < 0.05$). No detectable amounts resveratrol metabolites were found in the RSV1 group for the skeletal muscle. (Values <LOQ considered as the LOD value.)

body-fat-lowering effect of this polyphenol, such as adipose tissue, liver, and skeletal muscle,[30−32] and we carried out the present study in the same cohort of animals, which were fed an obesogenic diet and fasted overnight before sacrifice.

These experimental conditions were not ideal for this type of study, and obviously this is a limitation of this work. Nevertheless, an interesting aspect should also be emphasized. Usually, resveratrol metabolite distribution has been analyzed after a single dose of resveratrol, or repeated doses for a short time.[6,15,33,34] By contrast, in the present study resveratrol metabolite distribution was analyzed after a long-term treatment (6 weeks) and this can provide additional data on this issue.

With regard to diet composition, feeding rats an obesogenic diet (high-fat diet) can modify resveratrol absorption. Vaz-da-Silva et al.[35] showed that a high-fat breakfast delayed the absorption of *trans*-resveratrol compared with fasted intake, but it did not influence the extent of absorption in humans after a single dose of 400 mg. La Porte et al.[36] reported decreased and delayed absorption of *trans*-resveratrol in combination with a high-fat breakfast as compared with a standard breakfast in humans treated with 2000 mg of resveratrol twice daily. Despite this fact, we believe that results concerning resveratrol metabolite distribution obtained under the experimental conditions which allow resveratrol to show body fat-lowering properties, can be helpful to explain this biological effect. The distribution of resveratrol under other experimental conditions should also be analyzed in order to gain more insight into this issue.

In the present study, resveratrol metabolites, but not their parent compound, were detected in the three tissues analyzed by the method described. Taking into account that rats were fasted overnight, the results are to be expected because other authors have shown that after its administration, free resveratrol is either absent or present only as trace amounts in plasma and tissues and that resveratrol metabolite concentrations are much higher than the concentrations of the parent compound.[4,5] It has been also reported that resveratrol is detected in plasma as soon as 15 min after administration, reaching peak concentrations at 60 min and then sharply decreasing. In contrast, some resveratrol metabolites are detectable in plasma even 3 h after administration; at this time only trace amounts of free resveratrol are found in plasma.[3,37]

Our results are in line with those published by Wenzel et al.,[6] who found resveratrol metabolites, but not free resveratrol, in kidneys after an overnight fast when rats were administered 300 mg/kg/d of this polyphenol. In liver they found a small amount of free resveratrol and higher amounts of its metabolites but they used a dose far higher than that used in the present study. Nevertheless, the fact that resveratrol was present but below the LOD cannot be discarded.

Most of the studies devoted to analyzing resveratrol metabolic profile in tissues focus on liver, kidney, lungs, brain, and testis.[6,34,38,39] However, as far as we know, only one study has evaluated resveratrol metabolites in adipose tissue and skeletal muscle.[40] In recent years the effectiveness of resveratrol as a body-fat-lowering agent has been demonstrated in rodents.[21,30−32,41] Adipose tissue and skeletal muscle, as well as liver, are targets for resveratrol; in fact different actions of this polyphenol, such as decreasing lipogenesis, increasing lipolysis, and increasing fatty acid oxidation, take place in these tissues and organs and they are mechanisms of action that explain the effect of resveratrol on the reduction of body fat accumulation.[21,30−32,41−43] Thus, describing the resveratrol metabolic profile in these tissues is an important issue.

As expected, in general terms, resveratrol metabolite amounts increased with increasing concentrations of the polyphenol in the diet. A clear dose−response pattern was found in liver when the three experimental groups were analyzed. By contrast, this pattern was observed in adipose tissue only when RSV1 and RSV2 groups were compared. In skeletal muscle, resveratrol

metabolites were not detected in the RSV1 group, and very similar amounts were detected in the RSV2 and RSV3 groups. This could suggest that these two tissues have a maximal capacity for resveratrol metabolite incorporation, which cannot be exceeded by increasing resveratrol administration. In the present study, the highest amounts of total resveratrol metabolites for the three analyzed doses were detected in liver. Indeed, other authors have reported that this organ has been found to be subjected to an important accumulation of these compounds in rodents.[6,34,38,39] Resveratrol metabolite amounts in adipose tissue were much higher than those in skeletal muscle. It has been reported that resveratrol is mainly distributed in abundant blood-supplied tissues, such as liver, lung, and kidney.[34,40] This is logical, but other factors also may influence this process because although skeletal muscle has a more active blood flow than adipose tissue, lower amounts of resveratrol metabolites were detected in this tissue. Thus, the potential contribution of differences among tissues in terms of transporters that excreted xenobiotics from different organs (members of the family of ATP-binding cassette) cannot be discarded.[34]

In this work, the resveratrol metabolic profile evaluated in the three tissues under fasting conditions and during long-term treatment includes glucuronide and sulfate conjugates of resveratrol together with those derived from the microbiota. Although glucuronide and sulfate conjugates of resveratrol have blood and tissue peaks about 3−6 h postadministration, it could be hypothesized that resveratrol is accumulated in tissues and released slowly over a longer period of time, as previously published for resveratrol[44] or other polyphenols.[45]

Only a few studies have considered the determination of microbial metabolites after resveratrol administration: Azorín-Ortuño et al. in a great number of tissues[40] and Wang et al.[46] in urine samples. Dihydroresveratrol conjugates appear later in the organism after the colonic microbial degradation of resveratrol and further absorption and phase II metabolism. Thus, in our study, dihydroresveratrol as free or as glucuronide and sulfate conjugates was assessed. These were the main metabolites in liver, but not in adipose tissue and skeletal muscle.

When the distribution between glucuronide and sulfate metabolites was analyzed, it was noteworthy that while sulfates increased when resveratrol dosage was enhanced, glucuronides increased between 6 and 30 mg/kg/d, but remained unchanged when resveratrol dosage reached 60 mg/kg/d. These results may suggest that glucuronidation, but not sulfation, is a saturable metabolic pathway, at least in the range of doses used in the present study. Nevertheless, the potential degradation of glucuronide metabolites, as well as a more rapid elimination, cannot be discarded. Consequently, further studies are needed to better analyze this issue. In the literature there are other studies that provide data that lend support to this theory. Thus, Kapetanovic et al.[47] observed a shift from glucuronidation to sulfation of resveratrol possibly due to the saturation of the glucuronide pathway. Maier-Salamon et al.[48] have already described a possible saturation of the enzymes responsible for resveratrol glucuronidation (UDP-glucuronosyltransferases UGT1A7 and UGT1A10), when increasing administered resveratrol concentrations, while noncompetitive substrate inhibition was shown for resveratrol sulfation.

These data can provide a clue to explain the results that we obtained, by using this cohort of animals, when the effects of resveratrol on body fat accumulation were assessed.[21] As we have explained before in this section, while resveratrol reduced body fat at a dose of 30 mg/kg/d, it did not at 6 mg/kg/d. Surprisingly, rats treated with 60 mg/kg/d resveratrol did not show further reduction. This means that a plateau was reached when the dose of resveratrol increased. Moreover, in a previous experiment that we performed in 3T-L1 adipocytes, glucuronide, but not sulfate metabolites, were shown to be active in reducing triacylglycerol accumulation (data submitted). Thus, it can be hypothesized that the lack of increase in glucuronides, metabolites that could be responsible in part for the body-fat-lowering effect of resveratrol, when the dose of this polyphenol increases from 30 to 60 mg/kg/d could help to explain the above-mentioned plateau effect. Nevertheless, the levels of these metabolites detected in adipose tissue and skeletal muscle are very low and thus it is difficult to understand how they are contributing to the effects observed. Consequently, more studies are needed to explain the in vivo effects induced by resveratrol after a long-term treatment.

In conclusion, this study describes that the largest amounts of resveratrol metabolites were found in liver, intermediate amounts were observed in adipose tissue, and the lowest amounts were seen in skeletal muscle. In general terms, a dose−response pattern was found in sulfate conjugates but not glucuronides. Metabolites derived from microbiota were detected in greater amounts in liver than resveratrol conjugates were, whereas the opposite was the case in adipose tissue and skeletal muscle.

AUTHOR INFORMATION

Corresponding Author

*Tel: +34-945-013067. Fax: +34-945-013014. E-mail: mariapuy.portillo@ehu.es.

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Notes

The authors declare no competing financial interest.

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En aquest estudi pilot en humans s'ha estudiat l'exposició plasmàtica i l'excreció urinària de compostos fenòlics de les salses de tomàquet, amb especial atenció a l'efecte matriu que aporta l'oli. L'estudi de l'efecte matriu en un altre tipus d'aliment i en compostos de característiques similars (polifenols) va ser de gran ajut en el decurs de la Tesi Doctoral, així com en el desenvolupament d'un estudi clínic d'intervenció amb humans.

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Oil matrix effects on plasma exposure and urinary excretion of phenolic compounds from tomato sauces: Evidence from a human pilot study

Sara Tulipani [a], Miriam Martinez Huelamo [a], Maria Rotches Ribalta [a,e], Ramon Estruch [b,c], Elvira Escribano Ferrer [d], Cristina Andres-Lacueva [a,e], Montserrat Illan [a], Rosa M. Lamuela-Raventós [a,c,*]

[a] Department of Nutrition and Food Science-CeRTA, Faculty of Pharmacy, University of Barcelona, Barcelona, Spain
[b] Department of Internal Medecine, Clinical Hospital, Institute of Biomedical Investigation August Pi i Sunyer (IDIBAPS), University of Barcelona, Spain
[c] CIBER 06/003 Physiopathology of Obesity and Nutrition (CIBEROBN), and RETICS RD06/0045/0003, Institute of Health Carlos III, Spain
[d] Department of Pharmacy and Pharmaceutical Technology, Biopharmaceutics and Pharmacokinetics Unit, Faculty of Pharmacy, University of Barcelona, Barcelona, Spain
[e] Ingenio CONSOLIDER Program, FUN-C-FOOD (CSD2007-063), Barcelona, Spain

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ABSTRACT

The health-promoting effects attributable to dietary phenolic compounds strongly depend on their bioaccesibility from the food matrix and their consequent bioavailability. We carried out a pilot randomized controlled cross-over study to evaluate the effect of addition of an oil matrix during tomato sauce processing, on the bioavailability of tomato phenolics. Healthy subjects consumed a single dose of tomato sauce elaborated without oil (OO-F) and with the addition of 5% virgin olive oil (VOO-E) or refined olive oil (ROO-E). Plasma and urine samples were subjected to solid-phase extraction, followed by HPLC–MS/MS analysis. Six phenolic compounds, three aglycones (naringenin, ferulic and caffeic acids) and their corresponding glucuronide metabolites, were identified and quantified in urine after the ingestion of the tomato sauces. Two of the six phenolic urinary metabolites were also quantified in plasma samples. Only after ingestion of the oil-enriched tomato sauces, did the glucuronide metabolites of naringenin show a bi-phasic profile of absorption in plasma, suggesting that the lipid matrix added to the sauce may stimulate the occurrence of re-absorption events by enterohepatic circulation, potentially enhancing the apparent plasma half-life of the flavanone prior to excretion. The interindividual response variability observed underlies the need for further large-scale investigations.

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1. Introduction

The Mediterranen diet is an example of a dietary regime associated with a reported low incidence of cancers, CVD, and an overall reduction in mortality (Chrysohoou, Panagiotakos, Pitsavos, Das, & Stefanadis, 2004; Estruch et al., 2006; Knoops et al., 2006). The health-promoting effects of this diet have been mainly attributed to the high consumption of the typical fruit and vegetables of the Mediterranean basin (i.e. leafy green vegetables and tomatoes). Tomato (*Lycopersicon esculentum* Mill., Solanaceae) and tomato processed products (sauce, paste, puree and juice) are typical components, besides being a dietary staple for humans in many other parts of the world. Another major characteristic of the Mediterranean diet is a high consumption of olive oil as the main source of monounsaturated fatty acids, which has long been reported to exhibit numerous biological functions beneficial for the state of health (Cicerale, Lucas, & Keast, 2010). The combination of tomatoes with olive oil, in food preparation, is a typical daily habit, and tomato sauce, the most

Abbreviations: ALA, aldehydic form of ligstroside aglycone; ALA ox, aldehydic form of ligstroside aglycone oxidized; AOA, aldehydic form of oleuropein aglycone; AOA ox, aldehydic form of oleuropein aglycone oxidized; AUC_{last}, area under the plasma concentration-*versus*-time curve from time 0 until the last detectable concentration; CVD, cardiovascular disease; CAD, collision-activated dissociation; CE, collision energy; DP, declustering potential; 3,4-DHPEA, hydroxytyrosol or 3,4-dihydroxyphenylethanol; 3,4-DHPEA-Ac, hydroxytyrosol-acetate; 3,4-DHPEA-EDA, hydroxytyrosol-elenolic acid di-aldehyde; DLA, dialdehydic form of ligstroside aglycone; DOA, dialdehydic form of oleuropein aglycone linked to 3,4-DHPEA-EDA oxidized; FP, focusing potential; FS, full scan; *p*-HPEA, tyrosol or *p*-hydroxyphenylethanol; *p*-HPEA-EDA, tyrosol–elenolic acid di-aldehyde; LOD, limit of detection; C_{max}, maximum plasma concentration; MRT, mean residence time; MI, molecular ion; NL, neutral loss; OO-F, olive oil-free tomato sauce; PFD, polyphenol-free diet; PrIS, precursor ion scan; PIS, product ion scan; ROO-E, refined olive oil-enriched; SPE, solid-phase extraction; SEM, standard error; T_{max}, time to reach the maximum plasma concentration; TFD, tomato-free diet; Q_{ex}, the maximum excreted amount in the 24 h urine collection; VOO-E, virgin olive oil-enriched.

* Corresponding author at: Department of Nutrition and Food Science-CeRTA, Faculty of Pharmacy, University of Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain. Tel.: +34 934024508; fax: +34 934035931.
E-mail address: lamuela@ub.edu (R.M. Lamuela-Raventós).

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extensively consumed tomato product worldwide, is commonly elaborated and commercialized, both as an oil-free and oil-enriched product. The protective effects of a regular consumption of raw tomatoes and tomato sauce (Giovannucci, 1999) have long been attributed to the most pronounced bioactives in tomato, such as lycopene and other carotenoids (Agarwal & Rao, 2000). Recently, the high content of phenolic compounds such as flavonoids and hydroxycinnamic acids in tomato has been gaining interest because of the multiple biological effects they seem to express, ranging from free radical-scavenging, metal chelation, inhibition of cellular proliferation, and modulation of enzymatic activity and signal transduction pathways (Crozier, Jaganath, & Clifford, 2009). The flavanone, naringenin (4′,5,7-trihydroxyflavanone) and the hydroxycinnamate, chlorogenic acid (5-caffeoylquinic acid) (Slimestad, Fossen, & Verheul, 2008; Vallverdú-Queralt, Jáuregui, Medina-Remón, Andrés-Lacueva, & Lamuela-Raventós, 2010) are among the most abundant phenolics in tomato, and the most extensively studied.

It is known that the extent of the protective effect of the phenolic compounds *in vivo* strongly depends on their bioaccesibility and their consequent exposition in the organism, on their intestinal absorption and presystemic metabolism, up to their systemic distribution and potential interaction with target tissues. In turn, the food matrix, in which the phenolic compounds are contained, is one of the most important factors governing the *in vivo* bioavailability of these compounds. It plays a crucial role in determining their accessibility and extractability from food, and thus their absorption, metabolism and their final biological action in the human body (Ortuño et al., 2010).

Despite growing knowledge of the effects of the thermic and mechanical treatments involved in tomato sauce processing on carotenoid and vitamin bioavailability (Graziani et al., 2003; Unlu et al., 2007), little is known about the changes in the polyphenol/flavonoid content of tomatoes after their processing to tomato sauce (Bugianesi et al., 2004; Vallverdú-Queralt, Medina-Remón, Andrés-Lacueva, & Lamuela-Raventós, 2011). Furthermore, as the combination of tomatoes and tomato products with a lipid matrix has been reported to favour the extractability and bioaccessibility of tomato carotenoids (Fielding, Rowley, Cooper & O'Dea, 2005; Graziani et al., 2003), recent investigations have also shown that the fat content in a meal may enhance the bioavailability of the flavonol quercetin, due to a better solubility of the relatively lipophilic flavonoid in the intestinal tract in the presence of fat (Lesser, Cermak, & Wolffram, 2004). The typical addition of oil, during tomato sauce processing, may influence the bioavailability of the phenolics contained in tomato by modifying their bioaccessibility from the food matrix, modulating the gastric emptying and/or the intestinal and hepatic metabolism of the absorbed phenolic compounds. However, few studies have yet investigated the absorption and excretion of phenolic compounds from raw tomatoes and tomato sauces, and even less information is currently available on the impact of tomato–olive oil combination, during processing, on the bioaccessibility and human absorption of the phenolic compounds contained in the sauce (Capanoglu, Beekwilder, Boyacioglu, Hall, & de Vos, 2008; Graziani et al., 2003).

The aim of the present study was to investigate whether the absorption and metabolism of the tomato phenolics is influenced by the addition of a lipid matrix during tomato sauce processing, and eventually by the oil typology. For these purposes, we carried out a randomized controlled cross-over study, administering (to human volunteers) a single dose of tomato sauce without oil (OO-F) and tomato sauces elaborated with the addition of virgin olive oil (VOO-E) or refined olive oil (ROO-E) during processing.

2. Materials and methods

2.1. Standards and reagents

Chlorogenic acid, caffeic acid, ferulic acid, isoferulic acid, *p*-hydroxybenzoic acid, protocatechuic acid, *m*- and *p*-coumaric acids, gallic acid, naringenin-7-*O*-glucoside, naringin (naringenin-7-*O*-rhamnoglucoside), quercetin, rutin (quercetin-7-*O*-rutinoside), kaempferol and blank human plasma were purchased from Sigma–Aldrich (St. Louis, MO, USA). Naringenin (4′,5,7-trihydroxyflavanone), the internal standard ethylgallate (IS, added to sample before extraction) and the external standard taxifolin (ES, added to sample after extraction) were purchased from Extrasynthese (Genay, France). HPLC-grade methanol, acetonitrile and formic acid were purchased from Scharlau Chemie S. A. (Barcelona, Spain), and *o*-phosphoric acid (85%) and hydrochloric acid (37%) from Panreac Quimica SA (Barcelona, Spain). Ultrapure water (Milli-Q) was obtained from a Millipore system (Millipore, Bedford, MA, USA).

2.2. Tomato sauce material

A commercial tomato (*L. esculentum* L.) variety, suitable for tomato sauce elaboration, was used for the study. Virgin and refined olive oils were kindly furnished by Juan Ballester Rosés company (S.A., Tortosa, Spain). The OO-F, VOO-E and ROO-E tomato sauces were processed at Torribera campus, University of Barcelona (UB, Barcelona, Spain) by a standardized industrial scale-like making process. Fruits were washed, chopped in a breaker unit and weighed. The VOO and the ROO were rapidly heated up to 110 °C before adding the chopped tomatoes (5% of oil, w/w), and the mixture was cooked at 99 °C for 90 min. The chopped tomatoes were crushed into pulp, which was then separated from seeds and skin, cooled down, aliquotted and stored in vacuum bags at −20 °C until the day of the test. Approximately 1000 g of fresh tomatoes yielded 500 g of tomato sauce. Five percent of water was added to the final OO-F tomato sauce product, in order to standardize the amount of tomato compounds ingested by each intervention.

2.3. Preparation of extracts

For the extraction of phenolic compounds from the starting raw tomatoes and the tomato sauces, 5 ml of 80% ethanol in ultrapure water (v/v), acidified with 0.1% formic acid, were added to 0.5 g of sample. The mixture was vortexed for 1 min and then sonicated for 5 min on ice. After centrifugation at 900g for 20 min at 4 °C, the supernatant was collected; a further 5 ml of the acidified 80% ethanol/water solution were added to the pellet, and the extraction procedure repeated. The two supernatants were combined, and the ethanolic component was evaporated to dryness by a sample concentrator (Techne, Duxford, Cambridge, UK) at room temperature under a stream of nitrogen gas. After filtration of the acqueous extracts (~2 ml) with 4 mm, 0.45 μm PTFE syringe filters (Waters Corporation, USA), 50 μl of the resulting filtrate were injected into the LC/MS/MS system, in triplicate.

2.4. Subjects and study design

A total of five healthy men, aged between 25 and 36 (BMI = 25 ± 1.2 kg/m^2), volunteered for this randomised crossover dietary study. The study was explained to subjects through verbal and written instructions, and written informed consent was obtained before participation. Only male subjects were recruited, in order to reduce anthropometric variables and the menstrual cycle phase-related variability in premenopausal women, putatively affecting the absorption, metabolism and excretion of tomato

phenolics. Subjects were non-smokers, had no history of cardiovascular, hepatic or renal disease and had not adhered to any special diets at least 4 weeks prior to participating in the study. To avoid any acute peak in the plasma phenolic concentration and to standardize the baseline point, subjects were asked to follow a tomato-free diet (TFD) during the 3 days preceding the dietary intervention, and a low-polyphenol diet (LPD) during the 24 h immediately preceding the test. A list of acceptable and nonacceptable foods was provided to the subjects, together with two standardised polyphenol-free dinners to be consumed in the evening before the test, and on the day of the test, respectively. On the day of each intervention, the subjects were asked to fill in a 24 h food record to assess their compliance with the recommended LPD. No significant differences were observed in total energy or macronutrient and micronutrient intakes during the 24 h before the interventions, calculated using Food Processor software (data not shown).

Using a computer-generated randomisation number sheet, each volunteer was assigned to receive (in consecutive experiments) the OO-F, the VOO-E and the ROO-E tomato sauce interventions. Between the interventions, after a "washout" period of a minimum 3 days on their usual diet, the subjects repeated the 3 day TFD and the 1 day LPD before being subjected to the following test.

On the day of the intervention, the overnight-fasted subjects had an intravenous catheter inserted, and a baseline blood sample (5 ml) was collected. Then, subjects consumed 100 g of one of the three interventions (OO-F, VOO-E and ROO-E tomato sauces) within a space of 5 min, and refrained from consuming other foods for the following 6 h. A standardized phenolic-free lunch was distributed to the volunteers 6 h after the ingestion of the test sauces. Blood samples (5 ml) were collected into lithium heparin tubes at 15, 30 min and 1, 2, 3, 4, 5, 6, 8, 12 and 24 h after the ingestion of the tomato sauces, and immediately centrifuged at 1500*g* for 20 min at 4 °C. All plasma samples were subsequently aliquotted and stored in Eppendorf tubes at −80 °C prior to analysis. Urine was collected in plastic bottles 10–20 min before consumption of the tomato sauces (baseline, 0 h) and the 24 h-urine after the ingestion of the interventions was collected in four separate fractions (0–4, 4–8, 8–12 and 12–24 h), the participants being instructed to empty their bladders before a new fraction was collected. The urine bottles were stored at 4 °C and, immediately after the participants had started, to collect the next fraction; the amount of urine in each fraction was measured, acidified with 0.2 M HCl and stored at −80 °C prior to analysis. The study protocol was approved by the Ethics Committee of Clinical Investigation of the University of Barcelona (Spain), and the clinical trial was registered with the International Standard Randomized Controlled Trial Number (ISRCTN20409295). Informed consent was obtained from all participants.

2.5. Plasma and urine extraction procedure for tomato phenolic metabolites

Tomato sauce phenolic metabolites in plasma and urine were extracted by a solid-phase extraction (SPE) procedure, previously described and optimized by our work group (Mata-Bilbao et al., 2007), using a Waters Oasis HLB 96-well plate of 30 μm (30 mg) (Waters Oasis, Milford, MA, USA).

On the day of the analysis, the samples were defrosted on ice in the dark. Prior to SPE, urine samples (1 ml) were centrifuged at 15,000*g* for 4 min, at 4 °C, while plasma (1 ml) was acidified by adding 25 μl of *o*-phosphoric acid, to break down protein–polyphenol interactions and reach the optimum pH for phenolic retention in the solid-phase during the extraction procedure (Mata-Bilbao et al., 2007). One millilitre aliquots of plasma and urine samples were spiked with 100 μl of 2nM ethyl gallate as internal standard. After 2 min of vortex-mixing, samples were diluted 1:1 (v/v) with water. Plate activation was achieved by consecutively adding 1 ml of methanol and 1 ml of 1.5 M formic acid in water. After the sample loading into the 96-well plate, sample clean-up was performed with 1 ml of 1.5 M formic acid in water, followed by 1 ml of 5% methanol aqueous solution (v/v). Phenolic metabolites were then eluted with 1 ml of methanol acidified with 0.1% formic acid (v/v). The eluted fraction was evaporated to dryness by a sample concentrator (Techne, Duxford, Cambridge, UK), at room temperature under a stream of nitrogen gas, and reconstituted with 100 μl of water containing 0.1% formic acid and IS2 (500 ppb). The 96-well plate was then vortexed for 5 min and the reconstituted extracts transferred into a 96-well plate suitable for LC–MS/MS analysis.

For calibration, a standard mixture of commercially available naringenin, chlorogenic acid, caffeic acid, ferulic acid and quercetin was serially diluted, in both water and blank human plasma (Sigma–Aldrich, USA) (1–500 ppb). The standard solutions were treated exactly in the same way as the samples and subjected to the extraction procedure; therefore, the results could be read directly from the standard curve and no corrections due to the recovery losses had to be made.

Standard preparation and sample treatments were performed in a dark room with a red safety light to avoid oxidation of the analytes.

2.6. LC–MS/MS analysis

The native phenolic compounds present in raw tomatoes and in OO-F, VOO-E and ROO-E tomato sauces, as well as the corresponding phenolic metabolites putatively expected in urine and plasma samples, were identified and quantified by LC–MS/MS analysis.

Liquid chromatography separations were performed on an HP Agilent Technologies 1100 LC system (Waldbronn, Germany) equipped with a quaternary pump and a refrigerated autosampler. Coupled to the HPLC system, an Applied Biosystems API 3000 triple quadrupole mass spectrometer (PE Sciex, Concord, Ontario, Canada), equipped with a TurboIon spray source, was used in negative mode, to obtain mass spectrometry (MS) and MS/MS data. TurboIon spray source settings were as follows: capillary voltage, −4000 V; nebulizer gas (N_2), 10 (arbitrary units); curtain gas (N_2), 12 (arbitrary units); collision gas (N_2), 10 (arbitrary units); entrance potential, 10 V; drying gas (N_2), heated to 400 °C and introduced at a flow rate of 8000 cm^3/min. The system was controlled by the software analyst v. 1.4.2 supplied by Applied Biosystems (Foster City, CA, USA).

For both the analysis of the plant and the biological sample extracts, Phenomenex (Torrance, CA, USA) Luna C18 (50 × 2.0 mm i.d., 5 μm) and Phenomenex securityguard C18 (4 × 3 mm i.d.) columns were used at 30 °C, with a sample injection volume of 50 μl. Gradient elution was performed with a binary system consisting of [A] 0.1% acqueous formic acid and [B] 0.1% formic acid in acetonitrile. An increasing linear gradient (v/v) of [B] was used [*t*(min), %B], as follows: (a) (0, 5), (10, 18), (13, 100), (14, 100) and (15, 5), followed by a 5 min re-equilibration step, at a constant flow-rate of 400 μl/min; (b) (0, 5), (2, 25), (10, 90), (11, 100) and (12, 100), followed by a 5 min re-equilibration step, at a constant flow-rate of 600 μl/min, respectively, for the analysis of the tomato/tomato sauce extracts and the plasma/urine extracts.

Prior to the HPLC–MS/MS analysis of the samples, direct infusion experiments were carried out with each individual standard compound available. Briefly, a 50:50 (v/v) [A, B] solution of each standard compound (10 μg/ml) was injected into the mass spectrometer, using a model syringe pump (Harvard Apparatus, Holliston, MA, USA), at a constant flow rate of 5 μl/min, and manual tuning was performed in negative mode. For each standard com-

Table 1
List of the phenolic compounds identified in the three tomato sauces in study.

Peak number	Compound	Rt (min)	Molecular Formula	Ions full scan MS		MS/MS experiments			Comparison with standard
				$[M-H]^-$	Fragments	PIS	NL	PrIS	
1	Protocatechuic acid	2.08	$C_7H_6O_4$	153	109	–	–	–	Yes
2	3-Caffeoylquinic acid	2.94	$C_{16}H_{18}O_9$	353	191, 179, 135	353	162	–	No
3	Caffeic hexose II	3.27	$C_{15}H_{18}O_9$	341	179, 135	341	162	179	No
4	Caffeic hexose IV	4.19	$C_{15}H_{18}O_9$	341	281, 251, 221, 179, 161, 135	341	162	179	No
5	Homovanillic hexose 1	4.51	$C_{15}H_{20}O_9$	343	181, 137, 121	343	162	–	No
6	Ferulic hexose	4.75	$C_{16}H_{20}O_9$	355	193, 149, 134	355	–	193	No
7	Caffeic hexose II	4.94	$C_{15}H_{18}O_9$	341	179, 135	341	162	179	No
8	Homovanillic hexose 2	5.04	$C_{15}H_{20}O_9$	343	181, 1371	343	162	–	No
9	Coumaric hexose	5.25	$C_{15}H_{18}O8$	325	163, 119	–	162	–	No
10	5-Caffeoylquinic acid	5.38	$C_{16}H_{18}O_9$	353	191, 179, 161	353	162	–	Yes
11	3,4-Dihydroferulic hexose	5.77	$C_{16}H_{22}O_9$	357	195 , 135, 151	357	162	–	No
12	4-Caffeoylquinic acid	6.15	$C_{16}H_{18}O_9$	353	191, 179, 173, 155, 135	353	162	179	No
13	Quercetin dihexose deoxyhexose	7.08	$C_{33}H_{40}O_{21}$	771	609, 300	771	–	–	No
14	Rutin	11.40	$C_{27}H_{30}O_{16}$	609	301	609	–	–	Yes
15	Naringenin chalcone 3′,5′-di-C-hexose	12.09	$C_{27}H_{34}O_{15}$	597	477, 417, 387, 357	597	–	–	No
16	Dicaffeoylquinic acid	13.20	$C_{25}H_{24}O_{12}$	515	353, 179, 173	515, 353	162	353	No
17	Naringenin	13.87	$C_{15}H_{12}O_5$	271	151, 107, 119	–	–	–	Yes

Table 2
Quantification of the main identified phenolic compounds in the raw tomatoes and in OO-F, VOO-E and ROO-E tomato sauces in study. Values are means ± SEM. In each row, different letters correspond to significantly different concentrations ($p < 0.05$).

Compound	Rt (min)	MRM (m/z)	Raw Fruit (μg/g FW[A])	OO-F sauce (μg/g FW[A])	ROO-E sauce (μg/g FW[A])	VOO-E sauce (μg/g FW[A])
Protocateichuic acid		153 → 109	–	129 ± 6.7	144 ± 5.6	128 ± 14.4
3-Caffeoylquinic acid	2.94	353 → 191	4.3 ± 0.6b	60.3 ± 1.1a	61.2 ± 2.1a	59.8 ± 6.4a
5-Caffeoylquinic acid	5.38	353 → 191	234 ± 46.1c	814 ± 15.0a	552 ± 21.8b	652 ± 34.9b
4-Caffeoylquinic acid	6.15	353 → 191	149 ± 0.5 c	214 ± 6.9a	178 ± 0.3b	197 ± 21.8a,b
Dicaffeoylquinic acid	13.20	515 → 353	36.0 ± 7.9	54.3 ± 5.1	61.1 ± 1.4	48.4 ± 8.5
Caffeic hexose II	3.27	341 → 179	235 ± 44.9b	437 ± 9.4a	280 ± 4.0b	287 ± 34.0b
Caffeic hexose IV	4.19	341 → 179	98.1 ± 15.0b	151 ± 1.3a	106 ± 4.2b	107 ± 12.2b
Caffeic hexose II	4.94	341 → 179	5.5 ± 0.8	6.1 ± 0.1	7.4 ± 2.9	7.7 ± 0.7
Ferulic -hexose	4.75	355 → 193	104 ± 15.1b	239 ± 12.2a	242 ± 45.6a	157 ± 2.9a,b
	Total phenolic acids orally administered (mg)[B]			210.45	163.27	164.36
Naringenin	13.87	271 → 151	164 ± 12.1 b	1347 ± 63.6a	1296 ± 212a	1305 ± 238a
	Total flavanones orally administered (mg)[B]			134.7	129.6	130.5
Rutin	11.40	609 → 301	1.40 ± 0.19 c	3.01 ± 0.04a	2.16 ± 0.04b	2.23 ± 0.30b
	Total flavonols orally administered (mg)[B]			0.301	0.216	0.223

[A] FW: fresh weight.
[B] Corresponding to the dose orally administered (100 g) to the subjects.

pound, the molecular ion (MI) $[M-H]^-$ and the most abundant fragment ions (m/z) were easily detected, and the optimal focusing potential (FP), declustering potential (DP) and collision energy (CE) for the MI fragmentation were noted.

Phenolic compounds were identified by (a) chromatographic retention times, (b) full scan (FS) data, and (c) MS/MS experiments. In the plant extracts, the identity of the phenolic compounds was confirmed by combining product ion scan (PIS), neutral loss (NL), and precursor ion scan (PrIS) MS/MS experiments. In plasma and urine samples, due to the lower concentration of the phenolic metabolites investigated and consequently the need for greatest sensitivity, only PIS experiments were successfully used for identification purposes.

The PIS experiments were carried out at optimal and at double the CE values suggested by the direct infusion experiments in order to confirm the fragmentation pattern of each metabolite, using a cycle time of 2 s. MS/MS product ions were produced by collision-activated dissociation (CAD) of the selected precursor ions in the collision cell of the triple-quadrupole mass spectrometer, and the mass analysed using the second analyser of the instrument.

In parallel, the phenolic compounds in tomatoes/tomato sauce extracts, and the metabolites putatively expected in the biological fluids were quantified by multiple reaction monitoring (MRM), using suitable transitions (Jaganath, Mullen, Edwards, & Crozier, 2006). Phenolic standard curves were constructed with the standard solutions previously described, and conjugated metabolites of the available standard phenolic compounds were quantified using the calibration curves of the corresponding native unconjugated molecules.

The mean recovery of analytes ranged from 96% to 100% in plasma and 65–104% in urine, and the limit of detection (LOD) varied between 0.7 and 30 ng/ml, in plasma and urine. Precision met acceptance criteria (<8% RSD in plasma and <7% RSD in urine).

2.7. Calculation of pharmacokinetic parameters

The pharmacokinetic parameters were calculated by a non-compartmental analysis, using the WinNonlin Professional software version 2.0 (Pharsight Corporation, California, USA). For each phenolic metabolite detected and quantified, the maximum plasma concentration (C_{max}) and the time to reach it (T_{max}) were

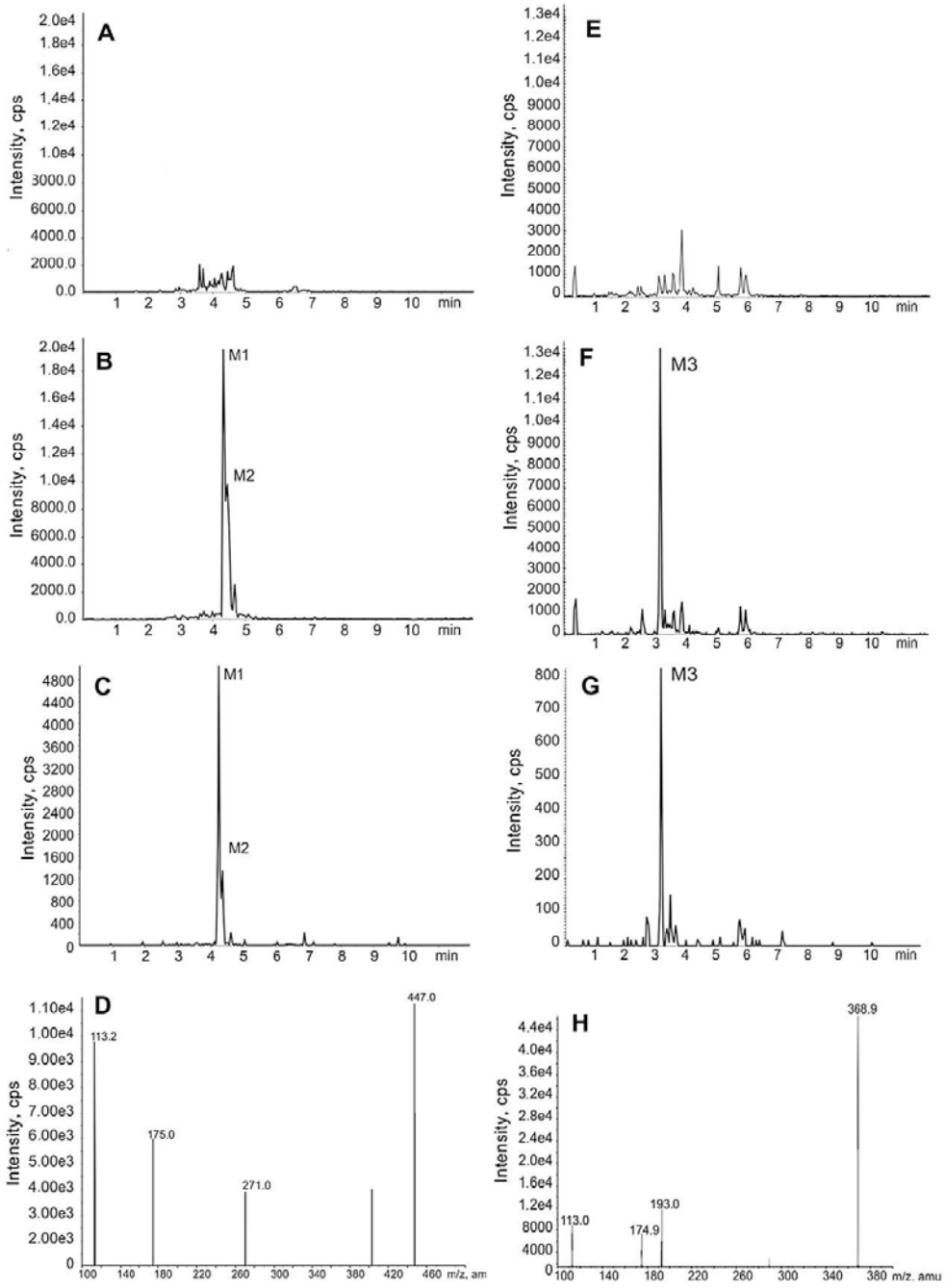

Fig. 1. MRM trace chromatograms of naringenin-7-*O*-glucuronide (M1) and naringenin-4′-*O*-glucuronide (M2) in urine at baseline (0 h) (A), at 4 h (B) and in plasma at 1 h (C) after tomato sauce consumption, and product ion spectra of M1 or M2 in urine (D). MRM trace chromatograms of (iso)ferulic glucuronide (M3) in urine at baseline (0 h) (E), at 4 h (F) and in plasma at 2 h (G) after tomato sauce consumption, and corresponding product ion spectra of M3 in urine (H).

taken directly from the observed data. The area under the plasma concentration-*versus*-time curve from time 0 until the last detectable concentration (AUC_{last}) was calculated using the linear trapezoidal method. The mean residence time (MRT_{last}) was estimated by means of the ratio AUMC/AUC, where AUMC is the first moment curve. The C_{max}-to-AUC_{last} ratio and the maximum excreted amount in the 24 h urine collection (Q_{∞}) were also calculated for each metabolite.

2.8. Statistical analysis

The statistical analysis of all data was performed using SPSS software (Version 17.0, Japan Inc., Tokyo, Japan). Differences at $p < 0.05$ were considered statistically significant. The phenolic composition of the three tomato sauces was subjected to one-way ANOVA analysis of variance for mean comparison. Data are reported as means ± standard deviation (SD). For each phenolic metabolite quantified in plasma and urine samples, the pharmacokinetic indices obtained, following the three dietary interventions, were compared and subjected to statistical analysis. All data are expressed as means ± standard error (SEM). C_{max}, AUC_{last}, MRT_{last} and C_{max}/AUC values were log-transformed and subjected to one-way ANOVA analysis of variance, while T_{max} data were compared by a non-parametric Kruskal–Wallis test.

3. Results and discussion

3.1. Phenolic composition of tomatoes and tomato sauces

The phenolic compounds identified in tomatoes and tomato sauces administered to the subjects are listed in Table 1, and the contents of the main quantified phenolic acids, flavanones and flavonols are listed in Table 2. Phenolic acids were the most abundant phenolic compounds found in all extracts, particularly hydroxycinnamic acids and their ester conjugates (mono- and di-caffeoylquinic acids, caffeic hexoses, ferulic and dihydroferulic hexoses, coumaric hexose). Chlorogenic acid (5-caffeoylquinic acid) was the most abundant phenolic acid in all three tomato sauces (Slimestad et al., 2008), while the flavanone naringenin (4′,5,7-trihydroxyflavanone) was the most abundant phenolic compound in all the samples, confirming its high content in tomato and tomato products.

When comparing the phenolic profile of the raw tomato fruits and the final tomato sauces, no qualitative differences were observed among them. However, from a quantitative point of view, the phenolic composition of the raw tomatoes significantly differed from the sauces in their lower contents of chlorogenic (5-caffeoylquinic acid) and neochlorogenic acids (3-caffeoylquinic acid), rutin (the major quercetin-glycoside in tomato), and especially in the 8-fold lower concentration of naringenin (Table 2). These data confirmed that heat and mechanical treatments, during the tomato sauce-making process) may induce biochemical changes in the food matrix, positively affecting the bioaccessibility of phenolics from the processed products. In addition, these findings seem to confirm that, as for carotenoids, naringenin bioaccessibility is particularly limited by interactions with the food matrix and improved by mechanical and heat treatments. Data on tomato fruit physiology are consistent with this consideration, since naringenin is trapped in the cutin matrix of the membrane of the ripe fruit where it strongly interacts with the insoluble polyesters of tomato fibre (Laguna, Casado, & Heredia, 1999). Mechanical and heat treatments may provide the energy necessary to break the interactions, potentially improving naringenin bioaccessibility *in vivo*.

When comparing the phenolic content of the tomato sauces, however, a quite homogeneous composition was observed among the three processed products (Table 2). The addition of olive oil during processing, did not seem to increase the extractability and accessibility of the tomato phenolic compounds. On the contrary, the OO-F tomato sauce revealed itself to be significantly richer in chlorogenic acid and caffeic hexose derivatives than the oil-enriched sauces, suggesting that the tomato-olive oil combination, during sauce elaboration, may not necesarily increase the phenolic content of the final product. For completeness, the oils added to the sauces were analyzed for their phenolic contents. The extraction and characterization of the phenolic fraction were achieved by HPLC with a diode array detector, as previously described (Boselli, Di Lecce, Strabbioli, Pieralisi, & Frega, 2009). As expected, no detectable phenolic compounds were observed in the ROO. In VOO, the phenylethyl alcohols typical of olive oil, *p*-HPEA (tyrosol), 3,4-DHPEA (hydroxytyrosol) and its acetate derivative (3,4-DHPEA-Ac), the flavones luteolin and apigenin, the aldehyde vanillin and eight secoiridoids, exclusively present in VOO and extra-VOO (3,4-DHPEA-EDA, DOA, *p*-HPEA-EDA, DLA, AOA, AOA ox, ALA, ALA ox), were present in the range of concentrations usually observed in VOO (0.9–22 ppm) (Boselli et al., 2009) (data not shown). As expected, none of the phenolic compounds observed in the tomatoes/tomato sauces were identified in the olive oils added to the sauce.

3.2. Identification of phase II phenolic metabolites in human plasma and urine

In total 134.7, 129.6 and 130.5 mg of flavanones (naringenin), 210.5, 163.3 and 164.4 mg of phenolic acids (predominantly caffeoylquinic acids) and 0.30, 0.21 and 0.22 mg of flavonols (rutin) were orally administered to the subjects through the 100 g dose of OO-F, VOO-E and ROO-E, respectively (Table 2). The presence of the most abundant phenolic compounds identified in the tomato sauces (naringenin, caffeoylquinic acids, caffeic acid, ferulic acid and rutin) and their most probably expected phase II conjugated forms (with methyl, glucuronic acid and sulfate groups) were investigated in both biological fluids.

With regard to the flavanone naringenin, the unconjugated native compound was detected only in urine samples. By contrast, two mass signals at m/z 447 → 271 were detected in both plasma and urine samples, indicating the presence of two partially co-eluting glucuronide (+176 amu) derivatives of naringenin (M1 and M2, Fig. 1A–C). These two metabolites were confirmed to be two isomeric naringenin glucuronides by their MS fragmentation patterns (MS2 ions at m/z 271, 175 and 113), as shown in Fig. 1D. It is known that naringenin has three possible sites for conjugation: 7-,4′- and 5-OH, with the 5-OH being the least reactive due to its low acidity (Zhang & Brodbelt, 2004). In view of the identification of naringenin-7-*O*-glucuronide and naringenin-4′-*O*-glucuronide in rat urine after the administration of naringenin chalcone from tomato skin extracts, and considering that the 7-*O*-glucuronide elutes from a reversed phase HPLC column before its 4′-isomer (Davis, Needs, Kroon, & Brodbelt, 2006), M1 was tentatively identified as naringenin-7-*O*-glucuronide and M2 as naringenin-4'-*O*-glucuronide. Nevertheless, the quantification of naringenin glucuronides was expressed as the sum of M1 and M2 in LC/MS/MS.

No mass signals from chrologenic acid (m/z 353 → 191) were detected in either plasma or urine samples, indicating the virtual absence of unmetabolized caffeoylquinic acids in the fluids. These findings are in keeping with previous studies on the urinary recovery of chlorogenic acid in humans and rats (Cremin, Kasim-Karakas, & Waterhouse, 2001), which failed to detect the native compound in urine after an acute dose, suggesting that it may undergo a more complete metabolism than the unesterified hydroxycinnamates. In fact, the gut microflora of animals and humans seem to be able to hydrolyze chlorogenic acid into caffeic acid and quinic acid (Plumb et al., 1999); thus, a combination of phase II conjugated metabolites

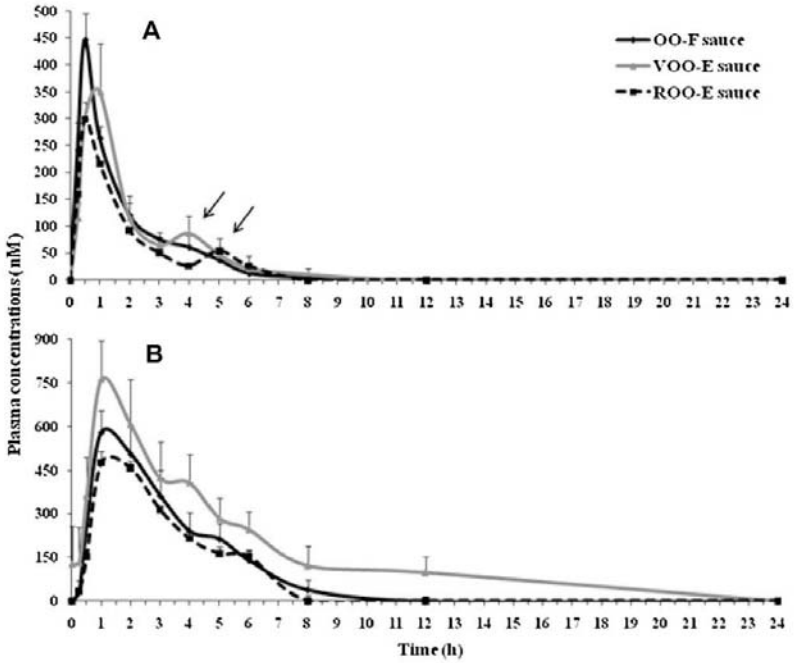

Fig. 2. Plasma naringenin (A) and ferulic (B) glucuronide concentrations in healthy subjects ($n = 5$) after single dose ingestions of OO-F, VOO-E and ROO-E tomato sauces. Values are means ± SEM.

of caffeic and ferulic acids (i.e. glucuronides and/or sulfates) would be identified in biological fluids after administration of chlorogenic acid (Azuma et al., 2000). In line with these findings, free caffeic (*m/z* 179 → 135) and ferulic (*m/z* 193 → 134) acids were clearly detected in urine samples after the ingestion of the tomato sauces, and peaks identification was confirmed by co-chromatography and fragmentation data with respect to the commercial standards. A mass signal at *m/z* 369 → 193 (Fig. 1E–G) and one at *m/z* 355 → 179 were also detected, both indicating the loss of a glucuronide unit (−176 amu) within the two molecules. The respective MS fragmentation pattern confirmed their identification as glucuronide derivatives of (iso)ferulic acid (Fig. 1H) and caffeic acid, respectively. Besides, being possible metabolites of the caffeoylquinic acids present in the sauces, these compounds may also originate from the metabolism of caffeic or ferulic hexoses originally contained in the tomato sauces (Stalmach et al., 2009). The mass signal for the ferulic glucuronide was also observed in plasma, and the metabolite peak was consistent with that found in urine. By contrast, the virtual absence of free naringenin, ferulic and caffeic acids and caffeic glucuronide in plasma samples might indicate that these compounds should be present at concentrations below the limit of detection of the instrument, probably because of their extensive first-pass metabolism via phase II conjugation, and their quick excretion via the kidney.

Rutin (quercetin-3-*O*-rutinoside) was the only flavonol found in the tomato sauces administered. As recently described (Jaganath et al., 2006), rutin is extensively metabolised and made bioavailable. Following the release of the aglycone, quercetin would in fact undergo glucuronidation and methylation, with quercetin-3-glucuronide and its methylated form isorhamnetin-3-glucuronide appearing in the circulatory and the excretory system. Further phase II metabolism could also occur in the liver and/or kidneys, resulting in the formation of quercetin-4′-glucuronide, quercetin-3′-glucuronide and combined conjugated forms, such as quercetin diglucuronide and methylquercetin diglucuronide. In the present study, all the above-cited phase II metabolites of rutin were investigated in plasma and urine of the subjects, but no mass signals from flavonols were detected after consumption of the three tomato sauces. The virtual absence of these compounds, expected mainly in urine samples, may be explained by the much lower amount of rutin ingested by the subjects in our study (an average of 246 μg among the three tomato sauces administered) compared to the previous investigation (176 μmoles, corresponding to 107.4 mg of rutin), which probably compromised the possibility of observing any metabolite peaks above the limit of detection of the HPLC–MS system. Additionally, it is known that derivatives containing rhamnose, such as rutinosides, are absorbed ~10 times less than are glucosides in the small intestine but, once transferred to the large intestine, they are degraded by the rhamnosidases of the intestinal microbiota (Manach et al., 1997). For these reasons, future research will include the study of the phenolic metabolites putatively originating from the intestinal microbiota, in order to fully evaluate the bioavailability of tomato and tomato sauce phenolics after oral ingestion.

Finally, no evidence of sulfation was detected in the present investigation. These findings confirmed previous observations that glucuronidation is the preferential conjugation for naringenin and rutin, although, until now, it has not been reported for the metabolism of chlorogenic, caffeic and ferulic acids.

3.3. Pharmacokinetics of naringenin glucuronides after ingestion of the three tomato sauces

The mean plasma concentration-*versus*-time curves for naringenin (A) and ferulic (B) glucuronides from the three tomato sauces

Table 3
Pharmacokinetic indices for naringenin and ferulic glucuronides after single ingestion (100 g) of OO-F, VOO-E and ROO-E tomato sauces by healthy subjects ($n = 5$). Values are means ± SEM.

		C_{max} (nmol/l)	T_{max} (min)	AUC_{last} (nmol/x min)	MRT (min)	C_{max}-to-AUC_{last} (ratio)
Naringenin glucuronide	OO-F Sauce	446 ± 48.8	30.0 ± 0.0	43559 ± 5630	99.6 ± 16.3	0.0105 ± 0.0011
	VOO-E Sauce	398 ± 86.8	45.0 ± 7.7	37649 ± 6794	91.7 ± 12.7	0.0106 ± 0.0013
	ROO-E Sauce	299 ± 30.1	30.0 ± 0.0	32009 ± 6705	106 ± 12.5	0.0113 ± 0.0025
Ferulic glucuronide	OO-F Sauce	580 ± 85.4	72.0 ± 12.0	118949 ± 26786	158 ± 10.6	0.0056 ± 0.0008
	VOO-E Sauce	727 ± 165	75.0 ± 13.4	197702 ± 53354	218 ± 56.6	0.0056 ± 0.0019
	ROO-E Sauce	544 ± 101	84.0 ± 14.7	96531 ± 20455	149 ± 14.0	0.0061 ± 0.0008

are shown in Fig. 2. The pharmacokinetic parameters for the two metabolites encountered in plasma are shown in Table 3.

None of the subjects had measurable concentrations of glucuronide metabolites of naringenin before ingestion of the sauces. By contrast, the two naringenin glucuronides identified already appeared in plasma 15 min after consumption (Fig. 2A), and their plasma levels decreased below the LOD within 12 h. The short T_{max} for naringenin glucuronides (Table 3) confirmed the rapid intestinal absorption and metabolism of naringenin to these phase II conjugates (Bredsdorff et al., 2010), and stressed the importance of an early collection of plasma samples during acute consumption studies, in order not to subestimate the plasma pharmacokinetics of these metabolites. When comparing the pharmacokinetic parameters obtained with the three interventions, higher C_{max} and AUC_{last} values were observed after the ingestion of the sauce without olive oil. However, the differences were not supported by the statistical analysis ($p > 0.05$, see Table 3). The absence of statistical significance in the differences observed among the three interventions was probably influenced by the great interindividual variability observed in the C_{max} and AUC_{last} values among the subjects (see SEM in Table 3). Also the apparent delay in the T_{max}, observed after the ingestion of VOO-E sauce, was only ascribed to two of the five subjects, resulting without statistical significance.

Interestingly, when comparing the plasma concentration-*versus*-time curves of the naringenin glucuronides in respect to the intervention administered, a typical biphasic absorption profile was only observed following the ingestion of the oil-enriched tomato sauces, with a first peak at 30 min and a second one at 4–5 h (Fig. 2A, see the arrows). These findings may suggest the occurrence of re-absorption events by enterohepatic circulation, specifically stimulated by the tomato–oil combination. It is known that polyphenols undergo repeated recycling through the digestive system, because of the enterohepatic and enteric recycling, which afford their apparent plasma half-life much longer than would be predicted from their intrinsic clearance value. A longer half-life may facilitate the pharmacological functions of polyphenols, since conjugated types may still be good bioactive compounds and target tissues often possess hydrolases (e.g. β-glucosidase or sulfatases) that can reconvert conjugates back to parent compounds. A recent dispositional study (Xu et al., 2006) revealed that large

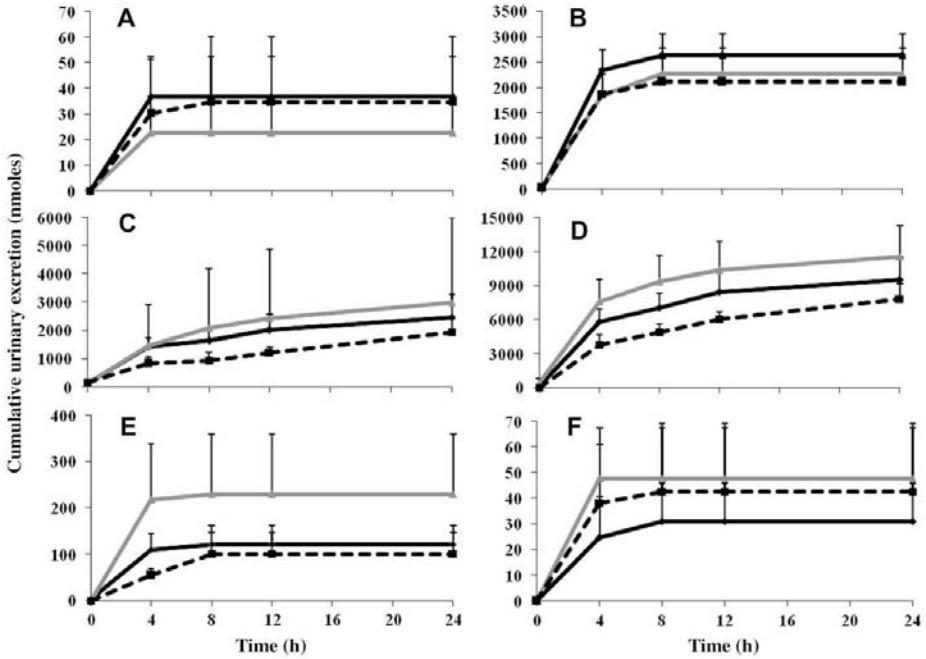

Fig. 3. Cumulative urinary excretion curves of healthy subjects ($n = 5$) for naringenin (A), naringenin glucuronides, (B) ferulic acid (C), ferulic glucuronide (D), caffeic acid (E) and caffeic glucuronide (F) after single dose ingestions of OO-F (black line), VOO-E (grey line) and ROO-E (black dashed line) tomato sauces. Values are means ± SEM.

amounts of one of the naringenin glucuronides (putatively M2) are found in the bile, suggesting that both intestine and liver organs are important contributors to the naringenin first pass metabolism. However, to our knowledge, no literature data are currently available on the extent and mechanisms of hepatic biotransformation of naringenin, particularly in the presence of a lipid matrix in the meal.

3.4. Pharmacokinetics of ferulic glucuronide after ingestion of the three tomato sauces

Higher C_{max}, T_{max}, AUC_{last} and MRT were observed for the ferulic glucuronide in respect to the naringenin glucuronides in plasma. The higher T_{max} and lower C_{max}-to-AUC_{last} ratio confirmed the slower rate of appearance in plasma of ferulic glucuronide (Fig. 2B), which is in agreement with the longer and more complex metabolism leading to the compound. On the other hand, the metabolite remains detectable in plasma for a longer period (MRT 2.5 h, in respect to 1.5 h of the naringenin glucuronide). When comparing the kinetic behaviour of ferulic glucuronide dependently on the sauce administered, apparently higher C_{max}, AUC_{last} and MRT were observed after the ingestion of VOO-E tomato sauce (Table 3). However, the differences were not statistically significant since, as for the plasma kinetics of naringenin glucuronides, high interindividual variations in the C_{max} and AUC_{last} were remarkable. The C_{max}-to-AUC_{last} ratio index did not reveal relevant variations among the three interventions, confirming no significant differences in the rate of appearance of ferulic glucuronide.

3.5. Urinary excretion of phenolic metabolites after ingestion of the three tomato sauces

The cumulative urinary excretion curves for the phenolic metabolites detected in urine are shown in Fig. 3. With the exceptions of ferulic acid and ferulic glucuronide (Fig. 3C and D), the phenolic compounds were mainly excreted into the 0- to 4 h urinary fraction collected after ingestion of the three tomato sauces (ranging from 54% excretion of ferulic acid derivatives up to 91%, for naringenin and naringenin glucuronides). The parent compound, naringenin, that was not observed in plasma after the tomato sauce ingestions, appeared in the 24 h urine to an extremely lower extent (Q_∞ = 37 nmoles) in respect to its glucuronide metabolites (Q_∞ = 2628 nmoles), confirming the rapid metabolism of the compound, and was rapidly excreted after ingestion (Fig. 3A and B). The free ferulic acid, not detected in plasma after the three interventions, was also excreted to a lesser extent than its glucuronide metabolite (Fig. 3C and D). However, both the free acid and its phase II conjugate showed slower urinary excretions than did the flavanone compounds (Fig. 3C and D), in keeping with the slower metabolism of ferulic acid and the longer plasma residence time of the glucuronide conjugate (Fig. 2B). Finally, both free caffeic acid and its glucuronide metabolite, not detected in plasma samples, appeared in urine at very low concentrations and were almost completely excreted within the first fraction of urine collected after ingestion (0–4 h). When comparing the mean urinary excretion curves, once again, no statistically different excretive behaviour was observed in respect to the intervention administered.

4. Conclusions

The present study confirmed that the flavanone naringenin and hydroxycinnamates, such as chlorogenic acid, caffeic and ferulic acid derivatives, are among the most abundant phenolics in tomato sauces. The phenolic content of the sauces in this study significantly differed from the starting raw tomatoes in the higher content of caffeoylquinic acid, rutin, but particularly in the 8-fold higher concentration of naringenin. These data confirmed how heat and/or mechanical treatments, during tomato sauce processing, may increase the phenolic compositions of the processed products.

Six phenolic compounds, namely three aglycones (naringenin, ferulic and caffeic acids) and their corresponding monoglucuronide metabolites, were detected in urine after the single ingestion of all three tomato sauces, reaching the maximum rate of excretion within 4 h from the intervention. Only two of the six urinary phenolic metabolites were also identified in plasma. The naringenin glucuronides rapidly appeared in plasma 15 min after consumption, while higher C_{max}, T_{max}, AUC_{last} and MRT were observed for the ferulic glucuronide, confirming the slower metabolism and urinary excretion of ferulic acid.

The pharmacokinetics of the naringenin glucuronides after ingestion of the oil-enriched tomato sauces let us to hypothesize that the lipid matrix in the sauces may stimulate the occurrence of re-absorption events by enterohepatic circulation, thus potentially affecting the apparent plasma half-life of these compounds. Nevertheless, the great interindividual response variability observed underlies the need for further large-scale investigations ideally involving a larger number of subjects, to confirm the hypothesis formulated through the present work.

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En aquest treball es presenta un mètode ràpid amb UPLC-DAD per a la quantificació de flavonoids en extractes de fruites cítriques, el qual va permetre coneixer la metodologia en cromatografia ultrarapida.

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A Fast Method Coupling Ultrahigh Performance Liquid Chromatography with Diode Array Detection for Flavonoid Quantification in *Citrus* Fruit Extracts

Alexander Medina-Remón,[†,‡] Sara Tulipani,[†,§] Maria Rotchés-Ribalta,[†,§] Maria De Lourdes Mata-Bilbao,[†] Cristina Andres-Lacueva,[†,§] and Rosa M. Lamuela-Raventos[*,†,‡]

[†]Nutrition and Food Science Department, XaRTA, INSA, Pharmacy School, University of Barcelona, Barcelona, Spain

[‡]CIBER 06/003 Physiopathology of Obesity and Nutrition, (CIBEROBN), and RETICS RD06/0045/0003, Instituto de Salud Carlos III, Spain

[§]Ingenio-CONSOLIDER program, FUN-C-FOOD, CSD2007-063, Barcelona, Spain

ABSTRACT: Flavonoids are a widely distributed group of polyphenolic compounds present in an extensive range of edible plants, notably *Citrus* species. This article reports a rapid, optimized, and validated method for the separation and quantification of flavonoids in three *Citrus* fruit extracts by ultrahigh performance liquid chromatography (UHPLC) using a photodiode array detector. This new procedure allowed the simultaneous separation and quantification of 11 selected flavonoids in 5.5 min, 8.2 times faster than that by HPLC analysis. The solvent consumption for each individual analysis was also reduced almost 6.2-fold. The most abundant component in the analyzed samples was naringin (299.06−544.36 mg 100 g^{-1}), followed by rutin (116.60−256.33 mg 100 g^{-1}) and quercetin (7.78−251.49 mg 100 g^{-1}). Isoquercitrin was found in a lower proportion (60.05−81.88 mg 100 g^{-1}). The method was completely validated, providing a sensitive analysis for flavonoid detection and showing satisfactory data for all the parameters tested. This methodology is cheaper, more environmentally friendly, and easier to perform than others previously described.

KEYWORDS: *Citrus*, flavonoids, *Citrus* fruit extracts, UHPLC, LC/MS/MS

INTRODUCTION

Flavonoids are a widely distributed group of polyphenolic compounds that have been reported to act as antioxidants in various biological systems.[1] They are present in a wide range of edible plants, fruits, vegetables, teas, wines, and fruit juices, especially those from *Citrus* species.[2,3] Flavonoids in plants usually occur in a glycosylated form, mainly with glucose or rhamnose, but they can also be linked with galactose, arabinose, xylose, glucuronic acid, or other sugars.[1,4] Because the solid parts of *Citrus* fruit have a very high flavanone content, the whole fruit may contain up to 5 times as much as a glass of orange juice.[5]

The amount of flavonoids present in *Citrus* fruit extracts varies according to the genetic background, the processing methods and maturity since immature fruits have higher amounts of polyphenols than mature fruits.[6] The concentrations also depend on the age of the plant, as the highest levels are detected in tissues showing pronounced cell divisions.[2,3,7] Because of the beneficial health effects of flavonoids present in fruits and vegetables,[8] their simultaneous identification and quantification is very important for many areas of science.

Liquid chromatographic (LC) methodologies represent, to date, the most widely used approach to phenolic analysis.[9] High performance liquid chromatography (HPLC) of *Citrus* fruit juices shows peaks for flavanone glycosides that vary from one fruit to another. Numerous methods for the detection and quantification of flavonoid compounds in *Citrus* fruit have already been developed.[10−13] According to Molnár-Perl et al.[14] and Kocevar et al.,[15] the number of flavonoids detected simultaneously by most HPLC methods are separated in 45 or 50 min.

However, Chen et al.[16] have developed a rapid ultrahigh performance liquid chromatography (UHPLC) method for the simultaneous determination of flavonoids in different species of *Epimedium* using a 12 min gradient elution. Spácil et al.[17] compared a conventional HPLC system and a UHPLC system, equipped with columns containing similar stationary phases, in the analysis of wine and tea samples. While both analytical methods gave good results, the UHPLC system appeared to be superior. UHPLC methods not only are faster, more sensitive, and more efficient but also are more reliable and ecological.[17]

This article reports a rapid, optimized, and validated method for the separation and quantification of flavonoids in *Citrus* fruit extracts by UHPLC using a photodiode array (PDA) detector with a very rapid sample preparation in order to decrease the time and cost of sample analysis; its many advantages include high resolution, speed, an exceptionally small sample volume, and short running time for the separation.[18] This method was applied in three *Citrus* fruits extracts to characterize the flavonoids with an efficient separation.

MATERIALS AND METHODS

Standards and Reagents. All samples and standards were handled with no exposure to light. Naringin (naringenin-7-*O*-rhamnoglucoside),

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quercetin, hesperidin (hesperetin-7-*O*-rhamnoglucoside), hesperetin, and rutin (quercetin 3-*O*-rutinoside) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); narirutin (naringenin-7-*O*-rutinoside), prunin (naringenin-7-*O*-glucoside), naringenin, tangeretin, sinensetin, neohesperidin (hesperetin -7-*O*-neohesperidoside), poncirin (isosakuranetin-7-*O*-neohesperidoside), didymin (isosakuranetin-7-*O*-rutinoside), neoeriocitrin (eriodictyol-7-*O*-neohesperidoside), and eriocitrin (eriodictyol-7-*O*-rutinoside) were purchased from Extrasynthèse (Genay, France). Isoquercitrin (quercetin 3-D-glucoside) was obtained from ChromaDex (LGC Promochem) (Barcelona, Spain). Methanol, acetonitrile, and formic acid were obtained from Scharlau Chemie, S.A. (Barcelona, Spain), and ultrapure water (Milli-Q) was obtained from Millipore System (Bedford, MA, USA). Naringin and quercetin standard solutions were prepared with acetonitrile at 0.1% of formic acid: water at 0.05% formic acid (30:70) with 30% of methanol; rutin and isoquercitrin were prepared with 100% methanol; and the rest of standards were prepared with water at 0.05% formic acid.

***Citrus* Fruit Extract Samples, Preparation, and Quantification.** The three *Citrus* fruit extracts Leben FC, Biocitro, and Trinken were manufactured by Quinabra (Química Natural Brasileira Ltd.a., Sao José dos Campos-SP-Brazil). The products are made from natural ingredients and do not contain genetically modified organisms, being composed of a mixture of four *Citrus* species: grapefruit (*C. paradisi*), sweet orange (*C. sinensis*), bergamot (*C. aurantium L.* subspecies *bergamia*), and mandarin (*C. white reticulate*). The fruits were crushed, fermented, and ultracentrifuged to remove the solid phase and keep the liquid phase, which was concentrated by evaporation with heat at 80 °C to obtain the final syrup. After these processes, vegetable glycerin (at 50%) and organic acids (citric and lactic acids, both at 1%) were added. Each extract contained the four *Citrus* species.

The samples were coded, packed, and preserved at 4 °C until analysis. They were transported from Brazil in a polystyrene box with dry ice to maintain their temperature. For flavonoid quantification, the different dilutions were prepared with Milli-Q water (4 g L^{-1} for Biocitro and 2 g L^{-1} for Leben FC and Trinken). Prior to UHPLC injection, they were filtered with 13 mm polytetrafluoroethylene (PTFE) and 0.22 μm filters from Waters (Milfords, MA, USA).

Instrumentation and Chromatography Conditions. Flavonoid quantification was accomplished with a Waters Ultra Performance Liquid Chromatographic Acquity system (UPLC) (Milford, MA, USA) equipped with a binary solvent delivery module, an autosampler cooler, a column heater, and a 2996 PDA detector, version 4.1 firmware a degassing system, and driven by Waters Empower software from Waters Corporation. Optimum separation was achieved with a binary mobile phase which consisted of (A) water at 0.05% of formic acid and (B) acetonitrile at 0.1% of formic acid, with a flow rate of 0.6 mL min^{-1}. The 5.5 min gradient was as follows: (t (min), % A), (0, 90), (0.1, 80), (1.7, 80), (1.8, 70), (2.1, 70), (2.3, 60), (3.8, 60), (3.9, 50), (4.5, 50), (4.6, 0), (5.4, 0), and (5.5, 90). The equilibration time for bringing the column to the initial conditions after gradient analysis was 1 min. All solvents were passed through a 0.22 μm pore diameter filter before use.

The autosampler was cooled to 15 °C, and 6 μL of samples were injected into the Waters Acquity UPLC system, combined with an Acquity UPLC BEH C_{18} column (1.7 μm, 2.1 × 100 mm) and protected with an Acquity UPLC BEH C_{18}Van Guard Precolumn (1.7 μm, 2.1 × 5 mm, 3/pk) (Waters, Milford, MA, USA); column temperature was thermostatted at 40 °C.

Detection was at 280 nm for narirutin, naringin, prunin, hesperidin, naringenin, hesperetin, sinensetin, and tangeretin, at 365 nm for rutin, isoquercitrin, and quercetin. They were identified by comparing the retention time and spectral characteristics of their peaks with those of standards, and they were quantified using the standards of each one.

Validation of the UHPLC Method. The analytical method was validated with naringin, quercetin, hesperidin, hesperetin, rutin, narirutin, prunin, naringenin, tangeretin, sinensetin, and isoquercitrin standards according to the recommendations of AOAC International,[19] and the following criteria were determined: linearity, precision, accuracy, and sensibility. To evaluate the linearity of the assay, a series of calibrators at different concentrations were prepared in Milli-Q water. The areas obtained were plotted against the corresponding standard concentrations, prepared in duplicate, in the range of 1−100 μg/mL for rutin, naringin, hesperidin, and hesperetin, 1−90 μg/mL for isoquercitrin, 1−80 μg/mL for narirutin, quercetin, and naringenin, 1−60 μg/mL for sinensetin, and 1−50 μg/mL for tangeretin, and 1−30 μg/mL for prunin.

Accuracy and precision were evaluated by repeatedly spiking the matrix of the *Citrus* fruit extracts, Trinken and Leben, with known levels of 11 standards at 3 different concentrations (80%, 100%, and 120% of expected value) of each flavonoid. The replicates were injected into the UHPLC system. The percentage of added analyte recovered from a *Citrus* fruit extract matrix was used as the index of accuracy. Precision is a function of concentration, and it was calculated by dividing the standard deviation (SD) by the means of concentration to obtain the coefficient of variation (CV), which when expressed on a percentage basis gives the relative standard deviation (RSD). The use of the RSD values facilitates comparisons of variabilities at different concentrations.

The sensibility of the method evaluated determining the limits of detection (LOD) and limits of quantification (LOQ). The LOD is the smallest quantity of analyte that can be shown to be significantly greater than the measurement (random) error of the blank at the prescribed level of confidence (usually 95%). It is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified, and it was calculated as 3 times the SD of the background noise. The LOQ is the smallest amount of analyte in a test sample that can be quantitatively determined with suitable precision and accuracy under previously established methods and conditions. It was taken as 10 times the SD of the background noise.[19]

UPLC-MS/MS Confirmation Analysis. The phenolic compounds present in the *Citrus* extracts were first identified by UPLC-MS/MS analysis. Online coupled to the UPLC system, an API 3000 triple quadrupole mass spectrometer (ABSciex, Concord, Ontario, Canada) equipped with a Turbo Ionspray source was used both in negative and positive ion modes to obtain mass spectrometry (MS) and MS/MS information. Turbo Ionspray source settings were as follows: capillary voltage, −3500 V; nebulizer gas (N_2), 10 (arbitrary units); curtain gas (N_2), 12 (arbitrary units); collision gas (N_2), 4 (arbitrary units); and drying gas (N_2), heated to 400 °C and introduced at a flow rate of 8000 cm^3/min. The system was controlled by software Analyst, version 1.4.2, supplied by Applied Biosystems (Foster City, CA, USA).

Prior to the UPLC-MS/MS analysis of the samples, direct infusion experiments were carried out with each standard compound commercially available. Briefly, 50:50 (v/v) [A, B] individual standard solutions (10 μg/mL) were infused into the mass spectrometer using a model syringe pump (Harvard Apparatus, Holliston, MA, USA), at a constant flow rate of 5 μL/min, and manual tuning was performed in positive and negative modes. For each standard compound, the molecular ion (MI) ($[M - H]^-$ and/or $[M + H]^+$) and the most abundant fragment ions (m/z) were easily detected, and the optimal focusing potential (FP), declustering potential (DP), and collision energy (CE) for the MI fragmentation were recorded.

Citrus phenolic compounds were identified on the basis of comparison of retention times (Rt), full scan (FS) data, and neutral loss (NL) and product ion scan (PIS) MS/MS spectra, with the commercial standards. Full data acquisition was performed scanning from 100 to 800 u in profile mode and using a cycle time of 2 s, with a step size of 0.1 u and a 2 ms pause between each scan. NL scans of 162 u (for the detection of glucosides) and 308 u (for the detection of rutinosides/rhamnosides) were carried out by scanning within the m/z range from 200 to 800 u.

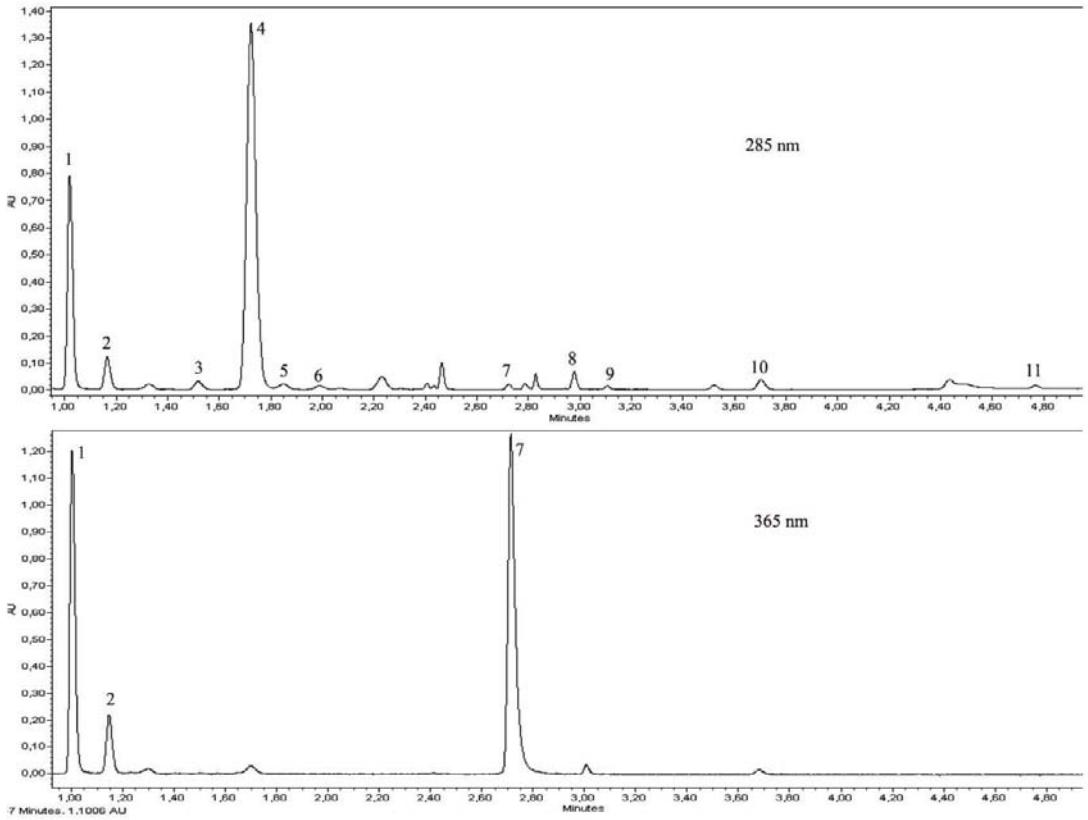

Figure 1. Chromatogram corresponding to Leben and Trinken extracts at 280 and 365 nm, respectively. Rutin (1), isoquercitrin (2), narirutin (3), naringin (4), prunin (5), hesperidin (6), quercetin (7), naringenin (8), hesperetin (9), sinensetin (10), and tangeretin (11).

PIS experiments were produced by collision-activated dissociation (CAD) of selected precursor ions in the collision cell of the triple quadrupole mass spectrometer and mass analyzed using the instrument's second analyzer. In all the experiments, both quadrupoles (Q1 and Q3) operated at unit resolution.

RESULTS AND DISCUSSION

In this work, the UHPLC method developed for the quantification of flavonoids in *Citrus* fruit extracts was found to be capable of giving a faster analysis with good resolution than that achieved with conventional HPLC. We developed a very rapid method especially adapted to analyze large batches of samples, with the potential for proportionally greater sensibility and with a methodology that is much friendlier to the environment due to its low consumption of organic solvents compared to that of other analytical techniques.

Flavonoid Samples. We separated the 11 selected flavonoids in only 5.5 min. The chromatograms for Leben and Trinken samples at 280 and 365 nm, respectively, are shown in Figure 1. The peaks separated for rutin, isoquercitrin, and quercetin reached their maximum at 365 nm, while narirutin, naringin, prunin, hesperidin, naringenin, hesperetin, sinensetin, and tangeretin reached their maximum at 280 nm. The classes of flavonoids that characterize *Citrus* species have their maximum absorption at specific wavelength ranges: flavanones (280−290 nm), flavones (304−350 nm), and flavonols (352−385 nm).[9]

Fujita et al.[6] measured the flavonoids of 12 immature *Citrus* fruit extracts using HPLC. In this assay, the samples were refluxed 3 times during 30 min at 80 °C, concentrated under vacuum, and dissolved in methanol. This laborious method required 45 min to obtain the chromatogram. Pupin et al.[20] used HPLC to analyze flavones in Brazilian orange juice. This technique is useful to distinguish orange juice (single strength or concentrated) from a second-pressure extract and can identify mixtures and determine the authenticity of pigmented orange juice.

Mata-Bilbao et al.[13] quantified flavonoids in a similar *Citrus* fruit extract composed of four species using HPLC with a photo diode array detector. This method required 45 min for the gradient elution and 15 min to equilibrate the HPLC column. Our UPLC analysis was faster in two steps: the run time was reduced, and the equilibration time for bringing the column to the initial conditions after gradient analysis was much shorter, allowing the separation of 11 selected flavonoids in only 5.5 min after the injection of less volume (6 μL).

In some European countries, there is a legal barrier against marketing some *Citrus* juice mixtures under the label orange juice, which the FDA conditionally allows for pasteurized and

Table 1. Linear Regression Data, LOD, and LOQ of the Investigated Compounds[a]

	parameter					
	linearity range (μg mL^{-1})	slope (b) ± SD	y-intercept (a) ± SD	correlation coefficient (R^2)	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
rutin	1−100	12378.50 ± 587.61	4237.65 ± 94.40	0.9996	0.02	0.08
isoquercitrin	1−90	14378.00 ± 33.94	10651.05 ± 1101.60	0.9994	0.23	0.77
narirutin	1−80	19511.00 ± 599.63	1146.00 ± 981.46	0.9995	0.15	0.50
naringin	1−100	16139.00 ± 257.39	11527.50 ± 1117.94	0.9996	0.21	0.69
hesperidin	1−100	15024.00 ± 728.32	18208.50 ± 440.53	0.9988	0.09	0.29
prunin	1−30	23451.00 ± 1112.98	9952.15 ± 394.35	0.9994	0.05	0.17
quercetin	1−80	35516.00 ± 1364.72	11574.50 ± 843.58	0.9992	0.07	0.24
naringenin	1−80	28722.50 ± 663.97	10378.05 ± 1098.77	0.9998	0.11	0.38
hesperetin	1−100	28298.50 ± 1802.42	10511.00 ± 493.56	0.9985	0.05	0.17
sinensetin	1−60	13743.00 ± 468.10	6797.70 ± 182.01	0.9992	0.04	0.13
tangeretin	1−50	22994.50 ± 409.41	35479.00 ± 1144.10	0.9969	0.15	0.50

[a] SD, standard deviation; LOD, limit of detection; LOQ, limit of quantification.

canned orange juice and frozen concentrated orange juice. A simultaneous LC method for the separation of flavonoid compounds allows *Citrus* juice to be characterized by its phenolic profile.[21] HPLC technology allows adulterated *Citrus* juices to be identified.[22,23] Ooghe et al.[24] established a procedure using HPLC equipped with a PDA detector to determine the juice addition to *C. sinensis* of up to 10% *C. reticulata* and hybrids thereof and up to 5% *C. aurantium*.

Spácil et al.[17] compared a conventional HPLC system and a UHPLC system and found that for flavonoid compounds (rutin, naringin, quercitrin, daidzein, quercetin, hesperetin, kaempferol, rhamnetin, and galangin), analysis duration was 2.5 times shorter with UHPLC, and solvent consumption was decreased by 5.5 times. The UHPLC system also showed very good sensitivity (1.7 times higher), allowing the injection of only 1.5 μL volume for reliable analysis results. Its peak capacity number was also significantly higher than that in the HPLC system. Cooper et al.[25] described a UHPLC method that can separate and quantify six of the major polyphenols in a wide range of chocolates from different countries in 3 min. UHPLC makes it possible to perform very high-resolution separations in short periods of time with little solvent consumption. Because of the hardware adjustments, it also allows work at extreme pressures of up to 100 MPa.[17,26,27] The run-time of analysis and the equilibration time for bringing the column to the initial conditions after gradient analysis are much shorter.[17] This new UHPLC analysis was performed 8.2 times faster than those by HPLC,[13] and solvent consumption for each individual analysis was reduced by almost 6.2 times. The pressure reached was 665 bar at 90% of mobile phase A and 332 bar at 100% of mobile phase B; hence, the pressures in the complete process were between these values at the flow rate of 0.6 mL min^{-1}.

Method Validation. For the validation of the method under the optimized conditions, the 7-point calibrator concentration showed a linear and reproducible curve for all standards, with good correlation coefficients (Table 1) indicated by $R^2 > 0.99$ values. LOD and LOQ were very low, less than 0.23 and 0.77 μg mL^{-1}, respectively, which confirmed the high sensitivity of the proposed method.

Precision was evaluated at three different concentration levels (Table 2) for all the standards found in the samples. Repeated aliquots were prepared for each concentration level. The spiked *Citrus* fruit extracts were prepared according to the general procedure and analyzed by the proposed method. Three recovery values were less than 80% but higher than 66%, while the others were between 80.11% and 107.00%; the AOAC International specifies that recovery should be within the range of 80−110%. The values obtained for precision RSD (%) were lower than those established by the AOAC International,[19] which would be 7.3% at this concentration. Obviously, the low RSD confirmed the high precision of the proposed method.

Similarly, levels of accuracy were determined by recovery studies of flavonoids from Trinken and Leben matrices spiked with different concentrations of flavonoids (80%, 100% and 120% of the expected value) covering the linear range of the method. Each flavonoid peak area versus the concentration of each flavonoid was then interpolated from the linear regression equation. The recovery values (presented as percentages) were calculated using the formula: calculated experimental concentration/nominal concentration × 100. The mean recovery value was found to be 94.99 ± 6.03%, indicating the accuracy of the proposed method (Table 2).

In our method, the speed of the analysis was improved in two steps: the run-time was reduced by 5.5 min, and the equilibration time for bringing the column to the initial conditions after gradient analysis was significantly shortened by 1 min. Solvent consumption was decreased by a factor of 8 in comparison with that in HPLC methods.[13] The UHPLC system also showed very good sensibility, allowing the injection of only 6 μL volume for reliable analysis results.

Confirmation Analysis. Ultraperformance liquid chromatography coupled to mass spectrometry (UPLC-QqQ-MS/MS) was applied to confirm the results obtained by UPLC/PDA analysis (Table 3). As with UPLC-PDA, 11 *Citrus* flavonoids were identified in the extracts: quercetin (m/z^- 301), isoquercetrin (m/z^- 463), rutin (m/z^- 609), naringenin (m/z^- 271), narirutin (m/z^- 579), naringin (m/z^- 579), hesperitin (m/z^- 301), hesperidin (m/z^- 609), tangeretin (m/z^+ 373), sinensetin (m/z^- 373), and prunin (m/z^- 433), with this last one only found in Biocitro and Leben *Citrus* extracts, showing an intense molecular ion $[M - H]^-$ at m/z 433 and the corresponding aglycone molecular ion at m/z^- 271 $[433 - 162]^-$. However, no neohesperidin (hesperitin-7-*O*-neohesperidoside) was observed in the extracts. Table 4 specifies the MRM transition and collision energy (CE) for each target analyte. Additional important MS parameters, namely, DP, FP, EP, and collision cell exit potential (CXP) optimized for each compound are also included in this table.

Table 2. Evaluation of Precision and Recovery Obtained from UHPLC for Different Compounds in Two *Citrus* Fruit Extracts[a]

	Trinken				Leben			
standard	added conc. mg g^{-1}	measured conc. (n = 3) mg g^{-1}	recovery (error) (%)	precision (RSD) (%)	added conc. mg g^{-1}	measured conc. (n = 3) mg g^{-1}	recovery (error) (%)	precision (RSD) (%)
rutin	3.39	3.07	90.55	5.69	3.54	3.79	107.00	5.66
	4.42	4.50	101.59	3.57	4.45	4.35	97.83	1.37
	5.77	6.00	103.99	1.45	5.09	4.78	93.83	1.72
isoquercitrin	0.69	0.56	80.66	2.37	0.73	0.72	99.75	3.66
	0.92	0.81	87.43	4.62	0.89	0.87	98.11	1.15
	1.15	1.19	102.75	2.91	1.02	0.97	94.92	2.44
narirutin	5.75	5.64	98.09	1.55	9.15	8.90	97.23	2.19
	7.19	7.05	98.09	0.86	11.44	11.27	98.51	3.59
	8.62	8.37	97.01	1.83	13.73	13.30	96.90	1.50
naringin	3.73	2.47	66.26	2.48	4.06	3.19	78.72	5.80
	5.05	4.03	79.74	2.49	4.90	4.03	82.14	2.24
	6.35	5.26	82.86	2.47	5.60	4.48	80.11	4.38
hesperidin	1.16	1.05	91.19	6.24	1.13	1.12	99.17	2.87
	6.24	1.37	94.92	6.59	1.41	1.16	82.61	2.72
	1.73	1.57	90.40	2.90	1.70	1.40	82.37	1.9
prunin	0.90	0.87	96.53	5.24	1.07	1.00	93.60	3.04
	1.13	1.07	94.99	4.94	1.34	1.17	87.37	3.63
	1.35	1.22	90.60	5.26	1.60	1.43	89.20	6.53
quercetin	4.51	4.27	94.45	6.06	4.72	4.77	101.0	3.72
	5.97	5.93	99.52	5.66	5.79	5.79	98.96	2.40
	7.50	7.58	100.96	2.92	6.61	6.66	100.59	1.69
naringenin	0.93	0.98	105.07	2.48	0.64	0.75	102.23	3.89
	1.16	1.16	100.22	3.23	1.00	0.98	97.92	2.41
	1.40	1.27	90.51	3.98	1.36	1.61	94.75	1.16
hesperetin	1.02	1.07	104.61	4.80	0.75	0.73	97.81	3.61
	1.27	1.13	89.36	2.63	0.93	0.82	88.39	5.42
	1.53	1.28	83.61	6.02	1.12	1.00	89.74	2.55
sinensetin	4.88	4.54	93.05	1.80	4.51	4.54	100.70	4.32
	6.10	5.62	92.21	3.22	5.63	5.72	101.53	1.99
	7.32	6.55	89.49	3.27	6.76	6.23	92.22	1.95
tangeretin	0.45	0.45	100.87	5.53	0.49	0.47	95.95	5.87
	0.57	0.56	98.96	6.96	0.62	0.62	99.79	6.66
	0.68	0.68	99.29	4.95	1.00	0.79	102.62	5.64

[a] SD, relative standard deviation.

The samples analyzed were composed of four *Citrus* species: *C. paradisi*, *C. sinensis*, *C. aurantium L.* subspecies *bergamia*, and *C. white reticulata*; each one is characterized by particular flavanone glycosides.[9] The presence of a relatively large number of flavonoids in *Citrus* juices is a result of the many different possible combinations between polyhydroxylated aglycones and a limited number of mono- and disaccharides.

Each species of *Citrus* is characterized by a particular flavanone glycoside pattern that can be separated by HPLC. The most abundant component in sweet orange juices (*C. sinensis*), regardless of variety, is by far hesperidin (286 mg L^{-1}), followed by narirutin (52 mg L^{-1}) and didymin (18.9 mg L^{-1}). Mandarin juice (*C. reticulata*) is quite similar to sweet orange juice, hesperidin (243 mg L^{-1}) again being the main component, followed by narirutin (39.2 mg L^{-1}) and didymin (14.4 mg L^{-1}).[9,28] Polymethoxyflavones have been reported over the years as minor components of orange juice, including sinensetin (3.7 mg L^{-1}), nobiletin (3.3 mg L^{-1}), tangeretin (0.4 mg L^{-1}), and 3,3′,4′,5,6,7,8-heptamethoxyflavone (0.8 mg L^{-1}).

Contrary to sweet orange, mandarin, and their closely related species, *C. aurantium* (Bergamot) is not generally suitable for direct consumption. Its juice composition is very different from that of sweet orange but quite similar to grapefruit, being rich in naringin (19.6 mg L^{-1}), neohesperidin (8.7 mg L^{-1}), and neoeriocitrin (7.7 mg L^{-1}).[9] Grapefruit (*C. paradisi*) juice can generally be found in three color varieties: red, pink and white, depending on the presence or absence of lycopene.[29] The main components of grapefruit juice are naringin (230 mg L^{-1}) and naringenin (27.0 mg L^{-1}), with narirutin also present in good amounts (76.0 mg L^{-1}).[9] Citrus extracts such as *C. paradisi* contain naringin and furanocoumarins in abundance.[6] *Citrus* flavonoid composition appears to vary greatly depending on genetic origin, the time of fruit collection, and the different parts of the fruit used (peel and edible parts).[30]

Table 3. List of the Flavonoid Compounds Identified in the *Citrus* Extracts[a]

compound	*Rt* (min)	ion mode	ion full scan MS MI	ion full scan MS fragment ions	MS/MS data PIS	MS/MS data NL	comparison with standard	Biocitro extract	Leben extract	Trinken extract
rutin	0.99	−	609.3	300.9	609	308	yes	yes	yes	yes
isoquercitrin	1.13	−	463.3	300.9, 179.1	463	162	yes	yes	yes	yes
narirutin	1.48	−	579.5	271.2, 151.1	579	308	yes	yes	yes	yes
naringin	1.68	−	579.5	459.6, 372.8, 271.3, 150.8	579	308	yes	yes	yes	yes
prunin	1.81	−	433.6	271.1	433	162	no	yes	yes	no
hesperidin	1.95	−	609	301.1	609	308	yes	yes	yes	yes
quercetin	2.70	−	301.3	178.9, 151.1, 121.3, 107.1	301	−	yes	yes	yes	yes
naringenin	2.96	−	271.1	150.9, 118,9, 107.1, 93.1	271	162	yes	yes	yes	yes
hesperetin	3.10	−	300.9	285.9, 242.3, 164.0, 150.9	301	−	yes	yes	yes	yes
sinensetin	3.70	+	373.1	358.1, 343.1, 329.1, 312.3	373	−	yes	yes	yes	yes
tangeretin	4.73	+	373	358.2, 342.9, 325.0, 297.3	373	−	yes	yes	yes	yes

[a] Rt: retention time; NL, neutral loss; PIS, precursor ion scan.

Table 4. MRM Transition, Declustering Potential (DP), Focusing Potential (FP), Entrance Potential (EP), Collision Energy (CE), and Collision Cell Exit Potential (CXP) Optimized for Each Analyte Searched

compound	ion mode	MRM (Q1→Q3)	DP (V)	FP (V)	EP (V)	CE (V)	CXP (V)
rutin	−	609→301	−60	−210	−10	−30	−15
isoquercetrin	−	463→301	−70	−230	−10	−30	−15
narirutin	−	579→271	−120	−200	−10	−40	−15
naringin	−	579→459	−130	−200	−10	−35	−15
prunin	−	433→271	−70	−200	−10	−30	−15
hesperidin	−	609→301	−70	−250	−10	−35	−15
quercetin	−	301→151	−60	−200	−10	−30	−15
naringenin	−	271→151	−80	−200	−10	−25	−15
hesperetin	−	301→286	−60	−220	−10	−35	−15
sinensetin	+	373→312	−50	−200	−10	−30	−15
tangeretin	+	373→343	−50	−190	−10	−30	−15

Table 5. Quantity of Each Flavonoid Identified in Different Samples[a]

flavonoids	Biocitro	Leben	Trinken
	Mean (mg 100 g^{-1} of sample) ± SD		
rutin	116.60 (5.75)	256.33 (26.79)	248.24 (9.64)
isoquercitrin	60.05 (0.75)	81.88 (7.92)	80.85 (2.32)
narirutin	5.54 (0.46)	14.17 (1.12)	11.51 (2.00)
naringin	299.06 (5.12)	544.36 (48.43)	515.78 (3.55)
hesperidin	1.27 (0.21)	2.20 (0.38)	2.31 (0.13)
prunin	1.89 (0.37)	1.85 (0.25)	nd
quercetin	251.49 (2.02)	7.78 (0.63)	179.88 (25.07)
naringenin	2.80 (0.55)	2.82 (0.32)	4.64 (0.56)
hesperetin	3.30 (0.28)	1.44 (0.14)	2.55 (0.17)
sinensetin	168.76 (10.06)	7.73 (1.58)	9.77 (1.18)
tangeretin	0.17 (0.01)	0.42 (0.07)	0.41 (0.24)

[a] SD, standard deviation; RSD, relative standard deviation; nd, not detected.

Table 5 shows the quantity of each flavonoid identified in three different samples (mg 100 g^{-1} of sample); the means ± SD represent the average of six replicated measures for each sample. The most abundant component in the analyzed samples was naringin (between 299.06 and 544.36 mg 100 g^{-1}) followed by rutin (between 116.60 and 256.33 mg 100 g^{-1}) and quercetin (between 7.78 and 251.49 mg 100 g^{-1}). Isoquercitrin was found in a lower proportion, between 60.05 and 81.88 mg 100 g^{-1}, similar to a previous study.[13] This is the second study to report the presence of isoquercitrin in samples of grapefruit (*C. paradisi*), sweet orange (*C. sinensis*), bergamot (*C. aurantium L. bergamia*), and mandarin (*C. white reticulate*).

In summary, we have developed a fast method combining UHPLC with a diode array detector, which was successfully applied in *Citrus* fruit extracts for the simultaneous quantification of flavonoids in only 5.5 min. This method required a rapid gradient that provides the flavonoid separation from the extracts. It is cheaper, more environmentally friendly, and simpler than other previously described methodologies. The excellent retention time and peak area repeatability of the UHPLC method are of particular importance for use in routine food analyses. The method was completely validated, providing a sensitive analysis for flavonoid detection and showing satisfactory data for all the parameters tested. Good results were obtained with respect to linearity and recovery as well as an excellent level of precision. UHPLC methods can also be rated as green methods.

AUTHOR INFORMATION

Corresponding Author

*Tel: +34-934034843. Fax: +34-934035931. E-mail: lamuela@ub.edu.

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■ ABBREVIATIONS USED

CV, coefficient of variation; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography−mass spectrometry; LOD, limits of detection; LOQ, limits of quantification; PDA, photodiode array; RSD, relative standard deviation; SD, standard deviation; UHPLC, ultrahigh performance liquid chromatography; UPLC, Waters Acquity ultraperformance liquid chromatography; UV, ultraviolet.

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En aquesta publicació es presenta la correlació entre l'excreció total de polifenols i la pressió arterial en subjectes amb elevat risc cardiovascular. Aquest treball va permetre ampliar el coneixement dels compostos fenòlics i els seus efectes en la salut.

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Total polyphenol excretion and blood pressure in subjects at high cardiovascular risk

A. Medina-Remón [a,b,c], R. Zamora-Ros [a,d], M. Rotchés-Ribalta [a,d], C. Andres-Lacueva [a,d], M.A. Martínez-González [c,e], M.I. Covas [b,f], D. Corella [b,g], J. Salas-Salvadó [b,h], E. Gómez-Gracia [c,i], V. Ruiz-Gutiérrez [c,j], F.J. García de la Corte [b,k], M. Fiol [b,l], M.A. Pena [m], G.T. Saez [c,n], E. Ros [b,o], L. Serra-Majem [c,p], X. Pinto [c,q], J. Warnberg [c,r,s], R. Estruch [b,c,t], R.M. Lamuela-Raventos [a,b,c,*], on behalf of the PREDIMED Study Investigators

[a] *Nutrition and Food Science Department, XaRTA, INSA, Pharmacy School, University of Barcelona, Barcelona, Spain*
[b] *CIBER CB06/03 Fisiopatología de la Obesidad y la Nutrición, (CIBEROBN), Spain*
[c] *RETICS RD06/0045, Instituto de Salud Carlos III, Spain*
[d] *Ingenio-CONSOLIDER Program, FUN-C-FOOD, Barcelona, Spain*
[e] *Department of Preventive Medicine and Public Health, School of Medicine, University of Navarra, Pamplona, Spain*
[f] *Cardiovascular Epidemiology Unit, Municipal Institute for Medical Research (IMIM), Barcelona, Spain*
[g] *Department of Epidemiology, Preventive Medicine and Public Health, School of Medicine, University of Valencia, Valencia, Spain*
[h] *Human Nutrition Unit, School of Medicine, IISPV, University Rovira i Virgili, Reus, Spain*
[i] *Department of Epidemiology, School of Medicine, University of Malaga, Málaga, Spain*
[j] *Instituto de la Grasa, Consejo Superior de Investigaciones Cientificas, Sevilla, Spain*
[k] *Department of Family Medicine, Primary Care Division of Sevilla, San Pablo Health Center, Sevilla, Spain*
[l] *Institut Universitari d'Investigació en Ciències de la Salut (IUNICS), Palma de Mallorca, Spain*
[m] *Clinical Trials Unit, Hospital Txangorritxu, Vitoria, Spain*
[n] *Department of Biochemistry and Molecular Biology Service of Clinical Analysis, CDB Hospital General Universitario, Universitat de Valencia, Spain*
[o] *Lipid Clinic, Endocrinology and Nutrition Service, Institut d'Investigacions Biomèdiques August Pi Sunyer (IDIBAPS), Hospital Clinic, Barcelona, Spain*
[p] *Department of Clinical Sciences, University of Las Palmas de Gran Canaria, Palmas de Gran Canaria, Spain*
[q] *Lipid Unit, Department of Internal Medicine, IDIBELL-Hospital Universitari de Bellvitge, L'Hospitalet de Llobregat, Fipec, Barcelona, Spain*
[r] *Immunonutrition Research Group, Instituto del Frío-ICTAN (CSIC), Madrid, Spain*
[s] *Department of Biosciences and Nutrition, Karolinska Institutet, Stockholm, Sweden*
[t] *Department of Internal Medicine, Hospital Clinic, IDIBAPS, University of Barcelona, Spain*

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Acronyms: GFR, glomerular filtration rate; BP, blood pressure; TPs, total polyphenols; TPE, total polyphenol excretion; F&V, fruit and vegetables; SPE, solid phase extraction; F–C, Folin–Ciocalteu; GAE, gallic acid equivalent; CHD, coronary heart disease; ORs, odds ratios.

* Corresponding author. Nutrition and Food Science Department, XaRTA, INSA Pharmacy School, University of Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain. Tel.: +34 934034843; fax: +34 934035931.
E-mail address: lamuela@ub.edu (R.M. Lamuela-Raventos).

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Mediterranean diet;
Biomarker

Abstract *Background and aims:* Dietary factors are critical for the prevention and treatment of hypertension, but data on the effects of specific nutrients on blood pressure (BP) are scarce. The aim of this study was to assess the relationship between total polyphenol excretion (TPE) in urine, as an objective measurement of total polyphenol intake and BP in an elderly population at high cardiovascular risk.

Methods and results: Cross-sectional substudy of 589 high-risk participants entering in the PREDIMED trial. BP was measured and TPE was determined in urine by Folin–Ciocalteu assay. A significant positive association was observed between TPE in urine and daily intake of fruit and vegetables (F&V), coffee or wine after adjusting for potential confounders. The intake of 100 g of F&V (Beta = 0.150; $P < 0.001$) had a greater contribution to TPE than 100 mL of coffee (Beta = 0.141; $P = 0.001$), and the latter two foods contributed more than the consumption of 100 mL of wine (Beta = 0.120; $P = 0.019$). An inverse association was observed between urinary TPE and the prevalence of hypertension. Participants in the highest quartile of urinary TPE had a reduced prevalence of hypertension compared to those in the lowest quartile (Odds Ratio = 0.64; 95% confidence interval 0.45 to 0.92; $P = 0.015$). Systolic and diastolic BP were inversely associated with urinary TPE after adjustment for potential confounders ($P = 0.024$ and $P = 0.003$, respectively).

Conclusions: Polyphenol intake, assessed via TPE in urine, was negatively associated with BP levels and prevalence of hypertension in an elderly Mediterranean population at high cardiovascular risk. Participants with the highest intake of polyphenol-rich foods showed the lowest BP measurements.

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Introduction

Hypertension is a major global public health problem [1]. A healthy diet and lifestyle modification are the first steps for the management of hypertension [2]. Adoption of a healthier food pattern such as the Mediterranean diet [3] or the DASH (Dietary-Approaches-to-Stop-Hypertension) diet has also been advocated. In addition, there is considerable evidence that the consumption of fruit and vegetables (F&V) is inversely associated with high BP, whereas a high consumption of refined cereals or of meat/meat products is associated with a higher risk [2,4,5].

However, besides sodium and potassium dietary intake, few studies have analyzed the effects of dietary nutrients, such as oleic acid [6,7] vitamin C or other antioxidant compounds [8,9] on BP. Some studies have also evaluated the effects of polyphenol-rich foods such as cocoa [10], however up to now no studies have evaluated the effects of total dietary polyphenol intake on this issue.

The total dietary amount of polyphenol is approximately 1 g/d [11], being an amount that is higher than any other known dietary antioxidants; in fact, it is around 10 times higher than dietary vitamin C and 100 times higher than vitamin E and carotenoid intake. On the other hand, epidemiologic data have shown an inverse association between the risk of overall mortality or cardiovascular disease and the consumption of polyphenol-rich foods such as fruit and vegetables, tea, olive oil and wine [12–14].

We undertook, therefore, a substudy within a larger clinical trial, the PREDIMED study [3,15], in order to evaluate whether polyphenol-rich food intake, assessed as the excretion of total polyphenols (TPs) in spot urine samples, was associated with average BP and/or with the prevalence of hypertension. To our knowledge this is the first study to assess this relationship using biochemical analysis of TPs in spot urine samples, and directly measuring BP in a large sample of elderly individuals at high cardiovascular risk.

Methods

Subjects and design

The PREDIMED (*PREvención con DIeta MEDiterránea*) study is a large, parallel-group, multicenter, randomized, controlled 5-year clinical trial aimed to assess the effects of the MedDiet on the primary prevention of cardiovascular disease (www.predimed.org; ISRCTN35739639). The detailed recruitment method and study protocol have been described previously [3].

From October-2003 to July-2004, we selected 612 potential participants in primary health centers affiliated with the Hospital Clinic of Barcelona and the University of Navarra (Pamplona) in Spain. Eligible participants were free of cardiovascular disease at baseline and fulfilled at least one of the following two criteria: (1) type-2 diabetes mellitus and/or (2) three or more coronary heart disease (CHD) risk factors [16]. The participants provided written informed consent and the study protocol was approved by the Institutional Review Boards of the two participating centres.

Measurements

At baseline, all participants completed a validated semi-quantitative food frequency questionnaire (FFQ) with 136-items [17], the validated Spanish version [18] of the Minnesota Leisure Time Physical Activity Questionnaire, and a 47-item questionnaire about education, lifestyle, history of illnesses and medication use. Trained nurses measured BP thrice with a validated semi-automatic oscillometer (Omron HEM-705CP [19]; Hoofddorp, The

Netherlands). Creatinine-estimated glomerular filtration rate (GFR) was calculated using the Modification of Diet in Renal Disease (MDRD) formula in all participants [20]. Energy and nutrient intake was derived from Spanish food composition tables [21]. The morning urine samples were collected; the aliquots were immediately prepared, coded, shipped to a central laboratory and frozen at −80 °C until analysis. TP consumption from plant food and beverages (mg/g fresh matter) was quantified according to Saura-Calixto F et al. [22] and Brat P et al. [23], from the FFQ.

Analysis of total polyphenols in urine

Solid Phase Extraction (SPE) with 96-well plate cartridges (Oasis® MAX) was performed in the urine samples to avoid any interference with F−C reagent. For all the spot urine samples TPs and creatinine were analyzed as described by Medina-Remón A. et al. [24]. TPE was expressed as mg gallic acid equivalent (GAE)/g of creatinine.

Statistical analysis

Analyses were performed using SPSS software v14.0 (Chicago, USA). The baseline characteristics of the participants were expressed as means or percentages and standard deviations (SD). Variables were examined for normality and skewness (Kolmogorov and Levene tests). The variable urinary TPE, which was not normally distributed and was not normalized by the logarithmic transformation, were Box−Cox transformed with $\alpha = 0.00001$ and $\lambda = 0.25$. Participants were divided into quartiles according to their urinary TPE (not normalized).

Pearson correlation coefficients were used to examine the unadjusted correlation between the urinary TPE and TP intake according to FFQ, as well as in the different food groups using units of 100 g/day. Multivariate linear regression models were used to assess the relationship between the TPE normalized in urine as the dependent variable, and TP intake (100 mg), total F&V (100 g), wine (100 mL) and coffee (100 mL), as the exposure variable with adjustment for potential confounders (see below). The relationship between the urinary TPE quartile and systolic or diastolic BP was assessed by linear regression models adjusted for potential confounders. Four models were fitted with each independent variable. Logistic regression models were fitted to assess the relationship between urinary TPE (categorized in quartiles) and hypertension status. Odds ratios (ORs) and 95% confidence intervals (CI) were calculated using the lowest quartile as the reference category. P-values < 0.05 (two-tailed) were considered to be significant. Diagnostics for the detection of outliers, multicollinearity, homoscedasticity, and the normality and independence of errors were assessed when appropriate.

Results

Participant characteristics

Twenty-three out of 612 presumably eligible participants were excluded from entry in the study; 11 declined to participate and 12 did not meet inclusion criteria. Thus, 589 participants entered the study, 263 men[mean age 66.3 (6.1) y] and 326 women[mean age 69.2 (5.6) y]. By study design, participants were older than 55 years, mostly overweight[mean body mass index 29.36 (3.37) kg/m^2], and with a sizeable burden of cardiovascular risk factors (32.6% diabetics, 80.8% hypertensive, 62.6% with dyslipidemia, 16.1% active smokers and 34.8 % had a family history of early-onset CHD).

Food and nutrient intake and urinary total polyphenol excretion

Almost all of participants adhered to a Mediterranean-style diet at baseline with a relatively high consumption of vegetables, fruits, legumes, olive oil and nuts, and a moderate intake of fish and wine, and lower, but also a relatively high intake of meat, meat products, pastries and dairy products. However, when nutrient intake was estimated, large between-subject variability in daily TPs intake was observed (from 122.96 to 3298.17 mg/day). This variability was consistent with the variation observed when the TPE in urine was analyzed. In fact, a weak but significant Pearson correlation was obtained between dietary intake of TPs from FFQ and urinary TPE measured ($r = 0.179$; $P < 0.001$).

Table 1 shows the average food consumption of study participants divided according to quartiles of urinary TPE expressed as mg GAE/g creatinine. Significant increasing trends across quartiles of TPE were observed for the intake of fruits, vegetables, total F&V, dairy products, fish and TP intake; whereas decreasing trends across TPE quartiles were observed for total alcohol intake, cereals, olive oil, pastries, cakes or sweets, as well as total energy intake.

The linear regression analyses of TPE in spot urine samples and TP intake (100 mg), total F&V intake (100 g), wine intake (100 mL) and coffee intake (100 mL) are presented in Table 2 with various models. The remaining variables are not shown because they did not exhibit any significant association with urinary TPE adjusted for potential confounding factors. We observed a significant positive association between urine TPE and daily intake of F&V, as well as with daily intake of coffee in the unadjusted model ($\beta = 0.131$;$P < 0.001$ and $\beta = 0.261$;$P < 0.05$, respectively). After adjusting for potential confounding factors in models 2, 3 and 4, the association remained statistically significant in the multivariate regression analysis. TPE concentration in spot urine samples showed a significant inverse association with the intake of wine, $\beta = -0.090$, $P < 0.05$ in the unadjusted model; but after adjusting for potential confounding factors this association reversed and became positive and statistically significant ($\beta = 0.121$, $P = 0.019$).

The standardized coefficients (Beta) are the regression coefficients obtained with the regression model using the standardized values (measured in standard deviation units), and are therefore independent of measurement units. The standardized coefficients (Table 2) from this model showed that total phenol intake (Beta = 0.283), F&V intake (Beta = 0.150) contributed more to urinary TPE than coffee (Beta = 0.141), and both contributed to a greater extent than wine (Beta = 0.120).

Table 1 Daily intake of selected foods according to quartiles of excreted total urinary polyphenols, expressed as mg GAE /g creatinine.

	Urine mg GAE/ g creatinine concentration quartile				
	Q1 (<89.0)	Q2 (89.1–119.5)	Q3 (119.6–160.2)	Q4 (>160.3)	*P* for trend[a]
Urine total polyphenol (mg GAE/ g creatinine)[b]	72.8 (11.6)	103.1 (8.2)	138.2 (11.1)	226.1 (69.8)	<0.001
No. of subjects	147	148	147	147	0.742
Olive oil (g)	48.7 (14.3)	47.3 (13.7)	45.1 (14.6)	45.0 (15.7)	0.014
Total nuts (g)	8.6 (10.7)	9.2 (11.3)	9.6 (12.2)	9.1 (10.9)	0.680
Vegetables (g)	253.1 (80.2)	261.1 (81.1)	262.6 (84.0)	272.7 (92.1)	0.053
Legumes (g)	17.9 (7.0)	17.9 (7.5)	17.7 (7.0)	16.5 (7.6)	0.107
Fruits (g)	304.6 (143.6)	319.3 (141.3)	315.1 (132.9)	360.8 (151.2)	0.002
Total fruits and vegetables (g)	557.7 (176.8)	580.4 (173.3)	577.7 (162.1)	633.5 (190.1)	0.001
Fish or seafood (g)	86.5 (34.0)	82.2 (35.8)	88.7 (36.9)	94.1 (39.0)	0.030
Meat or meat products (g)	132.0 (49.6)	129.7 (43.7)	134.3 (48.5)	134.2 (47.5)	0.531
Pastries, cakes or sweets (g)	34.8 (31.6)	27.9 (24.8)	25.3 (25.9)	23.7 (28.7)	<0.001
Cereals (g)	243.7 (106.7)	235.8 (91.6)	228.7 (98.3)	211.3 (83.3)	0.003
Milk and dairy products (mL)	352.1 (214.8)	378.8 (216.5)	396.5 (222.1)	403.1 (215.1)	0.033
Wine (mL)	121.1 (147.0)	81.5 (137.9)	101.1 (161.5)	81.3 (149.3)	0.071
Coffee (mL)	63.3 (47.7)	69.6 (52.9)	67.5 (48.3)	74.5 (56.0)	0.097
Tea (mL)	3.92 (16.4)	3.25 (11.4)	4.16 (12.6)	5.47 (20.4)	0.332
Chocolate (g)	3.1 (6.2)	2.4 (5.2)	2.3 (5.5)	1.9 (4.8)	0.063
Natural orange juice (mL)	23.3 (54.3)	25.19 (57.8)	13.4 (41.2)	19.1 (50.8)	0.199
Total polyphenol intake (mg GAE)	1075.6 (354.9)	1057.5 (320.2)	1086.2 (322.3)	1222.5 (439.8)	0.001
Alcohol (g)	15.7 (17.9)	10.2 (16.0)	12.2 (18.5)	9.9 (17.8)	0.018
Fibre (g)	22.1 (6.2)	22.0 (5.5)	21.9 (5.2)	22.5 (6.2)	0.606
Cholesterol (g)	353.6 (119.7)	328.6 (93.7)	340.5 (113.4)	342.1 (89.4)	0.561
Sodium (mg/d)	3347.7 (959.2)	3088.0 (905.4)	3123.2 (1006.5)	3145.4 (877.2)	0.100
Potassium (mg/d)	3926.7 (722.3)	3929.4 (700.9)	3994.8 (805.5)	4029.7 (659.7)	0.161
Total energy, Kcal/d	2380.1 (586.8)	2238.0 (472.0)	2205.1 (547.4)	2138.5 (476.8)	<0.001

[a] One-factor ANOVA was used for continuous variables and χ^2-test for categorical variables.
[b] Mean (standard deviation). GAE: gallic acid equivalent.

Urinary polyphenol analysis and cardiovascular risk factors

Table 3 shows the profile of cardiovascular risk factors of participants according to the quartile of urinary TPE. A higher polyphenol excretion in urine was associated with older age (P for trend < 0.001), lower systolic (P for trend $= 0.015$) and diastolic BP (P for trend < 0.001), and a lower, but not significant, use of anti-hypertensive medication. However, participants in the highest quartile of TPE reported a lower educational level than those in the lower quartile categories, had a higher prevalence of diabetes, but were less likely to be smokers, and to have a family history of CHD.

On multivariate linear regression analyses, systolic and diastolic BP exhibited a monotonic inverse association with TPE in spot urine samples (quartiles) after adjustment for potential confounders (Table 4). The non-standardized coefficients, $\beta = -1.731 (P = 0.024)$ and $\beta = -1.264 (P = 0.003)$ represent the expected change of systolic and diastolic BP, respectively, corresponding to an increase from a TPE to the upper quartile.

Logistic regression analysis showed an inverse association between urinary TPE (by quartile) and the prevalence of hypertension (Table 5). Compared to the participants in the lowest quartile of TPE (<88.99 mg GAE/g creatinine), participants in the highest quartile (>160.23 mg GAE/g creatinine) had a significantly reduced prevalence of hypertension (OR = 0.71, CI 0.53 to 0.95; $P = 0.022$) in the unadjusted model. In all three models, a significant difference was observed between the top and the bottom quartiles for the prevalence of hypertension, after adjustment for potential confounders. When the analysis was adjusted for all possible confounding factors (model 4), participants in the highest quartile had a 36% reduced odds of hypertension (OR = 0.64, CI 0.45 to 0.92; $P = 0.015$), compared to those in the lowest quartile. Finally, BP correlated better with urinary TPE than with TP intake assessed by FFQ. In fact, a highly significant association between polyphenol intake assessed via TPE in urine and

Table 2 Multivariate linear regression analysis with total polyphenol excreted normalized, in spot urine samples (mg GAE/g creatinine) as the dependent variable, and total polyphenol (100 mg/d), total fruit and vegetable (100 g/d), wine (100 mL/d) and coffee (100 mL/d) intakes, as exposure variable, adjusted for potential confounders.

	Model	β	SE	Beta	*P*	95 % CI
Total polyphenol intake (100 mg/d)	Model 1	0.073	0.017	0.179	<0.001	0.041 to 0.106
	Model 2	0.110	0.016	0.268	<0.001	0.078 to 0.141
	Model 3	0.117	0.017	0.286	<0.001	0.085 to 0.150
	Model 4	0.116	0.020	0.283	<0.001	0.077 to 0.154
Fruit and Vegetables (100 g)	Model 1	0.131	0.035	0.155	<0.001	0.064 to 0.199
	Model 2	0.112	0.033	0.132	0.001	0.047 to 0.177
	Model 3	0.107	0.034	0.126	0.002	0.040 to 0.174
	Model 4	0.127	0.036	0.150	<0.001	0.056 to 0.198
Wine (100 mL)	Model 1	−0.090	0.041	−0.090	0.029	−0.171 to −0.009
	Model 2	0.063	0.045	0.063	0.164	−0.026 to 0.152
	Model 3	0.075	0.047	0.075	0.107	−0.016 to 0.166
	Model 4	0.121	0.051	0.120	0.019	0.020 to 0.222
Coffee (100 mL)	Model 1	0.261	0.120	0.089	0.030	0.025 to 0.497
	Model 2	0.321	0.115	0.110	0.006	0.095 to 0.548
	Model 3	0.373	0.116	0.128	0.001	0.144 to 0.602
	Model 4	0.414	0.119	0.141	0.001	0.181 to 0.647

β: Non-standardized coefficient (regression line coefficient); SE: Standard error; Beta: Standardized coefficient; CI: Confidence interval; *P*: two-sided test of significance; Model 1: unadjusted; Model 2 was adjusted by sex, age and weight; Model 3 as in Model 2 plus smoking status, physical activity, educational level and energy expenditure in physical activity; Model 4 was adjusted as in Model 3 plus total fish/seafood, olive oil, beer, chocolate, natural orange juice, tea, cereals, milk and diary products, meat or meat products, nuts, pastries/cakes/sweets, fruits and vegetables, wine and coffee intake; GAE: Gallic acid equivalent.

systolic (*P* for trend = 0.024) and diastolic BP (*P* for trend = 0.003) was observed (Table 4), whereas polyphenol intake assessed via FFQ tended to be associated with BP values, but here the association did not reach the statistical significance.

Discussion

In the current study, we observed an inverse association between urinary TPE, an objective measurement of polyphenol intake, and the risk of hypertension in a large Spanish cohort of elderly participants at high cardiovascular risk. In addition, systolic and diastolic BP measurements correlated negatively with urinary TPE after adjustment for potential confounders. The results from the present study also provide evidence that total phenol, F&V, coffee and wine intake in the Mediterranean diet are positively correlated with the excretion of TPs in spot urine samples. The intake of F&V contributed more to urinary TPE than coffee, and wine consumption. In addition, the absolute daily consumption of F&V in grams was 781% and 852% higher than the daily intake of coffee and wine, respectively.

F&V, and beverages such as coffee, tea and red wine, constitute the main sources of polyphenols [25]. Polyphenols represent a wide variety of structures from different subclasses, that explain the difficulties to estimate the TP content in these foods, mainly F&V [23]. Polyphenols are quantitatively the main dietary antioxidant source; however, the biological effects of these compounds depends on their bioavailability, their kinetics and exposure time [26]. The most common polyphenols in the human diet are not necessarily the most active *in vivo*, either because they have a lower intrinsic activity than others or because they are poorly absorbed from the intestine, highly metabolized, or rapidly eliminated [25].

Most dietary polyphenols (75–99%) are not found in urine, and the quantities detected intact vary from one phenolic compound to another [11]. This fact may be due to their reduced absorption through the gut barrier, their excretion to the bile or their metabolization by the colonic microflora or our own tissues. To acquire high plasma concentrations requires repeated ingestion of polyphenol-rich foods [27].

The BP lowering effects of fruit- and vegetable-rich diets in hypertensive patients have mainly been attributed to the presence of multiple antioxidants. Two different double-blind cross-over trials [10,28] with polyphenol rich food (dark chocolate versus white and a rich versus low flavonoid sweet juice) showed that the polyphenols rich food reduced BP.

The clinical relevance of the endothelium-dependent effects of plant polyphenols is dependent upon their systemic availability. For this reason, intestinal absorption and metabolism of the plant polyphenols are a rate-limiting step for the protective effects of this class of compounds. In healthy volunteers, the coronary flow-velocity reserve increased 30 min after drinking red wine (1 g/kg ethanol), a polyphenol-rich beverage, but not after drinking the same quantity of alcohol as vodka or white wine, a polyphenol-free beverage or an alcoholic beverage with medium polyphenolic content, respectively [29]. Endothelium-dependent vasodilatation also improved in men, after the acute intake of 500 mL of red wine and de-alcoholized red wine [30]. In patients with mild isolated systolic

Table 3 Baseline characteristics of the study participants by quartile of total polyphenol excretion expressed as mg GAE/g creatinine.

	Urine mg GAE/ g creatinine concentration quartile				
	Q1 (<88.99)	Q2 (89–119.46)	Q3 (119.47–160.22)	Q4 (>160.23)	*P* for trend[a]
No. of subjects	147	148	147	147	
Age, (y) mean (SD)	66.0 (5.7)	68.3 (6.1)	68.0 (5.8)	69.3(6.0)	< 0.001
Women, n (%)	51 (34.7)	83 (56.1)	85 (57.8)	107 (72.8)	< 0.001
Weight (kg), mean (SD)	80.0 (11.1)	75.3 (11.2)	74.1 (10.3)	71.7 (11.2)	< 0.001
BMI, (kg/m^2), mean (SD)	29.6 (2.9)	29.6 (3.7)	29.2 (3.7)	29.1 (3.4)	0.171
Overweight or obese (BMI ≥25 Kg/m2), n (%)	138 (93.9)	137 (92.6)	128 (87.1)	135 (91.8)	0.263
Systolic BP (mm Hg), mean (SD)	156.0 (15.6)	152.2 (18.3)	155.0 (21.6)	149.1 (16.0)	0.015
Diastolic BP (mmHg), mean (SD)	88.9 (10.2)	86.2 (9.8)	85.5 (11.8)	83.4 (8.5)	< 0.001
Hypertension, n (%)	123 (83.7)	126 (85.1)	113 (76.9)	114 (77.6)	0.067
Diabetes, n (%)	31 (21.1)	47 (31.8)	52 (35.4)	62 (42.2)	< 0.001
Dyslipidemia, n (%)	78 (53.1)	103 (69.6)	93 (63.3)	96 (65.3)	0.097
Current smoker n (%)	39 (26.5)	20 (13.5)	22 (15.0)	14 (9.5)	< 0.001
Family history of CHD, n (%)	45 (30.6)	46 (31.1)	47 (32.0)	67 (45.6)	0.008
Medication, n (%)					
ACE inhibitors	119 (81.0)	114 (77.0)	106 (72.1)	107 (72.8)	0.063
Diuretics	11 (7.5)	7 (4.7)	7 (4.8)	9 (5.8)	0.639
Statins or other hypolipidemic drugs	42 (28.6)	70 (47.3)	69 (46.9)	65 (44.2)	0.01
Insulin	3 (2.0)	4 (2.7)	12 (8.2)	9 (6.1)	0.024
Oral hypoglycemic drugs	17 (11.6)	26 (17.6)	26 (17.6)	36 (24.5)	0.006
Aspirin or other antiplatelet drugs	29 (19.7)	28 (18.9)	37 (25.2)	30 (20.4)	0.582
Educational level, n (%)					
Primary school	104 (70.7)	112 (75.7)	108 (73.5)	121 (82.3)	0.041
High school	24 (16.3)	18 (12.2)	28 (19.0)	18 (12.2)	0.685
University	19 (12.9)	17 (11.5)	10 (6.8)	7 (4.8)	0.006
Energy expenditure in physical activity (kcal/d), mean (SD)	256.0 (215.5)	245.9 (220.7)	293.1 (231.0)	255.3 (205.3)	0.576
Glomerular filtration rate, mL/min	77.2 (16.4)	78.1 (14.6)	78.1 (18.1)	79.5 (18.9)	0.264

[a] ANOVA-one factor was used for continuous variables and χ^2-test for categorical variables. BMI: body mass index (calculated as weight in kilograms divided by height in square meters); BP: blood pressure; ACE: angiotensin-converting enzyme; CHD: coronary heart disease.

hypertension, intake of consecutive daily doses of 100 g of polyphenol-rich dark chocolate (500 mg of polyphenols), but not polyphenol-free chocolate, decreased both systolic and diastolic BPs within 10 days [31]. Dietary interventions with extra-virgin olive oil and a reduction in total and saturated fatty acid intake favorably affect BP control in pharmacologically treated hypertensive patients [32]. All these studies support the view that polyphenol-rich diets

Table 4 Multivariate linear regression analyses with systolic blood pressure and diastolic blood pressure as the dependent variables, and quartile of total polyphenol excreted in spot urine samples (mg GAE/g creatinine) as exposure variable, adjusted for potential confounders.

	Model	β	SE	Beta	*P*	95 % CI
Systolic blood pressure	Model 1	−1.743	0.712	−0.104	0.015	−3.141 to −0.345
	Model 2	−1.895	0.741	−0.113	0.011	−3.350 to −0.440
	Model 3	−1.895	0.743	−0.113	0.011	−3.354 to −0.436
	Model 4	−1.731	0.765	−0.103	0.024	−3.233 to −0.228
Diastolic blood pressure	Model 1	−1.705	0.397	−0.180	<0.001	−2.485 to −0.925
	Model 2	−1.438	0.408	−0.152	<0.001	−2.238 to −0.637
	Model 3	−1.405	0.409	−0.148	0.001	−2.208 to −0.602
	Model 4	−1.264	0.422	−0.133	0.003	−2.092 to −0.435

β: Non-standardized coefficient (regression line coefficient); SE: Standard error; Beta: Standardized coefficient; CI: Confidence interval; *P*: two-sided test of significance; Model 1: unadjusted; Model 2: adjusted by sex, age and weight; Model 3 adjusted as in Model 2 plus smoking status, physical activity, and educational level; Model 4 adjusted as in Model 3 plus additionally adjusted for medication use (angiotensin-convertor enzyme inhibitors, diuretics, statins or other hypolipidemic drugs, insulin, oral hypoglycemic drugs and aspirin or other antiplatelet drugs) consumed in the last month, sodium and potassium intake and glomerular filtration rate (GFR); GAE: Gallic acid equivalent.

Table 5 Multivariate adjusted odds ratios (95% confidence intervals) for cardiovascular risk factors (hypertension) according to quartiles of total polyphenol excretion expressed as mg GAE/g creatinine using the lowest quartile group as reference category.

	Urine mg GAE/ g creatinine concentration quartile				
	Q1 (<88.99)	Q2 (89–119.46)	Q3 (119.47–160.22)	Q4 (>160.23)	*P* for trend
Hypertension, n (%)	123 (83.7)	126 (85.1)	113 (76.9)	114 (77.6)	0.067
Model 1	1.00	1.82 (0.33–10.13)	0.67 (0.29–1.55)	0.71 (0.53–0.95)	0.021
Model 2	1.00	1.40 (0.21–9.24)	0.61 (0.25–1.47)	0.64 (0.46–0.89)	0.006
Model 3	1.00	1.29 (0.19–8.49)	0.55 (0.22–1.37)	0.65 (0.47–0.91)	0.007
Model 4	1.00	1.39 (0.19–10.34)	0.55 (0.20–1.48)	0.64 (0.45–0.92)	0.047

Model 1, unadjusted; Model 2 was adjusted for sex, age and weight; Model 3 adjusted as in Model 2 plus smoking status, physical activity, educational level and energy expenditure in physical activity; Model 4 was adjusted as in Model 3 plus medication intake: ACE inhibitor, diuretics, statins (hypolipidemic drugs), insulin, oral hypoglycemic drugs, aspirin or other antiplatelet drug supplements taken in the last month, sodium and potassium intake and glomerular filtration rate (GFR); GAE: Gallic acid equivalent.

may improve endothelium function and reduce BP in hypertensive subjects.

F&V seem to be essential items in the prevention of dietary diseases. Some epidemiological evidence supports that a diet rich in F&V may help prevent BP from increasing and help decrease elevated BP levels for those with high normal BP or hypertension [33]. In the SUN study [4], the prevalence of non-previously diagnosed hypertension was inversely associated with F&V consumption in a Mediterranean population with a very high intake of both fat- and plant-derived foods. In the Nurses' Health Study, intake of F&V was also inversely associated with systolic and diastolic BP, whereas the intake of cereals and meat was directly associated with systolic BP [34]. After the 8-year follow-up in 1714 employed middle-aged men in the Chicago Western Electric Study, vegetable protein, beta-carotene, and an antioxidant vitamin score based on vitamin C and beta-carotene were inversely and significantly related to an average annual change in BP [35]. Thus, in all mentioned studies a high consumption of F&V was associated with a decrease in BP levels. In the current study performed in a free-living Spanish high-risk population, we observed a similar effect when consumption of F&V was high. However, until now, no study has tried to correlate the biomarker of TP intake, determined in spot urine samples, with BP measurements or with the prevalence of hypertension. Taking into account that greater excretion of polyphenols in urine is determined by high TP consumption, we suggest that the observed independent inverse association of objectively measured TPE in urine samples with BP is very probably related to a favourable effect of TP intake in preventing raised BP levels.

Recently, in the SUN study [36] adherence to the Mediterranean diet was not associated with hypertension. However, it was associated with reduced changes in mean levels of systolic and diastolic BP after a 6-year follow-up, suggesting that adherence to a Mediterranean-type diet could contribute to the prevention of age-related changes in BP. Estruch et al. [3] compared the short-term effects of 2 Mediterranean diets versus those of a low-fat diet on intermediate markers of cardiovascular risk in an intervention feeding trial, participants included in the Mediterranean diet groups supplemented with either olive oil or nuts showed a significant decrease in systolic and diastolic BP measurements after 3-months of intervention compared to the low-fat diet group. Our results also add to the increasing evidence that polyphenol-rich diets decrease BP.

Our study has some limitations. First, the participants were elderly subjects at high risk for cardiovascular disease, thus the results can not be extrapolated to the general population. However, since most were hypertensive subjects, our results confirm the usefulness of polyphenol-rich diets in the management of hypertension. A second limitation of our study is its cross-sectional nature which does not allow definitive establishment of causal relationships. However, the current study has the strength of the objective measurement of polyphenol intake by determination of urinary TPE which allows a more precise correlation to be obtained with biological variables than data obtained by FFQ [37,38]. Another limitation is that the colonic microflora catalyze the breakdown of the polyphenol itself to more simple compounds, such as valerolactones or other chemical structures that are not able to further reduce the chemicals present in the F–C reagent and the behavior of these compounds in the SPE has not been studied.

In summary, we report that a high TPE in spot urine is negatively associated with BP levels in an elderly Mediterranean population. Thus, a high intake of polyphenol-rich diet may help to decrease BP in elderly hypertensive populations in order to lower their cardiovascular risk. Compared with the lowest quartile of TPE, in the participants in the highest quartile, the odds of hypertension were reduced by 36% after adjustment for potential confounding factors. However, the observation that systolic and diastolic BP decrease when TPE increases needs to be confirmed in further intervention studies. A longer follow-up of the whole PREDIMED trial will eventually provide the evidence to confirm our results.

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Appendix
Other PREDIMED Investigators

Nutrition and Food Science Department, XaRTA, INSA. Pharmacy School, University of Barcelona, Barcelona, Spain: Mireia Urpi-Sarda, PhD; Rafael Llorach, PhD; Carmen Lopéz-Sabater, PhD.

Hospital Clinic, Institut d'Investigacions Biomèdiques August Pi Sunyer, Barcelona, Spain: Mercè Serra, RD; Ana Pérez-Heras, RD; Concha Viñas, RD; Emilio Sacanella, MD, PhD; Saul Romero, MD; Rosa Casas, BS.

University of Navarra, Primary Care Division, Pamplona, Spain: Estefania Toledo, MD, PhD; Pilar Buil-Cosiales, MD , and Javier Diez-Espino, MD.

University of Valencia, Valencia, Spain: Olga Portolés, PhD; José Vicente Sorlí, MD.

University Rovira i Virgili, Reus (Tarragona), Spain: Mónica Bulló, PhD; Josep Basora, MD; Nancy Babio, PhD.

Instituto de la Grasa, Consejo Superior de Investigaciones Científicas, Sevilla, Spain: Javier S. Perona, PhD.

Municipal Institute for Medical Research, Barcelona, Spain: Montserrat Fitó, PhD; Jaime Marrugat, MD, PhD; Roberto Elosúa, MD, PhD.

University of Málaga, Málaga, Spain: Juan José Sánchez-Luque, MD, PhD; Virginia Velasco-García, MD.

University Institute for Health Sciences Investigation, Palma de Mallorca, Spain: Fernando Rigo, MD; Guillem Frontera, MD.

Primary Care Division, Catalan Institute of Health, Barcelona, Spain: Carmen Cabezas, MD.

Hospital Txagorritxu, Vitoria, Spain: E. Sanz MD.

Hospital Universitario Virgen del Rocío, Sevilla, Spain: Adoración Nieto, MD, PhD.

Department of Family Medicine, Primary Care Division of Sevilla, San Pablo Health Center, Sevilla: José Lapetra, MD, PhD; José Manuel Santos, MD, PhD; Pablo Iglesias, MD, PhD; Manuel Ortega, MD, PhD.

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L'efecte de la llet en la excreció urinària dels àcids fenòlics obtinguts per acció microbiana després del consum de cacau en pols en humans es va presentar en aquest treball. Com en la publicació IV, l'estudi de l'efecte matriu en un altre tipus d'aliment ric en polifenols va ser de gran ajut en el desenvolupament de la Tesi Doctoral; així com l'anàlisi per LC-MS/MS dels metabòlits obtinguts per acció microbiana i excretats en orina.

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ARTICLE

Effect of Milk on the Urinary Excretion of Microbial Phenolic Acids after Cocoa Powder Consumption in Humans

MIREIA URPI-SARDA,[†,‡,∥] RAFAEL LLORACH,[†,‡,∥] NASIRUDDIN KHAN,[†,‡] MARIA MONAGAS,[§] MARIA ROTCHES-RIBALTA,[†,‡] ROSA LAMUELA-RAVENTOS,[†,¶] RAMON ESTRUCH,[§,#] FRANCISCO J. TINAHONES,[⊥,#] AND CRISTINA ANDRES-LACUEVA*[,†,‡]

[†]Nutrition and Food Science Department, XaRTA, INSA, Pharmacy Faculty, University of Barcelona, Barcelona, Spain, [‡]INGENIO−CONSOLIDER Program, Fun-c-food (CSD2007-063), Ministry of Science and Innovation, Madrid, Spain, [§]Department of Internal Medicine, Hospital Clínic, Institut d'Investigació Biomèdica August Pi i Sunyer (IDIBAPS), University of Barcelona, Barcelona, Spain, [¶]RETICS RD06/0045/0003, Instituto de Salud Carlos III, Madrid, Spain, [#]CIBER 06/003 Physiopathology of Obesity and Nutrition (CIBEROBN) Instituto de Salud Carlos III, Madrid, Spain, and [⊥]Servicio de Endocrinología y Nutrición, Hospital Universitario Virgen de la Victoria de Málaga, Málaga, Spain. [∥] These authors contributed equally to this work.

Health effects of cocoa flavonols depend on their bioavailability, which is strongly influenced by the food matrix and the degree of flavanol polymerization. The effect of milk on the bioavailability of cocoa flavanoids considering phase II metabolites of epicatechin has been the subject of considerable debate. This work studies the effect of milk at the colonic microbial metabolism level of the nonabsorbed flavanol fraction that reaches the colon and is metabolized by the colonic microbiota into various phenolic acids. Twenty-one human volunteers followed a diet low in polyphenols for at least 48 h before taking, in a random order, 40 g of cocoa powder dissolved either in 250 mL of whole milk or in 250 mL of water. Urine samples were collected before the intake and during three different periods (0−6, 6−12, and 12−24 h). Phenolic acids were analyzed by LC-MS/MS after solid-phase extraction. Of the 15 metabolites assessed, the excretion of 9 phenolic acids was affected by the intake of milk. The urinary concentration of 3,4-dihydroxyphenylacetic, protocatechuic, 4-hydroxybenzoic, 4-hydroxyhippuric, hippuric, caffeic, and ferulic acids diminished after the intake of cocoa with milk, whereas urinary concentrations of vanillic and phenylacetic acids increased. In conclusion, milk partially affects the formation of microbial phenolic acids derived from the colonic degradation of procyanidins and other compounds present in cocoa powder.

KEYWORDS: Phenolic acids; milk effect; microbiota; cocoa powder; humans; LC-MS/MS

INTRODUCTION

The consumption of cocoa foods has been linked to short- and long-term health benefits, particularly related to cardiovascular diseases (*1*, *2*). Some studies have placed the polyphenolic content of cocoa foods behind these effects, showing that they are important sources of these compounds (*3*). Flavanols are the most abundant flavonoids in cocoa, occurring as monomers (mainly (−)-epicatechin) and as oligomeric (procyanidins B1, B2, and C1) and polymeric forms (procyanidins) (*4*). Monomers account for 5−10% of total cocoa polyphenols and oligomers and polymers for ≥90% (*5*). Requisites such as bioavailability, including microbiota degradation, have been shown as mandatory keys to these expected healthy effects (*4*, *6*).

The bioavailability of flavanols is strongly influenced by several factors, such as their degree of polymerization (*7*) and the food matrix. Whereas monomers seem to be readily absorbed in the small intestine, the absorption of dimeric procyanidins in humans seems to be very limited (*8*, *9*), and polymeric procyanidins are not well absorbed in their native form. These unabsorbed procyanidins reach the colon, where they are largely metabolized by the colonic microbiota, producing a complex phenolic acid profile from the qualitative and quantitative points of view (*10*−*13*). Once formed, they are absorbed, further metabolized in the liver, and excreted in urine. In vitro studies showed that phenolic acids inhibited the secretion of proinflammatory cytokine involved in atherosclerosis from LPS-induced human PBMC (*14*), and they showed the highest effect on modulation of NF-κB activity with strong inhibition of LPS-induced NF-κB activity (*15*). Gut microbiota has been largely related to some aspects of the metabolism and human health (*16*) due to their ability to recover bioactive substances from foods that would otherwise be washed out of the intestinal tract without benefit (*17*). In this context, microbial metabolome is very important given that a significant proportion of dietary polyphenols reaches the gut intact or scarcely metabolized, being further metabolized by colonic microbiota (*18*).

*Corresponding author (telephone +34-93-4034840; fax +34-93-4035931; e-mail candres@ub.edu).

The other important aspect that could modulate the bioavailability of polyphenols is the food matrix. Several studies have provided conflicting evidence about the effect of milk on the bioavailability of polyphenols, specifically flavanols, when looking at their phase II conjugate forms, from different dietary sources such as cocoa or tea (*19*−*22*). In this context, a recent study clarified the controversy, suggesting that the possible influence of milk on cocoa flavonoid absorption, and consequently on the urinary excretion of phase II metabolites, is more relevant for drinks with lower flavan-3-ol content, which is typical of many commercial cocoas, than for drinks with higher content (*23*). Therefore, bearing in mind that milk significantly lowered the excretion of urinary phase II metabolites of flavan-3-ol (*23*), there is a need to study the effect of milk on the colonic microbial metabolism of the nonabsorbed flavanol fraction that reaches the colon. As far as we know, little information is available about the evaluation of the impact of milk on phenolic acid excretion after consumption of flavan-3-ol-rich sources such as cocoa products. Studies related to the interaction between milk and cocoa polyphenol absorption are important because Spain has the largest consumption of cocoa powder products per person (1668 g/person/year), followed by Norway (1647 g/person/year) and Sweden (1288 g/person/year) [reports of ACNielsen, Euromonitor International, and Caobisco Association of the Chocolate biscuit and confectionery industries of the European Union (EU)], representing approximately 28% of the total cocoa consumption in this country (*24*). In addition, cocoa powder is mainly consumed with milk and during breakfast, and this represents the main source of flavonoids in the young population (Family Food Panel, Spain 2005−2006, Taylor Nelson Sofres).

In this context, the aim of this study was to evaluate the effect of milk at a colonic microbial level, studying the urinary excretion of microbial-derived phenolic acids after the intake of a standard portion of cocoa powder with either water or milk in an acute intervention using a targeted quantitative procedure.

MATERIALS AND METHODS

Standards and Reagents. The following compounds (% purity, when available) were used. 3,4-Dihydroxyphenylpropionic acid (≥98%), caffeic acid (≥95%), ferulic acid (≥98%), *p*-coumaric acid (≥98%), 3,4-dihydroxyphenylacetic acid (98%), 3-methoxy-4-hydroxyphenylacetic acid (99%), 3-hydroxyphenylacetic acid (≥97%), phenylacetic acid (≥98%), protocatechuic acid (>97%), 4-hydroxybenzoic acid (≥98%), 3-hydroxybenzoic acid (≥98%), hippuric acid (98% purity), ethyl gallate (≥96%), creatinine, and β-glucuronidase/sulfatase (from *Helix pomatia*) were purchased from Sigma-Aldrich (St. Louis, MO). 4-Hydroxyhippuric acid (>99%) was purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). Vanillic acid and *m*-coumaric acid were purchased from Extrasynthese (Genay, France). HPLC grade solvents methanol, acetonitrile, and formic acid were purchased from Scharlau (Barcelona, Spain). Hydrochloric acid was purchased from Panreac (Barcelona, Spain).

Subjects and Study Design. Twenty-one nonsmoking healthy volunteers (9 women and 12 men) between 18 and 50 years old with a body mass index of 21.6 ± 2.1 were recruited. None reported any history of cardiovascular risk factors, heart disease, or other medical disease, nor were receiving any medication or vitamin supplement. All gave written informed consent before their inclusion in the trial, and the Institutional Review Board of the Hospital Clinic of Barcelona (Spain) approved the study protocol.

The study was an open, prospective, randomized, crossover feeding trial. Participants were instructed to abstain from polyphenol-rich foods for at least 48 h before and during the intervention day. After overnight fasting, the 21 subjects were provided randomly with a single dose of 40 g of cocoa powder with 250 mL of water (hereafter termed CW) or with 250 mL of milk (hereafter termed CM). This protocol was repeated after 1 week following a crossover experimental design. Test meals were prepared each day of the study following a standardized procedure. Four hours after the cocoa intake, a light meal of bread and cheese was provided. Urine samples were collected before ingestion and in the periods of 0−6, 6−12, and 12−24 h. They were stored at −80 °C until analysis.

The CM and CW macronutrient composition (in 250 mL), respectively, was as follows: carbohydrates, 30.75 and 58.40 g; fat, 10.91 and 2.16 g; protein, 13.54 and 5.64 g; energy, 275.35 and 276.6 kcal. The phenolic composition (mean ± SD) of the cocoa powder was 0.71 ± 0.09 mg/g of (−)-epicatechin, 0.21 ± 0.01 mg/g of (+)-catechin, 0.64 ± 0.06 mg/g of procyanidin B2, 33.87 μg/g of isoquercitrin, 5.74 μg/g of quercetin, 4.33 μg/g of quercetin-3-glucuronide, and 36.32 μg/g of quercetin-3-arabinoside (*8*). The milk had no phenolic compounds.

Extraction of Phenolic Acids from Urine. The extraction of phenolic acids from the urine was carried out following a validated and published method (*8*). Briefly, after hydrolysis of the urine samples with β-glucuronidase/sulfatase, they were loaded into preconditioned Oasis MCX 96-well plates for solid-phase extraction (Waters, Milford, MA), washed, and extracted with methanol. Then, they were evaporated to dryness and reconstituted with 100 μL of mobile phase.

LC-MS/MS. The analyses were performed with liquid chromatography−tandem mass spectrometry (LC-MS/MS). LC-PAD analyses were performed using a Perkin-Elmer series 200 (Norwalk, CT) equipped with a quaternary pump and a refrigerated autosampler plate. An Applied Biosystems API 3000 triple-quadrupole mass spectrometer (PE Sciex, Concord, ON, Canada) equipped with a Turbo IonSpray ionizing in negative mode was used. A Phenomenex Luna C_{18} analytical column [50 × 2.0 mm i.d., 5 μm] (Torrance, CA) with mobile phases A (95% water, 5% acetonitrile, and 0.1% formic acid) and B (100% acetonitrile and 0.1% formic acid) was used. The gradient, at a flow rate of 400 μL/min, was as follows: 0−1 min, 4−40% B; 1−3 min, 40−100% B; 3−5 min, 100% B; 6−10 min, 4% B (*8*). Finally, the column was washed and re-equilibrated for 6 min. The injected volume was 15 μL. MS/MS parameters used were as follows: capillary voltage, −3700 V; focusing potential, −200 V; entrance potential, −10 V; declustering potential, −50 V; nebulizer gas, 10 (arbitrary units); curtain gas, 12 (arbitrary units); collision gas, 5 (arbitrary units); auxiliary gas temperature, 400 °C; auxiliary gas flow rate, 6000 cm^3/min. The collision energy for each compound was as published previously (*8*). For quantification of phenolic acids, data were collected in the multiple reaction monitoring (MRM) mode, tracking the transition of parent and product ions specific for each compound as follows: 3,4-dihydroxyphenylpropionic acid (181/137); caffeic acid (179/135); ferulic acid (193/134); *m*- and *p*-coumaric acids (163/119); 3,4-dihydroxyphenylacetic acid (167/123); 3-methoxy-4-hydroxyphenylacetic acid (181/137); 3-hydroxyphenylacetic acid (151/107); phenylacetic acid (135/91); protocatechuic acid (153/109); 3- and 4-hydroxybenzoic acids (137/93); 4-hydroxyhippuric acid (194/100); vanillic acid (167/152). A dwell time of 80 ms was used for each MRM transition. Hippuric acid was analyzed by PAD at 240 nm (*8*).

Human urine creatinine concentrations were measured by a colorimetric assay using picric acid (*25*).

Statistical Analysis. The SPSS Statistical Analysis System, ver. 15.0 (SPSS), was used to perform the statistical analysis. Because the data were nonparametric (Kolmogorov test) and presented nonhomogeneous variances (Levene test), the Wilcoxon test for related samples was used to compare changes in outcome variables in response to (A) both tested meals at each time period and (B) differences between time periods in each tested meal. Concentration levels were expressed as means (nmol/mg creatinine) ± standard error of the mean (SEM). Differences with $P < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

In this work we evaluated the excretion of 15 phenolic acids, which had been described in the microbial degradation pathway of flavanols and is well-known in the literature (*10*−*13*, *18*, *26*). The effect of milk on the urinary excretion of microbial-derived phenolic acids after 40 g of cocoa powder consumed with 250 mL of water or 250 mL of whole milk is presented in **Figure 1**. Of the 15 studied phenolic acids, 9 showed significant differences as a function of the consumption of CW or CM (**Figure 1** and **Table 1**).

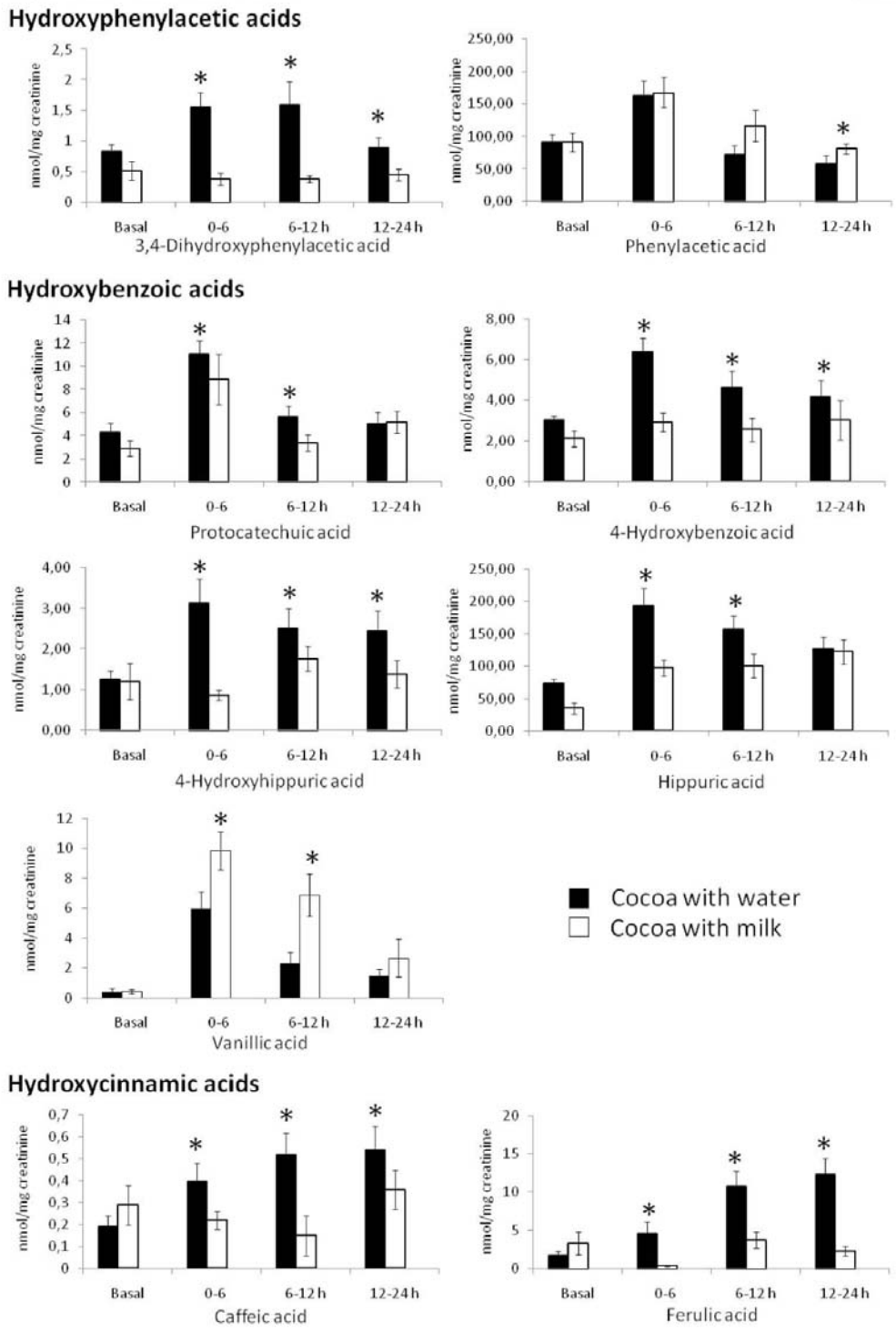

Figure 1. Urinary excretion (mean ± SEM) of the nine phenolic acids significantly affected by the consumption of cocoa powder with water or with milk. Bars with an asterisk are significantly different ($P < 0.05$; Wilcoxon's test; $n = 21$) in the same time period.

Table 1. Urinary Excretion of Phenolic Acid Metabolites after Consumption of Cocoa Powder with 250 mL of Water or Cocoa Powder with 250 mL of Milk[a]

	cocoa powder with water (nmol/mg of creatinine)				cocoa powder with milk (nmol/mg of creatinine)			
phenolic acid	baseline	0−6 h	6−12 h	12−24 h	baseline	0−6 h	6−12 h	12−24 h
hydroxyphenylpropionic acids								
3,4-dihydroxyphenylpropionic acid	5.96 ± 1.07 a	16.84 ± 2.81 b	7.92 ± 1.49 a	6.62 ± 1.67 a	3.37 ± 0.96 a	16.9 ± 4.00 b	5.19 ± 1.17 a	8.1 ± 1.21 c
hydroxyphenylacetic acids								
3,4-dihydroxyphenylacetic acid	0.84 ± 0.11 ac	1.55 ± 0.25 b	1.60 ± 0.37 b,c	0.89 ± 0.17 a	0.56 ± 0.15 a	0.38 ± 0.10 a	0.38 ± 0.05 a	0.45 ± 0.10 a
3-methoxy-4-hydroxyphenylacetic acid	4.37 ± 0.50 a	7.67 ± 1.05 ab	11.30 ± 1.63 b	8.45 ± 0.94 a	3.99 ± 0.31 a	6.95 ± 0.57 b	9.30 ± 0.74 b	8.05 ± 1.15 b
3-hydroxyphenylacetic acid	3.45 ± 0.73 a	5.25 ± 2.04 a	7.34 ± 2.23 a	10.1 ± 3.42 b	3.70 ± 1.16 ab	1.71 ± 0.23 a	4.12 ± 0.62 b	7.36 ± 1.61 b
phenylacetic acid	91.35 ± 11.36 a	163.77 ± 22.10 b	71.90 ± 14.78 ac	58.85 ± 11.94 c	91.80 ± 14.34 ab	167.72 ± 23.80 a	116.64 ± 24.47 b	81.37 ± 7.83 b
hydroxybenzoic acids								
protocatechuic acid	4.33 ± 0.77 a	11.07 ± 1.19 b	5.58 ± 0.97 a	5.05 ± 0.99 a	2.90 ± 0.65 a	8.88 ± 2.20 b	3.36 ± 0.69 a	5.18 ± 0.92 c
4-hydroxybenzoic acid	3.02 ± 0.20 a	6.39 ± 0.66 b	4.60 ± 0.85 a	4.16 ± 0.84 a	2.10 ± 0.40 a	2.92 ± 0.47 a	2.56 ± 0.58 a	3.01 ± 0.97 a
3-hydroxybenzoic acid	0.11 ± 0.03 a	0.24 ± 0.03 b	0.56 ± 0.24 abc	0.55 ± 0.17 c	0.18 ± 0.10 a	0.25 ± 0.41 a	0.41 ± 0.26 a	0.60 ± 0.28 b
4-hydroxyhippuric acid	1.26 ± 0.20 a	3.13 ± 0.60 b	2.51 ± 0.50 b	2.44 ± 0.51 b	1.21 ± 0.44 ab	0.87 ± 0.12 a	1.76 ± 0.30 b	1.39 ± 0.33 a
hippuric acid	73.05 ± 6.35 a	193.16 ± 27.89 b	156.14 ± 21.93 bc	126.55 ± 18.00 c	35.43 ± 8.32 a	97.92 ± 12.15 b	100.62 ± 18.63 b	122.82 ± 18.83 b
vanillic acid	0.41 ± 0.24 a	5.96 ± 1.15 b	2.29 ± 0.74 c	1.49 ± 0.41 c	0.43 ± 0.15 a	9.85 ± 1.27 b	6.88 ± 1.40 b	2.67 ± 1.26 c
hydroxycinnamic acids								
caffeic acid	0.19 ± 0.05 a	0.40 ± 0.08 b	0.52 ± 0.10 b	0.54 ± 0.11 b	0.29 ± 0.09 abc	0.22 ± 0.04 a	0.15 ± 0.09 b	0.36 ± 0.09 c
ferulic acid	1.72 ± 0.52 a	4.62 ± 1.48 a	10.72 ± 1.97 b	12.35 ± 2.07 b	3.27 ± 1.49 ab	0.39 ± 0.06 a	3.73 ± 1.03 b	2.31 ± 0.57 b
p-coumaric acid	0.004 ± 0.001 a	0.009 ± 0.003 b	0.004 ± 0.002 a	0.005 ± 0.002 a	0.004 ± 0.001 a	0.007 ± 0.002 b	0.003 ± 0.001 a	0.004 ± 0.002 a
m-coumaric acid	0.007 ± 0.001 a	0.024 ± 0.006 a	0.100 ± 0.060 a	0.014 ± 0.007 a	0.007 ± 0.003 a	0.006 ± 0.006 a	0.040 ± 0.015 a	0.013 ± 0.004 a

[a] All values are means ± SEMs; $n = 21$. Values in the same row and in the same intervention with different letters are significantly different, $P < 0.05$ (Wilcoxon's test).

With respect to the group of hydroxyphenylacetic acids, their colonic formation could occur mainly from the direct degradation of procyanidins (*13*). Among the group of hydroxyphenylacetic acids, 3,4-dihydroxyphenylacetic and phenylacetic acids showed significant changes as a function of the food matrix. Significantly lower levels of 3,4-dihydroxyphenylacetic acid were observed after CM when compared with CW in all of the urine fractions after intake. The major differences (~76%) were observed at 0−6 and 6−12 h fractions, and a lower difference (49%) was also observed at 12−24 h (**Figure 1**). Therefore, these results showed different excretion kinetics for this compound between the two intakes, with no increase in the excretion when CM was taken but increasing at 0−6 h and remaining constant up to 12 h after the consumption of CW (**Table 1**). This could mean that procyanidins from CW could be bioavailable for the intestinal microbiota, but not from CM, and degraded easily and rapidly, forming the 3,4-dihydroxyphenylacetic acid.

The excretion of phenylacetic acid showed a matrix effect in the last time period (12−24 h), although the same tendency was also observed at 6−12 h but was not statistically significant. Contrary to other hydroxyphenylacetic acids, its excretion at 12−24 h was significantly incremented at around 38% when volunteers consumed CM with respect those who consumed CW (**Figure 1**). Due to the major urinary excretion after CM, we could hypothesize that phenylacetic acid could come from cocoa phenylalanine, at least partially. During the roasting of cocoa there is a formation of biogenic amines (phenylethylamine) from cocoa amino acids (phenylalanine) (*27*). This phenylethylamine after CM could be absorbed more easily and rapidly than after CW. In the liver, phenylethylamine is oxidized by aldehyde dehydrogenase and aldehyde oxidase, producing phenylacetaldehyde, which is mainly metabolized to phenylacetic acid (*28*).

For the remaining hydroxyphenylacetic acids, such as 3-methoxy-4-hydroxyphenylacetic acid and 3-hydroxyphenylacetic acid, no significant changes were observed between the consumptions of CW or CM, although the same kinetic tendency was observed (**Table 1**).

With respect to hydroxybenzoic acids, the urinary levels of protocatechuic, 4-hydroxybenzoic, 4-hydroxyhippuric, hippuric, and vanillic acids were significantly affected by the matrix. The formation of protocatechuic acid has been described through β-oxidation of 3,4-dihydroxyphenylpropionic acid (*12*) or through α-oxidation of 3,4-dihydroxyphenylacetic acid (*18*). A similar kinetic pattern was observed for protocatechuic acid in both intakes, reaching the maximum concentration at 0−6 h (**Figure 1**). Moreover, protocatechuic acid excretion was affected by milk intake similar to the 3,4-dihydroxyphenylacetic acid. These data also suggest that although the microbial degradation pathway has been partially elucidated, the exact origin of certain specific phenolic acids such as protocatechuic acid remains to be studied. Lower levels of protocatechuic acid were observed after CM when compared with CW at 0−6 and 6−12 h periods (20 and 40%, respectively), whereas no significant differences were detected in the 12−24 h period. Concentrations of protocatechuic acid showed a significant increase in excretion at 0−6 h with CW, but with CM significant increases occurred at 0−6 and 12−24 h.

Microbial dehydroxylation of protocatechuic acid could give rise to 4-hydroxybenzoic acid derivative that may undergo glycination in the liver and kidney being converted into 4-hydroxyhippuric acid (*12*). Both 4-hydroxybenzoic and 4-hydroxyhippuric acids showed lower excretion after CM when compared with CW in all of the collected fractions (from 28 to 72% of diminution) (**Figure 1** and **Table 1**). When their kinetic patterns were taken into account, the excretion of both compounds did not vary in the time when CM was taken. However, variations in excretion were observed for 4-hydroxybenzoic acid when CW was consumed, reaching the maximum concentration at 0−6 h. Higher levels of 4-hydroxyhippuric acid were observed in all urine fractions after CW when compared with baseline. Both compounds, 4-hydroxybenzoic and 4-hydroxyhippuric, have been previously reported to arise from the microbial degradation of procyanidin dimer B3 in rats (*12*). Particularly, 4-hydroxybenzoic acid has also been detected after the administration of wine powder, catechin, and dimer B3 diets to rats (*12*, *26*).

The excretion of hippuric acid, which is most likely derived from the glycination of benzoic acid, was also diminished by the milk. The maximum difference between CM and CW was seen at 0−6 h (49% of diminution after CM), followed by a 36% of diminution at 6−12 h, whereas there was no significant difference

at 12−24 h. This implies that the difference in excretion of hippuric acid between CM and CW decreased over time and was less affected by milk as its metabolism progressed. Urinary excretion of hippuric acid has been associated with the microbial degradation of many phenolic compounds such as hydroxycinnamic acids (*29*) or flavanoids present in foods such as black or green tea (*29*, *30*), wine powder (*26*), and sorghum bran (*31*), and it is also common to the metabolism of other dietary components, which makes it difficult to pinpoint its origin.

With regard to hydroxycinnamic acids, caffeic acid and its methylated derivative (ferulic acid) arising from its subsequent liver metabolism were excreted to a lesser extent after CM than after CW. This behavior was observed in all collected urine fractions, representing 50% (mean value) and 80% (mean value) of diminution for caffeic acid and ferulic acid, respectively (**Figure 1**). As could be observed from **Table 1** and **Figure 1**, the increment in concentration found in the last fraction (12−24 h) for both caffeic and ferulic acids suggested that a longer period of urine collection was required for their complete excretion. Previously, Rios et al. (*11*) studied the urinary excretion of phenolic acids after acute consumption of 80 g of chocolate with bread and water. Similar to our results, they observed a significant increase of ferulic acid excretion at 9 h after consumption. According to the flavanol microbial degradation pathway partially elucidated by several authors (*10*, *12*, *13*) and the present results, it could be proposed that these compounds could come from several routes of metabolism, which could include dehydrogenation of 3,4-dihydroxyphenylpropionic acid as well as degradation from other phenolic derivatives present in cocoa powder, such as chlorogenic acid or *N*-phenylpropenoyl-L-amino acids (*11*). *N*-Phenylpropenoyl-L-amino acids are hydroxycinnamic acid amides of aspartic acid, tyrosine, and 3-hydroxytyrosine (*3*). In fact, Rios et al. (*11*) proposed that the urinary excretion of ferulic acid after consumption of chocolate was associated with the clovamide [(−)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine] content of cocoa foods.

Whereas a higher global urinary excretion of phenolic acids was observed with consumed CW, vanillic acid showed an excretion pattern contrary to those of other metabolites. This compound was excreted in higher significant amounts after consumption of CM at 0−6 and 6−12 h periods when compared with CW. This result could mean that liposoluble vanillin, when ingested with CM, could be absorbed more easily and rapidly than after its intake with CW. Vanillin, a flavoring product added in cocoa preparations (*10*, *11*), could be oxidized to vanillic acid by aldehyde oxidase of the liver (*32*), although it could also arise from the methylation of protocatechuic acid.

As far as we know, little information is available concerning studies about the effect of milk in the production of phenolic acids by colonic microbiota after polyphenol-rich food consumption. Recently, Serafini et al. (*33*) studied the effect of milk on the plasma antioxidant capacity and on the plasma levels of ferulic and caffeic acids after the consumption of 200 g of blueberries with 200 mL of water or with 200 mL of whole milk in human volunteers. They observed enhanced plasmatic concentration of caffeic and ferulic acids when blueberries were consumed with water, whereas no increase in plasma antioxidant capacity and reduced plasmatic levels of these metabolites were observed when consumed with milk (*33*). There is some controversy about the effect of milk on the plasma antioxidant capacity as Lotito et al. (*34*) have reviewed in 2006. It has been suggested that in whole milk, with a higher fat content than skimmed milk, polypeptide chains are located around the fat globule membrane, which promotes their linkage with phenolic compounds (*33*, *35*) and probably causes the decrease of accessibility by the colonic microbiota and therefore their degradation. Also, recently the same behavior was observed with yogurt. Roowi et al. (*36*) determined the excretion of 9 phenolic acids after the consumption of orange juice with and without natural fat yogurt, 5 of which were associated with orange juice consumption (3-hydroxyphenylacetic acid, 3-hydroxyphenylhydracrylic acid, dihydroferulic acid, 3-methoxy-4-hydroxyphenylhydracrylic acid, and 3-hydroxyhippuric). The 24 h excretion of these 5 phenolic acids diminished from 62 ± 18 to 9.3 ± 4.4 μmol when orange juice was ingested with yogurt.

In conclusion, among the 15 studied compounds, the excretion of 9 phenolic acids was significantly affected by the matrix in which the cocoa powder was dissolved (water or milk). According to the results, these 9 phenolic acids can be distributed in two groups: (1) positively affected by milk intake, vanillic acid and phenylacetic acid; and (2) negatively affected by milk intake, (2a) 3,4-dihydroxyphenylacetic acid, protocatechuic acid, 4-hydroxybenzoic acid, 4-hydroxyhippuric acid, and hippuric acid, and (2b) caffeic and ferulic acids probably related to the metabolism of hydroxycinnamic acid amides present in cocoa products as well as to other compounds. These results showed that milk had a partial effect on the phenolic acid excretion profile after the consumption of cocoa powder.

ABBREVIATIONS USED

PAD, photodiode array detector; MRM, multiple reaction monitoring; CM, cocoa with milk; CW, cocoa with water.

SAFETY

Guidelines for work with organic solvents and acids were respected. Universal precautions for the handling of chemicals and fluids were applied.

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ALTRES CAPÍTOLS DE LLIBRE

Capítol de llibre I: Trespalacios MP, Tulipani S, Garcia-Aloy M, Zamora-Ros R, Rotchés-Ribalta M, Rabassa M, Urpi-Sarda M, Boto M, Vázquez R, Llorach R, Andrés-Lacueva C. "Nutritional biomarkers: applications and challenges", dins el llibre "Food intake: regulation, assessing and controlling" (Nova Science Publishers, 2012).

Chapter book

NUTRITIONAL BIOMARKERS: APPLICATIONS AND CHALLENGES

María del Pilar Trespalacios, Sara Tulipani, Mar Garcia-Aloy, Raul Zamora-Ros, Maria Rotchés-Ribalta, Montserrat Rabassa, Mireia Urpi-Sarda, María Boto, Rosa Vázquez, Rafael Llorach, and Cristina Andrés-Lacueva*.

University of Barcelona, Spain.

ABSTRACT

Diet is implicated in a myriad of diseases, ranging from events in the first weeks of life to chronic and age-related illnesses, for example, cancer and cardiovascular disease. A primary goal of nutrition research is to optimize health via dietary interventions, by prevention, delay, or reduction in the severity of disease.

To date, several methods for dietary assessment have been used, such as food frequency questionnaires, food diaries, food records, or 24-h dietary recalls. However, they are associated with different measurement errors between diet observed and true intake, which may obscure some disease risk associations. Thus, nutrition epidemiologists started using different biomarkers in biological samples that are more objective measures and are independent of errors related to dietary assessment methods. A nutritional biomarker can be any biological compound that is an indicator of nutritional status with respect to the intake or metabolism of dietary constituents. An ideal nutritional biomarker would accurately reflect its dietary intake level and it would be specific, sensitive and applicable to many populations. It should also be reproducible, validated and reliable.

The discovery and assessment of nutritional biomarkers highly depend on appropriate analytical tools. An ideal technique for biomarkers discovery and monitoring, unfortunately, is

not currently available, the critical limiting factor being the lack of standardized technologies and methodologies in biomarker discovery and validation process.

Nevertheless, recent technological advances have improved the detection capabilities of the instrumental techniques commonly used in nutrition research. The current tendency is to make use of multiple analytical platforms to maximize the range of metabolic profile studied in a sample. Of the emerging multiple platforms for biomarker discovery, perhaps none has garnered more recent attention than metabolomics. The main analytical and statistical strategies used in nutritional metabolomics are discussed, and some applications to biomarkers discovery presented. Also the main factors affecting the utility of a biomarker in reflecting properly dietary exposures are discussed. Notwithstanding the current main challenges for metabolomics, several examples of successful applications to nutritional research exist. Concluding, the development of new robust nutritional biomarkers may help to improve disease risk stratification by better characterizing the metabolic phenotype at the individual level. Consequently, new nutritional biomarkers are needed to objectively evaluate the effect of diet on health and to check adherence to dietary recommendations and healthy eating patterns.

Capítol de llibre II: Rabassa M, Zamora-Ros R, Tulipani S, Khymenets O, Urpi-Sarda M, Garcia-Aloy M, Boto M, Vázquez R, Rotches-Ribalta M, Chiva G, Trespalacios MP, Llorach R, Andres-Lacueva C. "Flavonoids from food and its implication in human health", dins el llibre "Applications of natural products in food" (Nova Science Publishers, 2012).

Chapter

FLAVONOIDS FROM FOOD AND ITS IMPLICATION IN HUMAN HEALTH

Montse Rabassa[1,2], Raul Zamora-Ros[1,3], Sara Tulipani[1,2], Olha Khymenets[1,2], Mireia Urpi-Sarda[1,4], Mar Garcia-Aloy[1,2], Maria Boto[1,4], Rosa Vázquez[1,2], Maria Rotches-Ribalta[1,2], Gemma Chiva-Blanch[1,4], María del Pilar Trespalacios[1,2], Rafael Llorach[1,2], and Cristina Andres-Lacueva*[1,2]

[1]Nutrition and Food Science Department, XaRTA-INSA.
Pharmacy Faculty, University of Barcelona, Av Joan XXIII, s/n, Barcelona, Spain
[2]Ingenio-CONSOLIDER program, FUN-C-FOOD, Spain CSD2007-063.
Ministry of Science and Innovation, Barcelona, Spain
[3]Unit of Nutrition, Environment and Cancer, Cancer Epidemiology Research Programme, Catalan Institute of Oncology, IDIBELL, Barcelona, Spain
[4]Department of Internal Medicine, Hospital Clinic,
Institut d'Investigació Biomèdica August Pi i Sunyer (IDIBAPS),
University of Barcelona, Villarroel 170, 08036 Barcelona, Spain

ABSTRACT

Flavonoids comprise a large group of phenolic compounds synthesized by plants as secondary metabolites, with a common diphenylpropane skeleton (C6-C3-C6). Over 9,000 structurally distinct flavonoids have been identified in nature, although less than 200 were found in a sample French population. Flavonoids can be classified according to their chemical structure into eight subclasses: flavanones, flavones, dihydroflavonols, flavonols, flavan-3-ols or flavanols, anthocyanidins, isoflavones and proanthocyanidins.

Flavonoids occur ubiquitously in plants and plant products. They are generally found in glycosylated forms, mainly with glucose or rhamnose moieties, but other sugars may also be involved. Flavonoids in foods contribute to their flavor, providing color, astringency and bitterness. Flavonols are the most frequent flavonoids in foods, with onions, apples and tea being the main food sources. Flavones are widely distributed in plants, but in lower amounts than flavonols. The richest sources are herbs and spices. Dihydroflavonols have been identified in a few foods, such as wines and oregano. Flavanones are characteristic compounds of citrus fruits

* E-mail address: candres@ub.edu

and their derived juices. Anthocyanidins are responsible for the colours of fruits and vegetables, like pink, red, blue or purple. They are abundant in berries and their derived products, such as juices and wines. Flavan-3-ols are found as aglycones in foods. Catechin and epicatechin are the main flavan-3-ol contributors, with tea, apples, red wine and chocolate being their most common food sources. Proanthocyanidins are oligomeric and polymeric flavan-3-ols, also known as condensed tannins, and are responsible for the astringency of various fruits or beverages and for the bitterness of chocolate. Theaflavins and thearubigins are derived flavanol compounds that are only found in tea. Isoflavones are found almost exclusively in leguminous plants, particularly in soybeans and their processed products.

The bioavailability of flavonoids varies greatly among and within their subclasses, in terms of their chemical structure and sugar moiety type. Flavonoids are usually absorbed by both the small intestine and colon as aglycones after being hydrolyzed by either enzymes or the microbiota with a high intra- and inter-individuality. After absorption, they are quickly metabolized in the intestinal cells or in the liver. Therefore, the biological activity of flavonoids may be due to their conjugated forms or the aglycones that may be set free in target tissues. Finally, they are eliminated by renal and/or liver excretion. Most flavonoids are known to exert similar biological effects – such as antioxidant, anticarcinogenic, anti-inflammatory, antiobesity, antidiabetic, antiallergic, hepatoprotective, and gastroprotective activities – in vitro. But each individual compound has different effective concentrations, may use different mechanism of actions and even exert different effects.

Epidemiological studies have suggested that flavonoids may provide protection against several chronic diseases, such as cardiovascular and neurodegenerative diseases, diabetes, obesity and some cancers. However, scientific evidence on the role of flavonoid intake in disease prevention is promising but not conclusive, and further clinical and epidemiological studies are needed.

Capítol de llibre III: Urpi-Sarda M, Llorach R, Monagas M, Khan N, Rotches-Ribalta M, Roura E, Lamuela-Raventos RM, Estruch R, Andres-Lacueva C. "Effect of cocoa powder in the prevention of cardiovascular disease: biological, consumption and inflammatory biomarkers. A metabolomic approach", dins el llibre "Recent Advances in Pharmaceutical Sciences" (Transworld research Network, 2011).

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Trivandrum-695 023
Kerala, India

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5. Effect of cocoa powder in the prevention of cardiovascular disease: biological, consumption and inflammatory biomarkers. A metabolomic approach

Mireia Urpi-Sarda[1,2,5*], Rafael Llorach[1,4], Maria Monagas[2,3], Nasiruddin Khan[1,2], Maria Rotches-Ribalta[1,4], Elena Roura[1], Rosa Lamuela-Raventos[1,5], Ramón Estruch[2,5], Cristina Andres-Lacueva[1,4*]

[1] Nutrition and Food Science Department, XaRTA. INSA. Pharmacy Faculty, University of Barcelona (UB), Av.Joan XXIII s/n, 08028 Barcelona, Spain.
[2] Department of Internal Medicine, Hospital Clínic. Institut d'Investigació Biomèdica August Pi i Sunyer (IDIBAPS), UB, Barcelona, Spain
[3]Instituto de Fermentaciones Industriales (CSIC), Madrid, Spain.
[4]INGENIO-CONSOLIDER Program, Fun-c-food CSD2007-063
[5]CIBER de Fisiopatología de la Obesidad y la Nutrición (CIBEROBN), and RETICS RD06/0045, Instituto de Salud Carlos III, Spain

Abstract. Numerous health benefits have been attributed to cocoa and its derived products in the last decade including antioxidant, anti-platelet and positive effects on lipid metabolism and vascular function. Inflammation plays a key role in the initiation and progression of atherosclerosis. However, cocoa feeding trials focused on inflammation are still rare and the results yielded are controversial. Health effects derived from cocoa consumption have been partly attributed to its polyphenol content, in particular of flavanols. Bioavailability is a key issue in order for cocoa polyphenols could exert their biological activities. In the case of flavanols, bioavailability is strongly influenced by several factors, such as their degree of polymerization and the food matrix in which the polyphenols are delivered. Furthermore, gut has become an active site for the metabolism of procyanisins (oligomeric and polymeric flavanols). Estimation of polyphenol consumption or exposure is also a very challenging task in Food and Nutrition Science in order to correlate the intake of phytochemicals with *in vivo* health effects. In the area of nutrition, modern analytical techniques based on mass spectrometry are leading to considerable advances in targeted metabolite analysis and particularly in Metabolomics or global metabolite analysis.

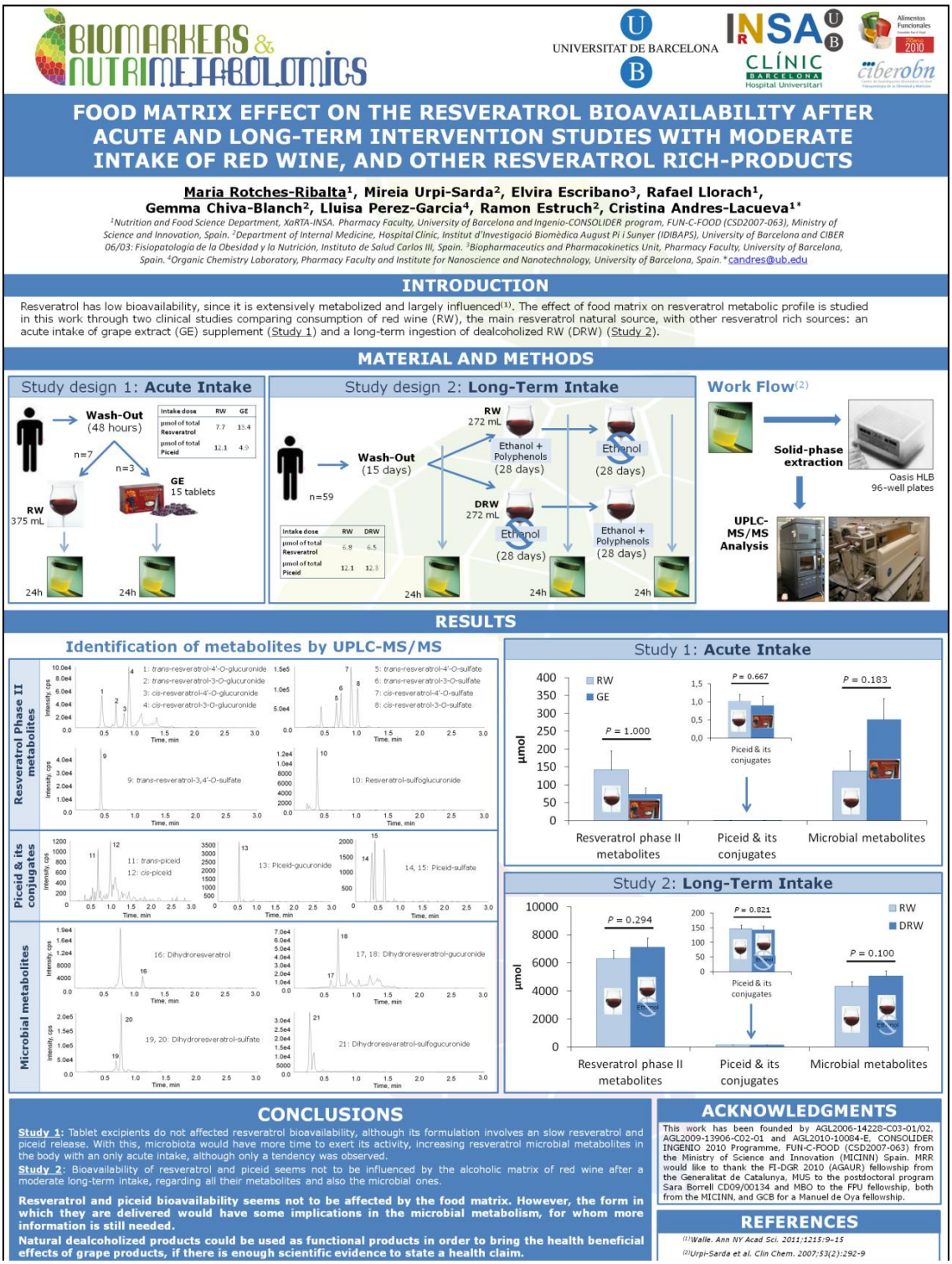
In the current chapter we have summarized the most relevant results of our recent research conducted on the bioavailability of cocoa polyphenols and the effect of the matrix in which cocoa polyphenols are delivered in humans considering both targeted analysis and a metabolomic approach. Furthermore, we have also summarized the effect of long-term consumption of cocoa powder in patients at high risk of cardiovascular disease (CHD) on the inflammatory biomarkers of atherosclerosis.

Correspondence/Reprint request: Dr. Cristina Andres-Lacueva and Dr. Mireia Urpi Sarda, *Nutrition and Food Science Department, XaRTA. INSA. Pharmacy Faculty, University of Barcelona (UB), Av.Joan XXIII s/n, 08028 Barcelona, Spain*. E-mail: candres@ub.edu; murpi@ub.edu

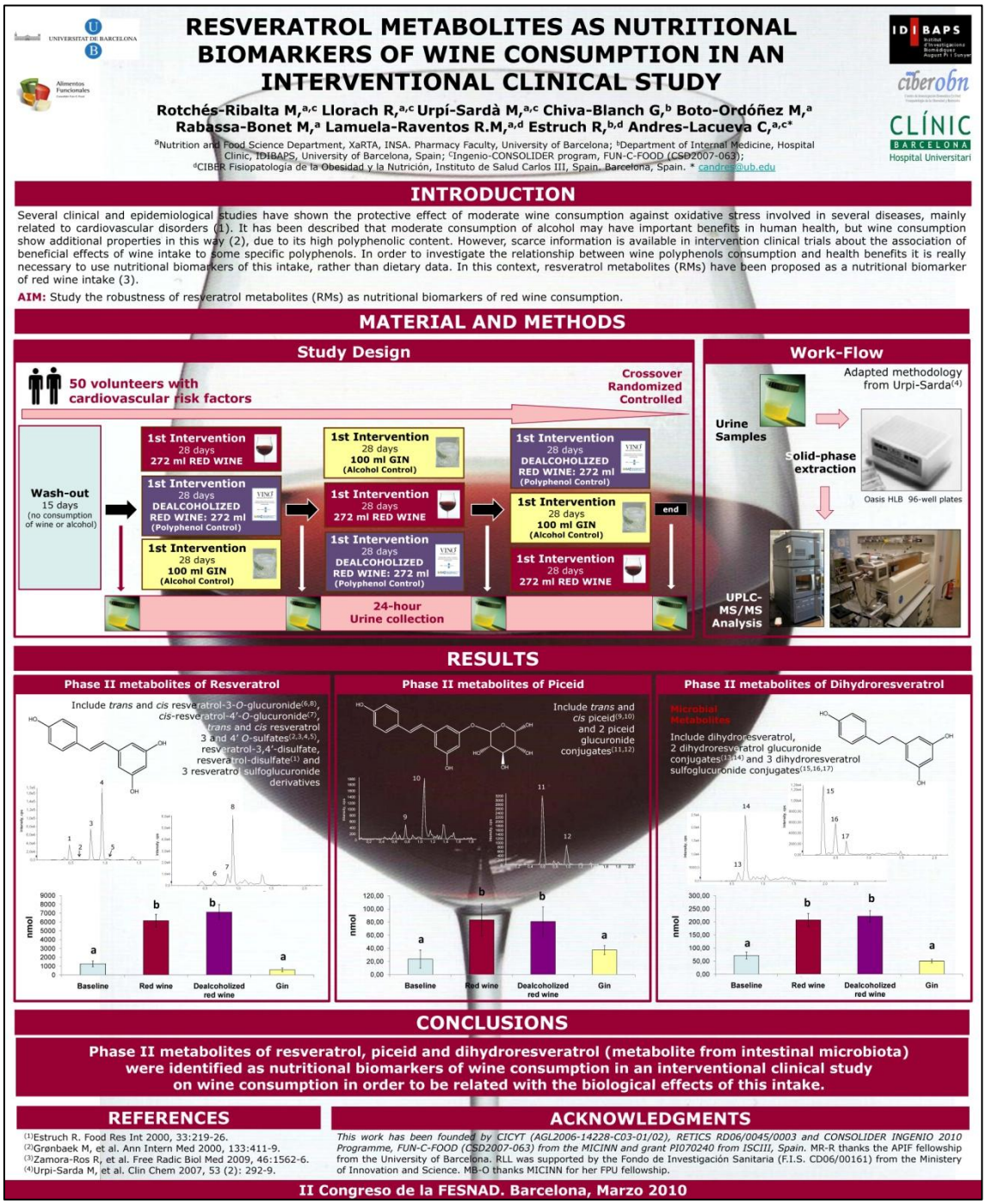
COMUNICACIONS A CONGRESSOS

Durant el període predoctoral s'han presentat fins a 20 comunicacions a congressos, 4 de les quals presenten alguns dels resultats que recull la present Tesi Doctoral. Aquestes 4 comunicacions s'han presentat com a poster en tres congressos internacionals: la IV i V edició de la "*International Conference on Polyphenols and Health*", celebrades a Harrogate i Sitges, respectivament, i el "II congrés de la FESNAD (Federación Española de Sociedades de Nutrición, Alimentación i Dietética)", que va tenir lloc a Barcelona. Més endavant, s'ennumera la resta de comunicacions presentades en diferents congressos.

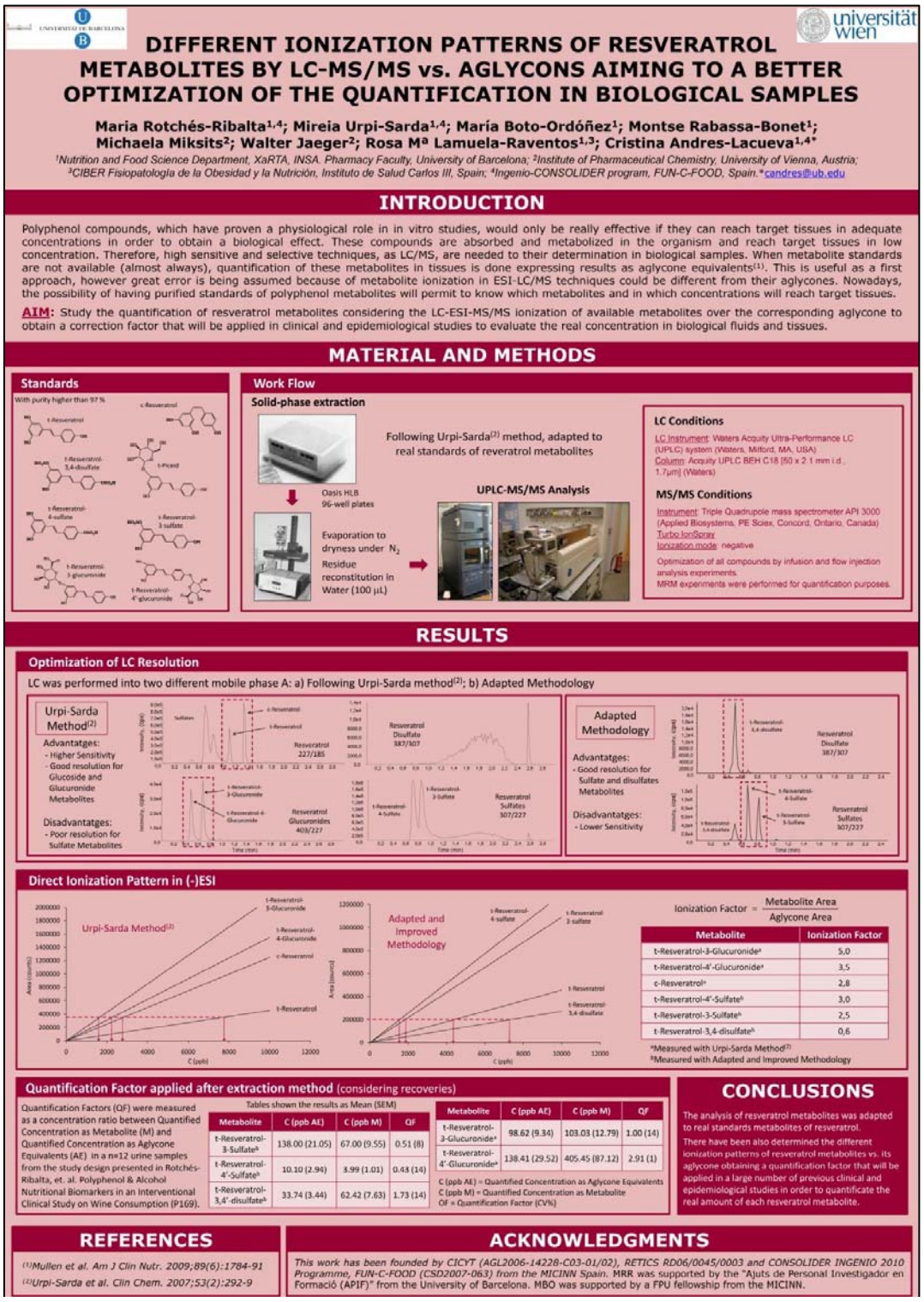
Poster: Maria Rotchés-Ribalta, Mireia Urpí-Sardà, Elvira Escribano, Rafael Llorach, Gemma Chiva-Blanch, Lluisa Perez-Garcia, Ramon Estruch, Cristina Andrés-Lacueva. Food matrix effect on the resveratrol bioavailability after acute and long-term intervention studies with moderate intake of red wine, and other resveratrol rich-products. 5th International Conference on Polyphenols and Health. Sitges - Barcelona (Espanya), Octubre 2011.



Poster: Maria Rotchés Ribalta, Mireia Urpi Sarda Rafael Llorach, Gemma Chiva Blanch, Rosa María Lamuela Raventos, Ramón Estruch, Cristina Andres Lacueva. Biomarcadores nutricionales: metabolitos de resveratrol tras el consumo de vino en un ensayo clínico de intervención. II Congreso FESNAD. Barcelona (Espanya), Març 2010.



Poster: Maria Rotchés-Ribalta, Mireia Urpi-Sarda, María Boto-Ordóñez, Montse Rabassa-Bonet, Michaela Miksits, Walter Jaeger, Rosa Mª Lamuela-Raventos, Cristina Andres-Lacueva. Different ionization patterns of resveratrol metabolites by LC-MS/MS vs. aglycons aiming to a better optimization of the quantification in biological samples. The 4th International Conferencia on Polyphenols and Health (ICPH). Harrogate (Anglaterra), Desembre 2009.



DIFFERENT IONIZATION PATTERNS OF RESVERATROL METABOLITES BY LC-MS/MS vs. AGLYCONS AIMING TO A BETTER OPTIMIZATION OF THE QUANTIFICATION IN BIOLOGICAL SAMPLES

Maria Rotchés-Ribalta[1,4]; Mireia Urpi-Sarda[1,4]; María Boto-Ordóñez[1]; Montse Rabassa-Bonet[1]; Michaela Miksits[2]; Walter Jaeger[2]; Rosa Mª Lamuela-Raventos[1,3]; Cristina Andres-Lacueva[1,4]*

[1]Nutrition and Food Science Department, XaRTA, INSA. Pharmacy Faculty, University of Barcelona; [2]Institute of Pharmaceutical Chemistry, University of Vienna, Austria; [3]CIBER Fisiopatología de la Obesidad y la Nutrición, Instituto de Salud Carlos III, Spain; [4]Ingenio-CONSOLIDER program, FUN-C-FOOD, Spain. candres@ub.edu

INTRODUCTION

Polyphenol compounds, which have proven a physiological role in in vitro studies, would only be really effective if they can reach target tissues in adequate concentrations in order to obtain a biological effect. These compounds are absorbed and metabolized in the organism and reach target tissues in low concentration. Therefore, high sensitive and selective techniques, as LC/MS, are needed to their determination in biological samples. When metabolite standards are not available (almost always), quantification of these metabolites in tissues is done expressing results as aglycone equivalents[(1)]. This is useful as a first approach, however great error is being assumed because of metabolite ionization in ESI-LC/MS techniques could be different from their aglycones. Nowadays, the possibility of having purified standards of polyphenol metabolites will permit to know which metabolites and in which concentrations will reach target tissues.

AIM: Study the quantification of resveratrol metabolites considering the LC-ESI-MS/MS ionization of available metabolites over the corresponding aglycone to obtain a correction factor that will be applied in clinical and epidemiological studies to evaluate the real concentration in biological fluids and tissues.

MATERIAL AND METHODS

Standards

With purity higher than 97 %

Work Flow

Solid-phase extraction

Oasis HLB 96-well plates

Evaporation to dryness under N_2

Residue reconstitution in Water (100 µL)

Following Urpi-Sarda[(2)] method, adapted to real standards of reveratrol metabolites

UPLC-MS/MS Analysis

LC Conditions

LC Instrument: Waters Acquity Ultra-Performance LC (UPLC) system (Waters, Milford, MA, USA)
Column: Acquity UPLC BEH C18 (50 x 2.1 mm i.d., 1.7µm) (Waters)

MS/MS Conditions

Instrument: Triple Quadrupole mass spectrometer API 3000 (Applied Biosystems, PE Sciex, Concord, Ontario, Canada)
Turbo IonSpray
Ionization mode: negative

Optimization of all compounds by infusion and flow injection analysis experiments.
MRM experiments were performed for quantification purposes.

RESULTS

Optimization of LC Resolution

LC was performed into two different mobile phase A: a) Following Urpi-Sarda method[(2)]; b) Adapted Methodology

Urpi-Sarda Method[(2)]

Advantatges:
- Higher Sensitivity
- Good resolution for Glucoside and Glucuronide Metabolites

Disadvantatges:
- Poor resolution for Sulfate Metabolites

Adapted Methodology

Advantatges:
- Good resolution for Sulfate and disulfates Metabolites

Disadvantatges:
- Lower Sensitivity

Direct Ionization Pattern in (-)ESI

Urpi-Sarda Method[(2)]

Adapted and Improved Methodology

$$\text{Ionization Factor} = \frac{\text{Metabolite Area}}{\text{Aglycone Area}}$$

Metabolite	Ionization Factor
t-Resveratrol-3-Glucuronide[a]	5,0
t-Resveratrol-4'-Glucuronide[a]	3,5
c-Resveratrol[a]	2,8
t-Resveratrol-4'-Sulfate[b]	3,0
t-Resveratrol-3-Sulfate[b]	2,5
t-Resveratrol-3,4-disulfate[b]	0,6

[a]Measured with Urpi-Sarda Method[(2)]
[b]Measured with Adapted and Improved Methodology

Quantification Factor applied after extraction method (considering recoveries)

Quantification Factors (QF) were measured as a concentration ratio between Quantified Concentration as Metabolite (M) and Quantified Concentration as Aglycone Equivalents (AE) in a n=12 urine samples from the study design presented in Rotchés-Ribalta, et. al. Polyphenol & Alcohol Nutritional Biomarkers in an Interventional Clinical Study on Wine Consumption (P169).

Tables shown the results as Mean (SEM)

Metabolite	C (ppb AE)	C (ppb M)	QF
t-Resveratrol-3-Sulfate[b]	138.00 (21.05)	67.00 (9.55)	0.51 (8)
t-Resveratrol-4'-Sulfate[b]	10.10 (2.94)	3.99 (1.01)	0.43 (14)
t-Resveratrol-3,4'-disulfate[b]	33.74 (3.44)	62.42 (7.63)	1.73 (14)

Metabolite	C (ppb AE)	C (ppb M)	QF
t-Resveratrol-3-Glucuronide[a]	98.62 (9.34)	103.03 (12.79)	1.00 (14)
t-Resveratrol-4'-Glucuronide[a]	138.41 (29.52)	405.45 (87.12)	2.91 (1)

C (ppb AE) = Quantified Concentration as Aglycone Equivalents
C (ppb M) = Quantified Concentration as Metabolite
QF = Quantification Factor (CV%)

CONCLUSIONS

The analysis of resveratrol metabolites was adapted to real standards metabolites of resveratrol.

There have been also determined the different ionization patterns of resveratrol metabolites vs. its aglycone obtaining a quantification factor that will be applied in a large number of previous clinical and epidemiological studies in order to quantificate the real amount of each resveratrol metabolite.

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ACKNOWLEDGMENTS

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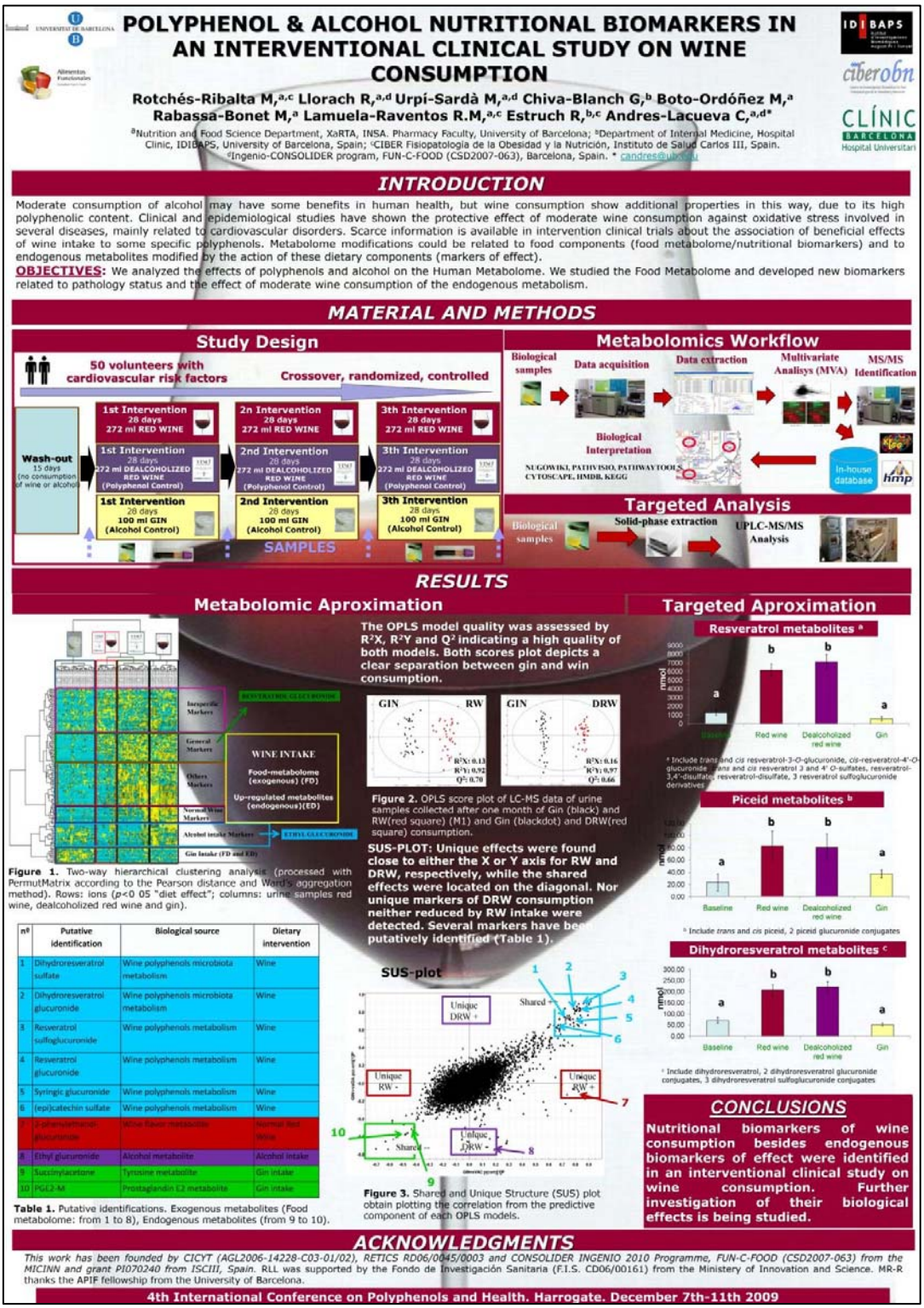

nº	Putative identification	Biological source	Dietary intervention
1	Dihydroresveratrol sulfate	Wine polyphenols microbiota metabolism	Wine
2	Dihydroresveratrol glucuronide	Wine polyphenols microbiota metabolism	Wine
3	Resveratrol sulfoglucuronide	Wine polyphenols metabolism	Wine
4	Resveratrol glucuronide	Wine polyphenols metabolism	Wine
5	Syringic glucuronide	Wine polyphenols metabolism	Wine
6	(epi)catechin sulfate	Wine polyphenols metabolism	Wine
7	3-phenylethanol glucuronide	Wine flavor metabolite	Normal diet Wine
8	Ethyl glucuronide	Alcohol metabolite	Alcohol intake
9	Succinylacetone	Tyrosine metabolite	Gin intake
10	PGE2-M	Prostaglandin E2 metabolite	Gin intake

Table 1. Putative identifications. Exogenous metabolites (Food metabolome: from 1 to 8), Endogenous metabolites (from 9 to 10).

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