UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA

DEPARTAMENT DE NUTRICIÓ I BROMATOLOGIA

PERFIL METABÒLIC DE RESVERATROL, FLAVANOLS I ISOFLAVONES EN TEIXITS BIOLÒGICS ADMINISTRATS A DOSIS DIETÈTIQUES. NOVES METODOLOGIES ANALÍTIQUES.

MIREIA URPÍ SARDÀ, 2008

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PERFIL METABÒLIC DE RESVERATROL, FLAVANOLS I ISOFLAVONES EN TEIXITS BIOLÒGICS ADMINISTRATS A DOSIS DIETÈTIQUES. NOVES METODOLOGIES ANALÍTIQUES.

Memòria presentada per Mireia Urpí Sardà per a optar al títol de doctor per la Universitat de Barcelona

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ABREVIATURES

13C-NMR	Ressonància Magnètica Nuclear de C-13
1H-NMR	Ressonància Magnètica Nuclear de H1
APCI	Ionització química a pressió atmosfèrica
APPI	Fotoionització a pressió atmosfèrica
ATP	Adenosil trifosfat
Bcl-2	Protooncogen de cèl·lules B limfoides
CAM	Molècules cel·lulars de l'adhesió
CID	Dissociació induïda en una cel·la de col·lisió
COMT	Catecol-O-metil transferasa
DAD	Detector FotodiodeArray
DESI	Desorció ESI
EC	Cèl·lules endotelials normals
ESI	Ionització en electrosprai
FDA	*Food and Drug Administration*
FIA	*Flow Injection Analysis*
GC-MS	Cromatografia gaseosa-Espectrometria de masses
HDL	Lipoproteïna d'alta densitat
HLB	Balanç Hidrofílic-Lipofílic
HMBC	Correlació heteronuclear a múltiples enllaços
JNK	Quinasa N-terminal de c-Jun
LC-MS/MS	Cromatografia líquida acoblada a espectrometria de masses en tàndem
LDL	Lipoproteïna de baixa densitat
LLE	Extracció líquid-líquid
LPH	Hidrolasa lactasa florizin
MALDI	Mètode de desorció i ionització per làser
MAPK	Proteïna quinasa mitogen activada
MAX	Mode mixte aniònic
MCP	Proteïna quimiotàctica monocitària
MCX	Mode mixte catiònic
M-LDL	Lipoproteïnes de baixa densitat modificades
MRM	*Multiple reaction monitoring*
mRNA	Àcid ribonucleic missatger
MRP2	Proteïna de transport multiresistent 2
NF-kB	Factor nuclear kB

NL	Pèrdua neutra
NO	Òxid nítric
ODMA	O-desmetilangolensina
PDGF	Factors de creixement plaquetaris
PIS	*Product ion scan*
PPT	Precipitació de proteïnes
PSA	Antigen específic de pròstata
Q1	Primer quadripol
Q3	Tercer quadripol
SGLT1	Transportador de glucosa sodi dependent
SIM	*Selected ion monitoring*
SIRT-1	Sirtuina-1
SPE	Extracció en fase sòlida
SRM	*Selected reaction monitoring*
SULT	Sulfotransferasa
TIC	Cromatograma d'ions totals
TOF	Temps de vol
UDP	Uridina-5'-difosfat
UGT1A	Uridina difosfoglucuronosil transferasa 1
UV	Ultraviolat
VLDL	Lipoproteïna de molt baixa densitat
VSMC	Cèl·lules musculars llises vasculars
WAX	Intercanvi aniònic dèbil
WCX	Intercanvi catiònic dèbil
XIC	Cromatograma d'ions extrets

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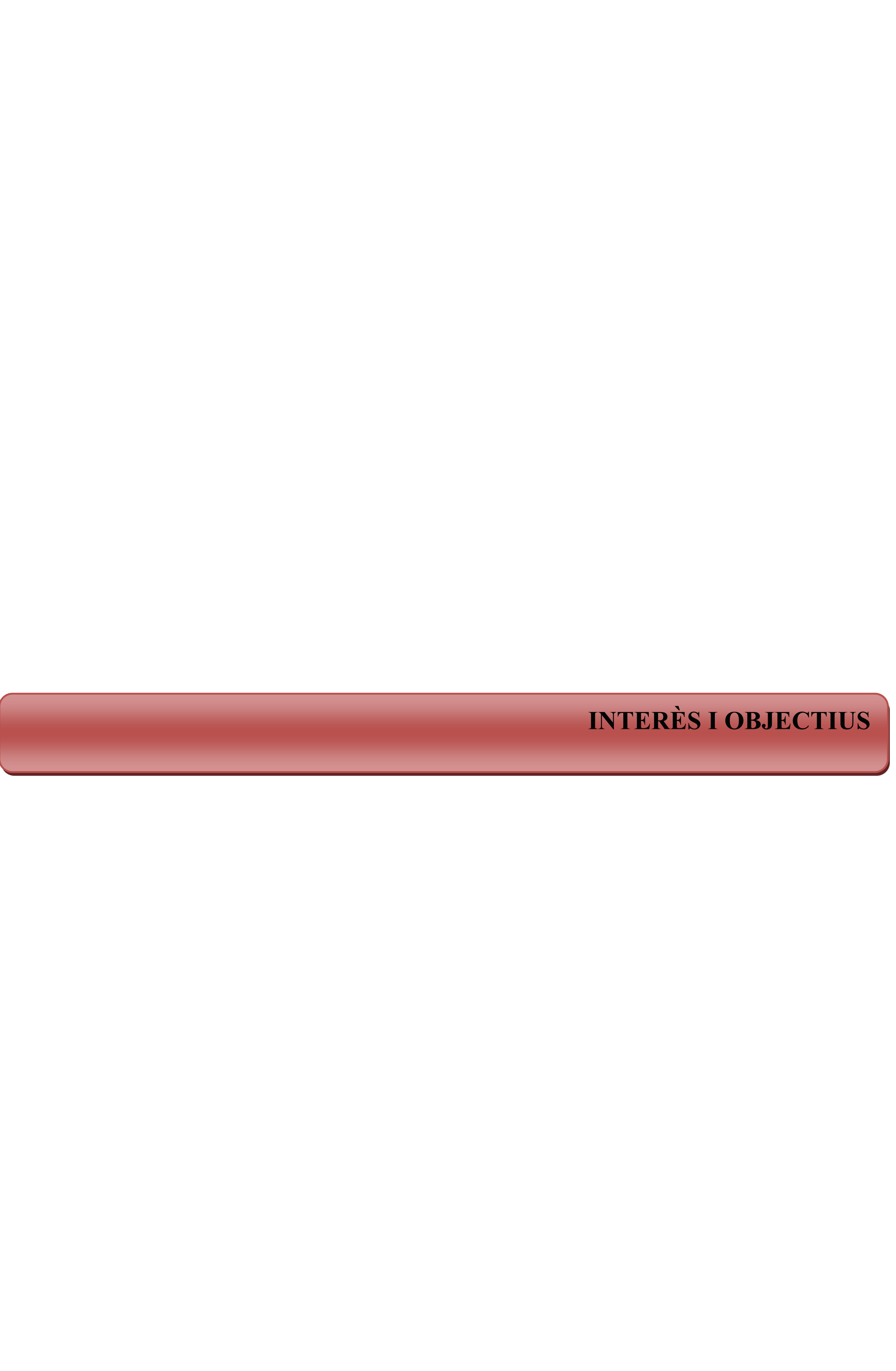

INTERÈS I OBJECTIUS

I. INTERÈS I OBJECTIUS

Estudis epidemiològics han posat en evidència la importància del consum de dietes riques en aliments d'origen vegetal en la prevenció de malalties cardiovasculars (Manach et al. 2005a), càncer (Yang et al. 2001) i malalties neurodegeneratives (Singh et al. 2008). Aquests efectes beneficiosos s'han relacionat amb l'existència de compostos bioactius en aliments vegetals coneguts també com substàncies fitoquímiques o fitonutrients. Dintre d'aquests fitonutrients s'engloben les substàncies fenòliques o polifenols que constitueixen un ampli grup de compostos que al seu torn es classifiquen per la seva estructura química.

Considerant estudis *in vitro*, aquests compostos fenòlics han demostrat un possible paper en la prevenció de determinades malalties, no obstant això, per a poder extrapolar aquests resultats i valorar els efectes fisiològics *in vivo* és necessari estudiar la seva absorció, metabolisme i capacitat d'excreció en l'organisme.

Encara és poc coneguda la biodisponibilitat dels fenols de la dieta, l'activitat dels metabòlits formats *in vivo*, la seva distribució i acció en teixits diana i la concentració suficient que és capaç de desencadenar un efecte biològic protector. Per tant, és essencial conèixer l'absorció, el metabolisme i acumulació d'aquests compostos polifenols en l'organisme humà i estudiar les bases científiques que sustentin els mecanismes implicats en el seu potencial benefici per a la salut així com en la prevenció de malalties mitjançant estudis de biodisponibilitat *in vivo*.

La biodisponibilitat dels compostos fenòlics ve condicionada principalment per la seva estructura química, per exemple una mínima modificació de la seva configuració pot modificar la seva absorció, aquest seria el cas de l'epicatequina, que sent un epímer de la catequina, s'absorbeix més eficaçment. També s'ha observat que el tipus de glicòsid unit a les aglicones, compostos majoritaris dels polifenols en els aliments vegetals, condiciona l'absorció.

Un cop absorbits, els polifenols es metabolitzaran en l'organisme donant lloc a metabòlits que podran mantenir i/o modificar substancialment l'activitat biològica dels compostos de partida. Però poc es coneix sobre les formes bioactives dels polifenols *in*

vivo i els mecanismes mitjançant els quals aquestes substàncies poden contribuir en la prevenció de malalties. A causa de l'escassa o nul·la disponibilitat de patrons comercials de metabòlits de polifenols d'elevada puresa, s'han portat a terme nombrosos estudis sobre la biodisponibilitat d'aquests, realitzant l'hidròlisi prèvia de la mostra i estudiant la recuperació de les aglicones en els teixits. Aquesta metodologia comporta la pèrdua de la possibilitat de l'estudi del perfil metabòlic i la seva singularitat específica.

Pocs treballs se centren en l'estudi dels metabòlits majoritaris formats *in vivo*. L'estudi del perfil metabòlic polifenòlic ajudarà a conèixer quins compostos fenòlics dels aliments seran responsables de la seva acció beneficiosa en l'organisme i ajudarà a destriar els mecanismes d'acció directes implicats. Paral·lelament, l'estudi quantitatiu del perfil metabòlic directe dels compostos fenòlics, també necessitarà per a una quantificació optimitzada, disposar de patrons de metabòlits per a poder avaluar la resposta directa i individualitzada de cadascun dels metabòlits en els diferents tipus de detectors utilitzats, ja sigui ultraviolat, espectrometria de masses o detector electroquímic.

El metabolisme dels polifenols ve també condicionat per l'estructura química de cada polifenol. Encara que s'ha estudiat que les formes conjugades majoritàries són els metabòlits glucuronidats i sulfatats, els polifenols amb un grup catecol també es poden metilar (Kroon et al. 2004). D'altra banda, per a les procianidines, dímers de catequines que constitueixen la fracció majoritària dels compostos fenòlics de la dieta i estan relacionades amb la protecció cardiovascular, l'absorció directa es troba molt limitada encara que es coneix que aquestes poden ser metabolitzades per la microbiota intestinal donant lloc a diversos àcids fenòlics, com per exemple l'àcid fenilpropiònic, fenilacètic i derivats d'àcid benzoic. Aquests àcids fenòlics podran ser absorbits (Gonthier et al. 2003a) i arribar als teixits diana on podran potencialment afavorir una acció. Són pocs els estudis que es focalitzen en l'estudi d'aquests metabòlits majoritaris formats *in vivo* després de la degradació de les procianidines per l'acció dels enzims bacterians presents en el còlon, així com la seva posterior absorció.

Estudis previs observen que després de l'administració de dosis dietètiques de polifenols, el que es considera després d'una ingesta habitual dels aliments, les concentracions presents en plasma o teixits es troben de l'ordre de nano o picomolar

(Manach et al. 2005b). Per a poder descriure el perfil metabòlic d'aquests compostos potencialment actius a concentracions fisiològiques és necessària la utilització de tècniques altament sensibles i específiques. L'espectrometria de masses, a més de ser una tècnica molt sensible i específica, també es combina fàcilment amb tècniques cromatogràfiques i fa possible la identificació i quantificació d'aquests metabòlits (Prasain et al. 2004) encara amb manca d'estàndards purs de metabòlits.

El fet de conèixer el tipus de metabòlit format i els nivells d'aquests conjugats i/o les seves aglicones presents en els teixits diana, serà útil per a dissenyar i interpretar estudis d'intervenció investigant els efectes beneficiosos dels polifenols.

Donada la importància de conèixer els metabòlits biològicament actius presents en fluids biològics i en teixits diana, l'objectiu principal de la present memòria ha estat posar a punt i validar metodologies analítiques altament sensibles i específiques per a la identificació i quantificació del perfil metabòlic polifenòlic *in vivo* de 3 tipus de compostos fenòlics rellevants en la dieta (estilbens, flavanols i isoflavones) després d'un consum d'aliments rics en aquests polifenols, ja sigui mitjançant estudis clínics d'intervenció o amb animals d'experimentació. Aquestes metodologies permetran conèixer aspectes rellevants de la seva biodisponibilitat específica que ens proporcionaran una eina necessària per a aportar evidència científica sobre l'efecte beneficiós del consum dietètic d'aliments rics en compostos polifenòlics.

En concret, aquesta tesi té els següents objectius:

1. Desenvolupar i validar un mètode sensible, ràpid i reproduïble per a determinar i quantificar el resveratrol i els seus metabòlits en mostres biològiques després de la ingesta d'un aliment ric en resveratrol.
2. Aplicar la metodologia validada en estudis d'intervenció per a determinar quins metabòlits del resveratrol arriben a la LDL i el perfil metabòlic urinari després d'un consum moderat de vi negre.
3. Desenvolupar i validar un mètode sensible, ràpid i reproduïble per a determinar el metaboloma urinari associat al consum regular de cacau.

4. Aplicar la metodologia validada en estudis d'intervenció per a identificar i quantificar els metabòlits urinaris després de la ingesta de cacau, considerant estudis amb animals d'experimentació i estudis clínics.
5. Determinar la concentració i el perfil metabòlic després del consum d’isoflavones en pròstata humana i la seva distribució tissular en teixits d'ovella.

ANTECEDENTS BIBLIOGRÀFICS

II. ANTECEDENTS BIBLIOGRÀFICS

1. ELS COMPOSTOS POLIFENÒLICS

1.1 Química, classificació i distribució de polifenols en aliments. Metodologia analítica per a la seva determinació en aliments i plantes.

La part que descriu la química i classificació dels compostos fenòlics, la seva distribució en els aliments i les tècniques utilitzades per a la seva determinació en aquests aliments, s'exposa en la present tesi en format de capítol de llibre "*Phenolic compounds. Chemistry and occurrence in fruits and vegetables"* enviat a l'editorial Blackwell Publishing (2009) del llibre "*Fruit and vegetable phytochemicals: Chemistry, nutritional value and stability"*.

Phenolic compounds. Chemistry and occurrence in fruits and vegetables.

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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

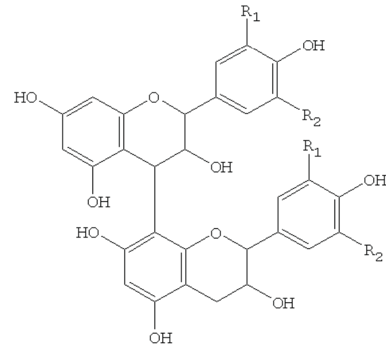
Flavonols

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

NO-FLAVONOIDS

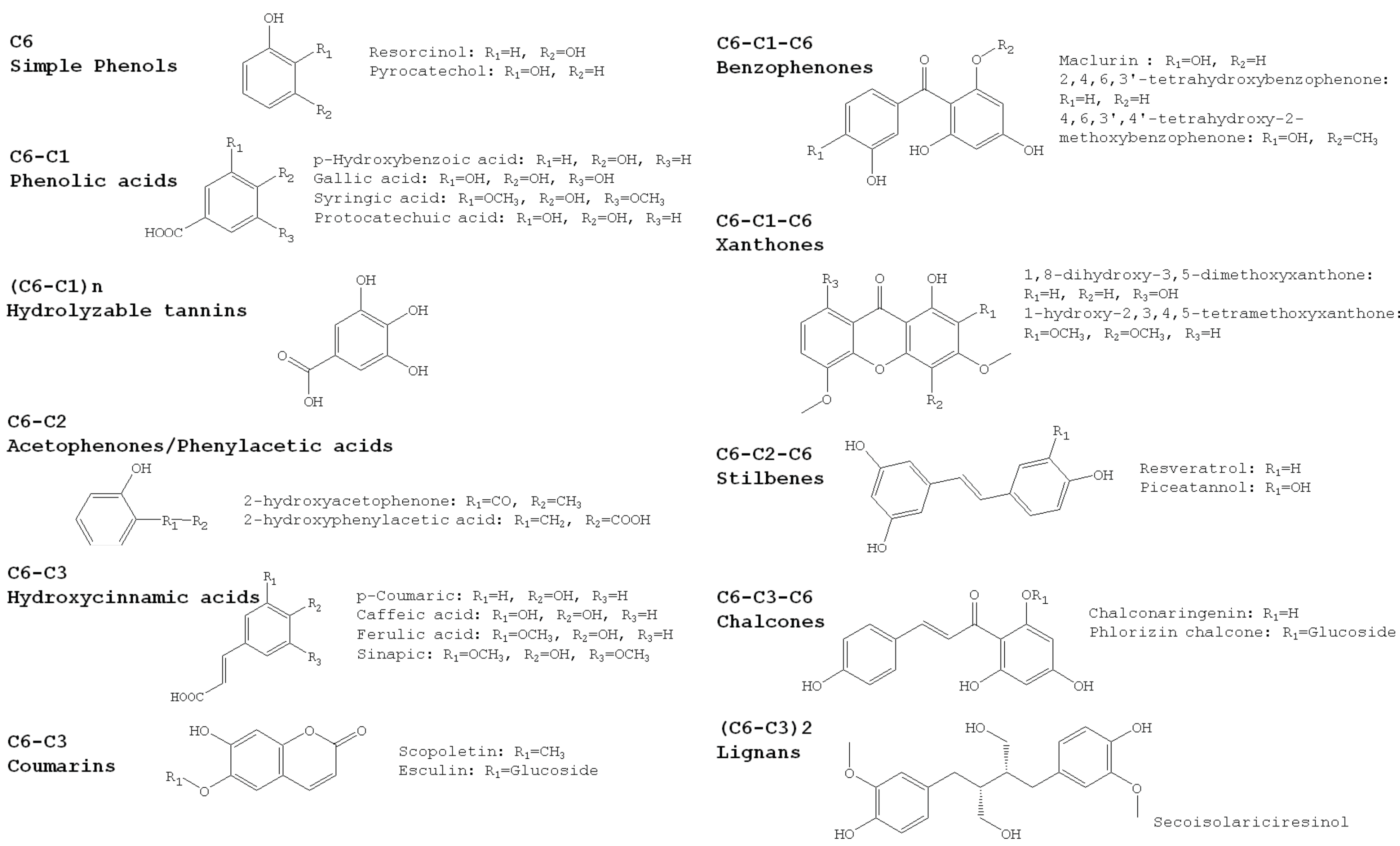


Figure 2. Chemical structures of no flavonoids.

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 Soja milk Tofu	13.8 (4.2) 0.6 (0.2) 2.3 (1.3)	(US Department of Agriculture 2007b)

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 Kiwi	35.3-216.4 21.9 (22.7) 33.8 (11.9) 20.8 (5.4) 5.6 (4.7) 2.6 (2.4)	(Gu and others 2004; US Department of Agriculture 2004)

	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 0.0089[£]	(Fernandez de Simon and others 2003)
	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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2. BIODISPONIBILITAT DE COMPOSTOS FENÒLICS

2.1 Generalitats

La biodisponibilitat dels polifenols varia àmpliament d'un polifenol a un altre. Aquesta depèn de la seva estructura química que determina la seva ràtio d'absorció a través del tracte gastrointestinal, metabolisme i finalment la seva activitat biològica. La majoria de polifenols s'absorbeixen poc a través de l'intestí i es metabolitzen extensivament o s'eliminen ràpidament.

Addicionalment, els metabòlits que trobem en sang i en els òrgans diana tindran activitats biològiques diferents en comparació de les seves formes natives. Recentment, s'ha descrit que els compostos glucuronidats/sulfatats de la quercetina tenen una activitat biològica menor, en concret en activitat anticarcinògena, quan es comparen amb l'aglicona quercetina en individus sans (Oi et al. 2008). Per tant, és essencial conèixer la biodisponibilitat dels polifenols si es volen entendre els seus efectes beneficiosos en la salut.

Absorció intestinal i metabolisme

El metabolisme dels polifenols presenta aspectes comuns en els diferents grups de compostos fenòlics (Scalbert and Williamson 2000). Les aglicones es poden absorbir a nivell d'intestí prim, no obstant això, la majoria dels polifenols estan presents en l'aliment sota la forma d'esters, glicòsids, o polímers que tenen una baixa absorció en la seva forma nativa.

El pas d'aquests glicòsids en l'estómac no està molt clar; encara que s'ha observat que algunes aglicones de flavonoids com la quercetina, la daidzeina i determinats antocians es poden absorbir en l'estómac de rata, no s'ha apreciat per als seus glicòsids (Crespy et al. 2002; Piskula et al. 1999; Passamonti et al. 2005). La majoria de glicòsids resisteixen probablement la hidròlisi àcida en l'estómac i arriben intactes al duodè (Gee et al. 1998; Rios et al. 2002). Només les aglicones i alguns glucòsids es podran absorbir en aquest intestí prim mentre que els polifenols units a altres sucres com per exemple la ramnosa

arriben al còlon on s'hidrolitzen per ramnosidases de la microbiota abans d'absorbir-se (Manach et al. 1995). Com a norma general, els glicòsids amb ramnosa s'absorbeixen més lentament i menys eficientment que les aglicones i els glucòsids (Manach et al. 2004). El mecanisme pel qual es facilita l'absorció dels glucòsids s'ha elucidat només parcialment. Els glucòsids es poden transportar dintre dels enteròcits per un transportador de glucosa sodi depenent (SGLT1) (Henry et al. 2005; Hollman et al. 1997) on es poden hidrolitzar a través de les beta-glucosidases cistòliques (Henry-Vitrac et al. 2006; Day et al. 1998). Una altra via d'absorció, inclou l'hidrolasa floridzina lactasa, una glucosidasa de la membrana del raspall de l'intestí prim que catalitza l'hidròlisi extracel·lular d'alguns glucòsids la qual està seguida per la difusió de les aglicones a través de la vora en raspall (Day et al. 2000b).

Efecte de la microbiota del còlon

Els polifenols que no són absorbits a l'intestí prim arriben al còlon. La microbiota colònica presenta tant enzims que li permeten hidrolitzar els glicòsids en aglicones com també altres enzims que li permet metabolitzar les aglicones. En aquest sentit, aquest metabolisme pot produir metabòlits específics de determinats polifenols com és el cas de l'equol a partir de daidzeina o hidroxifenilvalerolactones a partir de catequines. Junt amb això, també s'ha demostrat que la microbiota colònica pot metabolitzar àmpliament els polifenols produint així diversos àcids aromàtics (Gonthier et al. 2003a).

Les aglicones són hidrolitzades per l'obertura de l'anell heterocíclic en diversos punts depenent de la seva estructura química: els flavonols produeixen principalment àcids hidroxifenilacètics, les flavones i flavanones produeixen principalment àcids hidroxifenilpropiònics, i els flavanols produeixen principalment hidroxifenilvalerolactones i àcids hidroxifenilpropiònics. Posteriorment, aquests àcids es metabolitzaran en derivats de l'àcid benzoic. Els metabòlits microbians s'absorbeixen i es poden conjugar amb glicina, àcid glucurònic o sulfat. Encara que les rutes metabòliques estan bé establertes en animals, encara queden un poc limitades en humans, per tant, és possible que es puguin identificar nous metabòlits.

Un altre aspecte a considerar són les variacions interindividuals i la influència de la composició de la microbiota. Estudis recents han mostrat que les concentracions

plasmàtiques i l'excreció urinària dels metabòlits microbians en humans pot ser més elevada que aquells metabòlits tissulars, especialment per a polifenols com els polifenols del vi que no s'absorbeixen fàcilment (Gonthier et al. 2003a; Rechner et al. 2002a). En aquest sentit, Decroos et al (Decroos et al. 2005b) van mostrar que l'equol era més eficaçment absorbit a nivell del còlon que la daidzeina. Per tant, la identificació i quantificació dels metabòlits microbians constitueix un important camp d'investigació ja que alguns d'ells podrien tenir efectes fisiològics (Kim et al. 1998).

Conjugació i naturalesa dels metabòlits

Un cop absorbits, els principals metabòlits que es formen provenen del metabolisme de fase II, del qual, les conjugacions més destacades per als polifenols són: la metilació, la sulfatació i la glucuronidació, encara que també s'hauria de destacar que cada vegada més augmenten els articles relacionats amb altres conjugacions com per exemple la glutationització (Hong and Mitchell 2006).

La catecol-*O*-metil transferasa (COMT) catalitza la transferència d'un grup metil de la *S*-adenosil-*L*-metionina als polifenols que tenen un grup catecol (*p*-difenòlic) en la seva estructura. Aquesta reacció és ben coneguda per a la quercetina, catequina, àcid cafeic i luteolina i Wu et al. el 2002, van mostrar la metilació de la cianidina a peonidina en humans (Wu et al. 2002). La metilació generalment ocorre en la posició 3' del polifenol, però també es forma una menor proporció de conjugats 4'-metilats. Aquest enzim es troba en molts teixits però la seva activitat és major en el fetge i el ronyó (Piskula and Terao 1998). D'altra banda, les sulfotransferases (SULT) catalitzen la transferència d'un grup sulfat des del 3'-fosfoadenosina-5'-fosfosulfat a un grup hidroxil present en diversos substrats (esteroides, àcids biliars i polifenols, entre altres). Les UDP-glucuronosiltransferases són enzims de membrana localitzades en el reticle endoplasmàtic de molts teixits i que catalitzen la transferència de l'àcid glucurònic a esteroides, àcids biliars, polifenols i milers de constituent dietètics i xenobiòtics. La glucuronidació té lloc en els enteròcits i després en el fetge. S'han identificat al voltant de 15 isoformes UDP-glucuronosil-transferases en humans que tenen àmplies especificitats i diversa distribució en teixits (Fisher et al. 2001). La subfamília anomenada UGT1A es localitza en l'intestí i probablement juga un paper fonamental en el metabolisme dels polifenols. Aquests isoenzims tenen un ampli patró d'expressió

polimòrfic que podrien provocar les elevades variacions interindividuals. Encara que la naturalesa dels glucurònids formats és constant, la proporció dels metabòlits varia àmpliament depenent de les espècies i dels òrgans (Day et al. 2000a; Morand et al. 1998). S'han observat elevats ràtios de conjugació en la posició 7 i contràriament a això, la posició 5 no sembla ser emprada pels enzims en el procés de glucuronidació. Per a la majoria de polifenols, una elevada proporció de glucurònids que es formen en la mucosa intestinal es secreten al lumen intestinal reduint així l'absorció d'aglicones (Silberberg et al. 2006; Crespy et al. 1999). En aquest reflux estan involucrades la proteïna de transport multiresistent 2 (MRP2) o la *P*-glicoproteïna (Ayrton and Morgan 2001). La proporció secretada depèn del polifenol i varia entre 0-52% (Crespy et al. 2003).

La importància relativa dels 3 tipus de conjugacions sembla variar en funció de la naturalesa del substrat i de la dosi ingerida. La sulfatació té generalment una elevada afinitat i una baixa capacitat comparada amb la glucuronidació, per tant, quan la dosi ingerida augmenta hi ha un desplaçament de la sulfatació cap a la glucuronidació (Koster et al. 1981). El balanç entre la sulfatació i la glucuronidació de polifenols sembla que està afectat per l'espècie, el sexe i l'alimentació (Piskula 2000).

La identificació de metabòlits circulants ha anat augmentant en els últims anys a causa del desenvolupament de tècniques més sensibles i específiques com pot ser l'espectrometria de masses. Aquesta identificació inclou no només la naturalesa i el nombre de grups de conjugació sinó també la posició d'aquests grups en l'estructura polifenòlica degut al fet que aquestes posicions poden afectar a les propietats dels metabòlits (Day et al. 2000a). Els principals metabòlits circulants són generalment glucurònids.

Transport plasmàtic

Generalment, els metabòlits dels polifenols no es troben lliures en el torrent sanguini. L'albúmina és una de les proteïnes que actua com a transportador dels polifenols. L'afinitat dels polifenols per l'albúmina varia en funció de la seva estructura química. L'efecte de la sulfatació i la glucuronidació en la unió amb l'albúmina és desconegut però probablement depèn altament de la posició de la substitució (Manach et al. 2004).

El grau d'unió a l'albúmina pot tenir conseqüències per al ràtio d'eliminació dels metabòlits i de la seva distribució a cèl·lules i teixits. La concentració cel·lular és proporcional a les concentracions de metabòlits lliures. Variacions en el pH local de llocs específics podria induir a canvis conformacionals en l'albúmina que permeten la dissociació del complex lligant-albúmina. S'han aplicat canvis conformacionals en l'albúmina per a induir interaccions inespecífiques amb diverses membranes (Horie et al. 1988). Però es desconeix si aquests canvis podrien facilitar l'absorció cel·lular dels metabòlits de polifenols.

El coeficient de partició dels polifenols i els seus metabòlits entre fases aquoses i lipídiques es troba a favor de la fase aquosa a causa de la seva hidrofília i unió a l'albúmina. Encara que en alguns models de membrana lipofílics, alguns polifenols van mostrar la seva capacitat de travessar la membrana (Ollila et al. 2002). A pH fisiològic, la majoria de polifenols interaccionen amb el grup polar dels fosfolípids de membrana formant ponts d'hidrogen amb els grups hidroxils d'aquests polifenols (Verstraeten et al. 2003).

La LDL està composta d'una estructura lipofílica que una vegada oxidada participa en l'etiologia de l'aterosclerosi. Molts estudis han mostrat que diversos polifenols tenen la capacitat de protegir la LDL contra l'oxidació. No obstant això, una petita proporció dels polifenols del plasma estan associats amb la fracció de LDL després del consum de dosis nutricionals d'aquests compostos (Urpi-Sarda et al. 2005; Gimeno et al. 2002). Aquests estan associats amb les lipoproteïnes només per interaccions iòniques amb residus carregats en la superfície de les partícules.

Distribució tissular

La determinació de la biodisponibilitat dels metabòlits de polifenols en teixits podria ser molt més important que el coneixement de les seves concentracions plasmàtiques, però les dades encara són molt escasses, fins i tot en animals.

Molts dels estudis de distribució tissular s'han realitzat amb polifenols marcats radioactivament. Aquesta radioactivitat es recupera principalment en sang i en teixits del sistema digestiu com són l'estómac, intestí i fetge. Però també han estat detectats en un ampli rang de teixits com el cervell, cor, ronyó, pàncrees, pròstata, úter, glàndula mamària, testicles, ossos, pell, tant en rates com en ratolins (Coldham and Sauer 2000; Chang et al. 2000). Les concentracions obtingudes en aquests teixits varien de 30 a 3000 ng equivalents d'aglicona/g teixit depenent de la dosi administrada i del teixit considerat. El temps de presa de mostra també pot ser d'elevada importància degut al fet que no es té un coneixement de la cinètica de penetració i eliminació dels polifenols en teixits.

Resulta difícil dir si alguns polifenols s'acumulen en òrgans específics. Pocs estudis semblen indicar que algunes cèl·lules podrien incorporar polifenols per mecanismes específics. La microautoradiografia de teixits de rates després de l'administració de resveratrol indicava que la radioactivitat era incorporada de manera desigual en cèl·lules dels òrgans (Vitrac et al. 2003).

La naturalesa dels metabòlits circulants podria patir algunes variacions a causa de la distribució específica o eliminació d'alguns dels metabòlits tissulars o a causa del metabolisme intracel·lular.

Eliminació

Els metabòlits de polifenols poden seguir dos camins d'excreció, la via biliar o la via urinària. Els metabòlits d'alt pes molecular, que estan extensament conjugats, presenten una major probabilitat de ser excretats a la bilis, mentre que els conjugats de menor pes com els monosulfats s'excreten preferiblement en orina. En animals de laboratori, la relativa excreció urinària i biliar vària d'un polifenol a un altre (Crespy et al. 2003).

Les bactèries intestinals posseeixen beta-glucuronidases que són capaces d'alliberar aglicones dels metabòlits conjugats en la bilis podent aquestes aglicones ser

reabsorbides donant un cicle enterohepàtic. Estudis farmacocinètics en rates van mostrar un segon màxim plasmàtic a les 7h després de l'administració de genisteina el que és consistent amb la circulació enterohepàtica (Coldham and Sauer 2000).

L'excreció urinària ha estat altament determinada en estudis amb humans. El total de metabòlits excretats en orina està correlacionat aproximadament amb les concentracions plasmàtiques màximes. Aquesta excreció és bastant elevada per a flavanones dels fruits cítrics (4-30%) (Kaneko et al. 2006; Manach et al. 2003) i fins i tot més elevat per a isoflavones, 16-66% per a daidzeina i 10-24% per a genisteina (Setchell et al. 2003b; Kaneko et al. 2006; Xu et al. 1994). Sembla sorprenent que les concentracions plasmàtiques de genisteina siguin més elevades que les de daidzeina a pesar d'una excreció urinària més elevada de daidzeina. Això s'explica a causa de l'eficient excreció biliar de la genisteina.

Els percentatges d'excreció urinària d'altres polifenols com per exemple els antocians solen ser baixos (0.005-0.1%) (Cui et al. 2006; Cao et al. 2001; Matsumoto et al. 2001) encara que Lapidot et al. (1998) va reportar elevats percentatges d'excreció d'antocianins (fins a un 5%) després del consum de vi negre (Lapidot et al. 1998). Aquests valors baixos d'excreció podrien indicar una excreció biliar pronunciada o un excessiu metabolisme.

2.2 Biodisponibilitat i metabolisme de flavanols del cacau

La ingesta diària de flavanols està estimada entre 18-50 mg/dia sent les principals fonts en la dieta el te, la xocolata, les pomes, les peres, el raïm i el vi negre (Arts et al. 2000). Encara que es troben en gran quantitat en fruites i vi negre, la seva biodisponibilitat ha estat principalment estudiada després del consum de cacau, xocolata o te. El cacau és un aliment ric en flavan-3-ols on predominen els monòmers d'epicatequina, el majoritari, i catequina (23%), els dímers de procianidines (13%) i els 3-10mers (63%).

Molts estudis *in vitro* han caracteritzat als flavanols com a potents antioxidants (Rice-Evans 2001), no obstant això, la seva potencial capacitat antioxidant i la seva possible activitat en estudis *in vivo* depenen de la seva absorció, metabolisme, distribució i excreció després de la ingesta d'aliments rics en aquests compostos (Figura 1)(Spencer 2003). Per tant, s'ha realitzat una revisió bibliogràfica del comportament d'aquests flavanols del cacau a través del tracte gastrointestinal, el seu metabolisme, distribució i excreció.

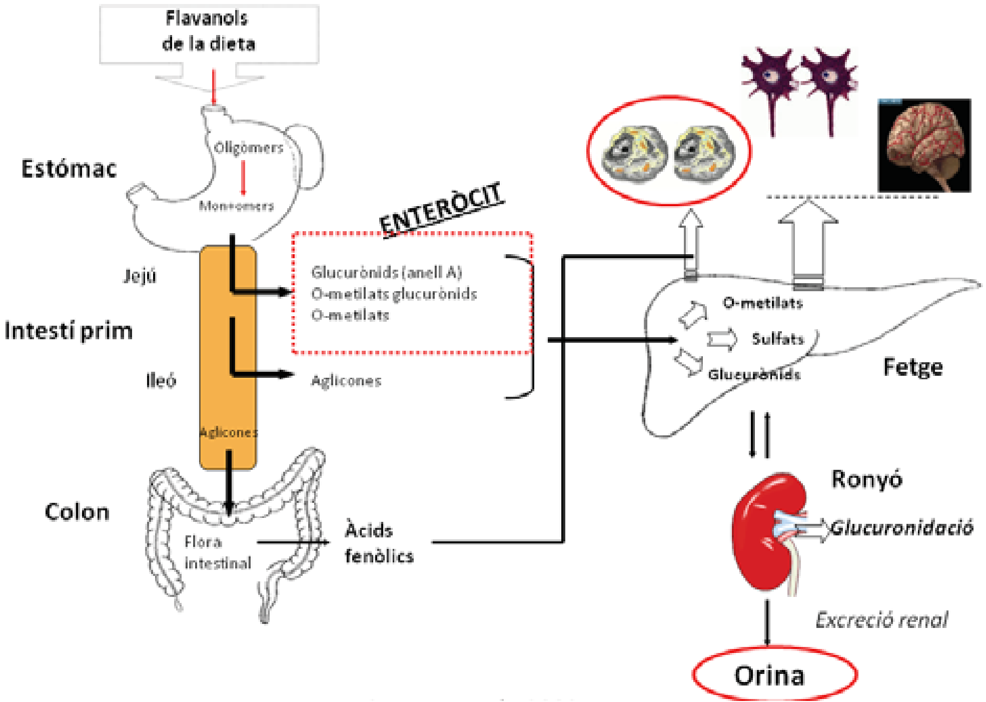

Figura 1: Metabolisme de flavanols

Existeixen pocs estudis sobre la capacitat que té la saliva d'alterar l'estructura dels flavonoids. S'ha observat que nivells molt baixos de diferents catequines del te verd (μg/mL) poden quedar retinguts fins a 1 hora en la saliva després d'esbandir-se amb una solució aquosa d'extracte de te verd (5 g/L) (Tsuchiya et al. 1997) i que els galats de flavanols podrien perdre el grup galat i absorbir-se en la mucosa oral (Yang et al. 1999). Contràriament, no s'ha observat cap degradació dels oligòmers de procianidines (dímers-hexàmers) cap a unitats oligomèriques menors a l'incubar-los amb saliva humana durant 30 minuts (Spencer et al. 2001b). A més, les proteïnes presents en la saliva podrien interaccionar amb els flavanols i procianidines. La catequina i les procianidines amb enllaços C4-C8 tenen major afinitat per les proteïnes riques en prolina de la saliva que l'epicatequina i les procianidines amb enllaços C4-C6, fet que demostra la importància de l'estereoquímica dels flavanols en la interacció amb proteïnes (De Freitas and Mateus 2001).

Els monòmers de flavanols sembla ser que es mantenen estables en les condicions d'acidesa de l'estómac, pel que arriben intactes a l'intestí prim per a ser absorbits. En canvi, s'ha observat que les procianidines, de dímers a decàmers, són inestables a condicions de pH àcides existents en l'estómac (Spencer et al. 2000). Això es va determinar després d'incubar les procianidines durant 0.1-3h amb un mitjà que simulava el suc gàstric i observar la descomposició dels oligòmers cap a monòmers i dímers d'epicatequina (Spencer et al. 2000). Posteriorment, Rios et al (2002), no van observar degradació de les procianidines en l'estómac de voluntaris després del consum de cacau (Rios et al. 2002). Per tant, el fet que aquestes procianidines arribin a l'intestí prim sense experimentar degradació alguna, concorda amb els resultats de Donovan et al (2002) en els quals no van trobar catequina en l'orina i plasma de rates després del consum d'una dieta amb procianidina B3 (Donovan et al. 2002). No obstant això, la descomposició química de les procianidines està bé caracteritzada i si les condicions de l'estómac són molt àcides haurà una degradació de procianidines a unitats menors de flavanols que entraran en l'intestí (Spencer 2003).

Els flavan-3-ols es troben en els aliments en forma d'aglicones, a diferència de la resta de flavonoids que es troben en forma glicosilada, i per tant, es podran absorbir directament sense necessitat de l'acció de la beta-glucosidasa.

Una vegada els flavanols deixen l'estómac i arriben a l'intestí prim es produeix un increment de pH en el mig (pH: 7), en el qual, els compostos fenòlics amb estructures catecols s'oxiden podent disminuir fins a un 82% (Yoshino et al. 1999). Encara que s'ha observat que l'epigalocatequinagalat es transforma en un compost dímer que posseeix una major activitat captadora de radicals lliures que el seu compost inicial (Yoshino et al. 1999). Un altre factor a tenir en compte és l'efecte de la matriu de l'aliment a causa d'unions entre flavanol i proteïnes de l'aliment o degut al fet que algunes substàncies com l'àcid ascòrbic poden estabilitzar als polifenols en aquestes condicions neutres o bàsiques (Chen et al. 1998).

Diversos estudis *in vivo* determinen que els flavanols del cacau s'absorbeixen en el tracte gastrointestinal, es troben conjugats en el plasma i augmenten l'activitat antioxidant en plasma (Wang et al. 2000; Baba et al. 2000a; Baba et al. 2000b). Baba et al. (2000) va determinar els nivells d'epicatequina i els seus metabòlits en plasma de rata després de l'administració oral de cacau en pols. Entre els 30 i 60 minuts van observar la màxima concentració de metabòlits sent els conjugats glucurònids d'epicatequina metilada i no metilada els majoritaris però també van detectar sulfats i sulfoglucorònids de l'epicatequina metilada i no metilada així com l'epicatequina lliure (Baba et al. 2000a). Posteriorment, Baba et al (2001) van comparar els nivells de catequina i epicatequina i els seus metabòlits en plasma i orina després de la seva administració oral com estàndards purs per separat o junts (grup mixt). Després de l'administració, els principals metabòlits trobats en plasma van ser els seus conjugats no metilats o els conjugats 3'-*O*-metilats. Per al grup de catequina i mixt, el principal metabòlit va ser el glucurònid en forma no metilada. Per al grup d'epicatequina i mixt, els metabòlits principals van ser els conjugats glucurònids i sulfoglucurònids de les formes no-metilades i el sulfat de la forma 3'-*O*-metilada. L'excreció urinària dels metabòlits en el grup d'epicatequina va ser major que la del grup de la catequina. A més els metabòlits excretats d'epicatequina i catequina en el grup mixt van ser menors que el dels grups purs de catequina i epicatequina concloent que la biodisponibilitat de l'epicatequina és major en rates i que la combinació d'ambdues podria produir una competició en l'absorció en el tracte gastrointestinal en rates (Baba et al. 2001a). De la mateixa manera, es va estudiar l'absorció i excreció de la procianidina B2 després de la seva administració en rates comprovant la presència de procianidina B2 i els metabòlits conjugats d'epicatequina i de metil-epicatequina en plasma i orina. L'excreció urinària

d'aquests metabòlits es va xifrar en un 0.48% de la dosi oral dada que indica la baixa biodisponibilitat de la procianidina B2 en rates (Baba et al. 2002b). A més, la biodisponibilitat de l'epicatequina en rates després de l'administració de diferents nivells de pols de cacau (150, 750 i 1500 mg/kg) o epicatequina pura (1, 5 i 10 mg/kg) és dosi depenent en plasma (Baba et al. 2001b). De manera similar, Wang et al (2000) van demostrar en humans que 2h després del consum de xocolata els nivells plasmàtics de l'epicatequina incrementen de manera dosi depenent (133, 258 i 355 nmol/L) després del consum de diferents dosis de xocolata (27, 53 i 80g) (Wang et al. 2000).

Baba et al (2000) van avaluar l'absorció i excreció en humans d'epicatequina present en el cacau i en la xocolata observant que els conjugats sulfats, glucurònids i sulfoglucurònids de l'epicatequina eren els principals metabòlits en plasma més que les formes conjugades metilades. La quantitat de metabòlits excretats en orina al cap de 24h es va xifrar en un 30% de la dosi ingerida (Baba et al. 2000b). Holt et al (2002) van estudiar l'absorció de les procianidines del cacau en humans després del consum de 375 mg/kg pes corporal i, van observar l'aparició a 0.5h i la seva concentració màxima a les 2h de procianidina B2 (16 i 41 nmol/L, respectivament), epicatequina (2.61 i 5.92 µmol/L, respectivament) i catequina (0.13 i 0.16µmol/L, respectivament) (Holt et al. 2002a). Sano et al (2003) van observar també l'absorció de la procianidina B1 en plasma de voluntaris després del consum d'extracte de raïm (Sano et al. 2003). És important comprovar que encara que en el cacau ingerit les proporcions d'epicatequina i catequina van ser iguals, les concentracions plasmàtiques de catequina van anar al voltant de 30 vegades menors (Holt et al. 2002a; Rein et al. 2000a). Aquest fet ha estat corroborat recentment en un estudi in vitro per Donovan et al. (2006) quan van comparar l'absorció de la catequina i de l'epicatequina i de mostres de xocolata després de la perfusió intestinal d'ambdues en el jejú i ili de rata observant la baixa biodisponibilitat de la catequina després del consum de xocolata (Donovan et al. 2006).

Roura et al (2005) van observar el perfil metabòlic de l'epicatequina en orina i plasma de voluntaris després del consum de 40g de cacau. L'epicatequina-glucurònid i tres epicatequina-sulfats van ser els metabòlits més abundants en orina, mentre que només es va detectar l'epicatequina-glucurònid en plasma a les 2h de consum a una concentració de 625.7 +/- 198.3 nmol/L (Roura et al. 2005).

Okushio et al. (1999), van identificar l'epicatequina, la 3'-*O*-metil-epicatequina, la 4'-O-metil-epicatequina en orina i plasma de rata després de l'administració oral d'epicatequina sent els dos primers metabòlits, els més abundants. També van identificar les dues formes conjugades de l'epicatequina més abundants en plasma i orina com l'epicatequina-5-*O*-glucurònid i la 3'-*O*-metil-epicatequina-5-*O*-glucurònid amb una excreció acumulada durant 24 h del 8% de la dosi administrada (Okushio et al. 1999). El 2003, Natsume et al., van purificar i van elucidar l'estructura química dels metabòlits de l'epicatequina en orina humana i de rata després de l'administració oral d'epicatequina. Els tres metabòlits purificats en l'orina humana van ser l'epicatequin-3'-*O*-glucurònid, el 4'-O-metil-epicatequina-3'-*O*-glucurònid i el 4'-*O*-metil-epicatequina-5 o 7-*O*-glucurònid segons assajos de ^{13}C-NMR, HMBC i LC-MS. En l'orina de rata van purificar el 3'-O-metil-epicatequina, epicatequina-7-*O*-glucurònid, i el 3'-*O*-metil-epicatequina-7-*O*-glucurònid (Figura 2). Aquests mateixos metabòlits van ser identificats en el plasma d'humans i de rates 1h després del consum d'epicatequina (Figura 2). La glucuronidació de l'epicatequina en rates es produeix principalment en la posició 7 de l'anell A, mentre que en humans, es produeix principalment en la posició 3' de l'anell B (Natsume et al. 2003). Més recentment, Schroeter et al (2006), van observar que l'epicatequina i l'epicatequina-7-*O*-glucurònid determinats en plasma de voluntaris després del consum de cacau estaven relacionats amb els efectes beneficiosos vasculars del consum de cacau encara que també van identificar els metabòlits 4'-metil-epicatequina, 4'-metil-epicatequin-*O*-glucurònid (Schroeter et al. 2006).

A

Epicatequina-3'-*O*-Glucurònid

4'-O-Metil-Epicatequina-3'-*O*-Glucurònid

4'-O-Metil-Epicatequina-5 o 7-*O*-Glucurònid

B

3'-O-Metil-Epicatequina

Epicatequina-7-*O*-Glucurònid

3'-O-Metil-Epicatequina-7-*O*-Glucurònid

Figura 2: Estructura dels metabòlits de l'epicatequina elucidats i purificats en orina d'humans (A) i de rates (B) per Natsume et al. (2003)

Estudis farmacològics van demostrar els efectes protectors de l'epicatequina i la seva conjugat 3'-*O*-metilat en la mort cel·lular cerebral induïda per l'estrès oxidatiu. Per tant, El Mohsen et al. (2002) van estudiar la capacitat de l'epicatequina per a travessar la barrera hematoencefàlica i arribar al cervell. Van identificar la presència de l'epicatequina-glucurònid i del 3'-*O*-metil-epicatequina-*O*-glucurònid en teixit cerebral després de l'administració oral de 100 mg/kg/dia d'epicatequina en rates durant 1, 5 i 10 dies (El Mohsen et al. 2002).

Les procianidines difereixen de la majoria de polifenols a causa de la seva naturalesa polimèrica i al seu elevat pes molecular. Aquest fet limita la seva absorció a través de la barrera intestinal fent que els oligòmers que són més grans que els trímers posseeixen una probabilitat més baixa de ser absorbits en l'intestí en les seves formes natives (Manach and Donovan 2004) i a més aquestes procianidines no es despolimeritzen en el tracte gastrointestinal. No obstant això, els efectes sobre la salut de les

proantocianidines podrien no requerir de la seva absorció a través de l'intestí. De fet, aquests compostos podrien tenir un efecte directe en la mucosa intestinal i protegir-la contra l'estrès oxidatiu o contra accions de carcinògens (Manach et al. 2005b). A més, el consum d'aliments rics en proantocianidines incrementa la capacitat antioxidant del plasma, té efectes positius en la funció vascular i redueix l'activitat plaquetària en humans (Williamson and Manach 2005). Per tant, les procianidines oligomèriques amb un grau de polimerització mig de 7 no s'absorbiran en l'intestí, de manera que arribaran intactes a l'intestí gruixut, on podran ser metabolitzades en diversos àcids aromàtics de baix pes molecular per la microbiota intestinal i posteriorment aquests compostos es podran absorbir (Rice-Evans 2001).

La incubació de polímers de procianidines i 14C-proantocianidines amb microbiota humana *in vitro* anaeròbiament durant 48h va permetre observar la degradació en un alt percentatge de les procianidines amb la conseqüent formació d'àcids fenilacètics, fenilpropiònics i fenilvalèrics monohidroxilats en les posicions meta i para (Figura 3) (Deprez et al. 2000). Groenewoud et al., ja el 1986, van observar la degradació de les catequines *in vitro* per la microbiota de rata amb la subsegüent formació de derivats d'àcid benzoic, àcid fenilacètic, àcid fenilpropiònic i 3-hidroxifenil-γ-valerolactona (Groenewoud and Hundt 1986). Els metabòlits microbians són fàcilment absorbits a través del còlon i poden ser transformats en els teixits, tant en el fetge com en el ronyó, per conjugació amb àcid glucurònic, sulfat i fins amb glicina produint metabòlits com els àcids 3 o 4-hidroxihipúric formats a partir de la unió de la glicina amb els àcids 3 o 4-hidroxibenzoics (Rechner et al. 2002b).

Gonthier et al (2003) van comparar el metabolisme urinari de la catequina, del dímer de procianidina B3, del trímer C2 i dels polímers administrats en rates durant 5 dies. En les rates alimentades amb procianidines no van observar cap metabòlit de catequina a diferència de les alimentades amb catequina on van observar una elevada excreció de catequina i de la seva conjugat 3'-O-metilat. D'altre banda, van identificar 16 metabòlits d'origen microbià derivats dels àcids fenilvalèrics, fenilpropiònics, fenilacètics i benzoics (Figura 3) en percentatges que disminuïen segons el grau de polimerització dels diferents tractaments amb catequina (10.6 +/- 1.1%), dímer de procianidina (6.5 +/- 0.2%), trímer (0.7 +/- 0.1%), i polímer (0.5 +/- 0.1%) podent explicar com el grau de

polimerització limita l'absorció i el metabolisme de les procianidines (Gonthier et al. 2003b).

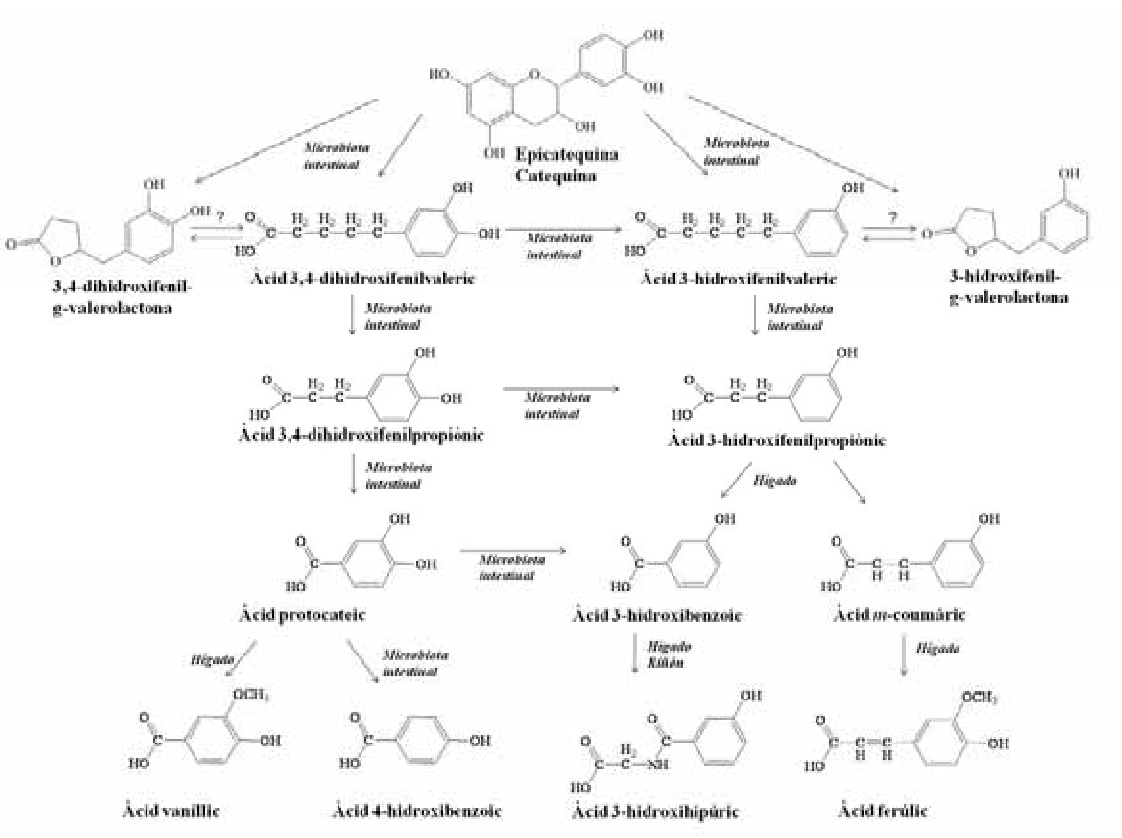

Figura 3: Ruta metabòlica de degradació d'epicatequina (catequina) a través de la microbiota intestinal (Gonthier et al. 2003b).

En un altre estudi de Gonthier et al (2003) van administrar catequina o un extracte de vi negre amb proantocianidines a rates durant 8 dies observant la formació d'àcids fenòlics en orina. Els principals metabòlits formats després de l'administració de catequina van anar l'àcid 3-hidroxifenilpropiònic, l'àcid 3-hidroxibenzoic i l'àcid 3-hidroxihipúric i després de l'administració d'extracte de vi negre van ser els mateixos però a més es van observar elevades excrecions d'àcid hipúric, àcid *p*-coumàric, àcid vanillic, àcid 4-hidroxibenzoic i àcid 3-hidroxifenilacètic (Figura 3). A més dels àcids aromàtics descrits van identificar mitjançant GC-MS tant fenilvalerolactones com àcids fenilvalèrics i van identificar per primera vegada un derivat metilat de fenilvalerolactona (Figura 3). L'elevada excreció d'àcids aromàtics després de

l'administració d'extracte de vi negre (9.2g/100g) comprada amb després de l'administració de catequina (4.7g/100g) es deu a la menor absorció de les procianidines en la part proximal de l'intestí i la seva elevada degradació i posterior absorció en el còlon (Gonthier et al. 2003a).

Unno et al. (2003) van observar la formació urinària de la 5-(3',4'-dihidroxifenil)-γ-valerolactona a partir de l'epicatequina administrada en rates i van demostrar que encara que aquest metabòlit microbià té capacitat antioxidant menor que l'epicatequina, també és un metabòlit actiu amb capacitat antioxidant (Unno et al. 2003).

Rios et al., en 2003, van estudiar l'excreció urinària dels àcids fenòlics formats per l'acció de la microbiota després del consum de 80 g de xocolata en voluntaris. El consum de xocolata va incrementar l'excreció urinària de l'àcid *m*-hidròxifenilpropiònic, l'àcid ferúlic, l'àcid 3,4-dihidroxifenilacètic, l'àcid *m*-hidroxifenilacètic, l'àcid vaníllic i l'àcid *m*-hidroxibenzoic (Rios et al. 2003).

2.3 Biodisponibilitat i metabolisme d’isoflavones

En la nostra alimentació, les isoflavones provenen principalment dels llegums sent la soja i dels productes derivats de la soja els més abundants en isoflavones. En la soja, les isoflavones es troben com aglicones o com glicòsids depenent del preparat de soja. El metabolisme de les isoflavones condicionarà el seu posterior efecte en la salut així com el seu mecanisme d'acció. S'han descrit nombrosos estudis del metabolisme de la daidzeina i la genisteina tant en animals d'experimentació com en humans. Els metabòlits de les isoflavones formats podrien tenir un important paper com transportadors de les seves aglicones a teixits diana com la pròstata i la glàndula mamaria.

Els glicòsids de les isoflavones, després de la ingesta de soja, s'absorbeixen poc en l'intestí prim a causa de la seva hidrofilicitat i al seu alt pes molecular (Liu and Hu 2002). Els glicòsids poden sofrir una hidròlisi abans d'absorbir-se a causa de l'activitat glicosidasa del mateix aliment (via enzimàtica d'origen endogen o afegida durant el procés), de les cèl·lules de la mucosa gastrointestinal o d'enzims de la microbiota colònica (Scalbert and Williamson 2000). Aquesta hidròlisi inicial és necessària per a alliberar les aglicones i permetre la seva absorció a través de l'intestí per difusió passiva (Scalbert and Williamson 2000). D'altre costat, recentment s'ha observat que els glicòsids de les isoflavones es poden absorbir i determinar en plasma de voluntaris i d'animals d'experimentació (Wen et al. 2008; Hosoda et al. 2008).

Les isoflavones ja es detecten en plasma als 30 minuts després de la ingesta de soja (King and Bursill 1998) amb un màxim d'absorció una hora després del consum (Richelle et al. 2002). Això podria ésser a causa de la presència de petites proporcions d’aglicones en l'aliment (King and Bursill 1998), o també, que durant la primera hora de digestió podria haver una hidròlisi i absorció inicial en el duodè i en el jejú proximal (Setchell et al. 2001; Rowland et al. 2003).

L'enzim hidrolasa lactasa florizina (LPH), present en la cara luminal de la vora en raspall de l'intestí prim, pot deglicosilar la genisteina-7-glucòsid i la daidzeina-7-glucòsid dintre del mateix lumen i llavors aquestes aglicones podrien absorbir-se mitjançant difusió passiva (Day et al. 2000b). Els glicòsids de isoflavones que no

s'hagin absorbit arribaran al còlon on els bacteris amb activitat beta-glicosidasa les hidrolitzaran (Parodi 1999).

Després de l'absorció inicial, les isoflavones sofreixen metabolisme de primer pas que fa disminuir la seva biodisponibilitat (Chen et al. 2003). Durant les biotransformacions de fase II, els grups hidroxils de les isoflavones es glucuroniden i es sulfaten a través de glucuronosil transferases o de sulfotransferases en el fetge (Xu et al. 1994) i/o intestí (Setchell et al. 2001). Les isoflavones segueixen la clàssica circulació enterohepàtica i es conjuguen en el fetge. Els metabòlits glucuronidats o sulfatats poden ser transportats via circulació sistèmica cap als teixits d'on eventualment s'excretaran via renal o podran ser secretats via biliar i tornar a l'intestí (Xu et al. 1994). Després de la desconjugació pels bacteris intestinals, les aglicones es podran reabsorbir, tornar al fetge via vena porta per a la seva reconjugació i recirculació enterohepàtica o excreció renal (Winter and Bokkenheuser 1987). Chen et al, 2003, van observar que la genisteina es glucuronidava i sulfatava en les cèl·lules intestinals *in vitro* i que aquests metabòlits s'excretaven en els costats apicals i basolaterals de l'enteròcit (Chen et al. 2003). Per tant, la conjugació intestinal de les isoflavones, la seva excreció en el lumen intestinal i la seva futura reabsorció i reconjugació constitueix la recirculació entèrica (Chen et al. 2003), la qual, combinada amb la recirculació enterohepàtica, perllonga significativament l'exposició a les isoflavones (Turner et al. 2003).

En la circulació sanguínia, les aglicones representen només una petita part de les isoflavones plasmàtiques generalment menys del 5% dels metabòlits totals (Doerge et al. 2000; Shelnutt et al. 2002). Els glucurònids (54%) són els metabòlits principals de les isoflavones (Spencer et al. al. 1999), seguits dels metabòlits sulfatats (13%), sulfoglucuronidats (0.9%) i diglucuronidats (0.4%) (Figura 4) (Kroon et al. 2004; Adlercreutz et al. 1995). També s'han observat diferències en el metabolisme de les isoflavones entre diferents espècies després del consum d'una dieta rica en soja. Gu et al (2006) van observar en plasma de dones un 75% de glucurònids, un 24% de sulfats i < 1% d'aglicones; en canvi, en micos, el perfil metabòlic canviava sent majoritari per als sulfats (64%) i amb un 30% de glucurònids i un 6% d'aglicones (Gu et al. 2006).

Holder et al (1999) van observar una formació majoritària de glucurònids en sang de rates suplementades amb diferents quantitats de genisteina i van identificar la posició d'aquests glucurònids per LC-MS i 1H-NMR veient que en el plasma de rata, la forma majoritària era la genistein-7-glucurònid i el seu isòmer en 4'- era el minoritari (Holder et al. 1999). Hosoda et al., el 2008 van investigar el perfil plasmàtic de 2 voluntaris després de 1 i 7 hores del consum de kinako (farina de soja torrada) observant la formació dels 7- i 4'- glucurònids i 7- i 4'- sulfats de la genisteina i de la daidzeina (Figura 4) (Hosoda et al. 2008).

Daidzeina-7-*O*-glucurònid

Genisteina-7-*O*-glucurònid

Daidzeina-4'-*O*-glucurònid

Genisteina-4'-*O*-glucurònid

Daidzeina-4',7-*O*-diglucurònid

Genisteina-4',7-*O*-diglucurònid

Daidzeina-7-sulfat

Genisteina-7-sulfat

Daidzeina-4'-sulfat

Genisteina-4'-sulfat

Daidzeina-4',7-disulfat

Genisteina-4',7-disulfat

Figura 4: Conjugats glucuronidats i sulfatats de la daidzeina i de la genisteina

Després del consum de soja, el percentatge total de daidzeina com glucurònids i sulfats (Shelnutt et al. 2002) sol ser major que el de la genisteina. Els sulfats d'ambdues isoflavones s'eliminen més ràpid que els glucurònids (Shelnutt et al. 2002). Mentre que la concentració de sulfat de daidzeina en plasma és més elevada que el de genisteina, s'elimina més ràpid encara que ambdós mostren recuperacions urinàries similars (Shelnutt et al. 2002). Això podria reflectir una major distribució tissular del sulfat de daidzeina.

Les concentracions màximes plasmàtiques de la daidzeina i la genisteina es troben generalment entre 6 i 8 hores després del consum de soja en humans (Setchell et al. 2003b; King and Bursill 1998; Setchell et al. 2003a). La daidzeina normalment arriba més tard a la concentració màxima però també desapareix abans que la genisteina (Shelnutt et al. 2002). El temps de vida mitja d'eliminació plasmàtic depèn del compost sent de 3-9 hores per a la daidzeina i de 8-11 hores per a la genisteina després del consum d'aliments de soja o d'estàndards purs de glicòsids d'isoflavones (Watanabe et al. 1998; Shelnutt et al. 2002; Setchell et al. 2003a).

La major part de les isoflavones s'absorbeixen i metabolitzen en l'intestí i només una petita part s'excreta per femta (Xu et al. 1995). Les isoflavones que arriben al còlon són degradades per la microbiota colònica cap a compostos més simples mitjançant reaccions d'*O*-desmetilació, reducció o trencament de l'oxigen heterocíclic que conté l'anell C (Scalbert and Williamson 2000). A través de la reacció d'O-desmetilació, les isoflavones metilades que provenen dels aliments com són la formononetina i la biochanina A es metabolitzen per a donar els compostos daidzeina i genisteina, respectivament (Roberts et al. 2004; Tolleson et al. 2002). Ambdues daidzeina i genisteina també es metabolitzen en el còlon per a donar metabòlits secundaris actius a través dels seus intermediaris dihidrodaidzeina i dihidrogenisteina, respectivament (Figura 5). La microbiota colònica metabolitza la daidzeina mitjançant una reacció de reducció per a obtenir el metabòlit equol o per trencament de l'anell C per a obtenir el metabòlit O-desmetilangolensina (ODMA), dels quals només l'equol és un compost biològicament actiu (Setchell et al. 2002). Sembla ser que el metabolisme de l'equol i de l'ODMA estan inversament relacionats suggerint que existeixen dues vies alternatives per al metabolisme de la daidzeína (Kelly et al. 1995).

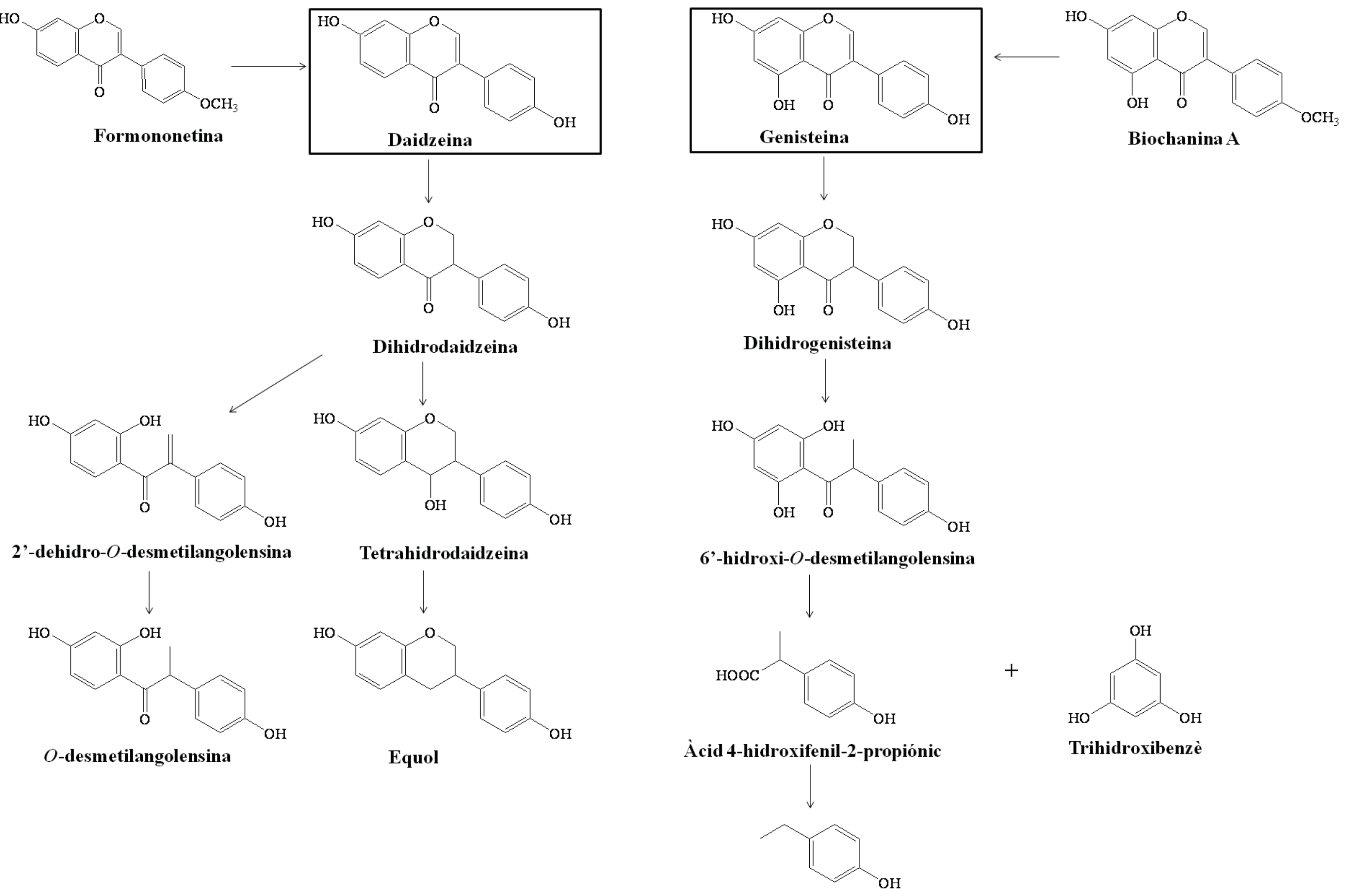

Figura 5: Metabolisme d'isoflavones

La genisteina es transforma a través d'una reacció d'hidrogenació per la microbiota colònica a dihidrogenisteina, la qual és hidrolitzada en l'anell C per a formar la 6'-hidroxi-ODMA, que pot degradar-se per la microbiota colònica a àcid 4-hidroxi-2-propiònic i trihidroxibenzè. Finalment la descarboxilació de l'àcid 4-hidroxi-2-propiònic podria conduir a la formació del producte metabòlic 4-etilfenol (Figura 5) (Heinonen et al. 1999; Kelly et al. 1993). La baixa solubilitat en aigua de la genisteina i el seu major pes molecular, podrien promoure l'excreció dels metabòlits de la genisteina via biliar, i per tant, sofrir un major metabolisme bacterià (Xu et al. 1994), mentre que la daidzeina sembla estar menys subjecta al metabolisme microbià *in vivo*, el que la fa potencialment més biodisponible (Decroos et al. 2005a).

En la majoria d'estudis, les isoflavones s'han quantificat en plasma, orina, bilis i femta, però també s'han trobat elevades concentracions acumulades en teixit mamari de dones i en glàndula prostàtica en homes (Maubach et al. 2003; Guy et al. 2008), i menors concentracions en cervell i placenta.

Les isoflavones s'excreten en orina principalment com glucurònids i en menor grau com sulfats o sulfoglucurònids (Adlercreutz et al. 1995). Després del consum de soja es va observar que la màxima excreció de la daidzeina i la genisteina s'arribava a entre les 7-8 hores després de la ingesta (Watanabe et al. 1998). La major part de l'excreció de la genisteina i daidzeina es dóna dintre de les 24h post-ingesta (Setchell et al. 2003a) encara que King et al. (King and Bursill 1998) van observar un ràtio constant d'eliminació entre 11 i 35 hores després de la ingestió i Watanabe et al. (Watanabe et al. 1998) van mostrar 2 o 3 pics d'excreció durant 48 hores després de la ingesta de soja la qual es podria atribuir al cicle enterohepàtic que sofreixen les isoflavones. La majoria d'estudis observen una major excreció urinària de daidzeina que de genisteina, al contrari del que s'ha descrit en plasma (Xu et al. 1994), per tant, la major excreció urinària de daidzeina reflecteix la major biodisponibilitat d'aquesta isoflavona comparada amb la genisteina (Xu et al. 1994). Aquesta diferència pot ser deguda que gran part de la genisteina consumida és eliminada, a causa de la seva baixa hidrofilicitat, per la via biliar tal com ha estat observat en rates (Sfakianos et al. 1997) i la daidzeina en orina a causa de la seva major solubilitat aquosa (Xu et al. 1994). La genisteina s'excreta majoritàriament com monoglucurònid (53-76%), com diglucurònid (12-16%) o com sulfoglucurònid (2-15%) (Adlercreutz et al. 1995).

D'altra banda, la daidzeina és excretada gairebé exclusivament com a monoglucurònid (80%), i en menys quantitat com a sulfats (13%), sulfoglucurònid (1-7%) i diglucurònid (0.4%) (Adlercreutz et al. 1995; Clarke et al. 2002). Yasuda et al (1996) van caracteritzar aquests conjugats en l'orina i bilis de rates després de l'administració oral de genisteina observant que els seus metabòlits majoritaris en mostres de bilis van ser el genistein-7-glucurònid, i el genistein-4'-sulfat-7-glucurònid com metabòlit majoritari i en orina van caracteritzar la genisteina-4'-sulfat, la genisteina-7-glucurònid i la genisteina-4'-sulfat-7-glucurònid (Yasuda et al. 1996). Posteriorment, Clarke et al (2002) van identificar els dos sulfats i glucurònids 4' i 7-monoconjugats, el 4' ,7-sulfat i glucurònid diconjugats, i dues barreges de 4',7-sulfoglucurònid per a ambdues daidzeina i genisteina en orina de voluntaris amb una ingesta regular d'una dieta rica en soja (Clarke et al. 2002).

Es va observar també una gran variabilitat entre espècies en el perfil d'excreció urinària d'isoflavones després del consum de soja. Mentre que en micos i rates s'excretaven elevades quantitats d'aglicones (>85% i >32%, respectivament), en porcs i dones la principal forma excretada van ser els glucurònids (> 80%) amb només un 10% d'aglicones (Gu et al. 2006).

La recuperació urinària de les isoflavones, comparada amb la quantitat ingerida, és generalment baixa (entre 10-50%) indicant que possiblement hi ha hagut degradació bacteriana colònica i/o metabolisme cap a altres compostos no identificats (Lampe et al. 1998). Hendrich et al. (1998) van suggerir que l'excreció biliar és el principal factor limitant pel que fa al percentatge d'isoflavones que són disponibles sistèmicament després de la seva ingesta (Hendrich et al. 1998).

L'equol sembla ser que té propietats antioxidants i estrogèniques equivalents o fins i tot més elevades (fins a 100 vegades) que la seva isoflavona precursora, la daidzeina, com s'ha observat en estudis *in vitro* i en models *in vivo* amb animals d'experimentació (Setchell et al. 2002). L'equol és un metabòlit exclusivament produït per la microbiota intestinal però existeix una elevada variabilitat interindividual en la capacitat de produir equol. Només un 30-40% de la població occidental són capaces de produir equol o ser "productors d'equol" després del consum de soja (Lampe et al. 2001) a diferència del 45-60% observat en la població asiàtica (Morton et al. 2002). La presència d'equol en orina o plasma s'ha utilitzat per a classificar als subjectes per la seva capacitat de

produir equol per a relacionar-lo amb diferents malalties en estudis epidemiològics. Un estudi cas-control va mostrar una reducció substancial del risc de càncer de mama entre dones que excretaven elevades quantitats d'isoflavones i aquesta reducció s'incrementava en els casos en els quals aquestes voluntàries excretaven equol (Ingram et al. 1997). Un altre estudi cas-control va mostrar un menor percentatge de productors d'equol en homes amb càncer de pròstata comparat amb voluntaris control (Akaza et al. 2002). Els productors d'equol podrien obtenir més beneficis del consum de soja que els no productors (Setchell et al. 2002) a causa de les seves propietats estrogèniques. Per tant, existeix un gran interès a trobar una via alternativa per a fer que els no productors es converteixin en productors, encara que segons s'ha revisat recentment, la majoria d'estudis que associen la producció d'equol amb beneficis en la salut, s'han portat a terme amb una baixa significació estadística (Atkinson et al. 2005). Per tant, es necessiten més estudis en aquest sentit a causa de la no concloent evidència científica.

La farmacocinètica de l'equol és molt semblant a la de les altres isoflavones encara que aquest presenta una menor eliminació plasmàtica (Lampe et al. 2001; Setchell et al. 2002) i un major temps de vida mitja que la daidzeina (Kelly et al. 1995) amb valors plasmàtics màxims entre les 24h i els 3 dies després de la ingesta (Kelly et al. 1995; Setchell et al. 2001). Al ser un metabòlit format a través de la microbiota colònica, la formació de l'equol a partir de la daidzeina és temps depenent començant a formar-se entre les 6 i 8 hores (Setchell et al. 2003b). Una vegada format, l'equol s'absorbeix a través de la paret del còlon (Decroos et al. 2005a) i es conjuga amb àcid glucurònic en el fetge així podent contribuir a allargar la seva farmacocinètica. La formació d'equol també depèn dels nivells inicials de daidzeina. S'ha observat que la formació d'equol és més elevada després del consum de daidzeina glucòsid que de daidzeina aglicona possiblement a causa de la major temps de trànsit del glucòsid (Zubik et al. 2003). Una vegada format, l'equol sembla ser que es manté metabòlicament estable només metabolitzant-se per reaccions de fase II, encara que recentment s'ha observat que podria metabolitzar-se en el fetge (Adlercreutz et al. 2004).

La biodisponibilitat i metabolisme de la gliciteina, un compost isoflavònic metilat, s'ha estudiat poc però estudis recents mostren com els seus metabòlits podrien ser semblats a l'equol. Heinonen et al (2003) van identificar elevades quantitats de 4',6,7-trihidroxiisoflavona en orina de voluntaris després del consum de soja (Heinonen et al.

2003). Aquest compost que conté un grup hidroxil més que l'equol ho van descriure com metabòlit de la gliciteina.

L'equol també roman elevat en orina durant llarg període de temps (Lampe et al. 2001) amb una excreció urinària màxima entre les 24 i 72 hores després del consum de soja (Xu et al. 1994; Kelly et al. 1995). S'excreta en orina gairebé exclusivament com monoglucurònid. Watanabe et al. (1998) van calcular el percentatge de conversió metabòlica de la daidzeina a ODMA (4%) i a equol (7%) i van mostrar que l'excreció fecal de l'equol era molt més elevada suggerint que la majoria de l'equol que s'obté en la femta representa l'excreció biliar (Figura 6) (Watanabe et al. 1998).

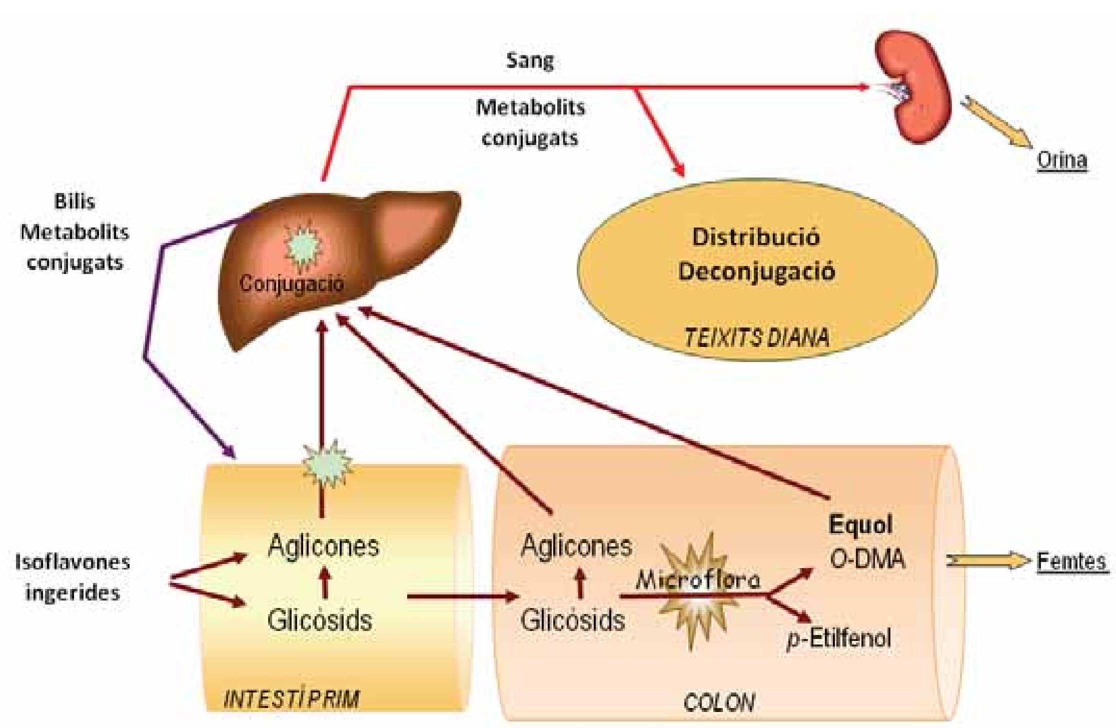

Figura 6: Metabolisme d'isoflavones (Setchell et al., 2002)

2.4. Biodisponibilitat del resveratrol

La biodisponibilitat del resveratrol s'ha concretat en un capítol de llibre "*Bioavailability and Metabolism of Resveratrol.* Cristina Andres-Lacueva, Mireia Urpi-Sarda, Raul Zamora-Ros, Rosa Mª Lamuela-Raventos" del llibre "*Phenolic compounds of plant origin and human health. The biochemistry behind their nutritional and pharmacological value*". Wiley Publishers. 2009 (en procés d'edició)

Bioavailability and Metabolism of Resveratrol

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Abtract

Resveratrol is a polyphenolic compound mainly present in grape and it by products juices and wines. Its importance remains in its described healthy properties. Bioavailability studies considering its absorption, distribution, metabolism and excretion are required to know the levels in biofluids and the major conjugated forms present in them, since they would be the molecules that would exert the biological activity. Here, we described the in vitro and in vivo bioavailability studies of resveratrol in both animal models and humans. Moreover, studies of other related compounds such as piceid, oxyresveratrol, piceatannol, pinosylvin and rhapontigenin were considered. Nowadays, the knowledge of resveratrol bioavailability has increased considerably due to modern specific and sensitive analytical techniques such as mass spectrometry. This has improved the detection of the major forms such as glucuronides and sulfates available at regular doses in the body.

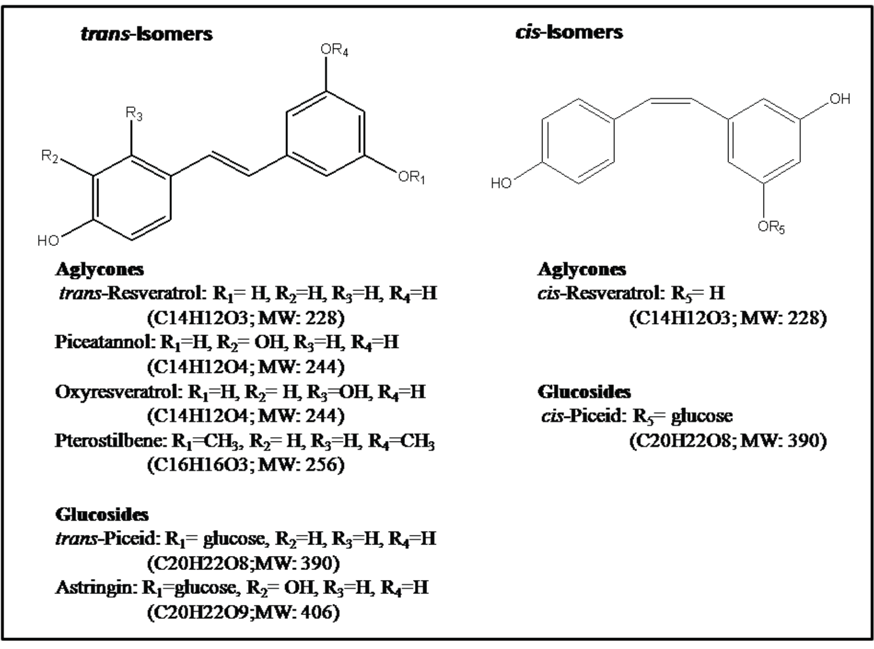
This chapter provides an overview of the bioavailability studies of resveratrol and the metabolites present in the body that will be the potential molecules for the physiological activity.

Introduction

The essential chemical structure of stilbenes is the *trans*-1,2-diphenylethylene. The parent molecule of this group is the resveratrol (3,4',5- trihydroxystilbene) that exists as two geometric isomers: *cis*- (*Z*) and *trans*- (*E*).The *trans* form can undergo isomerisation to the *cis*- form when exposed to ultraviolet irradiation. Piceid (resveratrol glucoside) is the major resveratrol derivative in plants. Other stilbenes in the vegetal kingdom are pterostilbene, piceatannol, astringin, and viniferins (Figure 1).

Resveratrol and piceid are mainly present in grape and grape products and its composition is affected by grape variety, maturity degree at harvest, fungal stress, climate, soil characteristics (*terroir*), and wine-making process as

well as technology [de Andres-de et al., 2007; Gonzalez-Barrio et al., 2006; Romero-Perez et al., 2001]. Other slight food sources of stilbenes are peanuts, pistachios and berries, such as bilberry, blueberry, and cranberry [Burns et al., 2002; Rimando et al., 2004; Sobolev and Cole, 1999; Tokusoglu et al., 2005]. In an adult Spanish cohort resveratrol and piceid, *trans*- and *cis*- forms, were evaluated. Estimated median and mean of resveratrol and piceid were 100µg/d and 933µg/d, respectively, of which 98.4%, 1.6% and less than 0.1% come from wines; grape and grape juice, and peanuts, pistachios and berries, respectively.



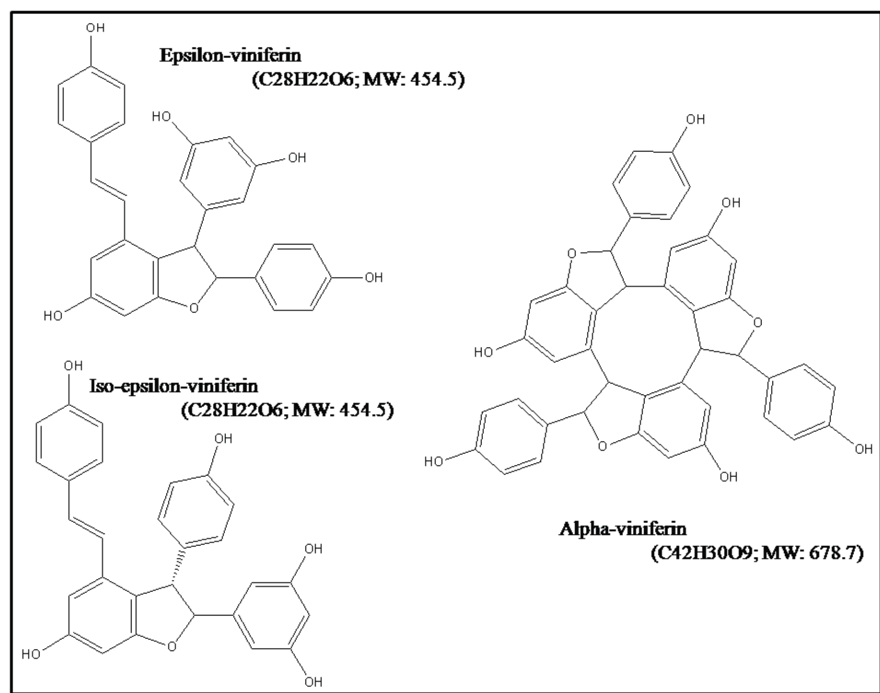


Figure 1: *Chemical structure of stilbenes present in foods.*

In vitro and ex vivo studies

Several studies have investigated the absorption, transport and metabolism of resveratrol in vitro and ex vivo. They are summarized in Table 1.

Caco-2 cells and isolated small intestine, are in vitro and in vivo models of basic nutrition that contributed to the understanding of resveratrol absorption and bioavailability. While the Caco-2 absorption model is a well defined cellular in vitro system based on a human colonic adenocarcinoma cell line, the isolated small intestine model is nearer to in vivo conditions and is also, simpler to handle. It also avoids the methodological problems of in vivo perfusion models [Barthe et al., 1998; Barthe et al., 1999].

The Caco-2 cells incubated in vitro with *trans*-resveratrol (5 to 40 µM) showed a concentration dependent transcellular absorption up to 3 h, with a linear rate for the first hour [Kaldas et al., 2003]. At 3 h of incubation, the concentration of resveratrol reached a plateau [Kaldas et al., 2003; Maier-Salamon et al., 2006]. However, incubations with higher amounts of this polyphenol (40µM) increased their concentration in the Caco-2 cells, so there was no saturation of the transport systems [Kaldas et al., 2003]. The cellular uptake of *trans*-piceid was also investigated in Caco-2 cells and its transport was slower and about 20% of the resveratrol aglycone [Henry et al., 2005]. *trans*-Resveratrol crosses the apical membrane of the Caco-2 cells using a passive transport, whereas *trans*-piceid seems to use both, the active transporter sodium-dependent glucose transporter 1 (SGLT1), and the multidrug resistance protein 2 (MRP2) [Henry et al., 2005]. After piceid absorption, it can be hydrolized to resveratrol by the cytosolic-β-glucosidase [Henry-Vitrac et al., 2006]. Another possible pathway to absorb piceid is through its deglycosilation by membrane-bound lactase phlorizin hydrolase, since it then goes across the apical membrane as resveratrol [Henry-Vitrac et al., 2006]. The basolateral to apical efflux also occurs at similar concentrations to apical and basolateral efflux [Henry-Vitrac et al., 2006; Kaldas et al., 2003]. After resveratrol absorption, this is conjugated rapidly in intestinal cells. At 10 µM concentrations, *trans*-3-*O*-sulfate was the main metabolite of resveratrol, however, its formation drastically decreased at higher resveratrol concentrations (200 µM), possibly due to saturation or inhibition of metabolism at higher stilbene concentrations [Maier-Salamon et al., 2006]. Glucuronidate forms such as *trans*-resveratrol-4'-*O*-glucuronide and *trans*-resveratrol-3-*O*-glucuronide were also released at less levels than sulfate forms (Figure 2) [Maier-Salamon et al., 2006].

Maier-Salomon et al. [2006] showed the high influence of metabolized resveratrol on the transepithelial transport of resveratrol, and its intracellular accumulation. At 10 µM concentration in the Caco-2 cells, resveratrol was 84%, 8% and 12% conjugated, transported and accumulated, respectively. Whereas at higher doses (200 µM) resveratrol was 8%, 26% and 61% conjugated, transported and accumulated, respectively. Moreover, Kaldas et al. [2003] also found a resveratrol accumulation higher than 35-fold when transported through the Caco-2 cells, which suggests enterocytes as a major target site for this polyphenol.

Table 1. *Metabolism of resveratrol in vitro and ex vivo models.*

*Normalized values.

Tissue	**Dose, Source (h)**	**Metabolites (C found)**	**Ref.**
Human erithrocytes (1.6x10^9)	21.9 nmol *t*- Resv (0.25h)	**t*- Resv :10.0 ± 1.7nmol/10^9	[Blanche et al., 1997]
Rat erithrocytes (1.6x10^9)		**t*- Resv 10.8 ± 2.2 nmol/10^9	
Rat platelets (10^9)		**t*- Resv 2.2 ± 1.2nmol/10^9	
Human LDL (0.5mg/mL)	17.5 µM *t*- Resv (0.5h)	**t*- Resv 3.8 ± 0.9 nmol/mg protein	
Jejunum and ileum of Sprague-Dawley male rats	200 µM Resv (1.5h)	Resv (0.03 nmol/cm jejunum) Gluc (1.19 nmol/cm jejunum)	[Kuhnle et al., 2000]
Small intestine of male Sprague-Dawley rats	28, 34, 57 µM Resv (1h)	Vascular effluent: Gluc (16.8%), Resv (3.4%), Sulf (0.3%); Luminal effluent: Gluc (11.2%), Resv (39.7%), Sulf (3.0%), Intestinal tissue: Gluc (0.1%), Resv (1.5%), Sulf (0.3%)	[Andlauer et al., 2000]
Human liver microsomes	1 mM *cis* and *t*-Resv	c-3-Gluc (+), t-3-Gluc (+), c-4’-Gluc (-), t-4’-Gluc (-)	[Aumont et al., 2001]
Rat hepatocytes	20 µM *t*- Resv (1h)		[Asensi et al., 2002]
Human liver microsomes	5mM Resv (1h)	Free Resv	[Yu et al., 2002]
Human Hepatocytes	0.1 mL of 0.1mM *t*-resveratrol (4h)	t-3-Gluc, t-4’-Gluc, c-3-Gluc, *t*-3-Sulf	
Caco-2 cells	5-40 µM Resv (6h)	Resv (200-4000 pmol)	[Kaldas et al., 2003]
Caco-2 cells	150-300 µM *t*- Resv and *t*-piceid (0.03-0.5h)	*t*- Resv > *t*-piceid	[Henry et al., 2005]
Caco-2 cells	10-200 µM Resv	*t*-4’-Gluc, *t*-3-Gluc, *t*-3-Sulf	[Maier-Salamon et al., 2006]
Human liver microsomes	500 µM *t*- Resv (5h)	3-Gluc > 4’-Gluc	[Brill et al., 2006]
Human intestional microsomes		3-Gluc < 4’-Gluc	

Resv: Resveratrol; Gluc: Glucuronide; Sulf: Sulfate; *t*-3-Gluc: *trans*-Resveratrol-3-*O*-Glucuronide; *t*-4’-Gluc: *trans*-Resveratrol-4’-*O*-Glucuronide; *c*-4’-Gluc: *cis*-Resveratrol-4’-*O*-Glucuronide; *c*-3-Gluc: *cis*-Resveratrol-3-*O*-Glucuronide, *t*-3-Sulf: *trans*-Resveratrol-3-Sulfate

INTESTINAL METABOLISM

trans-Isomers　　　　*cis*-Isomers

OR_3　OR_1　R_2O　OH　R_5O　OR_4

trans-Isomers

***trans*-Resveratrol-3-O-glucuronide: R_1= glucuronid acid, R_2=H, R_3=H (C20H20O9; MW: 404)**
***trans*-Resveratrol-4'-O-glucuronide: R_1= H, R_2=glucuronid acid, R_3=H (C20H20O9; MW: 404)**
***trans*-Resveratrol-3,4'-diglucuronide: R_1= glucuronid acid, R_2=glucuronid acid, R_3=H (C26H28O15; MW: 580)**
***trans*-Resveratrol-3-sulfate: R_1= SO_3H, R_2=H, R_3=H (C14H12O6S; MW: 308)**
***trans*-Resveratrol-4'- sulfate: R_1= H, R_2= SO_3H, R_3=H (C14H12O6S; MW: 308)**
***trans*-Resveratrol-3,4'-sulfate: R_1= SO_3H, R_2= SO_3H, R_3=H (C14H12O9S2; MW: 388)**
***trans*-Resveratrol-3,5-disulfate: R_1= SO_3H, R_2= H, R_3= SO_3H(C14H12O9S2; MW: 388)**
***trans*-Resveratrol-3,5,4'-trisulfate: R_1= SO_3H, R_2= SO_3H, R_3= SO_3H (C14H12O12S3; MW: 468)**

cis-Isomers

***cis*-Resveratrol-3-O-glucuronide: R_4= glucuronid acid, R_5=H (C20H20O9; MW: 404)**
***cis*-Resveratrol-4'-O-glucuronide: R_4= H, R_5=glucuronid acid (C20H20O9; MW: 404)**
***cis*-Resveratrol-3,4'-diglucuronide: R_4= glucuronid acid, R_5=glucuronid acid (C26H28O15; MW: 580)**
***cis*-Resveratrol-3-sulfate: R_4= SO_3H, R_5=H (C14H12O6S; MW: 308)**
***cis*-Resveratrol-4'- sulfate: R_4= H, R_5= SO_3H (C14H12O6S; MW: 308)**
***cis*-Resveratrol-3,4'-disulfate: R_4= SO_3H, R_5= SO_3H (C14H12O9S2; MW: 388)**

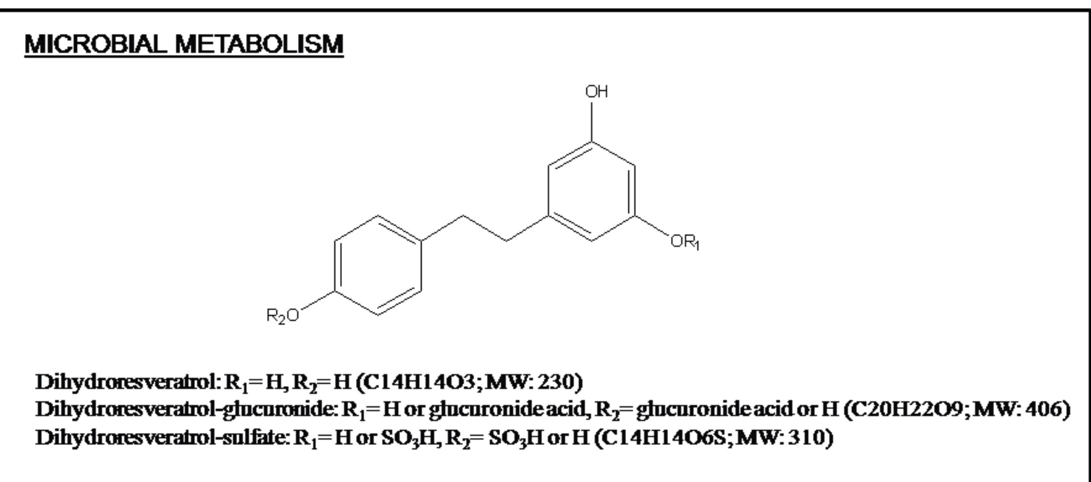


Figure 2: *Chemical structures of resveratrol metabolites.*

Perfusion of 200 µM *trans*-resveratrol in isolated jejunum and ileum only transferred 6% and 2% of the total amount of resveratrol available, respectively. Resveratrol passed across jejunum as a glucuronide form (1.1 nmol/cm) and in minor amounts as free resveratrol (0.03 nmol/cm) [Kuhnle et al., 2000]. In a single pass perfusion at low concentrations (28, 34 or 57 µM of *trans*-resveratrol) in the vascular and the luminar side, tissue and blood vessels of rat small intestine, 54%, 21%, 2% and 0.1% of the doses were recovered, respectively. In isolated luminal small intestine, the stilbene compounds found were free resveratrol (74%), 2-glucuronides (21%), and a sulfate (6%). Whereas in isolated vascular small intestine, the main metabolites were the glucuronidate forms (82%), free

resveratrol (17%) and the sulfate form (1.5%). No differences were found in the recoveries using several different doses of resveratrol [Andlauer et al., 2000]. *trans*-Resveratrol was able to bind to human and rat erythrocytes, rat platelets, and LDL as free resveratrol after in vitro incubations, confirming that resveratrol can diffused throughout the body by means of its incorporation into blood cells and lipoproteins [Blache et al., 1997].

Conjugation forms of resveratrol can occur in both enterocytes and hepatocytes. After intestinal microsome incubation with 500 μM *trans*-resveratrol, only glucuronides were formed, in which *trans*-resveratrol-4'-*O*-glucuronide was more abundant than *trans*-resveratrol-3-*O*-glucuronide. In comparison with liver ability to metabolize, glucuronides were formed at higher levels (up to 10-fold) by the intestinal microsomes than the liver microsomes [Brill et al., 2006]. Likewise in liver microsomes, only glucuronidate forms and aglycones were observed after incubation with *trans*- and *cis*-resveratrol. Glucuronidation in liver was stereoselective (*cis*-isomer was 5 to 10-fold faster than *trans*-isomer) at both positions and regioselective (3-position was greater than 4'-position) for both isomers [Aumont et al., 2001]. Moreover Aumont et al. [2001] and Brill et al. [2006] found that resveratrol was only glucuronidized by the UDP-glucuronosyltransferase family 1A (UGT1A). In particular, were UGT1A1 and UGT1A9 mainly involved in the formation of *trans*-resveratrol-4'-*O*-glucuronide and *trans*-resveratrol-3-*O*-glucuronide, respectively. However, in biopsies of human liver it was found that resveratrol is a better substrate for sulfotransferases (K_m 0.60 μM) [de Santi et al., 2000b] than glucuronosyl transferase (K_m 0.15 μM) [de Santi et al., 2000a].

trans-Resveratrol was rapidly metabolized in a dose-dependent manner in rat hepatocytes, approximately 80% and 100% of the resveratrol (20 μM) incubated were conjugated at 20 min and at 1 h, respectively [Asensi et al., 2002]. In rat hepatocytes the main metabolite formed was *trans*-resveratrol-3-*O*-sulfate followed by *trans*-resveratrol-3-*O*-glucuronide. In contrast, in human hepatocytes glucuronides, i.e. *trans*-resveratrol-4'-*O*-glucuronide, *trans*-resveratrol-3-*O*-glucuronide and *cis*-resveratrol-3-*O*-glucuronide, were more abundant than sulfates (*trans*-resveratrol-3-*O*-sulfate) [Yu et al., 2002]. Based on these results, sulfate forms seem to be a minor human hepatic metabolite.

In summary, resveratrol absorption is higher as aglycon than piceid, although piceid can be hydrolyzed by β-glucosidases in the enterocytes [Henry-Vitrac et al., 2006]. Enterocytes metabolize resveratrol up to certain extent. At low concentrations it seems that there is mainly a sulfate pathway, but at higher amounts glucuronidate forms become more abundant [Maier-Salamon et al., 2006]. Finally, the accumulation of resveratrol in Caco-2 cells suggests that enterocytes is an important target site [Kaldas et al., 2003]. Resveratrol is transported into the blood stream bounded to blood cells and lipoproteins [Blache et al., 1997]. Hepatocytes also metabolize resveratrol to facilitate its excretion, mainly to glucuronides and, in fewer amounts, to sulfates [Asensi et al., 2002; Yu et al., 2002].

Metabolism of resveratrol in vivo in animal models

There is an extensive amount of literature on the in vitro activities of resveratrol. Bertelli et al. were the first to study the bioavailability of resveratrol in rats. They assessed analysis of plasma and tissues from rats which were administered red wine in a single dose or a regular dose for 15 d. Results indicated that resveratrol is quickly absorbed with a maximum peak at 1 h in plasma as well as in liver and kidney, although in the heart it peak at 2 h. Values obtained after regular consumption of red wine were higher than without the regular consumption in the different organs studies, in particular in the liver. The kidney seemed to be the main route of excretion (Table 2) [Bertelli et al., 1996].

Table 2. *Tissue distribution in animal models*

Species	Source and dose	Administration	Mean (SD) nmol/g												Plasma (µmol/l)	Feces (%)	Urine (%)	Time	Ref.
			Brain	Lung	Liver	Kidney	Heart	Spleen	Gastrointestinal tract	Small intestine	Stomach	Testis	Colonic Mucosae	Bile					
6 Wistar rats	4 ml red wine (0.09mg/kg)	Intragastric	-	-	0.09	-	-	-	-	-	-	-	-	-	-	-	-	0.5h	(Bertelli et al. 1996)
6 Wistar rats			-	-	-	0.09	-	-	-	-	-	-	-	-	0.09	-	-	1h	
6 Wistar rats			-	-	-	-	0.009	-	-	-	-	-	-	-	-	-	-	2h	
36 Wistar rats	2 ml red wine/day (0.04mg/kg/day) during 15 days	Intragastric			0.23 (0.006)	0.19 (0.005)	0.01 (0.001)	-	-	-	-	-	-	-	0.03 (0.002)	-	0.29 (0.007)	24h	
5 C57BL/6J male mice	20 mg/kg *t*-Resv	Intragastric	0.50 (0.35)	0.40 (0.23)	1.03 (0.80)	0.17 (0.06)		-	-	-	-	-	-	-	1.7 (0.9)	-	-	5 min	(Asensi et al. 2002)
								-	-	-	-	-	-	-		-	2% Gluc	24h	
5 Wistar male rats		Intragastric	0.11 (0.05)	0.10 (0.05)	0.45 (0.11)	0.06 (0.03)		-	-	-	-	-	-	-	1.2 (0.4)	-	-	5 min	
								-	-	-	-	-	-	-		-	Gluc	24h	
5 ESD NZW male rabbits		Intragastric	0.08 (0.03)	0.62 (0.17)	0.35 (0.14)	0.05 (0.02)		-	-	-	-	-	-	-	0.7 (0.5)	-	-	5 min	
3 Mice per time point	240mg/kg *t*-Resv	Intragastric	1.2 (0.1)	50 (25)	51 (15)	16 (10)	75 (27)	-	-	960(100)	-	-	30 (5)	-	32 (14)	-	-	10 min	(Sale et al. 2004)
	240mg/kg *t*-3,4,5,4'-tetramethoxystilbene	Intragastric	5 (1)	11 (0)	8 (3)	11 (4)	10 (5)	-	-	7600 (6500)	-	-	330 (300) 30 min	-	5 (2)	-	-	10 min	
10 Male Wistar rats	300mg/kg/day *t*-Resv during 8 weeks	Oral	-	-	triSulf; n.d. diSulf: n.d. 3-Sulf: 1.92 (0.78) 4'-Sulf: 5.75 (2.08) 3-Gluc: 1.58 (0.59) Resv: 3.20 (1.01)	triSulf: n.d. diSulf: 5.00 (1.31) 3-Sulf: 1.10 (0.36) 4'-Sulf: n.d. 3-Gluc: 6.71 (1.49) Resv: n.d.		-	-	-	-	-	-	-	TriSulf: 7.0 (2.6) 3,4'-diSulf: 19.23 (6.0) 3,5-diSulf: 3.30 (0.8) 3-Sulf: 1.20 (0.3) 3-Gluc: 7.75 (2.2) Resv: n.d.	TriSulf: n.d. 3,4'-diSulf: 3.42% 3,5-diSulf: 0.80% 3-Sulf: 5.68% 4'-Sulf: 3.83% 3-Gluc: n.d. Resv: 17.2%	TriSulf: 1.42% 3,4'-diSulf: 3.5% 3,5-diSulf: 10% 3-Sulf: 5.93% 4'-Sulf: n.d. 3-Gluc: 28.6% Resv: 3.99%	24h	(Wenzel et al. 2005)
10 Male Wistar rats	50mg/kg/day *t*-Resv during 8 weeks	Oral	-	-	n.d.	n.d.		-	-	-	-	-	-	-	**n.d.**	TriSulf: n.d. 3,4'-diSulf: n.d. 3,5-diSulf: n.d. 3-Sulf: 1.73% 4'-Sulf: n.d. 3-Gluc: n.d. Resv: 13.1%	TriSulf: n.d. 3,4'-diSulf: 0.56% 3,5-diSulf: 0.26% 3-Sulf: 0.60% 4'-Sulf: n.d. 3-Gluc: 8.90% Resv: 5.03%		
18 Male Wistar rats	50mg/kg Piceid	Oral	15.56 (7.3)	26.72 (9.9)	11.46 (6.4)	6.62 (3.1)	1.28 (0.7)	71.87 (35.4)	-	278.60 (76.4)	432.78 (198.6)	13.60 (6.2)	-	-	-	-	-	10 min	(Lv et al. 2006)

6 Male Sprague-Dawley rats	50 mg/kg resveratrol + 1.85MBq [3H]resveratrol	Gavage	< 0.1% dpm	< 0.1% dpm	0.98% dpm	0.59% dpm	< 0.1% dpm	< 0.1% dpm	76.2% dpm	-	-	-	-	-	1.7% dpm	-	-	2h	(El-Mohsen et al. 2006)
			Gluc: 0.2 (0.0) Resv: 0.1 (0.0)	Gluc: 0.5 (0.2) Rev: 0.2 (0.2)	Gluc: 1.2 (0.3)	Gluc: 4.0 (0.6)	Gluc: 0.4 (0.0)	n.d.	-	-	-	-	-	-	Gluc: 7.0 (1.0) Resv	2 Gluc Resv	Resv 2 Gluc		
6 Male Sprague-Dawley rats	50 mg/kg resveratrol + 1.85MBq [3H]resveratrol	Gavage	0.35% dpm						5.1% dpm	-	-	-	1.55% dpm	-	0.48% dpm	-	3.3% dpm	18h	(El-Mohsen et al. 2006)
			Gluc: 0.13 (0.05) Resv: 0.07(0.05)	Gluc: 0.1 (0.03) Resv: 0.2 (0.03)	Gluc: 0.1 (0.03) Resv: 0.15 (0.03)	n.d.	Resv: 0.09 (0.02)	n.d.	-	-	-	-	-	-	n.d.	-	-		
3 Balb/c male mice	7.4 kBq ^{14}C- *t*-Resv (5 mg/kg)	Intragastric	n.c.	n.c.	441 (90) dpm/100 mg	342 (165)	n.c.	n.c.	-	2208 (1436) dpm/100 mg	-	n.c.	106 (80) dpm/100 mg	460 (70) $\cdot 10^3$dpm/ml	0.5 (0.2) $\cdot 10^3$dpm/ml	-	100 (26.7) $\cdot 10^3$dpm/ml	1.5h	(Vitrac et al. 2003)
3 Balb/c male mice			196 (47) dpm/100 mg	380 (148) dpm/100 mg	374 (48) dpm/100 mg	552 (51) dpm/100 mg	210 (132) dpm/100 mg	312 (62) dpm/100 mg	-	1841 (183) dpm/100 mg	-	**193 (9)**	300 (83) dpm/100 mg	1170 (500) $\cdot 10^3$dpm/ml	0.6 (0.8) $\cdot 10^3$dpm/ml	-	286 (16.7) $\cdot 10^3$dpm/ml	3h	
3 Balb/c male mice			n.c.	n.c.	189 (28) dpm/100 mg	263 (123) dpm/100 mg	n.c.	n.c.	-	933 (201) dpm/100 mg	-	n.c.	88 (26) dpm/100 mg	340 (330) $\cdot 10^3$dpm/ml	1.25 (0.8) $\cdot 10^3$dpm/ml	-	235 (23.3) $\cdot 10^3$dpm/ml	6h	
3 Balb/c male mice	74 kBq ^{14}C- *t*-Resv (50 mg/kg)	Intragastric	-	-	0.09 (0.03) cpm/mm^2	0.05 (0.02) cpm/mm^2	-	-	-	**n.d.**	1.09 (0.72) cpm/mm^2	-	-	-	-	-	-	1.5h	
3 Balb/c male mice			-	-	0.1 (0.01) cpm/mm^2	0.07 (0.02) cpm/mm^2	-	-	-	1.7 (0.16) cpm/mm^2	0.93 (0.55) cpm/mm^2	-	-	-	-	-	-	3h	
3 Balb/c male mice			-	-	0.05 (0.01) cpm/mm^2	0.03 (0.01) cpm/mm^2	-	-	-	0.2 (0.04) cpm/mm^2	0.4 (0.04) cpm/mm^2	-	-	-	-	-	-	6h	
3 Balb/c male mice	92.5 kBq ^{14}C- *t*-Resv (66 mg/kg)	Intragastric			^{14}C- *t*-Resv: 25 µmol/l	^{14}C- *t*-Resv: 30 µmol/l	-	-	-	-	-	-	-	-	-	-	-	3h	

TriSulf: resveratrol-3,4',5-trisulfate; diSulf: resveratrol-disulfate; Sulf: resveratrol-sulfate; Gluc: resveratrol-glucuronide; *t*-Resv: *trans*-resveratrol; Radioactivity was measured by dpm or cpm: dpm: desintegrations per minute and cpm: counts per minute; MBq: megabecquerel; kBq: kilobecquerel; n.d.: not detected; n.c.: not collected

Soleas et al. performed experiments using *trans*-resveratrol radiolabeled with [^{3}H] in a fixed position in the first benzene ring [Soleas et al., 2001a]. Male Wistar rats received 120 nCi by gavage that were added as part of three different matrices: 10% (v/v) ethanol, homogenized vegetable cocktail, and white grape juice. Short-term experiments at 2 h and longer-term experiments at 24 h were carried out. Over a 24-h period only trace amounts of resveratrol (<1%) were detected in tissues such as liver, kidney, heart or spleen. However, it appeared that 77-80 % of *trans*-resveratrol could be absorbed in the rat intestine, with no differences among the three food matrices when absorption was measured as the difference between the dose of radioactivity given and the recovered radioactivity in the stool (24 h feces plus colonic contents and colon). Nevertheless, values ranging from 49 to 61% were present in the urine, regardless the matrix. This 25% difference could be accounted for by excretion vía sweat and the respiratory system, metabolism to CO_2 and accumulation in fat rich tissues such as the brain and nervous system [Soleas et al., 2001a]. Short-term experiments (2 h) showed that over the first 30 min after administration, a significant amount of radioactivity accumulated blood and serum. The radioactivity remained at similar levels during the following 90 min. These observations included the parent compound as well as the metabolites (Table 3).

Experiments with unlabeled resveratrol were also conducted in rats after administration of 5 mg of resveratrol. *trans*-Resveratrol concentrations appeared in serum at 15 min, peaked at 30 min, and declined abruptly over the next 30 min. Soleas et al., in 2001a, also measured the competition of several polyphenols when catechin, quercetin and resveratrol (1-10 nM) were co-administered in rats. They found no competition between the three polyphenols and the absorption of *trans*-resveratrol was not saturable in the used concentrations [Soleas et al., 2001a].

In 2006, El-Mohsen et al. investigated the time-dependent appearance and disappearance in various organs of metabolic products of [^{3}H] *trans*-resveratrol (2 and 18 h following gastric administration of 50mg/kg + 1.85 MBq) to Sprague-Dawley rats. At 2 h post-gavage, most of the recovered radioactivity was still present in the gastrointestinal tract. The total dose administered reached only 1.7% in plasma. The only tissues with high concentrations were the liver and the kidney while the amount detected in other tissues was <0.1%. In contrast, at 2 and 18 h post-administration, approximately 11% of total dose was accounted for in all of the studied tissues (Table 2). They found that around 90% of the administered dose was absorbed, however only 3.3% of absorbed resveratrol was detected in urine. The undetected radioactivity is expected to be lost vía respiration and/or accumulation in other tissues, such as skeletal muscle and adipose tissue [El-Mohsen et al., 2006; Soleas et al., 2001a].

The metabolites of [^{3}H]*trans*-resveratrol detected in tissues and plasma were also investigated. In kidney, liver, heart, lungs, brain and plasma (2-h samples), the only metabolite found was resveratrol-glucuronide. Glucuronides in plasma and kidney disappeared completely at 18 h. In lungs, liver, heart and brain, the main detected metabolite at 18 h was the *trans*-resveratrol (Table 2). This study provided data on the metabolic fate of resveratrol. While glucuronides were predominant in plasma and tissues at the earlier times, the aglycone represented the main form at later times [El-Mohsen et al., 2006].

The kinetics of absorption, tissue distribution, and excretion was assessed after a single oral dose of ^{14}C-*trans*-resveratrol to male Balb/c mice. Blood and tissues samples were collected at 1.5, 3, and 6 h post-administration [Vitrac et al., 2003]. The auto radiographic examination of mice tissue sections and the radioactivity quantification revealed a higher fixation of ^{14}C-*trans*-resveratrol in the stomach, liver, kidney, intestine, bile and urine, and other organs of absorption and elimination. The concentration of radioactivity in blood was low and regular during the experimental period. During the entire experimental period, nearly complete absorption occurred in the small intestine as suggested by the higher concentration found in the proximal section compared to the distal section. After 6 h of oral administration the high concentration in stomach and duodenum was perhaps located in the mucosa, although this was also probably due to residual stomach content. The low concentrations found in the colon suggests that it was a minor way of elimination. The major concentrations found in urine and the decreasing concentrations in kidney showed that renal excretion was a major way of elimination. The kidney and the liver were the organs with

highest deposition of ^{14}C-*trans*-resveratrol. In contrast to the kidney in which the parent drug was the major radioactive product, liver extracts 3 h after administration showed the presence of ^{14}C-*trans*-resveratrol with a high concentration of radioactive glucuronide or sulfated conjugated [Vitrac et al., 2003].

Contrasting with other studies that showed the accumulation of labeled resveratrol in tissues, the following studies addressed the search for the formed metabolites in biological fluids and tissues. Yu et al. [2002] synthesized and identified for the first time, the mono sulfate isomers of resveratrol, resveratrol-3 and 4'-sulfate. These studies were performed in female Sprage-Dawley rats after intraperitoneal administration of 20 mg/kg of resveratrol with urine collection at 2 h, and in female Balb/c mice after intraperitoneal (20 mg/kg) and oral administration (20 mg/kg and 60 mg/kg) with collection of serum samples up to 4 h. The mass spectrometry analysis of rat urine samples only detected *trans*-resveratrol-3-glucuronide. In the mouse serum samples, after administration of 20 mg/kg vía intraperitoneal or oral, maximum concentrations of resveratrol-3-glucuronide and resveratrol-3-sulfate were observed at 15 min. The sulfate was almost 3-fold greater than the glucuronide (Table 3). Only traces of free resveratrol were observed. Furthermore, no resveratrol or metabolites were detected after 1 h. When higher doses were oral administered to mouse (60 mg/kg), the same metabolites as before were observed, but the maximum value for sulfate metabolite were reached after 30 min instead of 15 min, probably because more time was required to absorb the large volume that was administered. This was not the case for the minor dosage, both glucuronide and sulfate metabolite were still detected after 3 h suggesting that resveratrol was distributed to tissues and cleared slowly.

Wenzel et al. [2005] synthesized the same resveratrol-3 and 4'-sulfates as Yu et al [2002] but also the 3 and 4'-glucuronide, 3,4'-disulfate, 3,5-disulfate and 3,4',5-trisulfate metabolites of resveratrol. To search for these compounds in in vivo conditions, two experiments were carried out. Resveratrol aglycone was administered to male Wistar rats in a dosage of 50 mg/kg/d or 300 mg/kg/d during 8 w and urine, feces and tissue samples were collected. As shown in Table 1, the administration of 50 mg/kg of resveratrol resulted in the formation of *trans*-resveratrol-3-sulfate, *trans*-resveratrol-disulfate, *trans*-resveratrol-3-glucuronide and resveratrol in urine. Furthermore, an increase of the dosage (6-fold) showed the additional formation of *trans*-resveratrol-trisulfate. In both experiments, the 3-glucuronide was the main metabolite and sulfate (3-sulfate and 3,5-disulfate) derivatives were 100 and 50-fold less, in relation to the dosage, respectively. The total recovery in urine of rats on 50 and 300 mg/kg was 15 and 54%, respectively. In feces samples of rats of the 50 mg/kg group, only 3-sulfate and resveratrol were determined, and 300 mg/kg administration resulted in the formation of all possible sulfates except trisulfate metabolite. The total recovery in feces samples was 15 and 31%, respectively for both dosages [Wenzel et al., 2005]. They also studied the different distribution of these metabolites in plasma, kidney and liver tissues and only after feeding 300 mg/kg metabolites were observed in 50% of the animals. The main metabolite in plasma samples was 3,4'-disulfate followed by 3,4',5-trisulfate and 3-glucuronide metabolites (Figure 2). In liver samples only 3 and 4'-monosulfates and 3-monoglucuronide were identified. The main metabolite in kidneys was the 3-glucuronide metabolite, followed by disulfates and minor 3-sulfate. In contrast to plasma and kidney, free resveratrol was only observed in liver samples.

Table 3. *Metabolism of resveratrol in vivo in animal models.*

Animals	Source	Administration	Dose mg/Kg	Blood or serum or plasma	Urinary excretion		Time (h)	Ref.
				Metabolites (Concentration)	%	Metabolites		
8 Male Wistar rats	*trans*-Resveratrol tritiated in 4-position with 10%EtOH or V-8 homogenized vegetable cocktail or white grape juice	Intragastric	2µl of 120 nCi/mL	-	49-61%	-	24	(Soleas et al. 2001)
2 Male Wistar rats				Serum: 690 dpm/mL Blood: 600 dpm/ml	-	-	0.5	
Male Wistar rats	*t*-Resv	Intragastric	1.43 mg/kg	*t*-Resv: Serum: 0.01 µM Blood: 0.01 µM	-	-	1	
			4.29 mg/kg	*t*-Resv: Serum: 0.02 µM Blood: 0.01 µM	-	-	1	
			7.14 mg/kg	*t*-Resv: Serum: 0.03 µM Blood: 0.02 µM	-	-	1	
2 Male Wistar rats	*t*-Resv	Intragastric	14.28 mg/kg	*t*-Resv Serum: 1.44 µM Blood: 1.00 µM	-	-	0.5	
3 Sprague-Dawley female rats	Resv	Intraperitoneal	20 mg/kg	-		t-3-Gluc	2h	(Yu et al. 2002)
12 Balb/c female mice	Resv	Intraperitoneal	20 mg/kg	t-3- Gluc (3µM), t-3-Sulf (13µM)	-	-	0.25	
12 Balb/c female mice		Gavage	20 mg/kg	t-3- Gluc (1µM), t-3-Sulf (5µM)	-	-	0.25	
12 Balb/c female mice		Gavage	60 mg/kg	t-3- Gluc (175µM), t-3-Sulf (300µM)	-	-	0.25, 0.5	
5 male Sprague-Dawley rats	*t*-Resv	Oral	20 mg/kg	-	-	Gluc, Sulf, DHR, DHR-Sulf	-	(Wang et al. 2005)
Female CF-1 mice	Grape juice	Oral	~ 0.7 mg/kg Resv	n.d.	n.d.	-	-	(Meng et al. 2004)
			~ 1.4 mg/kg Resv	n.d.	1-2%	t-Resv *	24	
			~ 2.7 mg/kg Resv	n.d.	0.9-2.3%	t-Resv *	24	
2 Female Wistar rats	t-Resv	Intragastric	2mg/kg	Resv: 0.09 µM	-	-	4	
				Total: 1.2 µM	-	-	4	
			5mg/kg	Resv: 0.11 µM	-	-	1.5	
				Total:1.5µM	-	-	4	
5 Sprague-Dawley rats	*t*-Resv	Intravenous	5.13 mg/kg	*t*-Resv (~ 21.9 µM)	-	-	0.08	(Chen et al. 2007)
	c-Resv		4.87 mg/kg	*c*-Resv (~ 17.5 µM)	-	-	0.08	
Male Sprague-Dawley rats		Oral	2mg/kg	*t*-Resv (0.77 µM)	-	-	0.25	(Juan et al. 1999)

t-Resv: trans-resveratrol; t-3- Gluc: *trans*-resveratrol-3-O-glucuronide; t-3-Sulf: *trans*-resveratrol-3-sulfate; Gluc: resveratrol glucuronide; Sulf: resveratrol-sulfate; DHR: 7,8-dihydroresveratrol; DHR-Sulf: 7,8-dihydroresveratrol sulfate; Radioactivity was measured by dpm: desintegrations per minute. * Quantified after hydrolysis

Similarly in 2005, Wang et al. identified the microbial metabolites of resveratrol in rats (Table 3). Urine samples were obtained after oral administration of 20 mg/kg to Sprague-Dawley rats. They identified resveratrol-glucuronide, resveratrol-sulfate, 7,8-dihydroresveratrol, and dihydroresveratrol-sulfate as the main 12-h urinary metabolites in rats by mass spectrometry after a SPE treatment [Wang et al., 2005].

In 2004, Meng et al. investigated the urinary and plasma levels of resveratrol and quercetin after their administration as constituents of grape juice or as pure aglycones. The first study was carried out during 4 d with female CF-1 mice receiving solutions containing 18.4 and 36.8% of grape preparation. The urinary excretion of resveratrol increased gradually during the study period, excreting a cumulative amount of approximately 1-2% of the ingested dose when receiving 18.4% grape juice and 0.9-2.3% when the dose was 36.8%. The second study was done after the oral dose of 2 and 5 mg/kg resveratrol to female Wistar rats. In plasma for both doses, resveratrol were mainly present as conjugates and the resveratrol aglycone constituted around 10-11% of total resveratrol at the beginning and declined to 5-7% at 4 h (Table 3) [Meng et al., 2004].

Pharmacokinetic of resveratrol and derivatives

The evaluation of pharmacokinetic analysis of resveratrol was carried out in different animal models such as rats, mice and rabbits [Asensi et al., 2002; Chen et al., 2007; Juan et al., 2002; Marier et al., 2002; Sale et al., 2004]; furthermore, pharmacokinetic analysis was also evaluated with other resveratrol derivatives such as piceid [Lv et al., 2006; Zhou et al., 2007], a *Smilax china* root extract [Huang et al., 2007], 3,4,5,4'-tetramethoxystilbene [Sale et al., 2004], piceatannol, pinosylvin and rhapontigenin [Roupe et al., 2006] (Table 4).

In the first kinetic study, Juan et al. [2002] focused on the determination of the time course of *trans*-resveratrol level in plasma after the 2 mg/kg orally administrated dose to rats. Resveratrol had already reached the blood stream at 5 min, presented its maximum level at around 10 min and was still detected after 60 min [Juan et al., 2002].

Another pharmacokinetic study was carried out after intravenous administration of 4.87 mg/kg of *cis*-resveratrol and 5.13 mg/kg of *trans*-resveratrol to Sprague-Dawley rats. The study showed that both isomers showed a rapid eliminate disposition in 90 min [Chen et al., 2007].

There is a further pharmacokinetic study in which *trans*-resveratrol in the aglycone form and the glucuronide forms were examined following intravenous (15 mg/kg) and oral (50 mg/kg) administration of *trans*-resveratrol to rats [Marier et al., 2002]. After intravenous administration, the plasmatic resveratrol concentrations declined rapidly over the first 2 h. Then, concentration profiles of resveratrol and resveratrol-glucuronide from intravenous or oral administration increased abruptly due to enterohepatic recirculation over the 4- to 8-h time period that resulted in a significant maintenance in the terminal elimination half-life of resveratrol aglycone. The clearance of resveratrol after oral or intravenous administration was higher than that of resvertarol-glucuronide, which resulted in a systemic exposure of approximately 46- or 7-fold, respectively, lower than that of glucuronide.

Asensi et al. [2002] studied tissue levels and pharmacokinetic of resveratrol after intravenous (20 mg/kg) and oral (20 mg/kg) administration to rabbits, rats and mice. The highest concentration levels in plasma of *trans*-resveratrol were reached within the first 5 min in all animals studied but showed a short half-life and a rapid clearance. They found extravascular levels of resveratrol after its oral administration to rabbits, rats and mice with the highest levels occurring within the first 10 min in liver, lung, brain and kidney; therefore, it appears that resveratrol does not accumulate extravascularly and its presence in the tissues is parallel in time to its bioavailability in blood [Asensi et al., 2002]. They suggested that most or all circulating resveratrol may be removed by liver metabolism and if high doses are administered, high rates of hepatic metabolism could be occurring [Asensi et al., 2002].

Sale et al. [2004] studied the pharmacokinetic properties of 3,4,5,4'-tetramethoxystilbene compared with those of resveratrol in the plasma and mice tissues. This analogue compound was a novel congener of pharmacological interest and it was under preclinical evaluation as a potential antitumour prodrug. This tetramethoxystilbene was capable of preferentially interfering with the proliferation and survival of transformed human lung-derived cells, with much lower growth-inhibitory and apoptotic properties in its untransformed counterparts than resveratrol, which does not possess this discriminatory potential. Therefore, a kinetic evaluation and a tissue distribution for both were applied after a single dose (240 mg/kg) oral administration to mice. The results suggested that the introduction of four methoxy groups into the stilbene structure, three of which replaced the hydroxyl moieties in resveratrol, did not increase the systemic availability of the molecule in comparison to resveratrol [Sale et al., 2004].

Table 4. *Pharmacokinetic studies of resveratrol in animal models and humans.*

Species	**Source (dose)**	**Administration**	**Cmax (µmol/l)**	**Tmax (h)**	**$T_{1/2}$ (h)**	**$AUC_{0-\infty}$ (µmol·h/l)**	**Urinary excretion (%)**	**Ref.**
6 Male Wistar rats	Piceid (50mg/kg)	Oral	0.93 (0.39)	0.35 (0.14)	1.68 (0.3)	2.23 (0.69)		[Lv et al., 2006]
C57BL/6J male mice	*t*-Resv (20 mg/kg)	Intragastric	2.6 (1.0)	2.5	-	-	-	[Asensi et al., 2002]
Wistar male rats		Intragastric	1.2 (0.4)	5	-	-	2	
6 ESD NZW male rabbits		Intragastric	1.1 (0.8)	2.5	-	-	-	
		IV	-	-	0.24	-	-	
3 Mice	*t*-Resv (240mg/kg)	Intragastric	32	0.2	-	-	-	[Sale et al., 2004]
6 male Sprague-Dawley rats	*t*-Resv (15mg/kg)	Intravenous	-	-	Resv: 0.13 (0.02) Gluc:n.a.	Resv: 5.64 (0.5) Gluc:38.7 (5.5)	-	[Marier et al., 2002]
6 male Sprague-Dawley rats	*t*-Resv (50mg/kg)	Oral	Resv: 6.57(1.55) Gluc:105.2 (32.4)	Resv:0.29 (0.1) Gluc: 0.42 (0.3)	Resv: 1.48 (0.4) Gluc:1.55 (0.4)	Resv: 7.1 (2.0) Gluc:324.7 (57.6)	-	
5 Female Sprague-Dawley rats	1g/kg *Smilax china* root extract equivalent to 180 mg/kg OResv and 80 mg/kg Resv	Oral	OResv: 21.93 (3.1)	0.25	-	91.78 (13.7)	-	[Huang et al., 2007]
			Resv: 9.61 (1.5)	0.25	-	17.81 (0.6)	-	
Male Sprague-Dawley rats	*t*-Resv (2mg/kg)	Intragastric	Resv: 2.57	*t*-Resv 0.16	0.25	-	-	[Juan et al., 2002]
8 Wistar rats	*t*-Piceid (150 mg/kg)	Oral	t-Gluc: 64.85 (18.5)	2	4	-	-	[Zhou et al., 2007]
			t-Resv: 3.55 (0.7)	1	2	-	-	
			t-Piceid: 4.35 (1.3)	0.5	1	-	-	
5 Male Sprague-Dawley rats	Piceatannol : (10mg/kg)	Intravenous	-	-	4.23 (1.25)	34.75 (10.2)	32.8	[Roupe et al., 2006]
5 Male Sprague-Dawley rats	Pinosylvin: (10mg/kg)		-	-	0.82 (0.05)	24.67 (5.7)	9.46	

5 Male Sprague-Dawley rats	Rhapontigenin: (10mg/kg)		-	-	3.0 (1.35)	32.52 (0.4)	1.25	
10 Humans (45% males)	*t*-Resv (7.7mg/kg)	Oral	*t*-Resv:0.32 (0.16) Gluc 1 1.00 (0.35) Gluc 2 0.91 (0.36) 3-Sulf 3.69 (0.95)	*t*-Resv 0.83 (0.5-1.5) Gluc 1 2.00 (1.0-6.0) Gluc 2 1.50 (1.0-5.0) 3-Sulf 1.50 (1.0-5.0)	*t*-Resv 2.85 Gluc 1 2.85 Gluc 2 3.09 3-Sulf 3.21	*t*-Resv 0.98 Gluc 1 8.4 (0.1) Gluc 2 5.6 (0.1) 3-Sulf 17.8(0.1)	*t*-Resv 0.04 (0.05) Gluc 1 2.0 (0.4) Gluc 2 8.9 (2.6) 3-Sulf 11.4 (2.3)	[Boocock et al 2007]
10 Humans (45% males)	*t*-Resv (15.4mg/kg)		*t*-Resv 0.51 (0.38) Gluc 1 1.17 (0.90) Gluc 2 1.66 (1.35) 3-Sulf 6.82(21.39)	*t*-Resv 0.759 (0.5-4.0) Gluc 1 2.25 (1.0-6.0) Gluc 2 1.75 (1.0-5.1) 3-Sulf 2.00 (1.0-5.0)	*t*-Resv 8.87 Gluc 17.27 Gluc 2 6.64 3-Sulf 4.51	*t*-Resv 2.4 (0.3) Gluc 1 13.4(0.3) Gluc 2 11.4(0.3) 3-Sulf 44.1(0.3)	*t*-Resv 0.1 (0.1) Gluc 1 2.1 (1.1) Gluc 2 3.2 (1.7) 3-Sulf 7.3 (3.1)	
10 Humans (45% males)	*t*-Resv (38.5mg/kg)		*t*-Resv 1.18 (0.65) Gluc 1 2.16 (0.81) Gluc 2 4.02 (2.88) 3-Sulf 9.05 (2.46)	*t*-Resv 1.38 (0.5-4.0) Gluc 1 2.375 (1.0-8.0) Gluc 2 2.00 (1.0-6.0) 3-Sulf 2.00 (1.0-5.2)	*t*-Resv 4.22 Gluc 1 10.6 Gluc 2 8.42 3-Sulf 11.5	*t*-Resv 3.4 (0.2) Gluc 1 24.8(1.2) Gluc 2 18.9(0.1) 3-Sulf 74.5(0.2)	*t*-Resv 0.1 (0.1) Gluc 1 1.7 (1.7) Gluc 2 3.1 (1.4) 3-Sulf 5.2 (2.6)	
10 Humans (45% males)	*t*-Resv (76.9mg/kg)		*t*-Resv 2.36 (1.71) Gluc 1 3.18 (1.47) Gluc 2 4.29 (2.85) 3-Sulf 13.94(6.69)	*t*-Resv 0.83 (0.5-1.5) Gluc 1 2.00 (1.0-6.0) Gluc 2 1.50 (1.0-5.0) 3-Sulf 1.50 (1.0-5.0)	*t*-Resv 8.52 Gluc 1 7.90 Gluc 2 5.83 3-Sulf 7.71	*t*-Resv 5.8(0.3) Gluc 1 43.5(0.2) Gluc 2 37.5(0.3) 3-Sulf 135.5(0.2)	*t*-Resv 0.1 (0.1) Gluc 1 0.5 (0.3) Gluc 2 3.0 (1.4) 3-Sulf 5.0 (1.6)	

t-Resv: *trans*-resveratrol; OResv: oxyresveratrol; Gluc: resveratrol-glucuronide; 3-Sulf: resveratrol-3-sulfate

They also evaluated the pharmacokinetic properties of resveratrol compared to the synthetic analogue in different mice tissues in which resveratrol might prevent malignancy, or delay its onset. The availability of the synthetic analogue was inferior in plasma, liver, kidney, lung and heart than resveratrol meanwhile it was more available in intestinal and colonic mucosa and in brain [Sale et al., 2004]. These results provided a good argument to assess 3,4,5,4'-tetramethoxystilbene as a colorectal cancer chemo preventive agent. Furthermore, in the search for the main conjugated forms, resveratrol showed its glucuronided and sulfated conjugates, while, the 3,4,5,4'-tetramethoxystilbene underwent metabolic hydroxylation or single and double *O*-demethylation [Sale et al., 2004].

The pharmacokinetic dispositions of other stilbenes that are structurally similar to resveratrol and have pharmacological activity across many anti-cancer, anti-inflammatory and anti-oxidant assays have been studied. The pharmacokinetics was characterized in male Sprague-Dawley rats after single intravenous doses of 10 mg/kg of piceatannol, pinosylvin or rhanpontigenin. The detectable plasma half-lives of these compounds appear to be relatively short. The estimates of oral bioavailability characterize these stilbenes as poorly bioavailable compounds. All three stilbenes undergo extensive glucuronidation upon intravenous administration, as was determined by plasma and urine concentrations. The total amount excreted shows that the three stilbenes excreted in urine, 32.8%, 9.5% and 1.3%, respectively, are very small compared with the overall dose given of each one (3.5 mg). This indicates that, the three stilbenes in contrast to resveratrol are eliminated predominantly vía non-renal excretion [Roupe et al., 2006].

Recently, the pharmacokinetics of resveratrol from *Smilax china,* a rhizome extensively used in traditional Chinese medicine was evaluated [Huang et al., 2007, 2008]. Forty-five female rats were orally administered with 1 g/kg *Smilax china* extract equivalent to 180 mg/kg of oxyresveratrol and 80 mg/kg of resveratrol. The pharmacokinetic parameters showed that the two stilbenes were rapidly absorbed into the body fluid from the gastrointestinal tract and could still be detected in the plasma at least 6 h after the administration [Huang et al., 2008], probably due to the high dose administered.

Piceid, the glucoside of resveratrol

Various studies have focused on the metabolism of piceid, the glucoside of resveratrol. These studies are of great interest due to the higher amount of piceid compared to resveratrol in food. Therefore, bioavailability studies of this compound are required. In vitro studies have already observed the absorption of piceid in the enterocytes, but in vivo studies in animal models are still scarce.

The bioavailability and tissue distribution of *trans*-piceid was studied in Wistar rats after its oral administration [Lv et al., 2006; Zhou et al., 2007]. At present, the studies of the pharmacokinetics and distribution of piceid have been poorly documented. The first study with piceid administration to rats, measured its pharmacokinetics and its tissue distribution after a single oral administration of 50 mg/kg to 6 male Wistar rats. This was the first in vivo study that demonstrated the absorption of piceid with maximum plasma concentrations of 0.93 (0.4) µM at 21 min (Table 2) [Lv et al., 2006]. Another relevant result of this study was the diffusion of piceid to tissues. The highest concentrations were found in the stomach, then, in the small intestine, followed by spleen, lung, brain, testis, liver, kidney and heart at 10 min. At 30 min, high concentrations were still detected in stomach and relatively high concentrations were present at 120 min. The absorption, distribution and elimination of piceid were quick after the oral administration. The major distribution organs in rats were stomach, small intestine and spleen; furthermore, it was able to cross the blood-brain and blood-testis barriers. Nevertheless, no long-term accumulation of piceid in tissues took place [Lv et al., 2006].

A recent study showed the bioavailability of piceid after oral administration of 150 mg/kg of piceid to rats. *trans*-Piceid was absorbed, with maximum plasma levels at 30 min, and metabolized to *trans*-resveratrol, with a maximum plasma concentration at 60 min, and this to *trans*-resveratrol-glucuronide, with maximum concentration at

120 min [Zhou et al., 2007]. The resveratrol-glucoside was absorbed by transepithelial transport across the intestine with maximum concentration occurring at 30 min after administration. This glycosytated derivative is deglycosylated in *trans*-resveratrol in the intestine with a cleavage by the CBG after passing the brush-border membrane by SGLT1 or by the membrane-bound enzyme LPH followed by passive diffusion of the released *trans*-resveratrol, which is further metabolized inside the cells into glucuronoconjugates [Henry-Vitrac et al., 2006]. The constant absorption of piceid from the first minutes of ingestion is reflected in the *trans*-resveratrol-glucuronide with the highest concentrations (30-fold higher) taking place in plasma and having a relatively long elimination half-life. Furthermore, the parent drug and the metabolites, *trans*-resveratrol and *trans*-resveratrol-glucuronide were detected at 8, 12 and 24 h after the oral dose [Zhou et al., 2007].

In conclusion, resveratrol is absorbed and already shows plasmatic and serum levels between 5 min and 4 h depending on the dose and the animal species. Furthermore, plasmatic levels increase between 4-8 h due to the enterohepatic recirculation [Marier et al., 2002]. When absorbed, resveratrol is metabolized and the major conjugated forms in plasma consisted of sulfate conjugates, in which minor concentrations of the 3-glucuronide were observed. Further studies are required to obtain more standards of metabolites and more data about the major sulfate metabolite since Wenzel et al. [2005] and Yu et al. [2002] found major amounts for 3,4'-disulfate and 3-sulfate conjugates, respectively. Resveratrol is also distributed to different tissues such as liver and kidney, the major organs of deposition, and also in the lung, spleen, and heart. It crosses the blood-brain and blood-testis barriers showing major concentration levels in the intestinal tract. Maximum concentrations were found at early hours and traces amounts at later hours showing no accumulation extravascularly [Asensi et al., 2002]. The main metabolite found in tissues at early hours was the glucuronide form, and the free form of resveratrol predominated at later hours [El-Mohsen et al., 2006].

Renal excretion was the major way of elimination compared to the colonic one. Urinary excretion varied between 3 and the 61% depending on the study, animal species and dose. Some studies had also shown a possible excretion vía sweat and respiratory system and metabolism to CO_2 [El-Mohsen et al., 2006; Soleas et al., 2001a]. The major conjugated form present in urine is the 3-glucuronide metabolite [Wenzel et al., 2005; Yu et al., 2002] although mono-, di- and tri-sulfated metabolites and free resveratrol were also determined [Wang et al., 2005; Wenzel et al., 2005]. Furthermore, microbial metabolites such as dihydroresveratrol and its sulfate conjugate were also identified in 12 h rat urine [Wang et al., 2005].

Human studies

Studies that investigate the bioavailability of resveratrol in humans are scarce. Moreover, the research in this area is quite recently. It has been summarized in Table 5. The experimental approaches have been improved with the use of new analytical techniques such as mass spectrometry to identify and quantify metabolites present in very low concentrations. Resveratrol and its metabolites have been measured in several biofluids: plasma or serum, urine, LDL and feces.

In 2001, the group of Soleas, Yang & Goldberg [Soleas et al., 2001b] was the first to investigate the oral administration of resveratrol. Twenty-five mg of *trans*-resveratrol standard dissolved in 120 mL of white wine were consumed by 10 healthy volunteers. The analyses were performed by GC-MS after treatment with β-glucuronidases and sulfatases. After stilbene intake, free resveratrol and its conjugates were found in all subjects and at all times, even after an abstinence of at least 24 h from food sources of this polyphenol. In plasma samples, they found that the highest resveratrol concentration (345.1 μg/L) occurred at 30 min. Moreover, resveratrol conjugates were 20- to 50-fold more abundant than free resveratrol. After resveratrol intake, the recovery in urine of 24 h was 24.6% as free and

conjugated forms. Likewise, the urine concentrations of resveratrol conjugates were 30- to 50-fold higher than aglycone. At 2 h the highest concentration of resveratrol was observed, it was nearly 8 mg/L.

Three years later the same group [Goldberg et al., 2003] tested the absorptive efficiency of *trans*-resveratrol standard (25 mg) dissolved in three different matrices: white wine, grape juice and vegetable juice. The conditions of study were the same as in the previous work. The results were also similar and serum showed the highest level at 30 min.

Table 5. *Human bioavailability studies.*

Participants	Gender (%)	Age (y)	Resveratrol source	Dose (mg/kg body weight)	Concentration (µmol/L)	Time(h)	Urinary excretion (%) (Time, h)	Ref
10	Male (45%)	19-61	RESV Standard in 120mL of white wine	0.357mg/kg (25mg total)	Resv 0.031 Conjugated* 1.48	0.5-1	Total 24.6% (24h)	[Soleas et al., 2001]
4	Males (100%)	25-45	RESV Standard dissolved in 100mL vegetable juice	0.357mg/kg (25mg total)	Resv 0.037 Conjugated* 2.03	0.5	17.0 (24h)	[Goldberg et al., 2003]
4			RESV Standard dissolved in 100mL white wine	0.357mg/kg (25mg total)	Resv 0.031 Conjugated* 1.80	0.5	16.8 (24h)	
4			RESV Standard dissolved in 100mL grape juice	0.357mg/kg (25mg total)	Resv 0.035 Conjugated* 1.82	0.5	16.0 (24h)	
1	Male	30-50	RESV Standard	0.03mg/kg	n.d.		Gluc 52 (24h)	[Meng et al., 2004]
1				0.5 mg/kg	n.d.		Gluc 34 (24h)	
1				1 mg/kg	Gluc 1.86	1.5	Gluc 26 (24h)	
1			200mL grape juice	0.005mg/kg # (0.32mg total)	n.d.		n.d. (24h)	
1			400mL grape juice	0.009mg/kg # (0.64mg total)	n.d.		n.d. (24h)	
1			600mL grape juice	0.014mg/kg # (0.96mg total)	n.d.		Gluc (24h)	
1			1200mL grape juice	0.027mg/kg # (1.92mg total)	n.d.		Gluc 5.0(24h)	
6	3 Male (50%)	23-34	^{14}C- RESV standard ORAL	0.385mg/kg # (25mg total)			53.4-84.9% (72h)	[Walle et al., 2004]
5			^{14}C- RESV standard INTRAVENOUS	0.023mg/kg # (1.5 mg total)			42.3-83.2% (72h)	
1			RESV Standard ORAL	1.538mg/kg # (100mg total)	Tr.		Gluc 13 (1) Sulf 24 (3) Total 37 (12h)	
10	Males (100%)	30 (25-40)	300mL red wine Lambrusco + meal	0.0034µg/kg	Gluc 0.096	1		[Vitaglione et al., 2005]
5	1 Male (20%)	29 (24-38)	600mL red wine Cabernet Franc	0.0329µg/kg	Gluc 0.687	0.5-2		

10	3 Males (30%)	31 (24-54)	600mL red wine Aglianico + meal	0.0075µg/kg	Resv 0.004 Gluc 0.150	0.5 1-2		
10	Male (100%)	28.2 (25-41)	300mL/d sparkling wine (28d)	0.005mg/kg·d (0.357mg/d)#	n.d.		*t*-3-Gluc 4.8 (2.5) *c*-3-Gluc 2.4 (1.3) Total 7.2 (2.7) (morning urine)	[Zamora-Ros et al., 2006]
10	Women (100%)	38.1 (25-50)	200mL/d white wine (28d)	0.007mg/kg·d (0.398mg/d)#	n.d.		*t*-3-Gluc 11.7 (2.8) *c*-3-Gluc 3.3 (3.6) Total 15.0 (4.3) (morning urine)	
10	Women (100%)	38.1 (25-50)	200mL/d red wine (28d)	0.043mg/kg·d (2.56mg/d)#	n.d.		*t*-3-Gluc 4.2 (3.2) *c*-3-Gluc 1.2 (1.2) Total 5.4 (3.9) (morning urine)	
5	Male (100%)	25-28	250mL red wine	0.077mg/kg # (5.4mg total)			*t*-4' Gluc 0.13 (0.19) *t*-3 Gluc 0.38 (0.59) *c*-4' Gluc 0.75 (1.2) *c*-3 Gluc 1.9 (1.9) *t*-4' Sulf 0.01 (0.03) *t*-3 Sulf 0.16 (0.67) *c*-4' Sulf 19.6 (17.4) *c*-3 Sulf 0.47 (2.2) Total 23.4 (4h)	[Urpi-Sarda et al., 2007]
9	Males (100%)	23-41	PICEID Standard dissolved in 100mL ethanol (15%) + 400mL milk (1.5% fat)	1.22mg/kg (85.5mg/70kg)	*t*-3-Sulf 0.95 (0.16) *t*-3,4'-Disulf 0.33 (0.07) *t*-3,5-Disulf 0.94 (0.17) 3-Gluc 0.16 (0.04) 4'-Gluc 0.19 (0.05) (2) *t*-Digluc 0.35 (0.09)	*t*-3-Sulf 1 *t*-3,4'-Disulf 6- *t*-3,5-Disulf 8 3-Gluc 6-8 4'-Gluc 6 (2) *t*-Digluc 6	*t*-3-Sulf 4.53 *t*-3,4'-Disulf 1.71 *t*-3,5-Disulf 7.18 3-Gluc 2.99 4'-Gluc 0.69 (2) *t*-Digluc 2.65 Total 13.6-35.7 (48h)	[Burkon and Somoza 2008]

Furthermore, the total absorption curves were similar regardless the matrices. In this study slight amounts of resveratrol at basal time were detected too. Urinary 24 h resveratrol excretions were 17.0%, 16.8% and 16.0% after oral administration of vegetable juice, wine and grape juice, respectively. The results in plasma and urine supported that there were no differences in resveratrol absorption by using the different matrices.

In 2004, Meng et al. published the first paper that investigated the bioavailability of grape juice (ranged from 200 to 1200 mL) after oral ingestion, whose composition was 1.6 mg/L of stilbene, mainly as piceid [Meng et al., 2004]. Oral consumption of *trans*-resveratrol standard at several concentrations (0.03, 0.5, and 1 mg/kg) were also studied. In this case, the analyses were performed by LC-MS/MS. Resveratrol was found in plasma after enzymatic hydrolysis only at high doses (1 mg/kg). At lower concentrations as a standard or grape juice no peaks were reached. However urinary recoveries were 52%, 34% and 26% after 0.03, 0.5, and 1 mg/kg. These results could suggest an inversely dose-dependent manner. Furthermore with a dose of 0.03 mg/kg, resveratrol was mainly excreted in the first 2-3 h, however, with 1 mg/kg, 7-10 h were necessary to excrete most of the resveratrol. Resveratrol was mainly found in the conjugated form as glucuronide. After grape juice consumption, at low doses (200 and 400 mL) peaks of resveratrol in urine were not detected. Although at high doses (600 and 1200 mL) only conjugated forms were found. Moreover, after 1200 mL of grape juice, the recovery was only about 5% of the dose administered. This study showed that the glycoside forms are absorbed less than aglycones.

In the same year, Walle et al. [2004] were the first to administer intravenous and oral labeled resveratrol in humans. After 25 mg of an oral ^{14}C-resveratrol dose (6 healthy subjects), total radioactivity in plasma was maximum (491 ng/mL) at approximately 1 h after the intake, and then it keep falling during the following 72 h over the study. After a 1.5 mg intravenous^{14}C-resveratrol (5 healthy subjects) total radioactivity fell rapidly, but plasma radioactivity remained for the following 72 h. Moreover, both half lives ranged from 7 to 14 h after any dose. This data is important because a single high dose of resveratrol can be active in plasma at least half a day. After oral dosage, 53-85% and 0.3-38% radioactivity were recovered in urine and feces, respectively. Similar results were observed after intravenous doses: 42-83% and 0.6-23% of total radioactivity were found in urine and feces, respectively. High variability was observed in the urinary and fecal recoveries. Elimination half-lives in urine ranged from 6.5 to 18.8 h after oral or intravenous doses. The authors also tested the metabolites formed after a large unlabeled oral dose of 100 mg of resveratrol. The analyses were performed by LC-MS-UV. This was the first study of human urine that analyzed resveratrol metabolite profile, showing the presence of 2 monoglucuronides, a monosulfate, a dihydroresveratrol monoglucuronide, and a dihydroresveratrol monosulfate. Dihydroresveratrol metabolites could be formed by the intestinal microbiota as occurs with other polyphenols [Gonthier et al., 2003]. The sulfate and glucuronide conjugates excreted in the urine accounted for 24% and 13% of the dose, respectively. However in plasma resveratrol or its metabolites were not detected at any time. Only trace amounts (less than 5 ng/mL) could be found in plasma after an oral dose of the 100 mg.

Vitaglione et al. [2005] evaluated the bioavailability of red wine resveratrol consumed with several meals: standard, fat or lean meal. Identification and quantification of resveratrol and its metabolites in serums were done by LC-MS-MS. In the first experiment, 10 healthy males were involved in the assessment of the bioavailability of Lambrusco red wine (0.82 mg *trans*-resveratrol/L) consumed with a standard meal (milanese beef cutlet and chips). Only in 4 of the volunteers at 1 h, some amounts of resveratrol glucuronides that ranged from 15 to 168 ng/mL were found. In the second experiment, 5 healthy volunteers were recruited to intake 600 mL of Cabernet Franc red wine (3.2 mg *trans*-resveratrol/L) over night while fasting. Only three of the five subjects showed resveratrol free or metabolites in serum. In two volunteers resveratrol aglycone was detected but not in quantifiable amounts. Resveratrol glucuronides (isomers 3 and 4') were reached at different times (0.5 to 2 h) and different concentrations (77-900 ng/mL). In the third experiment, 10 healthy subjects conusmed 600 mL of aglianico red wine (0.8 mg *trans*-resveratrol/L) with either a lean meal or with a fat meal. Free resveratrol was detected in 2 of the subjects at

concentrations ranging 1-6 ng/mL at 30min after wine consumption. Resveratrol glucuronides were only detected in one subject of each intervention at 1-2 h after intake. This study clearly showed a high interindividual variation in the absorption and bioavailability of resveratrol.

Zamora-Ros [2006] carried out the first work that assessed the bioavailability of resveratrol (provided by different wines) in a regular intervention during 28 d. The analyses were performed by LC-MS/MS. In the first study, 10 healthy males were recruited to consume during 300 mL/day of sparkling wine (1.19 mg resveratrol/L). After 28 d of supplementation, urinary *trans*- and *cis*-resveratrol-3-*O*-glucuronides were 75 and 38 nmol/g creatinine, respectively. In the second study, 10 healthy females were selected to consume 200 mL of white wine (1.99 mg resveratrol /L) or 200mL of red wine (12.8 mg resveratrol/L) in a crossover clinical trial. Likewise after 28 d only resveratrol metabolites were detected in morning urine. *trans*- (205 and 473 nmol/g creatinine) and *cis*-resveratrol-3-*O*-glucuronides (58 and 140 nmol/g creatinine) were found after white and red wine intake, respectively. Those studies showed that urinary excretion was dose-dependent. Furthermore, slight amounts of resveratrol metabolites were also detected at baseline periods. No free resveratrol or piceid were detected in any of the studies.

Urpi-Sarda et al. [Urpi-Sarda et al., 2005; Urpi-Sarda et al., 2007] published the first works that investigated the presence of resveratrol in LDL. The analyses were performed by LC-MS/MS. Eleven healthy males were recruited to consume 250 mL of merlot red wine (10.2 mg resveratrol/L). Free resveratrol, glycoside, glucuronidate and sulfate forms were found in 24-h LDL. The detected metabolites were *cis*- and *trans*-, 3 and 4' position, glucuronides and sulfates. The more abundant metabolites were 88% glucuronides (*trans*-resveratrol-3-*O*-glucuronide, 112 pmol/mg LDL protein), 10.4% sulfates and 2.0% *trans*-aglycone. Piceid was also found in LDL 24 h in lower concentrations (1.1 to 28.5 pmol/mg LDL protein). In the second experiment it was assessed the metabolic profile at low resveratrol doses. Five healthy males were recruited to consume 250 mL of merlot red wine (10.2 mg/L). Only conjugated forms were detected in urine 4 h after wine consumption. The more abundant metabolites were *trans*-resveratrol-3-*O*-sulfate (9.3 mol/g creatinine) and *cis*-resveratrol-3-*O*-glucuronide (0.9 mol/g creatinine). Sulfation and glucuronidation represented 86.6% and 13.4% of total urinary resveratrol excretion, respectively.

In 2007, Boocock et al. were the first to publish a complete phase I dose pharmacokinetic study in humans [Boocock et al., 2007]. Ten healthy volunteers were recruited to consume single doses of oral resveratrol (0.5, 1, 2.5, or 5g). Consumption of resveratrol did not cause serious adverse events. Analyses of resveratrol and its metabolites were performed by LC-MS/MS. In plasma in all intake doses resveratrol-3-sulfate (56%) was the highest metabolite, the second and third metabolite were mono-glucuronides (17 and 23%, respectively) and, finally, the lowest was free resveratrol (5%). Resveratrol was rapidly absorbed, the T_{max} for all metabolites ranged between 0.8 and 2.4 h. Although the half lives of free resveratrol and the conjugated forms remained for long time in plasma, between 2.9 to 11.5 h.

Urinary excretion mainly took place in the first 4 h after consumption (77% of total excretion) although resveratrol metabolites remained in urine between 12-24 h after intake. Free resveratrol, 2 glucuronides, and the 3-sulfate excreted in the urine 24 h after intake were below 0.04%, 2%, 9%, 11% of the 0.5 mg provided, respectively. At higher dose (5 mg) resveratrol, glucuronides and sulfate recoveries in the urine at 24 h were 0.1%, 0.5%, 3% and 5% of the dose, respectively. In urinary excretion, the sulfate forms were also higher than the glucuronide and free forms.

The piceid absorption was recently investigated for the first time. Nine healthy males participated in this controlled trial, which consisted in the administration of a single oral dose of 85.5 mg piceid standard *per* 70kg. Resveratrol metabolites in plasma and urine were identified by LC-MS/MS, although these were quantified by HPLC-DAD. The same number of resveratrol metabolites were detected in both urine and plasma: *trans*-resveratrol-3-sulfate, *trans*-resveratrol-3,4'-disulfate, *trans*-resveratrol-3,5-disulfate, *trans*-3-*O*-glucuronide, *trans*-4'-*O*-

glucuronide and two resveratrol diglucuronides (*trans*-resveratrol-2-C-β-/4'-β-*O*-diglucuronide and *trans*-resveratrol-2-C-β-/5-β-*O*-diglucuronide). The two disulfates, previously identified in animals, and the two diglucuronides have been found in humans for the first time, thereby increasing the classical metabolic profile (monosulfates and monoglucoronides). The authors did not detect piceid nor resveratrol aglycone in any sample. Piceid was absorbed rapidly (1 h) in the form of *trans*-resveratrol-3-sulfate, whereas the other resveratrol metabolites reached their maximum concentration between 6 and 8 h after piceid administration. Sulfation pathway was more efficient than glucuronide vía. These authors also observed that 34%, 44% and 46% of sulfates, disulfates and diglucuronides, respectively, were noncovalently bound to plasma proteins; the rest of the percentage of conjugates were transported freely in plasma. After 24 h of piceid intake, no resveratrol metabolites were detected. The total urinary recovery ranged between 14 and 36%. The metabolic profile was approximately 15% and 8% as sulfate and glucuronide conjugates, respectively. Urinary excretion was completed within 48 h of oral piceid administration (Figure 3).

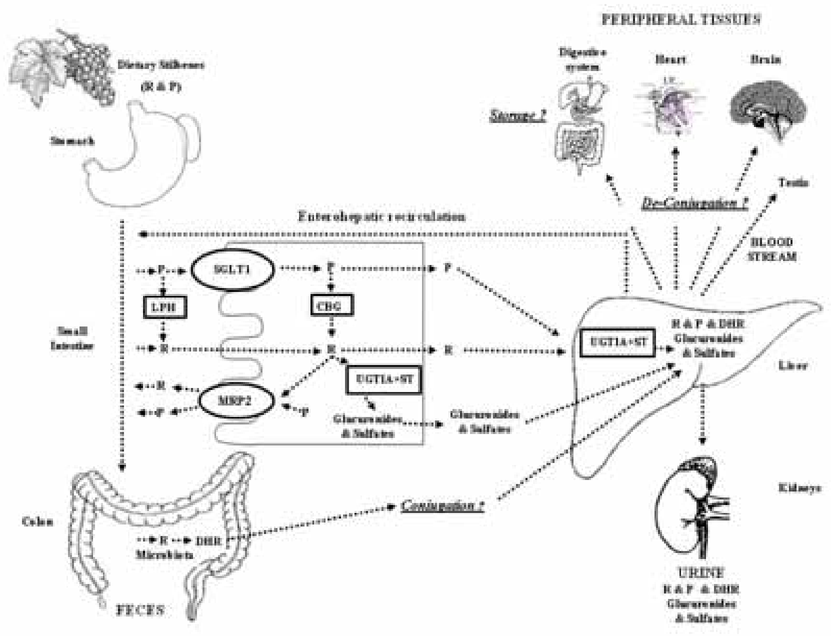


Figure 3*.Pathways of resveratrol absorption, distribution, metabolism and excretion.*

In conclusion, resveratrol seems to have a greater absorption than piceid at nutritional doses [Meng et al., 2004], although pharmacological doses of piceid standard is also recovered at similar percentages than resveratrol standard [Burkon and Somoza, 2008]. Moreover, resveratrol and piceid are absorbed and metabolized quickly. In blood samples, the highest resveratrol peak is detected at around 30-60 min [Boocock et al., 2007; Goldberg et al., 2003; Soleas et al., 2001b; Vitaglione et al., 2005] and 6 h [Burkon and Somoza, 2008] after consumption of resveratrol or piceid, respectively. Nevertheless traces of resveratrol could remain in plasma for at least 72 h after ingestion [Walle et al., 2004]. Resveratrol conjugates are more abundant than the free form. It seems that sulfation is a more efficient metabolic pathway than glucuronidation (56% vs 39%) [Boocock et al., 2007; Burkon and Somoza, 2008]. Part of resveratrol is transported through the body bound to LDL mainly as glucuronides (88) and sulfates (11%) [Urpi-Sarda et al., 2005; Urpi-Sarda et al., 2007]. In a recent study, it was shown that more than 50% of resveratrol conjugates (sulfates, disulfates and C/*O*-diglucuronides) were bound to proteins in plasma [Burkon and Somoza, 2008]. Urine (53-85%) and fecal (0.3-38%) were the most important ways of excretion of resveratrol measured by total radioactivity [Walle et al., 2004]. However, urinary recoveries by mass spectrometry ranged from 5 to 37%, depending on dose and the kind of resveratrol source [Boocock et al., 2007; Burkon and Somoza, 2008; Goldberg et al., 2003; Meng et al., 2004; Soleas et al., 2001b; Urpi-Sarda et al., 2007; Walle et al., 2004; Zamora-Ros

et al., 2006]. Metabolites identified in urine are four monoglucuronides, four monosulfates, two disulfates, two C/*O*-diglucuronides, free aglycone, dihydroresveratrol monogucuronide and monosulfate. Likewise in urinary excretion the sulfate forms seem to be higher than the glucuronide and free forms [Boocock et al., 2007; Burkon and Somoza, 2008; Urpi-Sarda et al., 2007; Walle et al., 2004]. The most important limitation of the bioavailability is the great individual variability; for this reason, further investigation with a higher number of volunteers is necessary in order to assess the percentage of absorption and excretion, and the metabolite profile of this polyphenol.

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3. ACTIVITAT BIOLÒGICA I EFECTES BENEFICIOSOS DELS COMPOSTOS POLIFENÒLICS

En l'actualitat, l'envelliment de la població, especialment en els països desenvolupats, està incrementant el nombre de persones que sofreixen diverses patologies crònic-degeneratives relacionades amb l'edat com són les malalties cardiovasculars, càncer i malalties neurodegeneratives. Aquest increment suposa una preocupació social i econòmica en aquests països. A Espanya, segons l'Institut Nacional d'Estadística (INE 2008), el grup de les malalties cardiovasculars va ser la primera causa de mort seguida del càncer el 2006 .

3.1 Polifenols i malaltia cardio i cerebrovascular

L'aterosclerosi (forma més comuna d'arteriosclerosi) és una malaltia de les artèries de mitjà i gruixut calibre en les quals es dipositen i infiltren substàncies lipídiques que constituïxen la placa d'ateroma. Aquestes plaques s'inicien amb el dipòsit de cristalls minúsculs de colesterol en l'íntima i en el múscul llis adjacent. Amb el temps, els cristalls creixen i s'uneixen per a formar xarxes cristal·lines. A més, els teixits fibrosos i el múscul llis proliferen fins a formar plaques cada vegada majors. Els dipòsits de colesterol més la proliferació cel·lular adjacent creixen en un punt que la placa sobresurt a la llum del vas i redueixen el flux sanguini fins a causar, de vegades, una obstrucció completa del vas sanguini (Guyton 2001).

Diversos factors de risc com l'obesitat, el sedentarisme, la diabetis o d'hipercolesterolèmia, entre uns altres, han estat relacionats amb el desenvolupament de la placa d'ateroma. Aquest procés patològic s'inicia amb el dany de l'endoteli de les artèries (Charo et al. 1998).

L'oxidació de la LDL que ha passat a través de l'endoteli disfuncional condueix a un procés inflamatori que involucra als monòcits, macròfags i limfòcits produint en última instància les cèl·lules espumoses. En l'actualitat, es coneixen fins a cinc mecanismes parcialment responsables del desenvolupament de la malaltia de l'artèria coronària:

- Disfunció endotelial
- Oxidació de LDL por radicals lliures
- Adhesió plaquetària i agregació
- Proliferació i migració de les cèl·lules musculars llises vasculars
- Inflamació implicant monòcits i limfòcits T

En la Figura 7 es mostra un esquema d'una artèria en secció longitudinal (Mann and Folts 2004) on en la meitat superior es representen les cèl·lules endotelials normals (EC) a l'esquerra, i les cèl·lules endotelials danyades o disfuncionals en el centre i a la dreta. Les cèl·lules endotelials sanes secreten òxid nítric (NO) i té diverses funcions útils: Inactiva les plaquetes i altres cèl·lules sanguínies blanques que s'adhereixen a la paret arterial, l'òxid nítric alliberat també difon cap a les cèl·lules musculars llises vasculars (VSMC) relaxant-les i, a més, quantitats fisiològiques d'òxid nítric poden inhibir la divisió i la migració de les cèl·lules musculars llises vasculars cap al lumen.

Quan les cèl·lules endotelials es danyen o es fan disfuncionals, o quan han mudat de la membrana subjacent, no produeixen suficient òxid nítric per a inhibir l'adherència de la plaqueta i l'agregació, i per tant, es formen els agregats plaquetaris en la paret. Les plaquetes (i altres cèl·lules) segreguen factors de creixement plaquetaris (PDGF), que estimulen les cèl·lules musculars llises vasculars per a dividir-se.

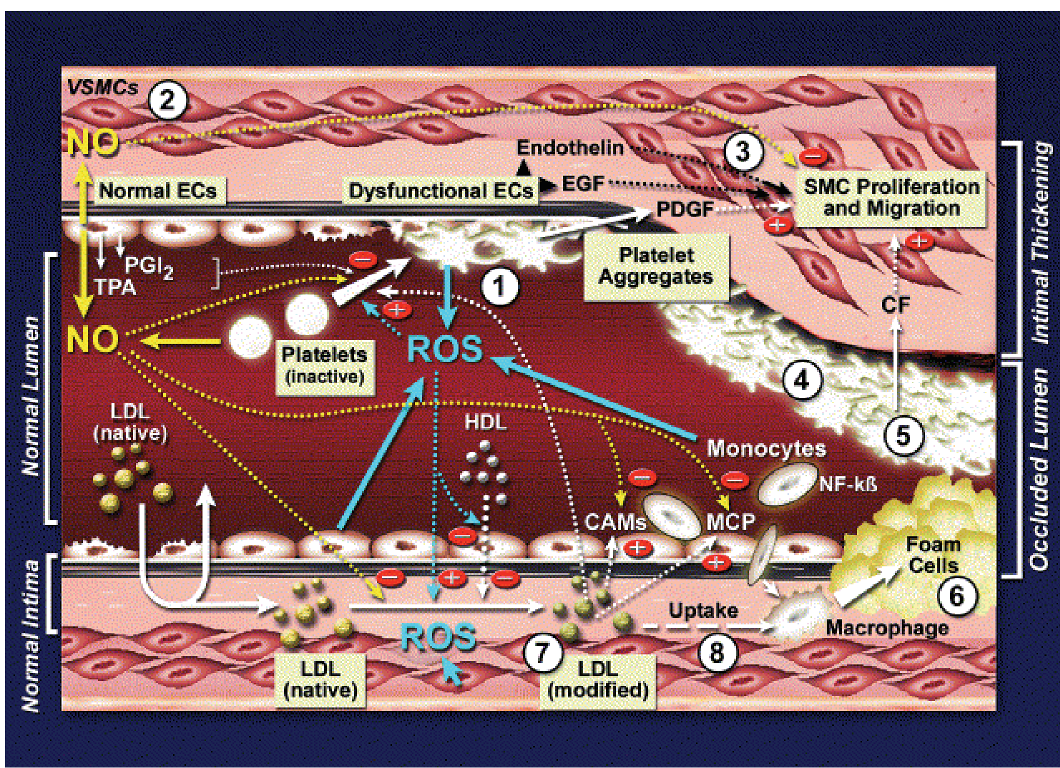


Figura 7: Malaltia cardiovascular (Mann and Folts 2004)

Les plaquetes també estimulen factors quimiotàctics que arrosseguessin les cèl·lules dividides cap al lumen arterial. Això produeix un espessiment de l'íntima modificant el lumen.

La meitat inferior de la figura mostra les lipoproteïnes de baixa densitat (LDL). Es coneix que aquestes poden entrar en la paret arterial per a contribuir al desenvolupament de les cèl·lules espumoses. Si les LDL s'oxiden pels compostos oxidants o pels radicals lliures alliberats per les cèl·lules endotelials, per les cèl·lules musculars llises vasculars, i per altres cèl·lules, es converteixen en LDL modificades (M-LDL). La LDL modificada pot estimular les cèl·lules endotelials per a alliberar les molècules cel·lulars de l'adhesió (CAM), que atreuen als monòcits i als limfòcits T per a fixar-se a la paret arterial. Les LDL modificades poden també estimular la producció de la proteïna quimiotàctica monocitària (MCP), que atreu als monòcits en l'espai íntim. Llavors aquests monòcits poden diferenciar-se cap a la formació de macròfags. Els macròfags accepten les M-LDL de manera no regulada, es recobreixen per aquestes LDL modificades i es converteixen en les cèl·lules espumoses.

Estudis *in vitro* han descrit com els polifenoles inhibeixen l'oxidació de la LDL. Aquest tipus d'oxidació és considerada com un mecanisme clau en l'aterosclerosi. L'efecte antioxidant d'aquests compostos pot disminuir l'oxidabilitat dels lípids que formen part de la LDL i de l'alfa-tocoferol associat (Zhu et al. 1999). Encara que hi ha evidències en humans que han mostrat efectes contraris, certs estudis han observat com el consum de begudes i aliments rics en polifenols (vi negre, xocolata, te o suc de magrana) redueixen la susceptibilitat de la LDL a la seva oxidació induïda *ex vivo* pel Cu (II) (Kondo et al. 1996). Els baixos nivells de productes d'oxidació de la fosfatidilcolina (el principal lípid oposat en LDL) observats després del consum de catequines del te verd en homes suggereixen que els polifenols protegeixen a la LDL contra la seva oxidació (Nakagawa et al. 1999).

Un gran nombre d'estudis en animals han observat que el consum de polifenols limita el desenvolupament de lesions ateromatoses. Una dieta suplementada amb vi desalcoholizat, suc de magrana, catequina o quercetina s'ha vist que redueix aquestes lesions en ratolins apoE deficients (Miura et al. 2001). Aquests efectes estan associats amb l'absorció reduïda de les lipoproteïnes de baixa densitat (LDL) pels macròfags, la baixa oxidació de la LDL i la susceptibilitat reduïda de la LDL a l'agregació. Resultats similars es van obtenir després de suplementar dietes riques en colesterol amb extractes de raïm riques en proantocianidines a conills (Yamakoshi et al. 1999).

3.1.1 Resveratrol

La importància del resveratrol com possible compost protector de la malaltia cardiovascular va sorgir a partir de diferents estudis epidemiològics. El més destacat va ser el de Renaud i Lorgeril, el 1992, també anomenat la Paradoxa Francesa (Renaud and Lorgeril 1992). La població francesa, que tenia una dieta rica en greixos saturats igual que altres poblacions europees, presentava una reduïda mortalitat per malaltia cardiovascular en comparació d'altres poblacions. Aquesta reducció es va relacionar amb el major consum de vi en la població francesa. Els possibles efectes cardioprotectors del vi se li van associar a la presència del resveratrol en el vi (Siemann EH. and Creasy LL. 1992). Posteriorment, diferents estudis han confirmat aquests

efectes beneficiosos del raïm i del vi en la malaltia cardiovascular (Baur and Sinclair 2006; Delmas et al. 2005; Fremont 2000). Els efectes estudiats corresponen a la disminució de l'agregació plaquetària, promoció de la vasorelaxació, reducció de la peroxidació lipídica i disminució de les concentracions plasmàtiques de colesterol i triglicèrids. A més, el resveratrol pot actuar en diferents estadis de l'aterogènesi: acumulació lipídica i oxidació de les LDL, infiltració de monòcits i limfòcits, proliferació i migració de les cèl·lules musculars llises i en l'agregació plaquetària (Baur and Sinclair 2006; Delmas et al. 2005; Fremont 2000).

En estudis *in vivo*, s'ha demostrat que el resveratrol no influencia significativament i de forma directa la disminució del colesterol i els triglicèrids plasmàtics (Wang et al. 2002b) encara que Zern et al el 2003 (Zern et al. 2003) va observar una reducció de triglicèrids i VLDL plasmàtics després de l'administració de raïm en conills porquins. A més, el tractament amb resveratrol en rates alimentades amb dietes riques en colesterol, va fer reduir la placa d'ateroma (Miura et al. 2003).

Frankel et al., van ser els primers investigadors a demostrar que el resveratrol podia prevenir l'oxidació de la LDL *in vitro* per la seva acció quelant sobre el coure i la seva capacitat antiradicalària (Belguendouz et al. 1998; Fremont et al. 1999). A més, el resveratrol té l'habilitat de modular diferents sistemes enzimàtics presents en les cèl·lules endotelials i macròfags implicats en l'oxidació de la LDL. El resveratrol podria prevenir o disminuir l'activitat de NADPH oxidasa, hipoxantina/xantina oxidasa, mieloperoxidasa i augmentar l'activitat de la superòxid dismutasa, catalasa, glutatió peroxidasa, glutatió reductasa i glutatió-*S*-transferasa (Delmas et al. 2005). L'acció d'aquests enzims consisteix a contribuir a la reducció de la formació intracel·lular en cèl·lules endotelials de les espècies reactives de l'oxigen i a la inhibició de l'adhesió leucocitària. L'oxidació induïda per les cèl·lules endotelials o pels macròfags depèn dels lipoperòxids generats intracel·lularment i posteriorment transferits a les LDL. Les lipooxigenases estan involucrades en aquest procés i diversos estudis han comprovat l'efecte inhibidor del resveratrol sobre aquestes.

3.1.2 Flavanols del cacau

En nombrosos estudis s'ha observat que els polifenols de cacau contribueixen a la integritat cel·lular, a augmentar la capacitat antioxidant del plasma, a regular el to vascular, a reduir la reactivitat plaquetària i per tant a disminuir la possibilitat de formació de trombes, i, com a conseqüència de tots aquests efectes, a reduir el risc de patir certes malalties cardiovasculars.

Els efectes dels flavonoids del cacau enfront de la prevenció de l'oxidació de les LDL ha estat àmpliament estudiada tant *in vitro*, per la seva habilitat d'eliminar les espècies radicals o per la seva capacitat de quelar metalls prooxidants (Wan et al. 2001) com *in vivo*. Kondo et al, ja el 1996 van demostrar *in vivo* que el cacau alentia l'oxidació de les LDL de manera directament proporcional a la concentració (Kondo et al. 1996). Waterhouse et al, en 1996 van observar que a 5µmol d'equivalents/L d'àcid gàlic, els fenols del cacau inhibien l'oxidació de les LDL en un 75% mentre que els del vi negre sol aconseguien una inhibició de 37-65% (Waterhouse et al. 1996). Wang et al. (2000) van descriure que un increment dosi-depenent d'epicatequina en plasma estava associat a un increment de la capacitat antioxidant del plasma i a una reducció en plasma de la peroxidació lipídica 2 i 6 hores després del consum de xocolata rica en procianidines (Wang et al. 2000).

Més recentment, Wiswedel et al. (2004) han confirmat l'acció antioxidant del cacau en un estudi *in vivo* en el qual en el plasma de voluntaris després de la presa d'una beguda de cacau disminuïa la concentració de les F2-isoprostans, disminució que reflecteix menor peroxidació lipídica (Wiswedel et al. 2004). El mateix any, Mursu et al. van afirmar que prendre 75g de xocolata negra diàriament durant 3 setmanes incrementava la concentració plasmàtica del colesterol HDL però que no es modificava la fracció LDL (Mursu et al. 2004). El 2005, Kurosawa et al. demostraven que la ingesta de fraccions riques en polifenols derivades del cacau augmentaven la resistència de les LDL a l'oxidació i suprimia la formació d'aterosclerosi en conills hipercolesterolèmics (Kurosawa et al. 2005). Finalment, en els estudis més recents realitzats en humans sobre l'efecte de cacau en les LDL i HDL, s'ha observat que la ingesta de cacau en pols diàriament per voluntaris sans i hipercolesterolèmics, provoca una disminució de les

concentracions de LDL colesterol, incrementa el colesterol HDL i genera una resistència a l'oxidació de les LDL (Baba et al. 2007a; Baba et al. 2007b).

Nombrosos estudis *in vitro* i *in vivo* han demostrat que els flavonoids del cacau poden modular la funció plaquetària i reduir la formació de trombes mitjançant diferents mecanismes, basats principalment en la inhibició de l'homeòstasi primària i moltes vies associades amb l'activació i agregació plaquetària (que inclouen la síntesi d'eicosanoids, peròxids d'hidrogen, mobilització del calci, inhibició de l'inositol fosfat i modulació de les concentracions de AMPc (Schramm et al. 2001; Murphy et al. 2003; Rein et al. 2000b; Holt et al. 2002b; Innes et al. 2003; Steinberg et al. 2003).

Murphy et al., 2003, van anar més enllà, després d'observar en diversos estudis que hores posteriors a un alt consum en flavanols del cacau s'aconseguia una clara disminució de l'activitat i agregació plaquetària, van voler veure si després de dosis baixes de cacau, però durant un període de temps més llarg (4 setmanes) també s'observaven els mateixos efectes. El resultat va ser una disminució de la funció plaquetària molt més modesta que en els estudis d'alt consum de cacau en agut. Però van concloure que el significat biològic d'aquests resultats era incert i que faria falta continuar realitzant estudis dosis-resposta per a poder determinar la ingesta de cacau més efectiva perquè sorgeixin efectes sobre la funció plaquetària (Murphy et al. 2003).

Innes *et al (2003) van concloure després de subministrar 100g de xocolata blanca, negre o amb llet a 30 voluntaris, que s'inhibia el col·lagen plasmàtic inductor de l'agregació plaquetària dels individus que havien pres xocolata negra i, per tant, que aquest podria exercir un paper protector front malalties cardiovasculars o altres d'origen tromboembòlic (Innes et al. 2003).

3.1.3 Isoflavones

Estudis en animals (Lin et al. 2004) i humans (Anthony et al. 1998) han mostrat que el consum de proteïna de soja o d'isoflavones tenen efectes beneficiosos en els factors de risc cardiovascular que inclouen una menor quantitat de nivells de triglicèrids hepàtics i plasmàtics, nivells de colesterol totals i en LDL, un increment del colesterol HDL i del ràtio de colesterol HDL/LDL. Estudis de biologia molecular i cel·lular han demostrat

que els components de la soja modulen els factors de transcripció implicats en la regulació del metabolisme lipídic i la seva expressió gènica en animals i en cèl·lules humanes *in vitro* a nivell transcripcional i post-translacional (Xiao et al. 2007; Huang et al. 2005).

La producció i el consum d'aliments derivats de la soja en països occidentals han experimentat un notable increment durant l'última dècada. Aquest increment ha estat clarament influenciat per l'aprovació, per part de la FDA (1999), de la inclusió en l'etiquetatge dels aliments rics en soja per a reivindicar la seva activitat preventiva en el desenvolupament de malalties coronàries (U.S.Food and Drug Administration. 1999).

La ingesta de soja modula favorablement els lípids plasmàtics (Anderson et al. 1995) però existeix una controvèrsia en si el component responsable d'aquest efecte és la proteïna de la soja o les isoflavones. Mantenint la quantitat de proteïna de soja constant i variant la concentració d'isoflavones en els aliments es va observar un efecte dosi-depenent en el colesterol plasmàtic i en el perfil lipídic (Merz-Demlow et al. 2000). En altres estudis amb micos, les isoflavones combinades amb caseina-lactoalbúmina sembla que no tenien cap efecte encara que al combinar la proteïna de soja amb isoflavones s'observava un efecte significativament favorable sobre el perfil lipídic (Greaves et al. 2000). En un altre estudi, només la proteïna de soja va mostrar un efecte moderat en el colesterol total i en la LDL i l'addició d'isoflavones no va canviar l'efecte (Lichtenstein et al. 2002).

Les isoflavones poden afectar favorablement la funció endotelial en rates (Squadrito et al. 2000) però no es van observar efectes en els nivells de lipoproteïnes o en la funció endotelial en dones postmenopàusiques amb evidència de disfunció endotelial (Simons et al. 2000). Per tant, els efectes protectors de les isoflavones en l'arteriosclerosi podrien tenir altres mecanismes degut al fet que els fitoestrògens de soja incrementen la resistència de la LDL contra l'oxidació i redueixen la LDL oxidada i els àcids grassos (Tikkanen et al. 1998; Wiseman et al. 2000).

3.2 Polifenols i càncer

Els efectes anticarcinogènics dels polifenols estan ben documentats en animals. Els polifenols quan es donen a rates o ratolins abans i/o després de l'administració d'un agent carcinogènic o de la implantació d'una línia cel·lular de càncer humà actuen protegint i induint una reducció del nombre de tumors o del seu creixement (Yang et al. 2001). Aquests efectes s'han observat en diversos localitzacions de l'organisme que inclouen la boca, l'estómac, el duodè, el còlon, el fetge, els pulmons, les glàndules mamàries o la pell.

Els polifenols com la quercetina, les catequines, les isoflavones, els lignans, les flavanones, l'àcid el·làgic, el resveratrol van ser provats i tots van mostrar efectes protectors en alguns models. S'han suggerit els següents mecanismes per a explicar els seus efectes anticarcinògens (Johnson et al. 1994).

1. Els polifenols poden actuar com agents bloquejants en estadis d'iniciació. Influencien el metabolisme de procarcinògens cap a carcinògens modulant l'expressió d'enzims del citocrom P450 involucrats en la seva activació. També poden facilitar la seva excreció incrementant l'expressió d'enzims de fase II. Aquesta inducció podria tenir el seu origen en la toxicitat dels polifenols. Els polifenols poden formar quinones tòxiques en el cos que són substrats d'aquests enzims (Baez et al. 1997).La ingesta de polifenols podria activar aquests enzims per al seu pròpia detoxificació i induir una estimulació de les nostres defenses contra xenobiòtics tòxics (Talalay et al. 1988).

2. Els polifenols poden actuar com agents de supressió i per tant inhibir la formació i el creixement de tumors de les cèl·lules d'iniciació, inhibeixen la proliferació cel·lular *in vitro* (Kuntz et al. 1999). També s'ha demostrat que alguns polifenols poden afectar als camins de creixement relacionats amb la transducció del senyal a través de la inhibició de l'activitat transcripcional de la proteïna quinasa C i AP-1 dependent (Dong et al. 1997). Ells inhibeixen l'expressió oncogènica 124 i l'activitat ornitin descarboxilasa, un enzim clau en la síntesi de poliamines associades a la proliferació cel·lular (Schneider et al. 2000). També poden

inhibir la proliferació cel·lular a través del seu efecte en el metabolisme de l'àcid araquidònic.

3.2.1 Resveratrol

El resveratrol pot inhibir la iniciació i el creixement de tumors en un ampli rang de models cancerígens en ratolins i rates. Entre diversos dels seus efectes, el resveratrol pot prevenir el càncer de còlon (Tessitore et al. 2000) i el càncer de pròstata (Harper et al. 2007), i incrementar la supervivència dels ratolins amb neuroblastomes subcutanis (Chen et al. 2004). Els seus efectes en el càncer de pit es troben en una fase de controvèrsia ja que s'han descrit des d'efectes no positius (Bove et al. 2002) fins a una prevenció en la progressió del càncer *in vivo* (Su et al. 2007; Whitsett et al. 2006).

Els múltiples mecanismes pels quals el resveratrol disminueix el desenvolupament de tumors inclou la inhibició de l'angiogènesi, les alteracions en el cicle cel·lular i l'apoptosi, així com els efectes antioxidants (Baur and Sinclair 2006). Alguns d'aquests mecanismes semblen ser deguts a la supressió de les ciclooxigenases per part del resveratrol i per l'expressió de l'ornitina descarboxilasa ja que normalment ambdues enzims promouen l'angiogènesi (Baur and Sinclair 2006). També s'ha descrit que el resveratrol inhibeix els enzims del citocrom P450 involucrades en el metabolisme de substàncies (medicaments, xenobiòtics, polifenols) considerats tots ells pro-carcinògens (Zhou et al. 2005).

Un altre mecanisme pel qual el resveratrol exerceix efectes protectors contra el càncer és el seu efecte antiproliferatiu i pro-apoptòtic (Aggarwal et al. 2004) permetent una subregulació del cicle cel·lular proteic i un increment de l'apoptosi de cèl·lules tumorals (Baur and Sinclair 2006).

Els efectes antioxidants del resveratrol podrien contribuir a les seves propietats anticarcinogèniques. Això es deu al fet que les espècies reactives d'oxigen (formades per danys en el DNA o en altres macromolècules), poden contribuir a la iniciació i progressió del càncer (Baur and Sinclair 2006). Però no està molt clar si aquests efectes

són directes o són conseqüència de la sobre-regulació dels enzims antioxidants per part del resveratrol.

3.2.2 Flavanols del cacau

L'efecte quimiopreventiu dels flavanols del cacau s'ha anat estudiat durant els últims anys. Carnésecchi et al. (2002) van descriure l'efecte beneficiós dels flavanols del cacau en la progressió tumoral. Van avaluar l'efecte dosi depenent d'uns extractes de cacau en la proliferació cel·lular i en l'efecte inhibidor de les cèl·lules Caco-2 (cèl·lules cancerígenes del còlon humà). Per a això, van addicionar 50µg/L de diferents tipus d'extractes de cacau; (un normal, un enriquit amb procianidines (501mg/g) del mateix extracte i un altre més enriquit en procianidines (941mg/g). El resultat va ser una reducció del 25% del creixement cel·lular amb l'extracte normal de cacau, però l'interessant va ser veure que aquest creixement disminuïa fins a un 75% quan l'extracte de cacau s'enriquia encara més en procianidines. Aquest efecte es va atribuir a la possible inhibició de la biosíntesis de poliamides per part dels flavanols de cacau (Carnesecchi et al. 2002).

Posteriorment, Yamagishi et al (2003) van observar un efecte similar amb les procinidines del licor de cacau. En aquest cas, durant 36 setmanes, es va addicionar a l'aigua de beguda de dos grups de rates una quantitat igual de compostos procancerígens. L'única diferència va ser que a les 2 setmanes del tractament procancerígen, a un dels 2 grups se li va enriquir la mateixa aigua amb procianidines (250mg/100ML) provinents del licor de cacau. Al final del tractament, la supervivència del grup suplementat amb procianidines va anar significativament major que en el grup sense el suplement. També van concloure que l'efecte quimioprotector de les procinidines es donava en el pulmó i no en altres òrgans (Yamagishi et al. 2003).

En estudis epidemiològics, Arts et al (2002) van establir una relació inversa entre el consum de catequines i la incidència de càncer rectal en dones post-menopàusiques (Arts et al. 2002).

3.2.3 Isoflavones

La similitud estructural de les aglicones isoflavòniques amb el 17b-estradiol els dóna la capacitat d'unir-se a receptors estrogènics i induir efectes hormonals. A causa de aquesta propietat, les isoflavones s'han estudiat per a protegir contra malalties que involucren una desregulació hormonal. Per tant, en diversos estudis amb humans i animals d'experimentació s'han determinat que existeix una associació entre el consum d'isoflavones i el càncer de pit i pròstata.

Existeix una recent controvèrsia de si la soja pot protegir contra el càncer de pit. Un estudi epidemiològic tipus cas-control va relacionar el consum elevat de soja en l'adolescència amb un menor risc de patir càncer de pit en adults (Shu et al. 2001). Però un estudi més recent de cohorts ha suggerit que el consum d'aliments rics en soja no té efectes protectors contra aquest tipus de càncer (Nishio et al. 2007). A més, en un altre estudi clínic, es va observar que les isoflavones poden estimular la proliferació de cèl·lules epitelials en pits de dones premenopàusiques (Michael-Phillips et al. 1998).

Les isoflavones disminueixen significativament el risc de càncer de pròstata en homes japonesos (Nagata et al. 2007). El suplement amb proteïna de soja o isoflavones de soja disminueixen els marcadors de desenvolupament de càncer i la progressió en cèl·lules prostàtiques incloent el PSA (antigen de pròstata específic), la testosterona i el receptor androgènic en pacients amb càncer de pròstata (Dalais et al. 2004; Kumar et al. 2004) o en homes amb un elevat risc de desenvolupar càncer de pròstata (Hamilton-Reeves et al. 2007). Encara que el consum de soja a nivells de 44g de proteïna de soja i 116 mg/dia d'isoflavones no mostra canvis significatius en el sèrum total o en el PSA lliure en homes sans de mitjana edat (Jenkins et al. 2003). Aquests resultats suggereixen que el consum de soja pot afectar només el PSA en pacients amb càncer de pròstata o al d'homes amb alt risc però no al de subjectes sans (Xiao 2008).

3.3 Polifenols i malaltia neurodegenerativa

L'increment de les expectatives de vida ha augmentat la prevalença de malalties cròniques i degenerativas. Al voltant del 15% de la població de més de 65 anys té Alzheimer i un 1% Parkinson (Cantuti-Castelvetri et al. 2000). La neurodegeneració en aquestes malalties sembla ser un procés multifactorial, a causa d'un complex nombre de reaccions tòxiques com la inflamació, la neurotoxicitat glutamatèrgica, increments en ferro i òxid nítric, deplecció d'antioxidants endògens, reducció de l'expressió de factors tròfics, disminució del sistema ubiquitin-proteasòmic i expressió de proteïnes proapoptòtiques que condueixen a la degeneració neuronal. Aquestes malalties depenen globalment de l'estrès oxidatiu que afecta particularment als teixits cerebrals (Halliwell 2001) i, per tant, els compostos antioxidants podrien contribuir a la seva prevenció (Cantuti-Castelvetri et al. 2000).

S'ha observat que rates d'edat avançada que s'alimentaven amb dietes suplementades d'extractes aquosos d'espinacs, maduixes o nabius milloraven la seva funció cognitiva i neuronal (Joseph et al. 1998).

Els efectes dels polifenols involucrats en la regulació de la proliferació cel·lular o apoptòtica podrien ser dosi depenent. A dosis baixes (0.1-10µM), l'epigalocatequin galat protegeix les cèl·lules neuronals contra el dany oxidatiu i millora la supervivència cel·lular, en canvi a dosis elevades (50 µM) és tòxic i podria actuar com prooxidant (Levites et al. 2002). Per tant, les concentracions baixes podrien ser més efectives per a prevenir les malalties neurodegeneratives. A causa de la dificultat d'aquest tipus d'estudis, es coneix poc sobre les concentracions que es troben en el cervell. La poca permeabilitat de la barrera hematoencefàlica per als polifenols s'ha confirmat en estudis amb naringin o quercetin (Mullen et al. 2002). S'ha observat que els conjugats glucurònids de l'epicatequina van ser incapaces de protegir a les neurones corticals contra l'estrès oxidatiu induït per H_2O_2 (Spencer et al. 2001a). No obstant això, les baixes quantitats de polifenols oposades en el cervell podrien ser sol de aglicones com s'ha descrit prèviament per a la genisteina i per als antocians a causa de la baixa permeabilitat de la barrera hematoencefàlica i a les conjugacions aniòniques (Mullen et al. 2002; Andres-Lacueva et al. 2005).

3.3.1 Resveratrol

El resveratrol confereix protecció neuronal com s'ha recollit recentment en estudis *in vitro* (Anekonda 2006). Diversos estudis *in vivo* van mostrar com el resveratrol podia actuar protegint el dany cerebral. En rates suplementades diàriament amb resveratrol intravenós durant 21 dies, van mostrar una millora motora i una disminució del volum de la zona afectada per l'isquèmia després de l'oclusió de l'artèria cerebral mitjana (Sinha et al. 2002). A l'injectar resveratrol a un tipus de rosegador (*jerb*) durant o immediatament a una isquèmia cerebral global transitòria, seguida per una segona dosi a les 24h, es va observar una disminució o un retard en la mort de cèl·lules neuronals i l'activació de cèl·lules glials en l'hipocamp (Wang et al. 2002a). Un tercer estudi va demostrar que el resveratrol administrat de manera intravenosa va reduir el volum isquèmic i el contingut d'aigua cerebral a dosis baixes (100 ng- 1000ng/kg pes) després de l'oclusió de l'artèria cerebral mitjana en rates (Wang et al. 2003). Aquests resultats suggereixen que el resveratrol té capacitat de travessar la barrera hematoencefàlica i exercir els seus efectes beneficiosos.

Altres autors (Chen et al. 2005b) van mostrar com el resveratrol a través de la inhibició de la SIRT-1, inhibia el senyal del factor nuclear kB (NF-kB) i protegia contra la toxicitat del β-amiloide en la malaltia d'Alzheimer. Un altre estudi recent (Kim et al. 2007) ha mostrat noves evidències científiques en dos models de ratolí amb malalties neurodegeneratives: Alzheimer i esclerosis amiotròfica lateral. En ambdós el resveratrol reduïa de manera significativa la mort neuronal i la neurotoxicitat, així com la neurodegeneració en l'hipocamp.

3.3.2 Flavanols del cacau

L'estrès oxidatiu s'ha associat amb diverses malalties neurodegeneratives i pèrdua de capacitats cognitives associades amb l'edat. Segons Spencer et al., tant l'epicatequina com el seu metabòlit el 3'-*O*-metilepicatequina podrien utilitzar-se com agents protectors front l'apoptosi neuronal causada per estrès oxidatiu, ja que van demostrar *in vitro* que eren capaços d'inhibir la toxicitat neuronal induïda per LDL oxidades i inhibir així l'activació de proteinquinases i l'activitat de proteases tipus caspasa-3 (Spencer et al. 2001a).

Aquesta protecció enfront d'apoptosi també va ser observada per Shimada et al mitjançant l'addició de catequina, epicatequina i procianidines B1 i B2 en un cultiu de cèl·lules granulars del cervell de rates on s'induïa la mort neuronal via glutamat (Shimada et al. 2001).

Per a dilucidar els efectes del cacau en la neurotoxicitat induïda per la proteïna beta-amiloide, Heo et al (2005), van tractar una línia cel·lular cancerosa (PC12), útil com model per a la diferenciació neuronal, amb el pèptid beta-amiloide tòxic i van estudiar els efectes de l'epicatequina i la catequina. Tal com s'esperava, es va observar un increment de la mort cel·lular neuronal quan les cèl·lules van ser tractades amb el beta-amiloide mentre que en aquelles cèl·lules tractades amb epicatequina i/o catequina es va evidenciar un efecte protector davant la mort neuronal, però addicionalment, es va observar una activitat sinèrgica quan els dos compostos es van administrar conjuntament (Heo and Lee 2005).

Cho et al., el 2008, van estudiar també sobre la línia cel·lular PC12, els efectes protectors que tenien la fracció de procianidines del cacau (1 i 5 µg/ml) i la procianidina B2 (1 i 5 µM) quan s'induïa la toxicitat amb peròxid d'hidrogen. Els resultats van indicar que els efectes protectors que produïen ambdues procianidines tant del cacau com la B2 pura, implicaven la inhibició de l'expressió de Bcl-X(L) i Bcl-2 a través del bloqueig de l'activació de JNK i p38 MAPK, i per tant, reduïen la mort cel·lular neuronal (Cho et al. 2008).

Bisson et al., en 2008, van observar que un extracte polifenòlic de cacau administrat oralment durant un any en rates a una dosi de 24 mg/kg/dia, millorava el funcionament

cognitiu, l'esperança de vida i conservava els nivells de dopamina urinària indicant que el cacau podria alentir l'aparició de malalties neurodegeneratives (Bisson et al. 2008).

3.3.3 Isoflavones

Els fitoestrògens tenen una estructura similar a l'estrògen 17β-estradiol i són substàncies que promouen activitats estrogèniques en mamífers. La seva elevada afinitat per al receptor estrogènic β, i el seu alt contingut en regions del cervell que expressen aquest tipus de receptor, suggereixen que les isoflavones poden regular determinats factors lligats al comportament com la memòria i l'aprenentatge. A més, existeixen diversos estudis que clarament mostren que les isoflavones poden influenciar els paràmetres del comportament neuronal intervinguts pel receptor estrogènic β (Lephart et al. 2002). Els fitoestrògens poden unir-se als receptors estrogènics per a activar la ruta de senyal intracel·lular que normalment està regulada per estrògen endogen permetent l'activació de la quinasa, l'activació del factor transcripcional i la transcripció gènica, les quals protegeixen la integritat de la membrana neuronal actuant com agonistes o antagonistes (Belcher and Zsarnovszky 2001). A més de les seves propietats antioxidants, com un estudi recent indica, la genisteina té propietats antiapoptòtiques intervingudes pel receptor estrogènic comparables amb el 17β-estradiol en neurones corticals primàries (Linford and Dorsa 2002).

Els fitoestrògens també podrien tenir l'efecte de disminuir la incidència o posposar l'inici de malalties neurodegeneratives com l'Alzheimer. Pedersen i Blusztajn van trobar que la genisteina augmentava el nivell d'acetilcolina *in vitro* bloquejant la degradació de l'acetilcolina induïda pel pèptid β-amiloide en cèl·lules de ratolins SN56 (Pedersen and Blusztajn 1997). A més, les isoflavones de soja poden incrementar els nivells de mRNA del factor neurotròfic derivats del cervell, la colina acetiltransferasa i el factor de creixement del nervi en el còrtex cerebral i en l'hipocampus, inhibir l'enzim acetilcolinesterasa (Zhao et al. 2002; Pan et al. 1999), incrementar el nivell de β-tubulina III (un marcador de diferenciació/supervivència neuronal) i disminuir el nivell de Bcl-2 associat amb la mort del promotor (un membre apoptòtic de la proteïna Bcl-2) de l'hipocamp i del còrtex cerebral.

Zhao et al. (Zhao et al. 2002) van investigar l'eficàcia neuroprotectora de 6 compostos fitoestrògens en cultius de neurones de l'hipocamp i van trobar que tots els fitoestrògens induïen una reducció significativa en la lactat deshidrogenasa alliberada després de l'exposició al glutamat i al β-amiloide a través de propietats antioxidants. A més, també poden revertir la deplecció de l'ATP induïda per la toxicitat del β-amiloide en la mitocondria i aquesta reducció de la neurotoxicitat induïda pel glutamat podria ser un dels mecanismes pels quals els fitoestrògens exerceixen les seves propietats neuroprotectores (Lecanu et al. 2005).

Les isoflavones de la soja podrien també millorar les funcions cognitives mimetitzant els efectes dels estrògens en el sistema nerviós central (Pan et al. 1999). No obstant això, les propietats agonistes de l'estrògen d'aquestes no expliquen tots els efectes en el cervell.

Encara que existeixen pocs estudis dels efectes de les isoflavones de la soja en la funció cognitiva en humans i animals d'experimentació, aquests han suggerit que les isoflavones milloren la funció cognitiva de les femelles independentment de la seva edat. Al contrari que en les femelles, els resultats obtinguts per als mascles han mostrat ser inconsistents tant en estudis amb humans com en animals (File et al. 2001; White et al. 2000; Lund et al. 2001). Les isoflavones de la soja poden afectar la funció del cervell per processos intervinguts pel receptor estrogènic o per inhibició de la tirosin-quinasa (Akiyama et al. 1987). Diversos estudis cel·lulars, amb animals i estudis comparables amb humans, indiquen que les isoflavones prevenen el dany neuronal i el declivi cognitiu (File et al. 2001; Pan et al. 2000). No obstant això, s'ha vist que la genisteina pot tenir una influència negativa en la funció cognitiva quan es troba present en elevades concentracions a causa de la seva acció com inhibidor de la tirosin-quinasa que la incrementa per a bloquejar la potenciació a llarg terme i la funció cognitiva (Pan et al. 2000; Lee et al. 2004).

4. METODOLOGIA ANALÍTICA

ANTECEDENTS BIBLIOGRÀFICS

L'anàlisi dels compostos polifenòlics en mostres biològiques per espectrometria de masses requereix una elevada sensibilitat, per tant, és fonamental eliminar els components de la matriu que podrien contaminar el sistema o causar supressió de la ionització (Guo et al. 2006).

4.1 Preparació de mostra

L'aïllament de compostos fenòlics i els seus metabòlits a partir de mostres biològiques és un aspecte de màxima importància tant per a l'anàlisi qualitatiu com quantitatiu. En primer lloc, trobem la complexitat de la matriu biològica. En la majoria de casos, no és possible analitzar directament les mostres biològiques ja que requereixen un tractament previ per a eliminar les proteïnes endògenes, carbohidrats, sals o lípids que podrien interferir en l'anàlisi. En segon lloc, els polifenols i els seus metabòlits normalment es troben en molt baixes concentracions requerint un alt nivell de sensibilitat. Per tant, la preparació de la mostra és el pas crucial en l'anàlisi de mostres biològiques.

La precipitació de proteïnes, l'extracció en fase sòlida i l'extracció líquid-líquid són les principals formes de preparació de mostra (extracció i aïllament) per a combinar amb l'anàlisi de LC-MS/MS per a analitzar compostos i metabòlits en biofluids (Xing et al. 2007).

4.1.1 Precipitació de proteïnes (PPT)

La precipitació de proteïnes és el tractament previ de la mostra més simple. Les mostres biològiques es caracteritzen per la presència de proteïnes que poden afectar a altres passos del procés tals com aïllament, purificació i anàlisi.

La tècnica de precipitació de proteïnes ha estat una de les més àmpliament utilitzades (Guy et al. 2008; Xing et al. 2005). El primer pas és l'eliminació d'interferències proteiques per precipitació i centrifugació. Diversos estudis han demostrat que la

desproteinizació amb acetonitril comporta una bona resolució i una bona recuperació (Xing et al. 2007). La precipitació de proteïnes amb àcid tricloracètic o altres àcids forts, pot provocar la hidròlisi d'alguns compostos conjugats com per exemple glucurònids o sulfats (Wilshire 2000).

4.1.2 Extracció líquid-líquid (LLE)

Tot i que l'extracció líquid-líquid està especialment aconsellada per als compostos lipofílics, s'ha utilitzat per a determinar els compostos polifenòlics en mostres biològiques. Els flavonoids de mostres biològiques s'han extret generalment amb acetat d’etil prèvia acidificació (Kanaze et al. 2004), mentre que els alcaloides s'extreuen amb cloroform o èter després de la seva alcalinització (Abe et al. 2006).

4.1.3 Extracció en fase sòlida (SPE)

En l'anàlisi de mostres biològiques, l'extracció en fase sòlida és la tècnica més freqüentment utilitzada per al tractament previ de la mostra. Comparat amb la PPT, quan s'analitzen mostres de plasma, el mètode SPE redueix notablement el soroll de fons. El SPE es tria per a l'extracció i la purificació dels analits a causa de la seva elevada selectivitat, a la velocitat d'extracció, al potencial per a la seva automatització i al fet que es requereixen volums molt més baixos de solucions orgàniques comparat amb l'extracció líquid-líquid (Kaneko et al. 2006). La preparació de la mostra utilitzant SPE de fase reversa ha estat molt utilitzada per a flavonoids, alcaloids, saponines i sesquiterpenoids (Felgines et al. 2003; Naik et al. 2005). En alguns casos és necessari acidificar o alcalinitzar les mostres biològiques abans de transferir-les als cartutxos SPE (Samanidou et al. 2005). Les mostres biològiques, tals com orina o plasma, és possible processar-les utilitzant SPE automatitzat en format de 96 pous per a la quantificació dels analits. El límit de detecció resulta ser més baix per als mètodes en línia que per als manuals, encara que la reproductibilitat és generalment millor per als mètodes manuals (Kuklenyik et al. 2004). Se sap que l'eficàcia del SPE depèn del tipus de sorbent, del volum de mostra, del pH, del contingut de la solució orgànica i del volum de la solució d’elució (Wang et al. 2006). A causa de l'àmplia gamma de cartutxos i de solucions que poden ser emprats, la SPE destaca per la seva alta versatilitat. Els cartutxos més

utilitzats són: Cartutxos SH (HySphere resina de poliestirè-divinilbenzè hidrofòbica forta), cartutxos GP, cartutxos C18, C8, C2 i cartutxos Oasis® HLB (de fase reversa), o de mode mixt tipus intercanvi catiònic (MCX i WCX) o de mode mixt tipus intercanvi aniònic (MAX i WAX). En la Figura 8 es mostra un esquema del funcionament els cartutxos Oasis®.

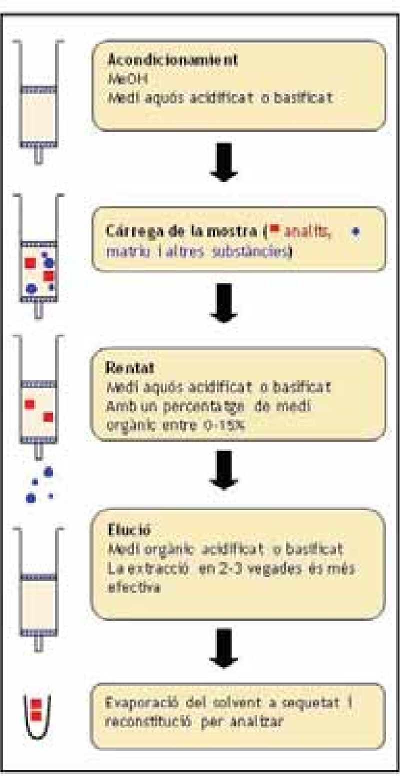


Figura 8: Extracció en fase sòlida (SPE)

La selecció de la tècnica del tractament previ de la mostra depèn de les concentracions previstes de l'analit i dels límits de detecció requerits. En l'orina, on normalment hi ha una acumulació de compostos i per tant alguns polifenols es poden trobar en elevades concentracions, es permet fer una PPT amb acetonitril ja que proporcionarà una suficient eliminació de proteïnes i donarà una bona mostra per a l'anàlisi qualitativa. Les dades obtingudes de la supressió de la ionització per l'anomenat efecte matriu indiquen que el SPE i el LLE són els mètodes més adequats per al bioanàlisi per espectrometria de masses (Dams et al. 2003). En altres casos, el sobrenadant obtingut després de la PPT pot ser utilitzat per a una SPE o també, poden processar-se amb ambdues SPE i LLE (Tawab et al. 2003).

4.2 Espectrometria de masses

L'espectrometria de masses és una de les tècniques més efectives en la investigació biomèdica, particularment en l'anàlisi de mostres biològiques des de fa dues dècades. És la tècnica utilitzada per molts analistes a causa de la seva elevada sensibilitat, especificitat i la seva fàcil combinació amb tècniques cromatogràfiques el que possibilita l'identificació, determinació i quantificació d'aquests compostos i els seus metabòlits (Prasain et al. 2004).

L'espectrometria de masses és considerada com la tercera tècnica espectral més poderosa en l'elucidació estructural de compostos orgànics desconeguts però té diferents avantatges front l'espectroscòpia NMR i la difracció en rajos X en el camp d'assaigs bioanalítics.

L'espectrometria de masses pot fàcilment ajuntar-se amb tècniques cromatogràfiques separatives de fase gasosa o de fase líquida per a l'anàlisi de mostres biològiques complexes. Té alta sensibilitat, baix consum de mostra i la informació requerida s'obté fàcilment seleccionant tècniques d'ionització i analitzadors de masses adequats. L'espectrometria de masses en tàndem, a més, juga un enorme paper en l'anàlisi i identificació de productes de transformació o metabòlits (Kamel and Prakash 2006). La LC-NMR té baixa sensibilitat però si s'uneix amb la LC-MS/MS genera dades superiors en l'elucidació estructural (Yilmazer et al. 2001).

4.2.1 Instrumentació analítica

Els espectròmetres de masses treballen amb molècules ionitzades on s'identifiquen els seus ions en funció de la seva relació massa/càrrega (m/z). Hi ha dos punts clau en aquest procés: la font d'ionització que genera ions i l'analitzador de masses. Depenent de la substància que es vulgui analitzar es combinaran ambdós.

4.2.1.1 Fonts d'ionització

Tenint en compte la gran varietat estructural dels compostos presents en les mostres biològiques, no existeix una sola tècnica d'ionització universal adequada per a totes elles encara que es podrien considerar diverses tècniques d'ionització depenent de l'estructura, polaritat del compost i pes molecular. En l'espectrometria de masses lligada amb la cromatografia líquida, existeixen tres tècniques d'ionització a pressió atmosfèrica que cobreixen l'ampli rang de polaritats i de pesos moleculars (Figura 9): ionització en electrospray (ESI), ionització química a pressió atmosfèrica (APCI) i fotoionització a pressió atmosfèrica (APPI). A més, la polaritat pot ser escollida en funció del caràcter àcid, neutre o bàsic dels analits. En alguns casos, la correcta tècnica d'ionització i la polaritat no és òbvia, per tant el primer pas a realitzar és l'optimització de les maneres d'ionització.

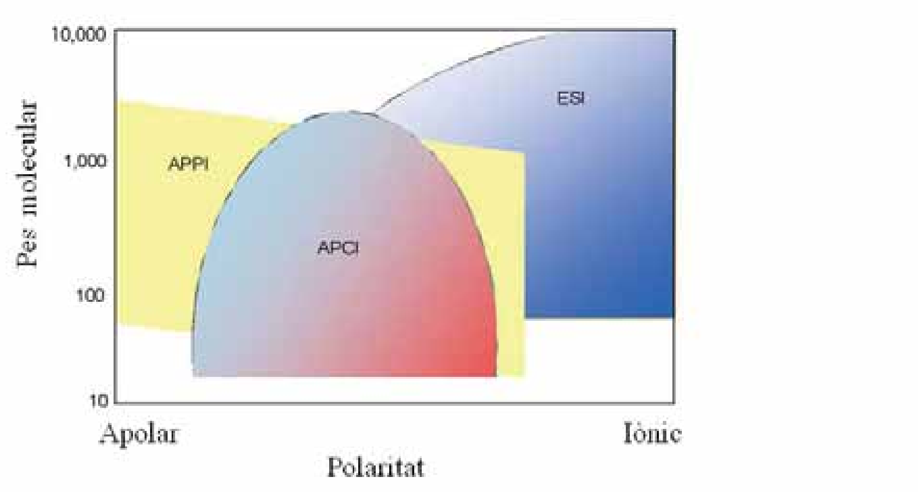


Figura 9: Tipus de compostos que és possible analitzar per LC-MS segons les diferents fonts d'ionització

La tècnica d'ionització més suau, ESI, és el mètode d'elecció per a compostos polars i iònics. Aquesta tècnica d'ionització ha tingut un gran reconeixement en la identificació de metabòlits degut al fet que, a diferència dels altres, permet una ionització suau dels metabòlits de fase II produint una informació fiable del pes molecular d'aquests conjugats. Per a la identificació dels analits i de metabòlits de fase I amb més baixa

polaritat, les ionitzacions APCI i APPI podrien proporcionar millor eficiència i sensibilitat (Kantharaj et al. 2005; Sheen and Her 2004).

Els APCI i APPI tenen millor tolerància per a les sals i per a l'efecte matriu comparats amb l'ESI (Schlusener and Bester 2005; Theron et al. 2007). L'APPI presenta un rang similar d'aplicació que l'APCI però una mica més estesa a compostos no polars (Cai et al. 2005).

Els ions són els mateixos per a totes les tècniques API: el pic base de l'espectre de masses en *full scan* quan s'ionitza en positiu és normalment l'ió $[M+H]^+$ acompanyat sovint d'adductes alcalins metàl·lics, $[M+Na]^+$ i $[M+K]^+$ (Nobilis et al. 2004), mentre que en ionització en mode negatiu els ions típics són la molècula desprotonada $[M-H]^-$.

L'APPI mostra més freqüentment la formació d'ions radicals moleculars M^+ a diferència de les altres tècniques API. Depenent de la composició de la fase mòbil, es poden observar adductes com els ions $[M+NH4]^+$ quan s'utilitzen fases d'acetat d'amoni (Nobilis et al. 2004). Rarament s'observen adductes amb fases mòbils d'acetonitril o metanol (Guan et al. 2003). Les abundàncies relatives dels fragments d'ions en espectres en *full scan* són baixes o inapreciables però hi ha excepcions en l'APCI on pot haver una extensiva fragmentació.

Recentment, s'ha descobert la desorció ESI (DESI) com una nova tècnica d'ionització desenvolupada per a la identificació de metabòlits (Williams et al. 2006). El seu principi es basa a polvoritzar el solvent a través del capil·lar d'acer inoxidable damunt de la mostra a analitzar. Aquesta tècnica no requereix preparació de la mostra i es pot utilitzar directament en teixits sencers o en organismes vius per a supervisar la localització dels metabòlits (Wiseman et al. 2008).

El MALDI (mètode de desorció i ionització per làser) és la segona tècnica d'ionització més important en proteòmica però el seu rol en la identificació de metabòlits no és molt elevat.

4.2.1.2 Analitzadors de masses

La funció d'un analitzador de massa és la separació d'ions en funció de la seva ràtio massa/càrrega (m/z). La qualitat de la separació de masses es caracteritza pel grau en el qual es poden separar valors molt pròxims de m/z. Els analitzadors de masses es classifiquen en dos grups en funció de la baixa o elevada resolució. En general, els analitzadors de baixa resolució (quadripols i trampes d'ions) tenen una resolució en un rang menor de 1000 mentre que els analitzadors d'alta resolució estan proveïts per una resolució mínima de 15000 per a analitzadors de temps de vol (TOF) o per sobre de 50000 per a analitzadors de masses Fourier (Peterman et al. 2006). L'avantatge de la trampa d'ions és la possibilitat de fer un escàner de masses, MS^n, molt útil per a estudiar el perfil de fragmentació dels compostos a analitzar (Anari et al. 2004; Tozuka et al. 2003; Yoo et al. 2006).

La determinació de la fórmula elemental permet obtenir la massa exacta de l'ió analitzat el que junt a experiments de MS/MS pot ajudar a l'elucidació estructural de metabòlits desconeguts i, especialment a la identificació de metabòlits inesperats o infreqüents.

Quadripol

L'analitzador de masses en quadripol se'l pot anomenar també “filtre de masses” i està format per quatre cilindres paral·lels formant un quadrat (Figura 10). Al combinar els potencials als cilindres del quadripol, es generaran camps electromagnètics, que permetran fixar els ions segons la relació massa/ràtio (m/z) i aquests podran passar pel centre del quadripol. La resta d'ions no presentaran una trajectòria estable a través del quadripol, xocaran amb les barres del quadripol i mai arribaran a el detector.

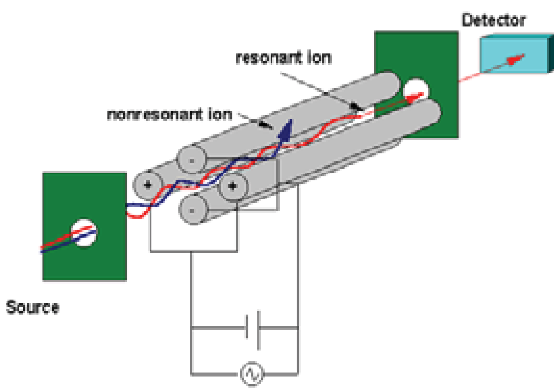


Figura 10: Diagrama esquemàtic d'un quadripol

Existeixen dues formes d'operar en aquest analitzador, en mode d'escaneig (*Scan mode* o *full scan*) o seleccionant i monitoritzant un ió en concret (*Selected ion monitoring*, SIM).

Temps de vol (*Time of flight*, TOF)

En aquest tipus d'analitzador s'aplica una força electromagnètica uniforme a tots els ions al mateix temps provocant una acceleració al seu pas a través d'un tub anomenat "*Time of flight*". Els ions més lleugers viatgen més ràpid i arriben primer al detector. Els ràtios de massa/carrega dels ions estan condicionats i es determinen segons el temps d'arribada. El temps de vol és l'analitzador que dóna una mesura més exacta de les masses dels ions arribant fins a una massa amb quatre decimals.

Trampa d'ions

Està format per una càmera amb dos elèctrodes i dues peces de finalització que atrapen els ions gràcies a una sèrie de camps electromagnètics. Aquest analitzador permet fer múltiples anàlisis o fragmentacions d'una mateixa molècula (MS^n) sense requerir un acoblament amb altres analitzadors de masses addicionals.

4.2.2 Utilitat de la CID (dissociació induïda en una cel·la de col·lisió)

Com ha estat comentat anteriorment, les tècniques d'ionització a pressió atmosfèrica són suaus i generen ions moleculars de càrrega positiva o negativa, adductes simples o ions que representen pèrdues moleculars simples com la perduda d'aigua. La informació sobre el pes molecular que s'obté és molt important per a determinar l'estructura dels compostos. Per a obtenir aquesta informació, les molècules ionitzades es fragmenten en una cel·la de col·lisió. Aplicant diferents voltatges a aquestes molècules ionitzades s'incrementa l'energia provocant una major fragmentació. Aquesta tècnica de dissociació o de fragmentació múltiple sol estar lligada a múltiples etapes de MS (MS/MS) on s'uneixen diferents analitzadors encara que també poden produir-se en un quadripol senzill o en un temps de vol.

CID en una etapa de MS

La col·lisió té lloc en la font d'ionització, denominant-se font de CID. Els ions procedents de la mostra ionitzada (precursor) s'acceleren i col·lisionen amb molècules residuals neutres per a donar fragments anomenats "ions producte" (*product ions*). L'avantatge de realitzar aquest procés en un únic analitzador és la seva senzillesa i el seu baix preu. L'inconvenient és que en aquest cas es fragmenten tots els ions. No es pot seleccionar un ió precursor específic de manera que no és possible saber de quin compost procedeix cada ió. Això no suposa un problema si l'analit és relativament pur però pot dificultar la identificació dels compostos en mostres biològiques complexes que no hagin tingut una separació cromatogràfica prèvia o mostres que provoquin un elevat soroll de fons.

CID en múltiples etapes

També es diuen experiments en tàndem (MS/MS o MS^n). Aquesta és l'eina realment útil per a poder determinar l'estructura dels compostos que analitzem. En un aparell que contingui com analitzador un triple quadripol (Figura 11) o en un quadripol/temps de vol, la dissociació es porta a terme en el segon quadripol, mentre que en el tercer quadripol o en el TOF es genera l'espectre d'ions resultants. El primer quadripol pot

estar estàtic o ser un seleccionador d'ions segons el tipus d'experiment que es porti a terme.

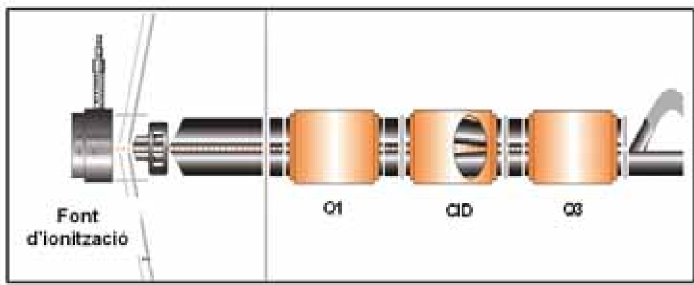
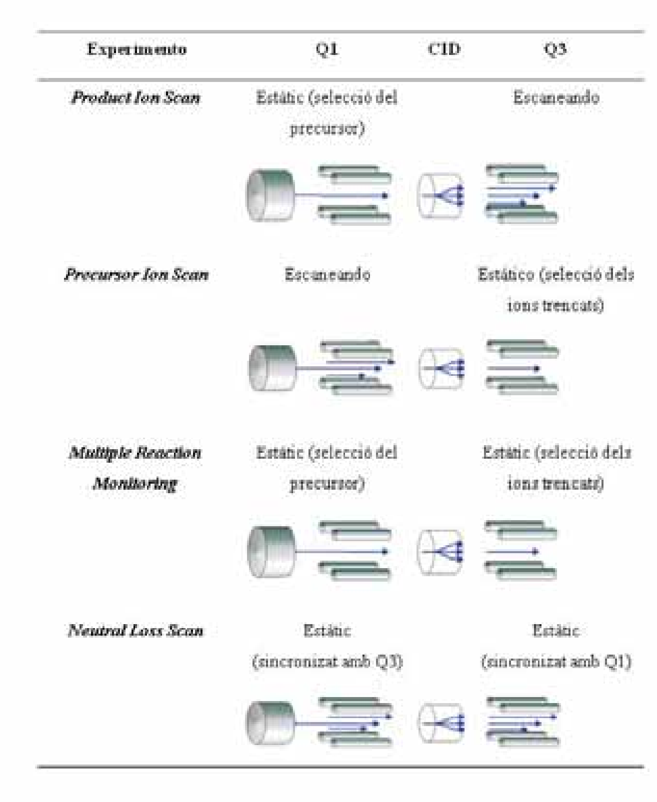


Figura 11: Triple quadripol.

En el cas del triple quadripol es poden realitzar quatre tipus d'experiments en MS/MS on les condicions Q1 i Q3 estan controlades segons l'experiment i tenen una funció concreta (Figura 12). Si es busca un compost o família específica de compostos s'utilitzaran experiments de precursor *ion scan* o de *neutral loss scan*, mentre que si es vol confirmar la identitat d'un compost determinat es portaran a terme experiments de *product ion scan*. Finalment, el MRM s'utilitzarà bàsicament per a obtenir millors límits de detecció en molècules conegudes i servirà per a quantificar els diferents analits. Les condicions de Q1 i Q3 estan controlades segons l'experiment i es resumeixen en la Figura 12:



Experimento	Q1	CID	Q3
Product Ion Scan	Estàtic (selecció del precursor)		Escaneando
Precursor Ion Scan	Escaneando		Estático (selecció dels ions trencats)
Multiple Reaction Monitoring	Estàtic (selecció del precursor)		Estàtic (selecció dels ions trencats)
Neutral Loss Scan	Estàtic (sincronizat amb Q3)		Estàtic (sincronizat amb Q1)

Figura 12: Condicions per a cada experiment de MS/MS.

En el cas de la trampa d'ions, es poden realitzar experiments múltiples sense necessitat de més analitzadors. Són experiments en múltiples etapes (MS^n) realitzats en el temps. Els ions seleccionats es queden en la càmera d'anàlisi i col·lisionen per a generar més ions. Els ions més abundants de cada etapa es fragmenten de nou. Aquest procés permet determinar l'estructura de les molècules que es volen identificar. Aquestes tècniques de múltiples processos de fragmentació permeten descartar en una primera etapa els ions que no interessen.

PART EXPERIMENTAL

III. PART EXPERIMENTAL

1. MÈTODES ANALÍTICS I INSTRUMENTACIÓ UTILIZADA

1.1 Extracció en fase sòlida (SPE)

En aquesta tesi s'ha treballat principalment en la posta a punt de metodologies analítiques mitjançant l'extracció en fase sòlida dels compostos fenòlics, en concret per al resveratrol i els seus metabòlits, els flavanols i els seus metabòlits i, els àcids fenòlics. També s'ha treballat amb la precipitació de proteïnes amb metanol acidulat per a la determinació de les isoflavones i els seus metabòlits.

L'extracció en fase sòlida s'ha portat a terme mitjançant cartutxos Oasis® de Waters (Mildford, Dt., EEUU). Existeixen diverses classes de farciment o sorbents segons el tipus de compost que es pretén estudiar (Figura 13).

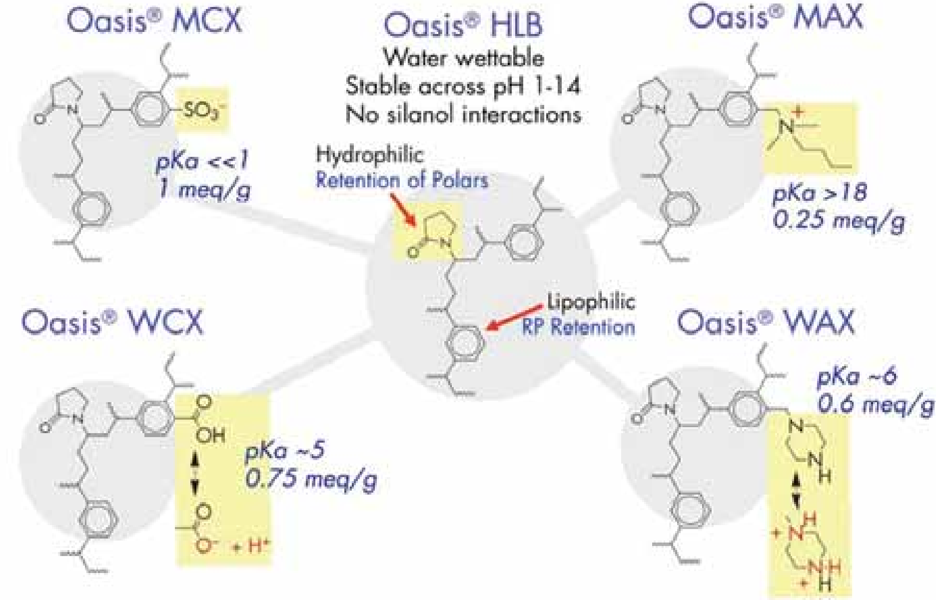


Figura 13: Tipus de farciment Oasis® (Waters)

Abans de provar qualsevol sorbent, s'ha de caracteritzar el compost o compostos a estudiar i després seleccionar el tipus de sorbent més adequat. Els sorbents MAX i

WAX es caracteritzen per posseir activitat d'intercanvi aniònic i fase reversa, els MCX i els WCX en canvi tenen intercanvi catiònic i fase reversa i els HLB només disposen de fase reversa. Per tant, per a compostos àcids o bàsics amb un pka de 2-8 s'hauria de seleccionar els cartutxos Oasis® MCX o MAX; per a àcids forts amb un pka <1.0 s'utilitzarien els WAX i per a bases fortes amb pka >10 s'utilitzarien els WCX. Per a compostos neutres es podria seleccionar qualsevol sorbent.

En el nostre cas, per al resveratrol i els seus metabòlits es van utilitzar els cartutxos HLB en format de placa de 96 pous, es va optimitzar el mètode i es va obtenir el resultat exposat en la Figura 14:

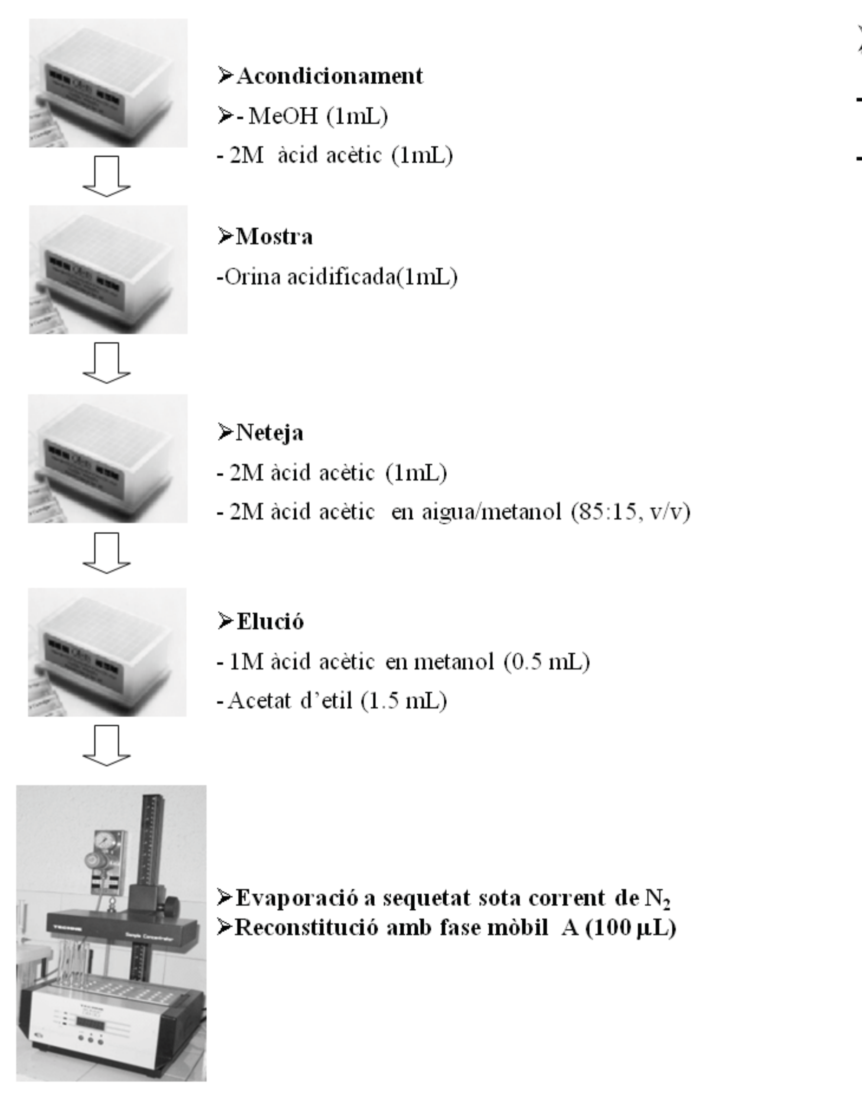


Figura 14: SPE per al resveratrol i els seus metabòlits

Per als àcids fenòlics i els flavanols després de la hidròlisi enzimàtica de la mostra biològica (plasma o orina), es van provar dos tipus de cartutxos: MCX i MAX. En espectrometria de masses es requereix una mostra neta amb quantitats mínimes de compostos interferents que produeixin un efecte matriu i per tant, es disminueixi la ionització dels compostos d'interès. Després de provar ambdós cartutxos, el sorbent MCX va demostrar millors resultats d'ionització per tenir un menor efecte matriu (Figura 15):

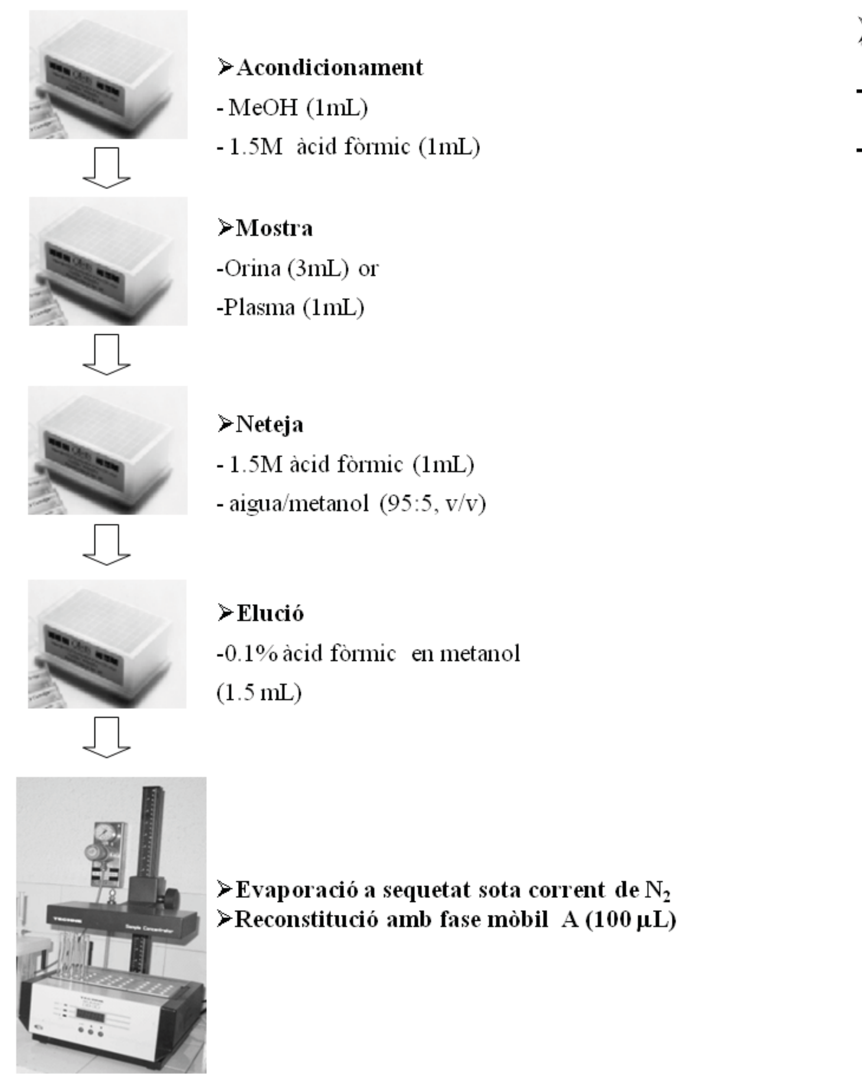


Figura 15: SPE per als àcids fenòlics i flavanols

1.2 Cromatografia líquida d'alta resolució acoblada a espectrometria de masses. El triple quadripol.

En aquesta tesi doctoral s'ha treballat principalment en cromatografia líquida d'alta eficàcia lligada a l'espectròmetre de masses triple quadripol API 3000 d'Applied Biosystems (PE Sciex, Concord, Ontario, Canadà) (Figura 16). Aquest està equipat amb una font d'ionització TurboIonspray (electrospray assistit per nebulització en calent) operant amb nitrogen d'elevada puresa com gas de nebulització, com gas auxiliar i com gas cortina. Per als compostos polifenòlics, normalment es treballa en manera negativa encara que per a alguns compostos, el treballar en manera positiva augmenta la seva ionització millorant la sensibilitat.

Figura 16: Espectròmetre de masses triple quadripol API 3000 amb font d'ionització electrospray.

El triple quadripol consta de tres quadripols en sèrie. El primer i el tercer quadripol, anomenats també Q1 i Q3, actuen com analitzadors, mentre que el segon (Q2) actua com cel·la de col·lisió en mètodes tàndem i com filtre en mètodes Q1 Scan o Q3 Scan o

SIM. Els mètodes en tàndem requereixen la CID (*collision induced dissociation*) en el Q2 .

En el triple quadripol es poden realitzar vuit tipus d'experiments diferents incloent experiments MS o MS/MS els quals són útils per a identificar compostos o metabòlits.

Experiments MS:

Full scan (Q1 *scan* o Q3 *scan*): experiment que escaneja ions en un interval o rang determinat de m/z. Aquesta és la tècnica utilitzada per a fer un escombrat de la mostra i tenir una primera aproximació dels metabòlits que es poden trobar en la mostra analitzada. Però per a la identificació de metabòlits es requereix utilitzar experiments MS/MS.

SIM (*Selected ion monitoring*): medeix un nombre determinat d'ions seleccionats en Q1 o en Q3. Aquest experiment fixa un ió en Q1 o en Q3. Aquest experiment s'utilitza principalment quan es disposa d'un quadripol o quan no es coneixen bé els fragments obtinguts per a una molècula. No dóna informació espectral.

Experiments MS/MS:

Precursor Ion Scan: experiment que escaneja a Q1 els possibles ions precursors d'un determinat ió en Q3. És molt útil quan volem conèixer nous metabòlits que provenen d'un compost conegut.

Product Ion Scan: experiment que aïlla un ió a Q1, ho trenca en la cel·la de col·lisió (Q2) i escaneja els seus fragments en Q3. Aquest és el mètode tàndem d'anàlisi qualitativa per excel·lència. És la tècnica més utilitzada per a identificar/confirmar compostos o metabòlits comparant l'espectre de masses del patró amb el compost de la mostra. A continuació es mostra un exemple d'un *product ion scan* del cis-resveratrol-3-*O*-glucurònid (Figura 17):

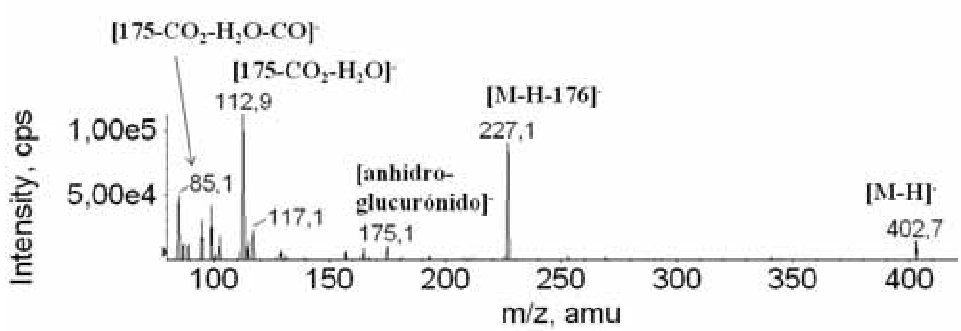


Figura 17: Product ion scan del cis-resveratrol-3-*O*-glucurònid en orina després del consum de vi negre

<u>*Neutral Loss Scan*</u>: experiment que busca tots els parells d'ions precursors i productes que discrepen per una massa neutra constant. Per exemple, els compostos glucuronidats sempre tindran una pèrdua constant de 176 u i els sulfatats de 80 u. En la Figura 18 es mostra un exemple de *neutral loss* del resveratrol-sulfat. El TIC o *total ion chromatogram* indica tots els pics que perden les 80 u. El pic amb una m/z del 307 dóna en l'espectre de masses la massa 307 u.

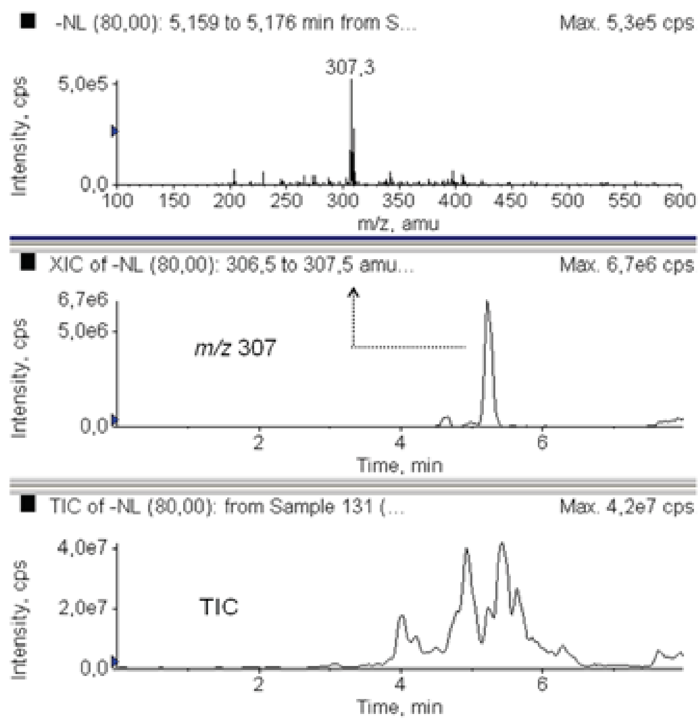


Figura 18: Neutral loss de 80u per al sulfat del resveratrol determinat en orina de voluntaris després de consum de vi negre

MRM (*Multiple Reaction Monitoring*): aquest experiment fixa un ió en Q1 (precursor) i un altre en Q3 (producte). És l'experiment d'anàlisi quantitativa per excel·lència a causa de la seva elevada sensibilitat, encara que no dóna informació espectral. En la Figura 19, es mostra un exemple del metabolisme urinari del resveratrol seleccionant els seus glucurònids (403/227), sulfats (307/227) i el resveratrol (227/185):

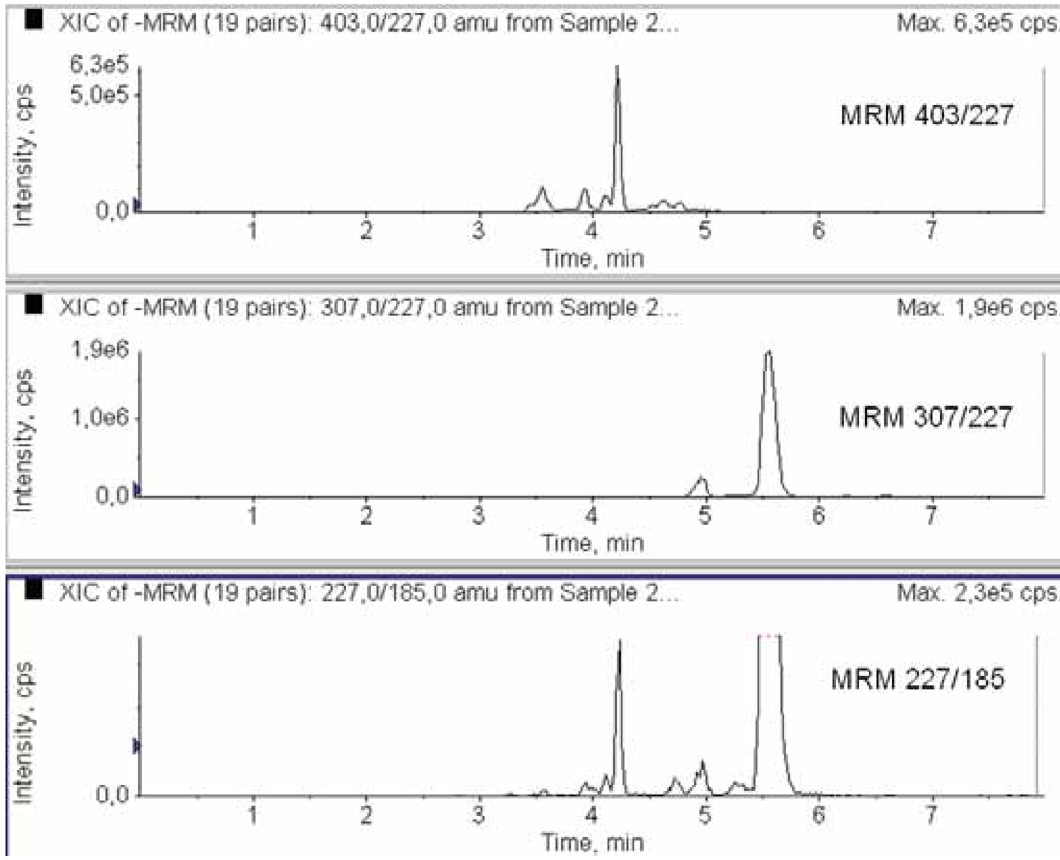


Figura 19: MRM del perfil metabòlic del resveratrol: glucurònids (403/227), sulfats (307/227) i del resveratrol (227/185) en orina després del consum de vi negre

1.2.1 Eines per a la identificació de metabòlits per LC-MS/MS

Tot procés metabòlic per a xenobiòtics tendeix a augmentar la solubilitat, polaritat i la hidrofilia dels compostos facilitant així la seva eliminació. Això resulta en un augment de la polaritat que en cromatografia de fase reversa es transforma que aquests metabòlits tindran un temps de retenció menor que el seu compost pare. Però existeixen algunes excepcions com l'acilació (Nobilis et al. 2004) i la metilació entre unes altres. Encara que existeix aquesta tendència en el comportament del temps de retenció dels metabòlits, existeixen diversos factors que també poden provocar variacions com són la composició de la fase mòbil, el gradient, la columna utilitzada, la isomerització posicional i l'estructura del compost.

En la majoria de casos, en el procés d'identificació de metabòlits, l'estructura del compost pare és coneguda, per tant, el comportament de la ionització i de la fragmentació es poden estudiar primer amb el compost pare i després, amb estàndards de metabòlits si són disponibles. Després, aquest coneixement s'aplicarà per analogia per a la identificació de metabòlits.

Des del punt de vista de l'espectrometria de masses les reaccions metabòliques poden traduir-se en un canvi característic en el pes molecular dels metabòlits el qual és la primera indicació per a la seva identificació.

L'espectrometria de masses no és aplicable per a distingir isòmers posicionals (excepte per a isòmers amb grups funcionals en anells diferents (Lampinen-Salomonsson et al. 2006), estereoisòmers i enantiòmers, per tant es recomana el reforç d'altres tècniques de separació i espectrals (Prakash et al. 2007). Una tècnica establerta per a estudis metabòlics *in vivo* és unir el compost amb isòtops radioactius com el ^{14}C o el ^{3}H (Nassar et al. 2003) on la detecció per comptador de radioactivitat ajuda positivament a identificar metabòlits provinents del compost pare. Posteriorment, els metabòlits detectats per comptador de radioactivitat s'identifiquen per tècniques de LC-MS/MS i per LC-NMR (Shaffer et al. 2005). Actualment s'estan desenvolupant tècniques de derivatització post-columna amb cobalt per a determinar la posició dels glucurònids en alguns polifenols com les flavanones (Davis et al. 2006).

Reaccions de fase II per LC-MS/MS

Les principals reaccions que s'han estudiat en la present tesi són reaccions de fase II degut al fet que els polifenols es troben majoritàriament en plasma i orina conjugats pel tipus de reaccions que s'expliquen a continuació.

La glucuronidació és la major reacció metabòlica de fase II en vertebrats. El lloc de glucuronidació és normalment un heteroàtom (O, N, S) nucleòfil ric en electrons (Holcapek et al. 2008). La segona biotransformació més important és la conjugació amb sulfat en grups hidroxils o amines. Aquestes dues reaccions poden ocórrer múltiples vegades formant per exemple diglucurònids o disulfats.

La Taula 1 resumeix les reaccions de biotransformació més comunes i més estudiades en animals i humans. Les reaccions metabòliques poden ocórrer múltiples vegades o en combinació amb altres reaccions encara que aquestes últimes no s'han inclòs aquí. Si un compost té ambdós conjugats sulfat i glucurònid, llavors el glucurònid té una retenció més baixa que el corresponent sulfat en la mateixa posició (Chen et al. 2005a). Però aquesta regla no és generalment aplicada a conjugats en diferents posicions degut al fet que la posició de conjugació té un efecte igual o superior en la retenció. La formació d'aquests conjugats afecta a la seva polaritat, a la retenció cromatogràfica, a la seva ionització i a la seva fragmentació (Levsen et al. 2005). Els compostos de fase II normalment es poden identificar en funció del seu increment de massa, massa exacta, i també per la seva pèrdua característica (Taula 1).

Reacció de conjugació	Substituient	Diferencia de massa nominal (ΔDa)	Pèrdua característica	Massa de la pèrdua neutra de la $[M+H]^+$	Massa de la pèrdua neutra de la $[M-H]^-$
Metilació	CH_3	+14	Radical metilo	15	15
Acetilació	$COCH_3$	+42	Keteno	42	42
Conjugació amb glicina	C_2H_3ON	+57	Glicina	75	
			$CO + H_2O$	46	44
Sulfatació	SO_3H	+80	SO_3	80	80
Conjugació amb cisteïna	$C_3H_5O_2NS$	+119	cisteína	121	121
Conjugació con *N*-acetilcisteïna	$C_5H_7O_3NS$	+161	NAcCys	163	163
Glucosilació	$C_6H_{10}O_5$	+162	Gluc anhidro	162	162
Glucuronidació	$C_6H_8O_6$	+176	GlucA anhidro	176	176
Cisteína-glicina	Gly-cisteinil	+176	Cis-Gli	178	176
Conjugació amb glutatió	$C_{10}H_{15}O_6N_3S$	+305	GSH	307	306

Taula 1: Reaccions metabòliques de fase II més comunes del comportament en espectrometria de masses

Tal i com s'aprecia en la Taula 1, existeixen diverses reaccions de conjugació. En aquesta memòria aprofundirem en les tres més comunament estudiades i majoritàries per als polifenols: la glucuronidació, la sulfatació i la metilació.

Els mètodes per a la separació dels glucurònids s'han revisat recentment. Depenent de la composició de la fase mòbil, es pot observar la formació d'adductes de $[M+NH_4]^+$, $[M+Na]^+$ i $[M+K]^+$ a més de la molècula protonada $[M+H]^+$, mentre que en manera negativa preval la molècula desprotonada $[M-H]^-$ (Nobilis et al. 2004). La pèrdua neutra més característica de tots els glucurònids és la pèrdua de l'àcid glucurònic anhidre (*m/z* 176) que s'observa en ambdós maneres. Aquesta diferència de massa (*Δ m/z* 176) es pot utilitzar en mode de *selected reaction monitoring* (SRM) per a la quantificació de glucurònids o per a una detecció més sensible dels glucurònids en mostres biològiques complexes. La identificació de la posició de conjugació en masses és una tasca difícil i la diferenciació s'aconsegueix només per a conjugats en anells aromàtics. Recentment s'ha descrit un sistema interessant per a diferenciar entre glucurònids units a grups hidroxils o a grups acils que està basat en la presència d'ions $[M-H-CO_2]^-$ per a glucurònids units a grups hidroxils i absència d'aquests ions per a glucurònids acilats (Jaggi et al. 2002). Encara que no està molt clar si aquesta regla pot ser generalitzada a

causa de l'absència d'altres referències. Altres maneres per a determinar la posició de conjugació es basen en derivatitzacions químiques amb de 2-cloro-1-metilpiridina iodat (Lampinen-Salomonsson et al. 2006) o per la formació d'adductes amb cobalt i amb fragmentacions característiques (Davis et al. 2006). En general, l'espectre obtingut per ionització en manera negativa conté principalment la molècula desprotonada $[M-H]^-$, l'àcid anhidroglucurònic desprotonat amb una *m/z* 175 així com subseqüents pèrdues de CO_2 i H_2O (*m/z* 113) i CO (*m/z* 85) (Nobilis et al. 2004; Levsen et al. 2005).

Els conjugats amb sulfat solament poden ser ionitzats intactes utilitzant el mètode en electrospray (Keski-Hynnila et al. 2002). La manera electrospray en negatiu és preferible encara que hi ha alguns estudis en ionització en positiu (Keski-Hynnila et al. 2002). La introducció d'un grup sulfat incrementa la *m/z* del metabòlit en 80. La pèrdua típica per a la conjugació amb sulfat (SO_3) és de *m/z* 80, la conjugació amb SO_2 és de *m/z* 64 i menys freqüent la H_2SO_4 és de *m/z* 98 (Guan et al. 2003).

La conjugació per metilació redueix la polaritat dels metabòlits. Els grups amino, els grups fenòlics en compostos que contenen grups dihidroxifenils i també grups tiol es metilen per reaccions de metilació de fase II. La conjugació d'aquests metabòlits es reflecteix per l'increment de massa de 14 Da. Si un anell fenil conté dos grups hidroxils, com és el cas d'un flavanol com l'epicatequina, les dades de masses poden provar que existeix la metilació en un grup hidroxil però normalment no és possible diferenciar entre els dos llocs de metilació ja que els espectres de masses dels isòmers són gairebé indistingibles. Els metabòlits de polifenols de fase II formats per metilació no tenen normalment una pèrdua característica degut al fet que la fragmentació del grup metil només apareix en absència d'altre grup polar funcional en la molècula. Per exemple, la *N*-metilanalina produeix una pèrdua neutra del radical metil (Levsen et al. 2005) i en canvi no es produeix per a la 4'-metil-epicatequin-7-*O*-glucurónido (Schroeter et al. 2006).

1.3 Cromatografia líquida d'alta resolució acoblada a detector electroquímic. El coularray.

Els detectors electroquímics medeixen les propietats químiques dels compostos, no les propietats físiques com ho fa l'absorció UV. L'electroquímica inclou reaccions químiques en les quals els electrons es transfereixen d'un compost a un altre. Per tant, l'electroquímica és un mètode destructiu on pocs compostos poden tornar a l'estructura original una vegada han estat oxidats.

A continuació es mostren les reaccions típiques per a la parella redox hidroquinona (HQ)/quinona (Q):

HQ → Q + 2e- + 2H+ (oxidació)

Q + 2e- + 2H+ → HQ (reducció)

L'anàlisi electroquímica està afectada per una reacció a un temps, que normalment és l'oxidació i aquesta energia s'obté en forma de potencial elèctric. Aquest potencial s'aplica directament en l'elèctrode i, quan els compostos elueixen de la columna cromatogràfica, entren en contacte amb aquest elèctrode i sofreixen un potencial suficient per a conduir la reacció, llavors, té lloc una reordenació molecular en la superfície de l'elèctrode amb una pèrdua (oxidació) o un guany (reducció) d'electrons. Els electrons es detecten a temps real. Depenent del disseny de l'elèctrode que treballa, el detector electroquímic pot ser amperomètric o coulomètric.

El detector electroquímic utilitzat en els treballs de la present memòria ha estat un detector colorimètric o CoulArray (model 5600, Eurosep, Cergy, Francia) (Figura 20).

Figura 20: CoulArray (model 5600, Eurosep, Cergy, Francia) amb les seves bombes, injector i electrodes en sèrie

En un detector electroquímic els compostos flueixen a través de l'elèctrode constituït per porus de grafit. A causa de l'elevada superfície de contacte que presenten els elèctrodes, el 100% dels compostos que entren s’oxiden (o redueixen) sent la magnitud de la quantitat detectada proporcional a la quantitat injectada del compost. Amb els mètodes electroquímics es poden analitzar una àmplia varietat de compostos però l’electroactivitat depèn de la presència de grups funcionals electroactius com són els grups hidroxils dels fenols o grups aminos d’anilines.

El potencial utilitzat per a un analit és important. Cada compost es caracteritza per un potencial òptim d'oxidació o reducció. Aquest potencial depèn de diversos factors que inclouen la naturalesa de la superfície dels elèctrodes, el pH, la composició de la fase mòbil especialment la seva força iònica, i per descomptat l'estructura química dels compostos a estudiar. El potencial òptim es pot determinar amidant l'oxidació (o la

reducció) obtinguda a una concentració constant d'un analit conegut en un rang de potencials d'elèctrodes.

Els detectors electroquímics es dissenyen normalment amb dos o més elèctrodes. Això és molt útil per a identificar compostos que poden coeluir però que tenen diferent oxidació o reducció i a més eliminar compostos interferents.

El detector electroquímic utilitzat en aquest treball conté dues cel·les col·locades en sèrie i cadascuna d'elles conté quatre porus de grafit amb elèctrodes amb un elèctrode de referència de pal·ladi. S'apliquen diferents potencials sobre aquests elèctrodes, per tant es recolliran vuit cromatogrames simultàniament (Figura 21). En el cas de les isoflavones hem utilitzat els potencials a 200, 280, 450, 550, 600, 650, 700 i 750 mV. En la majoria de casos, els analits que elueixen de la columna s'oxiden en tres o més elèctrodes successius. Encara que cada elèctrode té un 100% d'eficiència, l'analit reacciona a través dels elèctrodes adjacents degut al fet que cap d'ells es posa al seu potencial màxim d'oxidació.

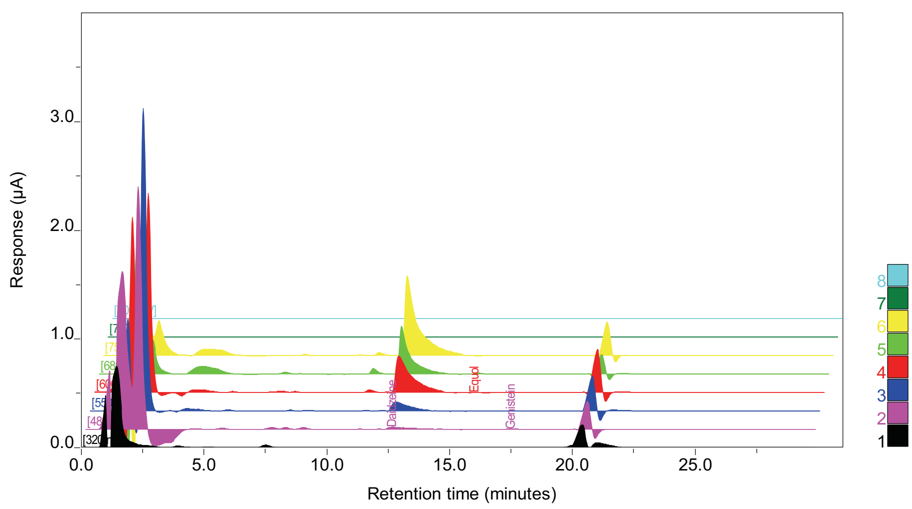


Figura 21: Cromatograma obtingut per a l'estàndard de daidzeina

Els compostos desconeguts presents en les mostres es podran identificar comparant-los amb estàndards purs coneguts, basant-nos en el temps de retenció i en el seu comportament electroquímic. Si existeixen diferències en els ràtios observats dels compostos desconeguts i de l'estàndard significa que existeix alguna contaminació, o

coelució d'algun compost desconegut o que el compost que estem determinant no és el mateix.

RESULTATS

IV. RESULTATS

En aquesta secció s'exposen els resultats obtinguts durant la tesi doctoral en funció dels diferents objectius i tenint en compte el compost fenòlic considerat en cadascun d'ells. Tots aquests resultats de la tesi doctoral queden recollits en 5 treballs publicats en revistes del *Science Citation Index* i 2 treballs actualment en fase de revisió. Previ a cada publicació hi ha un resum on s'exposen els objectius, el disseny experimental, la metodologia i els seus principals resultats i conclusions.

1. RESVERATROL

1.1 Desenvolupament i validació d'un mètode analític dirigit reproduïble, sensible i ràpid per espectrometria de masses per a determinar i quantificar el perfil metabòlic del resveratrol en mostres biològiques després d'un consum dietètic de resveratrol.

Publicació I: Ingesta de resveratrol dietètic i estudi de teixits diana (LDL). Identificació i quantificació de metabòlits del resveratrol.

Mireia Urpi-Sarda, Olga Jauregui, Rosa Lamuela-Raventos, Walter Jaeger, Mikaela Miksits, Maria Isabel Covas, Cristina Andres-Lacueva. Uptake of diet resveratrol into the human low-density lipoprotein. Identification and quantification of resveratrol metabolites by liquid chromatography coupled with tandem mass spectrometry. *Analytical Chemistry*. **2005**, 77 (10): 3149-55

Resum:

L'objectiu d'aquest treball ha estat desenvolupar un mètode analític sensible i selectiu per a la identificació i quantificació de metabòlits del resveratrol en lipoproteïna de baixa densitat després del consum moderat de vi negre per espectrometria de masses en tàndem (LC-MS/MS).

Per a la posta a punt de la metodologia per a la identificació del perfil metabòlic del resveratrol en LDL es van estudiar i van avaluar els tres mètodes clàssics d'extracció. Aquests van anar: extracció líquid-líquid, precipitació de proteïnes i extracció en fase sòlida. Es va seleccionar l'extracció en fase sòlida degut al fet que presentava un menor efecte matriu i una màxima sensibilitat en mostres de LDL per espectrometria de masses. Després d'optimitzar els paràmetres de l'espectròmetre de masses per infusió i *flow injection analysis* (FIA) del resveratrol es va procedir a la validació del mètode. Els valors de precisió i exactitud per al resveratrol van ser menors del 6%, les corbes de calibrat van ser lineals per al rang entre 0.44-438.6 pmol/mL, i el mètode va permetre uns límits de detecció i quantificació en LDL de 0.15 i 0.44 pmol/mL, respectivament.

L'estudi va consistir en 11 voluntaris que van prendre un consum moderat de vi negre de la varietat Merlot (250 ml) en dejú i després de 10 dies d'una dieta pobra en polifenols. Les mostres de LDL van ser obtingudes abans del consum i 24 hores després del consum moderat de vi negre (Figura 22).

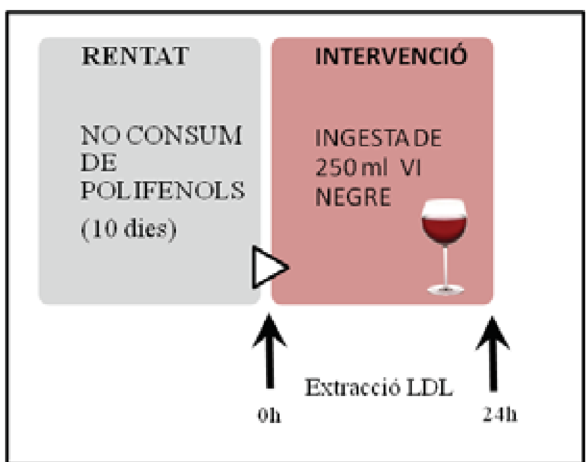


Figura 22: Esquema de l'estudi

Es van identificar i van quantificar en LDL diversos metabòlits: el trans-resveratrol-3-*O*-glucurònid, el cis-resveratrol-3-*O*-glucurònid, el cis-resveratrol-3-*O*-glucòsid, i el *trans*-resveratrol lliure.

Aquesta va ser la primera vegada que es van identificar i van quantificar el resveratrol i els seus metabòlits en LDL humana després del consum moderat de vi negre. Això suggereix que aquests compostos podrien provocar efectes antioxidants sobre la LDL i per tant prevenir la seva oxidació podent actuar sobre la malaltia cardiovascular.

Anal. Chem. **2005**, *77*, 3149−3155

Uptake of Diet Resveratrol into the Human Low-Density Lipoprotein. Identification and Quantification of Resveratrol Metabolites by Liquid Chromatography Coupled with Tandem Mass Spectrometry

Mireia Urpí-Sardà,† Olga Jáuregui,‡ Rosa Maria Lamuela-Raventós,† Walter Jaeger,§ Michaela Miksits,§ María-Isabel Covas,⊥ and Cristina Andres-Lacueva*,†

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In this paper, a sensitive, precise, and selective analytical method has been developed for the identification and quantification of resveratrol metabolites in human low-density lipoprotein (LDL) after moderate consumption of red wine, using high-performance liquid chromatography electrospray in tandem mass spectrometry (LC−ESI-MS/MS). From different extraction procedures tested, solid-phase extraction was selected to minimize matrix effects reaching the highest sensitivity. Standard calibration curves prepared in human LDL for *trans*-resveratrol were linear over a range of 0.44−438.59 pmol/mL. The accuracy and interassay precision of this LC−MS/MS assay for resveratrol showed a coefficient of variation of <6.0%. The method allows detection and quantification limits for resveratrol in LDL at 0.15 and 0.44 pmol/mL, respectively. Results to date indicate that resveratrol metabolites were incorporated into LDL after a moderate intake of red wine. The metabolites identified in LDL were *trans*-resveratrol-3-*O*-glucuronide, *cis*-resveratrol-3-*O*-glucuronide, and *cis*-resveratrol-3-*O*-glucoside, as well as free *trans*-resveratrol. To our knowledge, it is the first time that a polyphenol from red wine, specifically resveratrol, has been identified in human LDL after moderate intake of red wine. Furthermore, these findings suggest that these compounds may deliver their antioxidant effect to LDL.

Resveratrol is a stilbenic phenolic compound mainly present in grapes and wine. In red wine, resveratrol occurs free, and as glucoside in their respective isomeric forms (Figure 1). Resveratrol may contribute to the cardioprotective effect of red wine because it has been shown to inhibit low-density lipoprotein (LDL) oxidation, lipid peroxidation, platelet aggregation, and eicosanoid synthesis,[1] the initial stage of pathogenesis of atherosclerosis.[2] Oxidation of LDL is a hallmark for atherosclerosis development.[3]

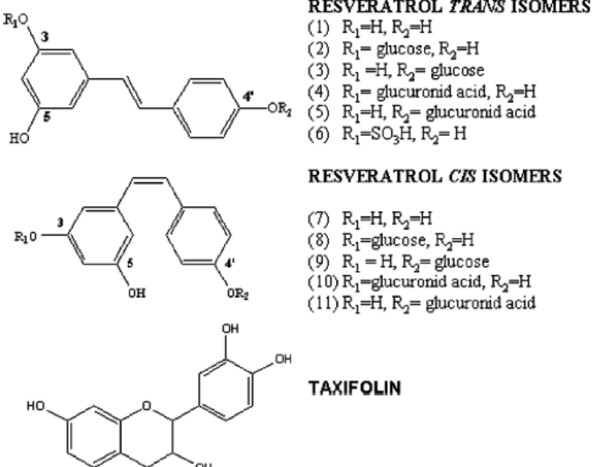

Figure 1. Structures of resveratrol, resveratrol metabolites, and taxifolin (internal standard): (1) *trans*-resveratrol, (2) *trans*-resveratrol-3-*O*-glucoside (*trans*-piceid), (3) *trans*-resveratrol-4′-*O*-glucoside, (4) *trans*-resveratrol-3-*O*-glucuronide, (5) *trans*-resveratrol-4′-*O*-glucuronide, (6) trans-resveratrol-3-sulfate, (7) *cis*-resveratrol, (8) *cis*-resveratrol-3-*O*-glucoside (*cis*-piceid), (9) *cis*-resveratrol-4′-*O*-glucoside, (10) *cis*-resveratrol-3-*O*-glucuronide, and (11) *cis*-resveratrol-4′-*O*-glucuronide.

In LDL, some compounds have already been described, such as vitamin E (α-tocopherol), retinol, β-carotene,[4] tyrosol, and hydroxytyrosol.[5] In quantitative terms, lipid-soluble α-tocopherol is the major antioxidant among those present in LDL at levels[4] up to 18.44 ± 4.7 nmol/mg LDL protein. Thus, it is considered

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⊥ ULEC-IMIM.

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the first line of defense against oxidation. Retinol and β-carotene are present in lesser quantities in LDL particles at levels[4] of 0.35 ± 0.035 nmol/mg LDL protein and 0.187 ± 0.019 nmol/mg LDL protein, respectively. After olive oil intake, LDL tyrosol and hydroxytyrosol presented even lower concentrations, with maximum levels[5] in females of 93.11 ± 93.11 and 87.93 ± 85.35 pmol/mg cholesterol, respectively, and major levels in male volunteers of 3.71 ± 3.71 nmol/mg cholesterol for tyrosol observing great variability among subjects.[5] In vitro studies showed the affinity of resveratrol for pig lipoproteins with a higher lipid content.[6] The majority of studies examine the effect of total polyphenols of red wine in LDL oxidation in vitro,[7] and in vivo studies[8] have shown that red wine consumption increased total plasma polyphenols and enhanced antioxidant activity.

Phenolic compounds are the subject of an extremely extensive first pass intestinal/hepatic metabolism in the human body. After ingestion of phenolic-rich food, the main forms present in plasma and urine are metabolites of the primary species present in the ingested food.[9] Thus, the target level of the available compound, the metabolite, will determine the biological activity.[10,11]

Various methods for the determination of resveratrol and its metabolites (glucuronides and sulfates) in biological matrixes have been reported, including GC/MS,[12] LC–MS,[13,14] and LC–MS/MS[15] methods. To our knowledge, bioavailability studies quantified resveratrol metabolites after enzymatic hydrolysis,[12,13,15–17] not giving any information about the resveratrol metabolite profile. However, this profile has been studied in vitro and in vivo in human serum after intake of 1 g of resveratrol,[11] the results describing the glucurono conjugation in two major forms (3- and 4′-*O*-glucuronides), the preferred position being at C-3,[18] the *trans*-resveratrol-3-sulfate,[15] and the resveratrol in free form.[12] The low expected concentration (pmol/mL) of resveratrol[19] in human LDL requires a very sensitive and selective technique, such as liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) after an adequate extraction procedure to minimize matrix effects.[20] LC–MS/MS has emerged as the preferred technology for quantitative determination of metabolites in biomatrixes.[21]

Polyphenols from red wine can inhibit the oxidation of human LDL.[8] However, to our knowledge, there are no studies describing which specific phenols from wine reach and bond to the LDL. Phenolic compounds which could bind LDL would have a greater accessibility to lipid peroxyl radicals within LDL particles and would be likely to exert their peroxyl scavenging activity in the arterial intima, where oxidation of LDL commonly occurs in microdomains sequestered from antioxidants of plasma.[3] The primary aim of this study was to develop a rigorous and sensitive method for the identification of low concentrations (pmol/mL) of resveratrol and its metabolites. Therefore, we investigated the quantitative binding of resveratrol and its metabolites in the LDL of human volunteers after a moderate intake of red wine.

EXPERIMENTAL SECTION

Standards and Reagents. Standards of *trans*-resveratrol (99% purity), *trans*-3,4′,5-trihydroxystilbene-3-β-D-glucopyranoside (*trans*-piceid) (97% purity), and LDL from human plasma were purchased from Sigma-Aldrich (St. Louis, MO). The internal standard (i.s.) taxifolin (purity >90%) was purchased from Extrasynthese (Genay, France). Methanol and acetonitrile of HPLC grade were purchased from SDS (Peypin, France); glacial acetic acid, ethyl acetate, ammonium acetate, and *o*-phosphoric acid from Panreac (Barcelona, Spain); and formic acid from Sigma-Aldrich Co. (Steinheim, Germany). Ultrapure water (Milli-Q) was obtained from a Millipore System (Bedford, MA). All the standards were prepared as stock solutions at 4.39 μM in 80% (v/v) methanol.

Biochemical Synthesis of Resveratrol Metabolites. Livers of male Wistar rats (223–261 g), raised at the Institut für Versuchstierzucht und -haltung (University of Vienna, Himberg, Austria), were perfused with 20 μM of *trans*-resveratrol in a recirculating system as described previously.[22] Bile samples were collected over a time period of 60 min.

Structural Identification of Isolated Metabolites. Bile samples (360–600 μL) were diluted with distilled water (1:2; v/v), and aliquots (200 μL) were injected onto a Hypersil C18 column (10 μm, 250 × 10 mm i.d., Astmoor, England) preceded by a Hypersil C18 guard column (10 μm, 10 × 3 mm i.d.) at a flow rate of 5 mL/min. HPLC analysis was performed using a Merck "La Chrom" System (Merck, Darmstadt, Germany) equipped with an L-7250 injector, an L-7100 pump, a D-7000 interface, and an L-7400 UV detector set at a wavelength of 307 nm. Gradient elution was carried out with 10 mM ammonium acetate/acetic acid buffer, pH 5.0, and methanol. The linear gradient profile with the following proportions (v/v) of methanol was applied (*t* (min), % MeOH): (0, 10), (10, 20), (22, 60), (25, 60), (27, 10). The column was reequilibrated for 13 min between runs. The peaks corresponding to *trans*-resveratrol-3-*O*-glucuronide and *trans*-resveratrol-3-sulfate from each chromatographic run were collected, pooled, and lyophilized. Structures were confirmed by nuclear magnetic

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resonance (NMR) measurements. Spectra were recorded on a Varian UnityInova (Varian, Palo Alto, CA) 600-MHz instrument, and the following data were obtained.

trans-Resveratrol-3-*O*-glucuronide: ^{1}H NMR (CD_3OD) δ 6.77 (d, J = 8.6 Hz, H-3′,5′), 7.36 (d, J = 8.6 Hz, H-2′,6′), 6.98 (d, J = 6.5 Hz, *trans*-vinyl), 6.85 (d, J = 16.5 Hz, *trans*-vinyl), 6.77 (tr, J = 1.9 Hz, H-6), 6.52 (tr, J = 1.9 Hz, H-4), 6.62 (tr, J = 1.9 Hz, H-2).

Resveratrol-3-sulfate: ^{1}H NMR (CD_3OD) δ 6.80 (d, J = 8.5 Hz, H-3′,5′), 7.38 (d, J = 8.5 Hz, H-2′,6′), 7.06 (d, J = 16.3 Hz, *trans*-vinyl), 6.90 (d, J = 16.3 Hz, *trans*-vinyl), 6.77 (tr, J = 1.7 Hz, H-6), 6.69 (tr, J = 1.7 Hz, H-4), 7.01 (tr, J = 1.7 Hz, H-2).

Subjects and Study Design. Eleven healthy male volunteers (aged 18−50), nonsmokers (or ex-smokers for more than 1 year) were recruited. All volunteers could be considered healthy on the basis of a physical examination and standard biochemical and hematological tests. Subjects had an average weight of 83.2 ± 4.4 kg and a body mass index of 25.9 ± 3.29 kg/m^2.

Subjects with any of the following conditions were excluded from the study: intake of antioxidant supplements; intake of acetosalicylic acid; intake of any other drug with established antioxidative properties; obesity (body mass index > 30 kg/m^2); physical activity greater than or equal to 3000 kcal/week; drug-addiction, including alcohol ingestion higher than 20 g/day; and any condition that limited mobility.

The study was done in accordance with the Helsinki Declaration of 1975, as revised in 1996. The ethical committee (CEIC-IMAS) approved the protocol and participants signed an informed consent. Exercise was monitored with the Minnesota Leisure Time Physical Activity Questionnaire, which has been validated for Spanish males.[23]

Red wine of the Merlot variety was selected because of the major amount of total resveratrol (*trans* and *cis*-resveratrol and *trans* and *cis*-piceid), as compared with other red varieties,[24] white wines,[25] and sparkling wines.[26]

Before wine administration, volunteers followed a 10-day washout period in which from day 1 to 7, volunteerers followed a controlled diet, avoiding an excess of antioxidant consumption. Three days before and on the intervention day, volunteers followed a strict very low phenolic compound diet. A nutritionist instructed them on excluding several foods from their diet (coffee, tea, fruit, vegetables, peanuts, soy, wine, grape juice, and olive oil). On the intervention day at 8 a.m. at fasting, they were provided with a single dose of 250 mL of red wine. At 6 h, a meat and starch-based diet was provided in the Centre. EDTA blood was collected at baseline and at 24 h after wine administration according to Miyagi et al.[27]

LDL Samples. EDTA plasma was separated by centrifugation at 1000*g* at 4 °C for 15 min. LDL was isolated by sequential flotation ultracentrifugation.[28] All LDL samples were immediately frozen and stored at −80°C. Immediately prior to analysis, we thawed them. The freeze and thaw stability of resveratrol had been checked.[29] The long-term stability of the resveratrol glucuronide in the rat bile extract (1/100) stored at −80 °C was checked for up to 1 year; the relative standard deviation (RSD (%)) was 4.7% (n = 5). Protein content was determined by the red pirogalol method (Sigma, St. Louis, MO).

Sample Extraction Procedures. Three common sample preparation methods were tested: solid-phase extraction (SPE), liquid−liquid extraction using ethyl acetate, and protein precipitation (n = 5 for each one). The main objective was to reach the higest sensitivity while minimizing the matrix effect. This parameter has become highly recommended in bioanalytical method development by LC−MS/MS using electrospray ionization.

Since resveratrol and its metabolites are liable to oxidation in light conditions, extraction procedures were carried out in the dark.

Before each procedure, 20 μL of *o*-phosphoric acid 85% (v/v) was added to 1-mL aliquots of LDL and mixed in a vortex for 2 min.

SPE was performed using a Visidry Drying Attachment from Supelco (Bellefonte, PA). Acidified LDL was loaded onto an activated Oasis HLB (60 mg) cartridge from Waters (Mildford, MA). To reduce interfering components, the cartridges were then washed with 2 mL of 1 M acetic acid and with 2 mL of 5% methanol in 1 M acetic acid. Resveratrol and its metabolites were eluted with 1 mL of 1 M acetic acid in methanol, followed by 2 mL of ethyl acetate.

Liquid−liquid extraction with ethyl acetate was carried out as described by Soleas et al.[12] Protein precipitation samples were prepared with acidified methanol (200 mM), widely used in biological analysis, according to Morand et al.[30]

All the final organic solutions were evaporated in a Techne sample concentrator (Duxford, Cambridge, England) at 25 °C under a stream of nitrogen. Residues were immediately reconstituted in chromatographic initial conditions up to 100 μL. Then, taxifolin was added at 65.79 pmol/mL as an additional standard to assess the performance of the mass spectrometer. All the samples were filtered through PTFE 4-mm filters, 0.45 μm, from Waters (Mildford, MA) into amber vials for LC−MS/MS analysis.

Instrumentation. *LC−DAD.* Analysis of resveratrol and piceid in red wine and the preliminary recovery analysis of the extraction procedures were carried out in a Hewlett-Packard (HP) 1050 (Palo Alto, CA) liquid chromatograph equipped with an automatic injector, HP 1050, and a HP diode array, 1050 M, at 280 and 306 nm. HPLC conditions were as described previously.[24] Briefly, this method consisted of a gradient profile with 1.5 mL/min in a Tracer Nucleosil column C_{18} 120 (250 × 4 mm i.d., 5 μm) maintained at 40°C. Volume injected was 100 μL.

LC−MS/MS. Identification and quantification of resveratrol metabolites were carried out by LC−MS/MS analysis of rat bile and human LDL samples. LC analyses were performed using a Perkin-Elmer series 200 (Norwalk, CT) equipped with a quaternary pump and an autosampler. A triple quadrupole mass spectrometer, API 3000 (Applied Biosystems, PE Sciex, Concord, Ontario, Canada), equipped with a Turbo IonSpray source was

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Table 1. Description of Molecular Weight, Retention Time, Multiple Reaction Monitoring Transitions in Negative Mode, and Quantification in pmol Resveratrol/mg LDL Protein of Resveratrol and Metabolites in LDL Samples

compd	peak no.[a]	M_W	R_t (min)	MS/MS ions (m/z)	pmol resveratrol/mg LDL protein[a]
trans-resveratrol-3-*O*-glucoside	2	390	8.20	389 → 227	n.d.
trans-resveratrol-4′-*O*-glucoside	3	390	9.48	389 → 227	1.08–28.47
taxifolin	i.s.	304	10.20	303 → 285	i.s.
resveratrol-4′-*O*-glucuronide	5/11	404	11.10	403 → 227	7.14–69.39
cis-resveratrol-4′-*O*-glucoside	9	390	12.89	389 → 227	n.d.
cis-resveratrol-3-*O*-glucoside	8	390	13.32	389 → 227	n.q.
trans-resveratrol-3-*O*-glucuronide	4	404	14.69	403 → 227	2.08–278.32
trans-resveratrol	1	228	14.86	227 → 185	1.43–8.76
cis-resveratrol	7	228	16.35	227 → 185	n.d.
cis-resveratrol-3-*O*-glucuronide	10	404	16.58	403 → 227	3.78–12.18
trans-resveratrol-3-sulfate	6	308	30.37	307 → 227	n.q.

[a] i.s.: internal standard; n.d.: not detected; n.q.: not quantificable.

used to obtain the MS and MS/MS data. Prior to its use, the instrument was checked to meet the acceptance specifications defined by the manufacturer. The triple quadrupole mass spectrometer was calibrated with the Turbo IonSpray using a test mixture solution of poly(propyleneglycol) obtained from Applied Biosystems. The mass spectrometer was calibrated so that mass accuracy specifications and sensitivity were achieved over the entire mass range. A Luna C_{18} column (150 × 2.0 mm i.d., 5 μm) from Phenomenex (Torrance, CA) was used at room temperature, and the injected volume was 40 μL. Gradient elution was carried out with acetic acid of 0.05% and acetonitrile at a constant flow rate of 400μl/min. The linear gradient profile with the following proportions (v/v) of acetonitrile was applied (t (min), % acetonitrile): (0, 15), (2, 15), (10, 40), (15, 55), (15.5, 100), and (38, 100). The column was reequilibrated for 15 min between runs. Full-scan data acquisition was performed by scanning from m/z 100 to 600 in profile mode, using a cycle time of 2 s with a step size of 0.1 u and a pause between scans of 2 ms.

For MS/MS, product ion scan utilizing a cycle time of 2 s was used. To identify the characteristic ions for each compound, standards were injected in product ion scan mode of the $[M - H]^-$ ion. Both the spectra generated for *trans*-resveratrol and the available metabolite in the negative ion mode gave the deprotonated molecule $[M - H]^-$ and some fragment, even at relatively low declustering potentials. *trans*-Resveratrol-3-*O*-glucoside showed the deprotonated molecule (m/z 389) and the neutral loss of the glucose moiety (−162 u) from the glucoside (m/z 227). No ions characteristic of the sugar part were observed in the negative ion mode. Similarly, the glucuronide derivatives showed as characteristic ion the cleavage of the glucuronide unit (−176 u). For sulfates, the loss of the sulfate group (−80 u) is proven to be the main characteristic fragmentation pathway. The multiple reaction monitoring (MRM), the method of choice due to the highest selectivity and sensitivity in LC−MS/MS,[31] monitored five transitions for each analysis: resveratrol, m/z 227 → 185; resveratrol glucosides, m/z 389 → 227; resveratrol glucuronides, m/z 403 → 227; resveratrol sulfates, m/z 307 → 227; taxifolin, m/z 303 → 285 (Table 1) with a dwell time for each transition of 350 ms, and a pause between mass ranges of 5 ms. The criterion for identification of resveratrol metabolites was retention time, if possible, the MRM transition above-mentioned, and because no other fragmentations were observed for the studied compound, the transition 227 → 185 (at a higher DP value) was chosen as confirmation MRM trace for each metabolite in CID-MS/MS (collisionally induced dissociation-MS/MS) experiments.[32,33]

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Method Optimization. Taking into account that the mobile phase composition has a significant influence on the ionization efficiency, it was chosen according to the literature,[15−17,34] and the relative ion abundance (%) of resveratrol was examined in selected ion monitoring (SIM) and MRM modes. These phases included 0.05% (v/v) acetic acid, 0.1% (v/v) formic acid, and ammonium acetate (5 mM), pH 5.5, as the aqueous solvent A and acetonitrile 100% (v/v) as solvent B in gradient elution (see LC−MS/MS Instrumentation). They were tested in both positive and negative ion mode using the Turbo IonSpray source to obtain the maximum signal-to-noise ratio.

Once the mobile phase and the ion mode were selected, MS/MS parameters for resveratrol and its metabolites were optimized. Capillary voltage, focusing potential, entrance potential, declustering potential (DP), and collision energy (CE) were optimized in infusion experiments of resveratrol (4.39 μM) in mobile phase A/B (1:1) at a constant flow rate of 5μL/min into the mass spectrometer using a model 11 syringe pump (Harvard Apparatus, Holliston, MA). Nebulizer, curtain, collision, and auxiliary gas (nitrogen) flow rates and also the temperature of the auxiliary gas were optimized in flow injection analysis (FIA) experiments after injection of 5 μL of standard solution of resveratrol (4.39 μM) in mobile phases A/B (1:1) at 400 μL/min.

For quantitative analysis of *trans*-resveratrol, resveratrol-*O*-glucosides, and resveratrol-*O*-glucuronides, calibration curves were constructed by plotting the LC−MS/MS peak area ratio of *trans*-resveratrol to the internal standard taxifolin (at 65.79 pmol/mL) against the analyte concentration. The concentrations of resveratrol metabolites were estimated using the *trans*-resveratrol standard curve.[15]

Safety Considerations. Human LDL samples were considered as potentially infectious. We respected general guidelines for work with organic solvents and acids. Universal precautions for the handling of chemicals and fluids were applied.

RESULTS AND DISCUSSION

Wine Samples. The results of the LC−DAD quantitation of resveratrol and piceid in Merlot red wine were found to have 10.24 ± 0.05 mg/L of *trans*-piceid, 7.83 ± 0.74 mg/L of *cis*-piceid, 1.73

± 0.29 mg/L of *trans*-resveratrol, and 1.71 ± 0.40 mg/L of *cis*-resveratrol.

Optimization of Resveratrol Extraction. In the first step, we compared the recovery of resveratrol from a spiked LDL blank matrix (4.39 μM) using three extraction methods described in the Experimental Section. The LC−DAD analysis of the samples gave the best result for protein precipitation (112 ± 3.0%), widely used in biological analysis,[30] whereas SPE (87 ± 1.0%) and liquid−liquid extraction (65 ± 6.0%) presented slightly low recoveries. After these preliminary findings, the liquid−liquid extraction was frowned upon because of the lowest recovery. For further experiments, only protein precipitation and SPE were used. LC−MS/MS analysis of these two sample extraction methods were then applied to human LDL samples spiked with 0.22 μM of resveratrol. Recovery results showed differences between SPE (87 ± 6.0%) and protein precipitation (57 ± 2.0%), detecting a high suppressive effect when protein precipitation was applied. These observations indicated that the extraction method influenced resveratrol ionization's obtaining the best signal-to-noise ratio with SPE and supporting the hypothesis that nonvolatile sample components in biological samples were mainly responsible for ionization suppression.[20]

Therefore, SPE was the selected method used for the LDL volunteers' samples, standard calibration curves in LDL blank matrix, and rat bile.

Quality Parameters of the Method. To evaluate the method, the following criteria were used: selectivity, linearity, sensitivity, recovery, precision and accuracy.

LC−MS/MS Optimization. This was achieved by selecting the best mobile phase, the ionization mode, and mass spectrometer parameters. In these preliminary studies, we used infusion and FIA experiments to optimize resveratrol conditions.

Infusion experiments using three different mobile phases were performed in order to study MS and MS/MS behavior of resveratrol. No adducts were observed in any of the tested conditions. The main ion in full scan positive mode was m/z 229 ($[M + H]^+$), and in negative mode was m/z 227 ($[M - H]^-$).

Injection (5 μL) of resveratrol standard (4.39 μM) in the different mobile phase was performed to determine the best signal-to-noise ratio. In a first step, positive and negative ion modes were compared after injection in SIM mode (m/z 229 in positive mode and m/z 227 in negative mode). The negative mode presented a 6-fold higher signal-to-noise ratio than the positive one. Therefore, all MS/MS data were collected in negative ion mode.

A product ion scan of m/z 227 ion was carried out in each mobile phase, resulting in a stable fragment ion at m/z 185 through the loss of C_2OH_2, so MRM acquisition was done by using the 227 → 185 transition. Injection of 5 μL of resveratrol (0.22 μM) provided a signal-to-noise ratio for 0.05% acetic acid between 11 and 14 times higher than that obtained for 0.1% formic acid and 5 mM ammonium acetate, respectively. Therefore, 0.05% (v/v) acetic acid was selected for further experiments.

Finally, the optimum parameters for detection of resveratrol were the following: capillary voltage −3500 V, focusing potential −200 V, entrance potential −10 V, nebulizer gas 10 (arbitrary units), curtain gas 12 (arbitrary units), collision gas 4 (arbitrary units), auxiliary gas heated to 400 °C, and introduced at a flow rate of 6000 cm^3/min. The DP and CE for the standards were *trans*-resveratrol (−60, −25), *trans*-piceid (−60, −25), taxifolin (−60, −25). A higher DP value (−70 V) was used for monitoring the 227 → 185 transition in CID-MS/MS conditions.

Selectivity. 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 human LDL samples. Endogenous peaks at the retention time of the analytes were not observed in LDL evaluated after injection of blank samples in full scan and MRM mode.

Linearity. The linearity of the method was investigated by spiking blank human LDL with known concentrations of *trans*-resveratrol at six concentration levels from 0.44 to 438.59 pmol/mL. The six-point calibrator concentrations showed a linear and reproducible curve with a correlation coefficient of 0.9912 or higher obtained for the relationship between peak area ratios (analyte/internal standard) and the corresponding calibration concentration. The sample concentrations were determined by weighted ($1/x^2$) linear regression of the standard line. The residual analysis for this concentration range was the following: mean (SD); 100.1% (± 15.3).

Sensitivity. The sensitivity of the method was evaluated by determining the limit of detection (LOD) and the limit of quantitation (LOQ). LOD was defined as the concentration of resveratrol with a signal-to-noise ratio of at least 3, whereas LOQ was the lowest standard with a signal-to-noise ratio of at least 10. The LOD and the LOQ were 0.15 and 0.44 pmol *trans*-resveratrol/mL LDL, respectively. To our knowledge, this is the lowest LOD measured for resveratrol in biological samples.

Recovery. The extraction efficiency (%) was investigated by spiking blank LDL samples with *trans*-resveratrol at six different concentration levels within the linear range of the calibration curve (0.44, 2.19, 4.39, 21.93, 43.86, and 438.59 pmol/mL), processed as described above and then analyzed using LC−MS/MS. The recovery of known amounts of resveratrol added to blank LDL was an average of 87 ± 4.0%.

Precision and Accuracy. The bioanalytical precision and accuracy were assessed through replicate analysis of samples containing known amounts of *trans*-resveratrol and taxifolin prepared in blank human LDL by SPE. Analysis of standard calibration curves resulted in the precision and accuracy data found in Table 2. The criteria for acceptance[35] precision and accuracy were accepted at all concentration levels. Following this criteria, the lowest standard (0.44 pmol/mL) was accepted as the LOQ (Table 2).[35]

The retention time of *trans*-resveratrol and taxifolin was also evaluated for the precision in repeatability and reproducibility. The within-day precision (n = 10) for the two was 0.3 and 0.8%, respectively, and the between-day result when it was evaluated over a period of 3 days (n = 30) was 1.3 and 5.6% for *trans*-resveratrol and taxifolin, respectively.

Resveratrol Metabolites Characterization. The confirmation of resveratrol metabolites in LDL samples was based on their retention times and ion fragmentation (Table 1) in the MS/MS mode, as compared with those of standards available and rat bile.[22]

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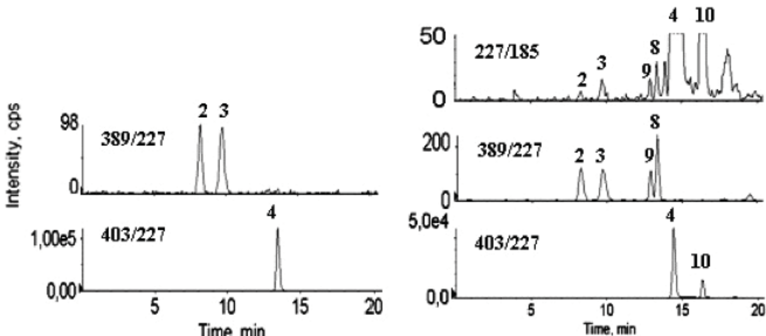

Figure 2. Multiple reaction monitoring chromatogram of the rat bile extract (1/100) before and after UV light exposure. Peaks: (2) *trans*-resveratrol-3-*O*-glucoside (8.20 min); (3) tentatively, *trans*-resveratrol-4′-*O*-glucoside (9.48 min); (4) *trans*-resveratrol-3-*O*-glucuronide (14.69 min); (8) *cis*-resveratrol-3-*O*-glucoside (13.32 min); (9) *cis*-resveratrol-4′-*O*-glucoside (12.89 min); and (10) *cis*-resveratrol-3-*O*-glucuronide (16.58 min).

Table 2. Precision and Accuracy Data Obtained from the LC−MS/MS of *trans*-Resveratrol in Blank Human LDL

std (pmol/mL)	mean (pmol/mL) (n=5)	RSD (%)	accuracy (%)
0.44	0.45	4.5	103
2.19	2.15	9.1	98
4.39	4.56	11.2	104
21.93	22.81	9.7	104
43.86	48.42	9.5	110
87.72	90.61	7.6	103
219.30	220.18	2.2	100
438.60	393.42	8.6	90

As mentioned in the Experimental Section, metabolites were identified by MRM metabolite transition (403 → 227; 389 → 227; 307 → 227) and confirmed by monitoring the MRM transition of resveratrol (227 → 185) itself in CID-MS/MS experiments.

Study of Standards. *trans*-Resveratrol and *trans*-piceid standards (commercially available) eluted at retention times of 14.86 and 8.20 min, respectively.

Study of Rat Bile Extract. An extract from rat bile was obtained after SPE as described in the Experimental Section. After the dilution of the extract 1/100 (v/v), it was injected into the LC−MS/MS system to identify resveratrol metabolites (Figure 2). The major peak (4) in rat bile showed m/z values of 403 and 227 for their parent and product ion, respectively. This peak was identified as *trans*-resveratrol-3-*O*-glucuronide, a metabolite fully structurally characterized by NMR. Peaks 2 and 3, in lower concentration levels, were identified as resveratrol glucosides with m/z value of 389 and 227 for their parent and product ion, respectively. Peak 2, at 8.20 min, corresponded to *trans*-piceid by comparison with the commercial available standard. Peak 3, at 9.48 min, after being ruled out as the *cis*-piceid, could be tentatively assigned as a 4′-*O*-glucoside substitution. The 4′-position has been already considered in the identification of resveratrol-4′-*O*-glucuronide in human hepatocytes.[15] The study of the MRM transition m/z 307 → 227 revealed a peak at a retention time of 30.37 min showing a poor chromatographic peak shape. As can be seen in Figure 2, all described metabolites were confirmed by the presence of a peak in the 227 → 185 trace at the same retention time of the metabolite. It should be mentioned that the intensity of the 227 → 185 transition is ~5- to 10-fold lower than the transition corresponding to the loss of the glucoside unit and between 15- and 20-fold lower than the transition corresponding to the loss of the glucuronide unit, so it is possible that a metabolite in the LDL sample at a very low concentration could not be confirmed by the second MRM transition. Then the confirmation of this metabolite was made by the metabolite transition and its retention time.

cis Isomers. The cis isomers of the standards and the rat bile extract were obtained after exposure to UV light ($\lambda = 254$ nm for ~10 min).[15] The exposure of *trans*-resveratrol and *trans*-piceid standards to sunlight gave as a result their cis isomers at retention times of 16.35 min (peak 7) and 13.32 min (peak 8), respectively. Peak 7 was also observed after the sunlight exposure of the rat bile extract. Under these conditions, compounds 9 and 10 were formed in this extract. Peak 9, in the transition m/z 389 → 227 at 12.89 min, could be tentatively the *cis*-resveratrol-4′-*O*-glucoside, and for peak 10 in the transition m/z 403 → 227 at 16.58 min, the formed compound could corresponded to the *cis*-resveratrol-3-*O*-glucuronide. The presence of two new peaks (9 and 10) in the 227 → 185 transition in rat bile confirmed these two cis metabolites of resveratrol.

Analysis of Resveratrol Metabolites in LDL Samples. Volunteers for this study consumed 250 mL of red wine, and LDL samples were obtained at 24 h. Table 1 shows the concentration ranges of metabolites and free resveratrol; Figure 3 shows the LDL MRM chromatograms of three volunteers. The wide range of each metabolite from all individuals allows us to observe the quantitative variability among subjects already noted in similar studies.[5,36] *trans*-Resveratrol-3-*O*-glucuronide reached the highest value in LDL (Table 1). Individual differences in resveratrol bioavailability from wine or in LDL composition could account for the differences observed in the metabolites of resveratrol incorporated into the LDL after a single dose of 250 mL of red wine containing 5.38 mg of total resveratrol content.

This great variability of profile and quantity observed may be due to the fact that the different polymorphisms of intestinal enzymes or transporters between individuals can affect the

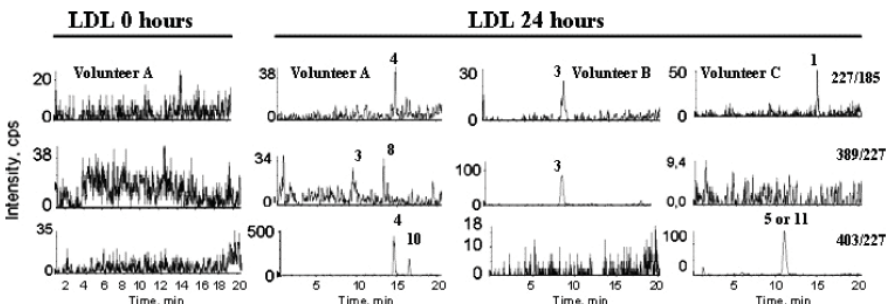

Figure 3. Multiple reaction monitoring chromatogram of 0-h LDL (volunteer A) and 24-h LDL after the intake of 250 mL of red wine (volunteers A, B, and C). Peaks: (1) *trans*-resveratrol; (3) tentatively, *trans*-resveratrol-4′-*O*-glucoside; (4) *trans*-resveratrol-3-*O*-glucuronide; (5 or 11) trans or cis resveratrol-4′-*O*-glucuronide; (8) *cis*-resveratrol-3-*O*-glucoside; and (10) *cis*-resveratrol-3-*O*-glucuronide.

phenolic absorption.[36] Further investigation in this regard is needed.

Figure 3 shows the LDL of volunteer A before consumption of the wine and 24-h LDL levels of resveratrol and glucuronidated and glucosidated metabolites after a single dose of red wine from three volunteers (A, B, and C), representing the different profile that can be observed. No resveratrol or resveratrol metabolites were detected in any LDL before consumption except for one volunteer who showed low baseline levels of *trans*-resveratrol-3-*O*-glucuronide and *cis*-resveratrol-3-*O*-glucuronide. The identification of baseline levels before intervention in humans has been reported[10] and reviewed.[36]

Four metabolites were identified in volunteer A (Figure 3). Peaks 4 and 10 corresponded to trans-resveratrol-3-*O*-glucuronide (the main metabolite in accordance with Yu et al.[13]) and its *cis*-isomer, respectively, both identified also in rat bile. Peak 4 can be confirmed by the presence of a peak in the transition 227 → 185 at the same retention time but with 15-fold lower levels than in the transition 403 → 227, then peak 10, with lower concentration levels, is confirmed by retention time from rat bile. Peak 3 and 8, with *m/z* 389 and 227 for the parent and product ion, corresponded to *trans*-resveratrol-4′-*O*-glucoside (tentatively identified in rat bile) and *cis*-resveratrol-3-*O*-glucoside, respectively. Compound 8 has only been identified but not quantified because it is below LOQ of the technique. Volunteer B only presented the *trans*-resveratrol-4′-*O*-glucoside (3) metabolite. This compound is confirmed by the presence of a peak at the same retention time in the transition 227 → 185 with 5-fold lower levels. Although literature data about glucoside formation in humans is rare, it has been found[37] that in human liver microsomes, uridine diphosphate glucuronosyltransferases catalyze the transfer of glucuronic acid and glucose from the uridine diphosphate glucuronic acid (UDPGA) and uridine diphosphate glucose (UDPG), respectively. Furthermore, this study also showed that in human liver microsomes, glucosidation may serve as an alternative detoxification pathway when levels of UDPGA are depleted due to toxicological or pathogical consequences or in cases in which aglycones preferentially undergo glucosidation. The use of resveratrol glucosides, which showed antimetastatic activity,[38] therefore seems to be a promising approach in resveratrol application, because resveratrol glucosides are absorbed[13] from the intestine, possibly by the same glucose transport system also responsible for the absorption of other glucosided phenolic compounds in rats.[19] However, considering activity, specific structural determinants, such as the presence of 4′-OH together with stereoisomery in the trans conformation, are absolutely required for inhibition of cell proliferation.[39] Volunteer C showed peak 1, identified as the aglycone *trans*-resveratrol at retention time of 14.86 min. Free resveratrol could be found in LDL either due to direct absorption[13] or due to the β-glucuronidase activity in liver, which has already been described.[40] This volunteer also showed a peak eluting at 11.10 min in the transition 403 → 227 that could be a potential resveratrol-*O*-glucuronide. This compound was retained for a shorter time than *trans*-resveratrol-3-*O*-glucuronide and could correspond to resveratrol-4′-*O*-glucuronide in trans (5) or cis (11) form, which has already described in human hepatocytes.[15] This is a tentative metabolite, because its confirmation with a peak in the transition 227 → 185 is not possible due to its low concentration. The identification of resveratrol sulfates in LDL samples was performed in trace chromatogram 307 → 227. Taking into account that *trans*-resveratrol-3-sulfate in rat bile extract gave a poor peak shape, the presence of some wide peaks in the 307 → 227 trace for the LDL extract needs further investigation.

In summary, the metabolites found in 24-h LDL samples from volunteers who consumed 250 mL of red wine were *trans*-resveratrol-3-*O*-glucuronide, *cis*-resveratrol-3-*O*-glucuronide, *cis*-resveratrol-3-*O*-glucoside, free *trans*-resveratrol, and tentatively, resveratrol-4′-*O*-glucuronide and *trans*-resveratrol-4′-*O*-glucoside.

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1.2 Adequació i posta a punt de la metodologia analítica per espectrometria de masses per a l'estudi dirigit del perfil metabòlic del resveratrol en LDL i orina després del consum moderat de vi aplicat a estudis clínics i epidemiològics amb un gran nombre de mostres.

Publicació II: Posta a punt d'una metodologia per a la determinació del resveratrol i els seus metabòlits en orina i LDL i la seva adequació a estudis clínics i/o epidemiològics amb un gran nombre de mostres

Mireia Urpi-Sarda, Raul Zamora-Ros, Rosa Lamuela-Raventos, Antonio Cherubini, Olga Jauregui, Rafael de la Torre, Maria Isabel Covas, Ramon Estruch, Walter Jaeger, Cristina Andres-Lacueva. HPLC-tandem mass spectrometric method to characterize resveratrol metabolism in humans. *Clincal Chemistry*. **2007**, 53 (2): 292-9

Resum:

L'objectiu d'aquest treball va ser aprofundir i ampliar el perfil metabòlic del resveratrol en orina i LDL, augmentar la sensibilitat del mètode i adaptar-lo a estudis amb un gran nombre de mostres com seria el cas d'estudis epidemiològics.

Les mostres de LDL utilitzades en aquest estudi provenien de 11 voluntaris amb un consum puntual de 250 ml de vi negre i les mostres d'orina es van recollir després de 4h del consum de 250 ml de vi negre (Figura 23).

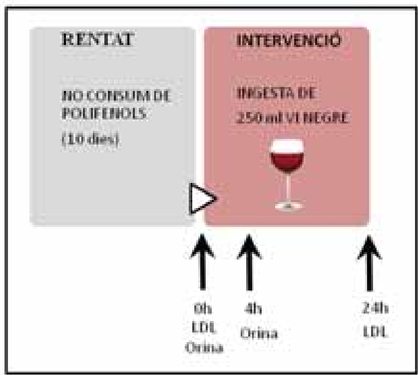

Figura 23: Esquema de l'estudi

Es va determinar el resveratrol i els seus metabòlits glucuronidats i sulfatats després de l'optimització de l'extracció en fase sòlida en placa de 96 pous HLB (Oasi®, Waters, Mildford, Dt., EEUU). Posteriorment, es va optimitzar també l'anàlisi per cromatografia liquida d'alta eficàcia acoblada a la espectrometria de masses en tàndem per a reduir el temps cromatogràfic i obtenir una bona ionització dels metabòlits sulfatats. El mètode desenvolupat va ser validat per al resveratrol i el piceid utilitzant l'hexestrol com estàndard intern**.**

El mètode validat va mostrar uns límits de detecció en funció de la mostra estudiada: 0.2 nmol/L per a LDL, 0.3 nmol/L per a orina sintètica i 4.0 nmol/L per a orina, les corbes de calibrat van ser lineals entre 4.4-3289.5 nmol/L, es va obtenir una anàlisi de residuals de 100% (3.2) per al resveratrol i de 100 (11.1) per al piceid, la precisió a totes les concentracions estudiades va ser menor del 10.8%. També es van realitzar tests d'estabilitat de la mostra biològica i no es va observar degradació ni dels estàndards ni de les mostres. Es va demostrar l'estabilitat de la mostra durant un període de 5 anys.

Les principals millores respecte el mètode anterior estan en el processat de 96 mostres al mateix temps a causa de l'extracció i optimització en placa de 96 pous, la utilització d'una columna que permet una anàlisi cromatogràfic de 10 minuts, i la determinació de metabòlits sulfatats del resveratrol al canviar la fase mòbil orgànica.

Les principals conclusions del mètode és la quantificació, per primera vegada, de sulfats de resveratrol en LDL i la caracterització completa dels metabòlits sulfatats i glucuronidats del resveratrol tant en orina com en LDL, la qual pot utilitzar-se com biomarcador nutricional per a avaluar els efectes biològics del consum moderat de vi en humans.

Aquesta metodologia ofereix una eina de treball indispensable per a la valoració del consum de resveratrol.

L'aplicació d'aquesta metodologia en estudis clínics i epidemiològics queda recollida en 2 publicacions de l'annex realitzades en col·laboració amb el Dr. Raul Zamora-Ros:

- Raul Zamora-Ros, Mireia Urpi-Sarda, Rosa M. Lamuela-Raventos, Ramon Estruch, Monica Vazquez-Agell, Manuel Serrano-Martinez, Walter Jaeger, Cristina Andres-Lacueva. Diagnostic performance of urinary resveratrol metabolites as a biomarker of moderate wine consumption. *Clinical Chemistry*. **2006**. 52 (7):1373-80

- Raul Zamora-Ros, Mireia Urpí-Sardà, Rosa M. Lamuela-Raventós, Ramon Estruch, Miguel Ángel Martínez-González, Mònica Bulló, Fernando Arós, Antonio Cherubini and Cristina Andres-Lacueva. Resveratrol metabolites in urine as biomarker of wine intake in free-living subjects: the PREDIMED Study. *Free Radical Biology and Medicine*. **2008**. En revisió.

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Automation and
Analytical Techniques

HPLC–Tandem Mass Spectrometric Method to Characterize Resveratrol Metabolism in Humans

Mireia Urpi-Sarda,[1] Raul Zamora-Ros,[1] Rosa Lamuela-Raventos,[1] Antonio Cherubini,[2] Olga Jauregui,[3] Rafael de la Torre,[4] Maria Isabel Covas,[5] Ramon Estruch,[6] Walter Jaeger,[7] and Cristina Andres-Lacueva[1*]

Background: Nutritional biomarkers are alternatives to traditional dietary assessment tools. We sought to develop a method for nutritional analysis of resveratrol, a phenolic compound with purported health-promoting properties, and to determine all resveratrol metabolites.
Methods: We obtained LDL and urine samples from 11 healthy male volunteers who had consumed 250 mL of Merlot red wine. We measured resveratrol and its metabolites with 96-well solid-phase extraction plates coupled with HPLC-tandem mass spectrometry. Hexestrol was used as the internal standard. Gradient chromatography in multiple reaction monitoring mode was performed on a Luna C_{18} column, maintained at 40 °C; *m/z* transitions were as follows: resveratrol, 227/185; resveratrol glucosides, 389/227; resveratrol glucuronides, 403/227; resveratrol sulfates, 307/227; taxifolin, 303/285; and hexestrol, 269/134.
Results: Standard calibration curves were linear at 4.4–3289.5 nmol/L. Residual analyses were 100% (3.2) for *trans*-resveratrol and 100% (11.1) for *trans*-piceid. In both matrices, imprecision (CV) was <10.8% at all concentrations. Detection limits for resveratrol were 0.2 nmol/L (LDL), 0.3 nmol/L (synthetic urine), and 4.0 nmol/L (blank urine). Resveratrol and metabolites were checked for stability, and no degradation was observed.
Conclusions: The HPLC–tandem mass spectrometry method enabled us to identify resveratrol sulfates in human LDL and to characterize the complete profile of resveratrol metabolism in human LDL and urine. This method provides an accurate index of exposure to resveratrol and its metabolites, which can be used as nutritional biomarkers for evaluating the biological effects of moderate wine intake on human health.
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Resveratrol is a phenolic compound that has been linked to the beneficial effects of red wine *(1)* (Fig. 1), which have been proposed to be mimetic of caloric restriction in mammals *(2)*. In red wine, resveratrol occurs predominantly as its glucose derivative, piceid. Several in vitro studies have demonstrated that resveratrol acts as an antioxidant *(3)*, reduces the synthesis of proatherosclerotic substances *(4)*, is a potential cancer preventative *(5)*, and acts as a neuroprotector *(6)*. Few authors, however, have studied resveratrol metabolism in humans. As with many polyphenols, resveratrol is reasonably well absorbed but has low bioavailability *(7)*. Therefore, the health benefits attributed to the ingestion of resveratrol are most likely related to biologically active metabolites. In vivo characterization of resveratrol's metabolic profile may reveal which metabolites act as signaling molecules within tissues *(6)* or reach target organs and account for the health benefits of resveratrol *(8)*.

Nutritional biomarkers of nutrient exposure may be useful alternatives to traditional dietary assessment tools but require a clear understanding of the metabolism of the specific phytochemical. The metabolism of resveratrol has been partially characterized *(9–13)*. After resveratrol ingestion, the main metabolites found in biological fluids are glucuronide and sulfate conjugates *(9–12)*. Resveratrol glucuronide was reported to be a nutritional biomarker of wine consumption *(13)*, but underestimation of

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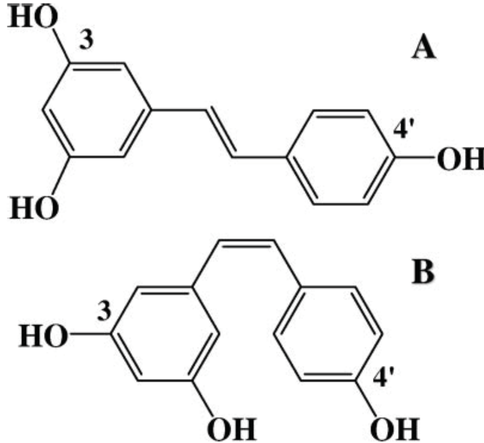

Fig. 1. Structure of *trans*-resveratrol (*A*) and *cis*-resveratrol (*B*).

sulfate conjugates due to poor chromatographic behavior has limited the analytical methods used for the analysis of resveratrol metabolites *(9–13)*. Other drawbacks included rather laborious sample preparation *(14–16)*, long total analysis time *(9–20)*, and the use of enzymatic hydrolysis that precluded direct detection of conjugates *(14, 19, 20)*.

We describe an HPLC–tandem mass spectrometry (HPLC-MS/MS)[1] method to characterize the metabolic profile of resveratrol in human urine and LDL after sample clean-up with solid-phase extraction (SPE).

Materials and Methods

STANDARDS AND REAGENTS

All samples and standards were handled with no exposure to light. Standards of *trans*-resveratrol (99% purity), *trans*-3,4′,5-trihydroxystilbene-3-β-D-glucopyranoside (*trans*-piceid) (97% purity), diethylstilbestrol (≥99% purity), diethylstilbestrol dipropionate, dienestrol, hexestrol (≥98% purity), and human blank LDL were purchased from Sigma-Aldrich. Trismethoxy resveratrol (≥98% purity) was purchased from Cayman Chemical, diethylstilbestrol-d6 from RIVM, taxifolin (>90% purity) from Extrasynthese, and creatinine from Fluka.

Methanol, acetone, and acetonitrile of HPLC grade were purchased from SDS. Glacial acetic acid, ethyl acetate, and *o*-phosphoric acid were purchased from Panreac. Ultrapure water (MilliQ) was obtained from Millipore. Synthetic urine was prepared as previously described *(21)*.

We purified standard resveratrol metabolites from the livers of male Wistar rats raised at the Institut für Versuchstierzucht und-haltung (University of Vienna). Ethics Review Board approval was obtained for the animal studies. The animals were humanely treated. The livers were perfused with 20 μmol/L of *trans*-resveratrol in a recirculating system as previously described *(22)*. We purified resveratrol metabolites from multiple bile samples collected over a time period of 60 min. After collection the samples were pooled and lyophilized. Chemical structures were confirmed by nuclear magnetic resonance *(10)*.

STUDY DESIGN AND SAMPLES

We obtained human LDL samples from 11 healthy male volunteers (ages 18–50) before and 24 h after the consumption of 250 mL of Merlot red wine *(10)*. All volunteers were considered healthy based on the results of physical examination and standard biochemical and hematological tests. The study was performed in accordance with the Helsinki Declaration of 1975, as revised in 1996. The Ethics Committee of our institution (Comité Ético de Investigación Clínica–Institut Municipal d'Investigació Mèdica) approved the protocol, and all the participants provided signed informed consent. Exercise was monitored with the Minnesota Leisure Time Physical Activity Questionnaire *(23)*.

Before administration, the volunteers followed a 10-day washout period in which they consumed a controlled diet from days 1 to 7, avoiding excess intake of antioxidants. During the immediate 3 days before and on the intervention day, the volunteers consumed a standardized low phenolic compound diet. On the intervention day they drank a single dose of 250 mL of red wine. We collected EDTA blood at baseline and at 24 h after wine consumption. LDL was isolated by sequential flotation ultracentrifugation *(24)*. We immediately froze all LDL samples at −80 °C, with thawing immediately before analysis. Protein content was determined with the red pirogalol method (Sigma-Aldrich).

We obtained urine samples from 5 healthy male volunteers (ages 25–28 years). The study design and conditions were similar to those of Meng et al. *(9)*, with the exception that urine was collected at baseline and during the 4 h after wine consumption. Urine creatinine was measured by a colorimetric assay using picric acid *(25)*.

We used the same red wine in both studies and analyzed resveratrol by HPLC *(26)*. The mean (SD) amount of total resveratrol consumed was 5.4 (0.4) mg, corresponding to 2.6 (0.0) mg of *trans*-piceid, 2.0 (0.2) mg of *cis*-piceid, 0.4 (0.1) mg of *trans*-resveratrol, and 0.4 (0.1) mg of *cis*-resveratrol.

SAMPLE EXTRACTION

LDL (1 mL) was treated with 20 μL of *o*-phosphoric acid (850 mL/L) and vortex-mixed. Urine was centrifuged at 10 000*g* at 4 °C for 3 min and then vortex-mix mixed after addition of 20 μL of the hexestrol as internal standard (92.6 μmol/L) to 1 mL of sample. Samples were then loaded onto a Waters Oasis® HLB 96-well SPE plate (30 mg) that had been preconditioned with 1 mL of methanol and equilibrated with 1 mL of 2 mol/L acetic acid in

[1] Nonstandard abbreviations: MS/MS, tandem mass spectrometry; SPE, solid-phase extraction; LC, liquid chromatography; MS, mass spectrometry; MRM, multiple reaction monitoring; DP, declustering potential.

water. The plate was 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 1mol/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. We reconstituted the residue with 100 μL of taxifolin (1.64 μmol/L) dissolved in mobile phase as an additional the external standard.

HPLC-MS/MS ANALYSES

We performed liquid chromatography (LC) analyses using a Perkin-Elmer series 200 system equipped with a quaternary pump and a refrigerated plate autosampler. An Applied Biosystems API 3000 triple quadrupole mass spectrometer, equipped with a Turbo IonSpray source ionizing in the negative mode, was used to obtain the mass spectrometry (MS) and MS/MS data. A Phenomenex Luna C_{18} column, 50 × 2.0 mm i.d., 3 μm, maintained at 40 °C, was used for chromatographic separation. The injection volume was 15 μL, and the flow rate was 550 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. We applied a linear gradient profile with the following proportions (v/v) of phase B [t(min), %B]: (0, 15), (1, 15), (1.5, 40), (2.5, 100), (4.5, 100), (4.8, 15), (10, 15). The column was reequilibrated for 6 min. The MS and MS/MS parameters were as previously described *(10)*.

The identification of metabolites in biological samples was based on 3 indicators *(10, 27)*: (*a*) comparison of retention time of available standard, (*b*) multiple reaction monitoring (MRM) of metabolite and resveratrol transitions [with higher declustering potential (DP) in collision-induced dissociation MS/MS conditions], or (*c*) product ion spectra. For MS/MS, a product ion scan was used at a cycle time of 2 s. The product ion spectra of metabolites showed the deprotonated molecule (*m/z* 403 or *m/z* 307, respectively) and the ion corresponding to resveratrol (*m/z* 227) through the neutral loss of the glucuronide or sulfate unit (−176 u or −80 u, respectively) from the glucuronide or sulfate. MRM mode was used with a dwell time of 200 ms, monitoring 6 transitions for each analysis: resveratrol (227/185), resveratrol glucosides (389/227), resveratrol glucuronides (403/227), resveratrol sulfates (307/227), taxifolin (303/285), and hexestrol (269/134). The concentrations of resveratrol metabolites were expressed as *trans*-resveratrol equivalents *(10, 20)*.

EVALUATION OF INTERNAL STANDARDS

Several compounds, structurally similar to resveratrol, were evaluated as possible internal standards. MRM transitions were 267/237 for diethylstilbestrol, 273/254 for diethylstilbestrol-d6, 269/134 for hexestrol, and 265/93 for dienestrol. Trismethoxy resveratrol and diethylstilbestrol dipropionate were not ionizable in negative mode.

ASSAY VALIDATION

We assessed endogenous interference by analyzing blank human LDL, synthetic urine, and blank urine samples (n = 5) collected from volunteers after the washout period. Recovery and linearity were investigated by adding *trans*-resveratrol and *trans*-piceid, at 10 concentrations, to blank urine (Table 1). The limit of detection was defined as the concentration of analyte that produced a signal-to-noise ratio of 3. The lowest standard on the calibration curve was accepted as the limit of quantification *(28)*. Within- and between-day imprecision and recovery were evaluated with use of 10 different concentrations of resveratrol and piceid (n = 3) over a 10-day period. We evaluated stability during the analytical process, after freeze and thaw cycles, and after short-term and long-term storage. Control materials with resveratrol concentrations of 219.3 nmol/L and 2193.0 nmol/L, and piceid concentrations of 140.8 nmol/L and 1145.6 nmol/L, in the proper matrices, were stored under the same conditions (−80 °C) as biological samples. We assessed the stability of metabolites with urine from volunteers who had consumed red wine.

After we had validated the analytical method for routine use, we used resveratrol at concentrations of 21.9, 219.3, and 2193.0 nmol/L and piceid at concentrations of 12.8, 128.2, and 1282.0 nmol/L in duplicate as QC samples *(28)*.

STATISTICAL ANALYSIS

SPSS statistical software, Windows version 11.5.1, was used. Kolmogorov–Levene and a paired Student t-test were employed. A weighted least-squares regression analysis was used to obtain correlation coefficients and slopes. Statistical significance was defined as $P < 0.05$. Data are shown as the mean (SD).

Results

SELECTIVITY

Under the chromatographic and MS/MS conditions used for the assay, metabolites and standards were well resolved (Fig. 2, Table 2). Endogenous peaks at the retention time of the analytes of interest were not observed in blank human LDL or in synthetic urine. Blank urine from volunteers showed some endogenous peaks, but none at the same retention time of the analytes.

EXTRACTION RECOVERY AND LINEARITY

The mean (SD) recoveries of known amounts of *trans*-resveratrol and *trans*-piceid added to blank matrices were 92 (11.5)% and 89 (6.3)%, respectively. The 9-point calibrator concentrations showed a linear and reproducible curve for standards. Weighted ($1/x^2$) least-square regression analysis yielded equation regression lines and residual analysis [mean range (SD)] as follows: $y = 35.2x - 0.07$ ($r^2 = 0.996$) and 100% (3.2) for *trans*-resveratrol and $y = 19.3x + 1.3$ ($r^2 = 0.967$) and 100% (11.1) for *trans*-piceid.

Table 1. Within- and between-day precision and recovery data obtained from the LC-MS/MS of *trans*-resveratrol and *trans*-piceid in blank human urine.

	trans-Resveratrol				*trans*-Piceid			
Imprecision	Added, nmol/L	Mean, nmol/L	Precision (RSD), %	Recovery (error), %	Added, nmol/L	Mean, nmol/L	Precision (RSD), %	Recovery (error), %
Within-day (n = 3)								
	4.4	4.4	4.5	99.8				
	21.9	22.8	2.1	104.0	12.8	13.0	8.7	101.6
	43.9	40.9	3.2	93.2	25.6	27.0	2.8	105.3
	87.7	90.6	2.8	103.3	51.3	52.6	8.7	102.6
	219.3	226.1	3.7	103.1	128.2	133.6	0.7	104.2
	329.0	318.7	10.5	96.9	192.3	209.4	9.4	108.9
	438.6	475.6	6.0	108.4	256.4	264.1	6.0	103.0
	1096.5	1106.4	8.1	100.9	641.0	658.3	5.5	102.7
	2193.0	2022.6	6.8	92.2	1282.0	1287.2	4.6	100.4
	3289.5	3441.1	3.1	104.6	1923.1	1857.8	8.5	96.6
Between-day (n = 10)								
	4.4	4.7	10.8	106.6				
	21.9	24.2	10.5	110.3	12.8	12.5	9.8	97.3
	43.9	39.4	8.1	89.9	25.6	27.7	10.5	108.0
	87.7	95.5	10.1	108.9	51.3	52.8	4.4	102.9
	219.3	227.2	4.4	103.6	128.2	137.8	8.2	107.5
	328.9	295.7	10.2	89.9	192.3	211.2	10.4	109.8
	438.6	475.7	9.2	108.5	256.4	247.0	9.5	96.3
	1096.5	1076.5	8.4	98.2	641.0	705.1	10.5	110.0
	2193.0	1994.1	8.5	90.9	1282.0	1193.3	9.6	93.1
	3289.5	3605.9	10.4	109.6	1923.1	1727.0	8.8	89.8

PRECISION, RECOVERY, AND DETECTION LIMIT

Precision and recovery (Table 1) met acceptance criteria *(28)* at all concentrations. According to these criteria, the lowest standards of *trans*-resveratrol and *trans*-piceid, 4.4 and 12.8 nmol/L, respectively, were accepted as the limit of quantification in human blank urine, and 0.4 and 1.9 nmol/L, respectively, in the LDL matrix *(10)*. Limits of detection for *trans*-resveratrol and *trans*-piceid were 0.2 and 1.2 nmol/L, respectively, in LDL matrix, 4.0 and 8.4 nmol/L, respectively, in human blank urine, and 0.3 and 1.9 nmol/L, respectively, in synthetic urine.

STABILITY

To evaluate short-term temperature stability, 3 aliquots of each concentration were thawed at room temperature, maintained at this temperature for 3 h, and then analyzed. This time represents the average sample preparation time for 96-well plates. The aliquots were then put in a refrigerated autosampler and analyzed at 10 and 25 h, the average time required to analyze 96 samples. Under these conditions, and after freeze and thaw cycles, we observed differences <5% for *trans*-resveratrol and *trans*-piceid. Evaluation of the long-term stability of resveratrol glucuronide stored at −80 °C for 5 years yielded an observed CV of 10.8% (n = 5). After testing the stability of human urine after moderate consumption of red wine, we observed no statistically significant differences in glucuronided and sulfated metabolites at freeze and thaw

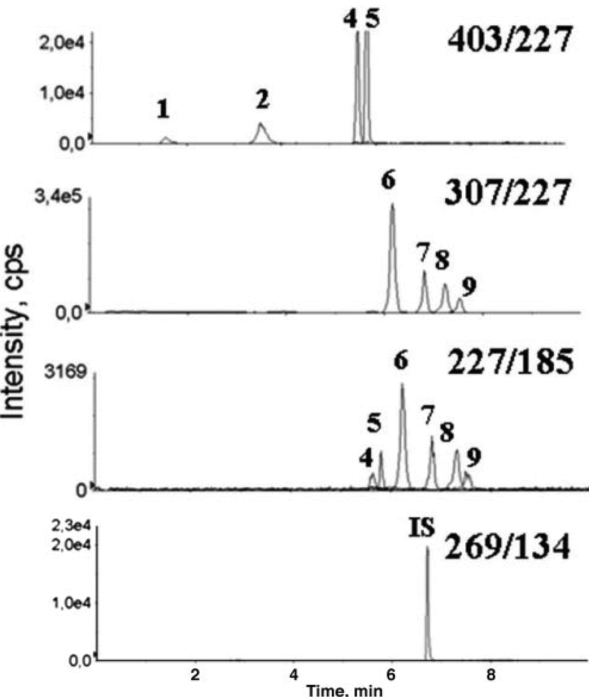

Fig. 2. MRM trace chromatogram of sulfated and glucuronidated standards of resveratrol and MRM of 227/185 (DP = −80) in LC-MS/MS conditions as described in the text.
Numbered peaks refer to Table 2.

Table 2. Description of relative molecular mass, retention times, negative mode multiple reaction monitoring transitions, mean concentrations (SD) of 24-h LDL and 4-h urine after moderate consumption of red wine, and percentage of volunteers who demonstrated each metabolite.

					LDL samples		Urine samples	
Compound	Peak no.	M_r	R_t, min	MS/MS ions, *m/z*	pmol resveratrol/mg LDL protein, mean (SD)	Volunteers, %	nmol resveratrol/g creatinine, mean (SD)	Volunteers, %
trans-Resveratrol-4′-*O*-glucuronide	1	404	1.6	403/227	37.8 (43.6)	27	59.6 (88.7)	80
Taxifolin	ES[a]	304	2.4	303/285	ES		ES	
trans-Resveratrol-3-*O*-glucuronide	2	404	3.3	403/227	111.7 (126.0)	36	179.2 (276.0)	80
trans-Resveratrol	3	228	5.5	227/185	3.5 (4.6)	73	ND	ND
cis-Resveratrol-4′-*O*-glucuronide	4	404	5.6	403/227	ND	ND	355.8 (567.4)	80
cis-Resveratrol-3-*O*-glucuronide	5	404	5.8	403/227	7.1 (5.8)	27	893.5 (894.6)	100
trans-Resveratrol-4′-sulfate	6	308	6.2	307/227	2.0 (1.9)	36	2.4 (14.8)	40
Hexestrol	IS	270	6.7	269/134	IS		IS	
trans-Resveratrol-3-sulfate	7	308	6.8	307/227	4.0 (5.4)	36	74.7 (339.0)	40
cis-Resveratrol-4′-sulfate	8	308	7.3	307/227	7.1 (5.2)	64	9294.2 (8219.2)	100
cis-Resveratrol-3-sulfate	9	308	7.5	307/227	5.4 (2.9)	36	221.2 (1010.1)	40

[a] ES, additional external standard; IS, internal standard; ND, not detected.

cycles and after short- and long-term stability. We concluded that the metabolites were stable under the storage and sample handling conditions used for this assay.

INTERNAL STANDARD EVALUATION

Diethylstilbestrol and diethylstilbestrol-d6 showed 2 unstable peaks over time. Hexestrol and dienestrol, both veterinary synthetic products, were absent in human nutritional and body fluids. Their mean recoveries (n = 11) at the concentrations used in the assay procedure (1851.8 and 1879.7 nmol/L, respectively) were 96% and 89%, respectively. Although the mean recoveries were acceptable for both, dienestrol showed a higher variability (CV >15%) than hexestrol (CV = 11.2%). Hexestrol was selected as the internal standard.

QUALITY CONTROL RESULTS

trans-Resveratrol showed that 83% of QC were within 15% of their nominal value. *trans*-Piceid showed that 67% of QC were within 15% of their nominal value.

APPLICATION TO LDL SAMPLES

To identify sulfated metabolites of resveratrol and to complete its metabolic profile *(10)*, we analyzed LDL samples with this LC-MS/MS method. Three different profiles of 24-h LDL glucuronide and sulfate conjugates of resveratrol after a single dose of red wine are shown in Fig. 3. Six metabolites were identified in volunteer A, 5 in volunteer B, and 4 in volunteer C. Volunteer B showed several peaks with 403/227 transition, but only 2 of them were positively identified as resveratrol glucuronides. Mean (SD) concentrations are shown in Table 2.

In addition to the well-described phase II metabolites of resveratrol, we also screened phase I metabolites, such as methylated (241/227) and hydroxylated (243/159) resveratrol, and their respective phase II metabolites, such as hydroxyresveratrol-glucuronide (419/243) and hydroxyresveratrol-sulfate (323/243). We also screened microflora metabolites, such as dihydroresveratrol-glucuronide (405/229) and dihydroresveratrol-sulfate (309/229) *(12)*. After checking for these

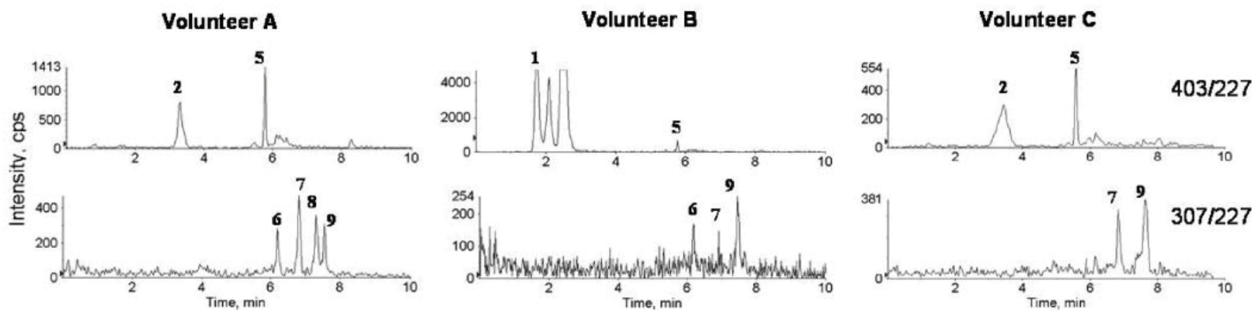

Fig. 3. MRM trace chromatogram of LDL after the intake of 250 mL of red wine (volunteers A, B, and, C).
Numbered peaks refer to Table 2.

transitions, some peaks were observed but were below the limit of detection.

APPLICATION TO URINE SAMPLES

Glucuronided and sulfated metabolites were characterized in human urine by LC-MS/MS. MRM chromatograms of sulfates (307/227) and glucuronides (403/227) in urine from 4 volunteers are shown in Fig. 4. As can be seen in Fig. 4, the application of a higher DP (−80) in the collision-induced dissociation MS/MS experiment allowed the confirmation of all the metabolites through the characteristic 227/185 transition for resveratrol. The means (SD) of the metabolites for these volunteers are presented in Table 2 as nmol resveratrol/g creatinine.

Discussion

We have developed a new method to evaluate resveratrol metabolism in human samples. With this HPLC-MS/MS method, we determined the resveratrol metabolic profile in 10 min in different types of matrices. We emphasize that because of the observed differences in limits of detection, human blank urine is a better tool than synthetic urine because it shows the real matrix effect *(29)*.

Investigations on human resveratrol metabolism have only recently been performed. In 2003, Goldberg et al. *(30)* were the first to administer resveratrol to humans. Subsequent published studies have shown glucuronides and sulfates to be the main metabolites of resveratrol. Only the glucuronide metabolites have been well characterized because of the poor chromatographic behavior of resveratrol sulfates *(12)*.

We have circumvented the drawbacks of previous methods. To improve the resolution of the sulfates *(10, 12)*, acetone was incorporated into mobile phase B. Acetone allows better resolution of sulfates by improving the peak shape and reducing the relative retention time. The incorporation of a shorter chromatographic column also reduced the chromatographic time to 10 min *(9–20)*. The use of a 96-well SPE plate helped avoid laborious sample preparation *(14–16)*, requiring ~3 h of preparation per plate. The use of LC-MS/MS avoids the need to perform enzymatic hydrolysis *(14, 19, 20)*, thus simplify-

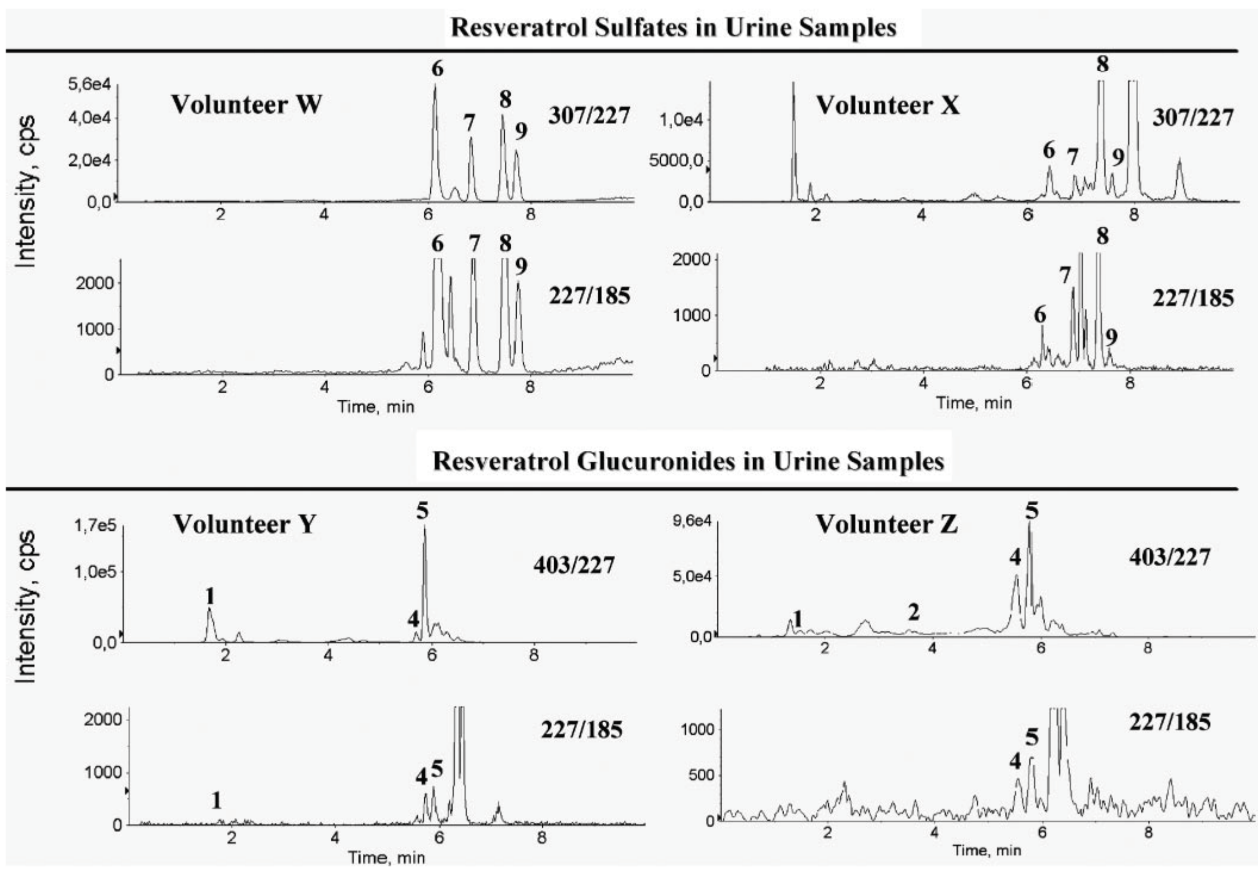

Fig. 4. MRM trace chromatogram of resveratrol sulfates (307/227), resveratrol glucuronides (403/227), and resveratrol (227/185; DP −80) in urine samples of representative volunteers after the consumption of 250 mL of red wine.

Numbered peaks refer to Table 2.

ing the quantitative and qualitative profiling of the resveratrol metabolites.

Another highlight of the present method is the ability to differentiate between the *trans* and *cis* isomers of resveratrol-4′-*O*-glucuronide, resveratrol-3-*O*-glucuronide, resveratrol-4′-sulfate, and resveratrol-3-sulfate. This method is the first to identify the entire profile of resveratrol sulfates in human LDL and urine (Figs. 3 and 4).

There was variability between volunteers (Table 2), but all sulfates were found in similar concentrations in LDL. The main sulfate in LDL was the *cis*-resveratrol-4′-sulfate, and the main glucuronide was *trans*-resveratrol-3-*O*-glucuronide. The *trans*-resveratrol-*O*-glucuronides were in greater concentrations than sulfates. Resveratrol can be glucuronidated at 2 positions on the molecule. Although the 3 position seemed to be the preferential glucuronidation site in vitro in human liver microsomes, the 4′ position is also a possible site of metabolism in humans in vivo *(11)*. Considering activity, the presence of the 4′-OH is a requisite for inhibition of cell proliferation *(31)*. Our results show major glucuronidation of resveratrol in 3-position at 24 h maintaining the 4′-OH free. Although the glucuronide metabolites of resveratrol have previously been described in LDL *(10)*, this new method is able to determine resveratrol sulfates without reducing the resolution of glucuronides.

After successful characterization of the resveratrol metabolites profile in LDL, we applied the method to urine samples. Urine is a more adequate sample to be used in large-scale population studies to establish nutritional biomarkers *(32)*. Meng et al. *(9)* described the rapid excretion of resveratrol in urine (after 2–3 h) when low amounts are consumed. In this study, the urine was collected during the 4 h after moderate red wine intake. When absorbed, resveratrol is rapidly cleared through the glucuronidation and sulfation pathways, and metabolites are principally excreted in urine *(9, 12)*. All the resveratrol metabolites previously described were found in these urine samples. Concerning the stereoselectivity of glucuronidation, *cis*-isomers were glucuronidated faster than *trans*-isomers *(15)*. This observation is in accordance with our results of our study, in which greater amounts of *cis*-*O*-glucuronide are obtained. Because this is the first time that sulfates of resveratrol have been well characterized, there are no published data about sulfate stereoselectivity. Taking into account the concentration results (Table 2), however, the behavior of sulfates seems similar to that of glucuronides, showing higher amounts for *cis* isomers. The variability shown in these results has been seen previously in LDL *(10)* and is attributable to polymorphisms of intestinal enzymes *(33)* or to interactions with other compounds *(34)*. Further investigations on resveratrol variability with more volunteers are needed.

This method can be used in future epidemiological and clinical intervention trials. In studies aimed at evaluating the biological effects of resveratrol intake via moderate wine consumption, knowledge of the resveratrol profile may facilitate better estimation of resveratrol consumption than dietary data obtained by food frequency questionnaires.

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2. FLAVANOLS

2.1 Desenvolupament i validació d'un mètode reproduïble, sensible i ràpid per a determinar el metaboloma urinari associat al consum regular de cacau

Publicació III: **Posta a punt d'una metodologia per espectrometria de masses per a l'anàlisi de compostos fenòlics derivats de la microbiota intestinal després d'un consum de cacau.**

Mireia Urpi-Sarda, Maria Monagas, Nasiruddin Khan, Rosa M. Lamuela-Raventos, Celestino Santos-Buelga, Emilio Sacanella, Margarida Castell, Joan Permanyer, Cristina Andres-Lacueva. Epicatechin, procyanidin and phenolic microbial metabolites after cocoa intake in humans and rats.

Analytical and Bioanalytical Chemistry (En procès de revisió)

Resum:

Les proantocianidines constitueixen la major fracció de flavonoids ingerits en la dieta. Encara que s'absorbeixen poc, poden ser metabolitzats per la microbiota intestinal donant lloc a diversos tipus d'àcids fenòlics (Gonthier et al. 2003b).

L'objectiu d'aquest estudi és la posta a punt i validació d'un mètode analític per a l'anàlisi de 19 metabòlits fenòlics microbians, així com els monòmers i dímers de flavanols per extracció en fase sòlida en placa de 96 pous i determinació per espectrometria de masses en tàndem.

Per a posar a punt aquesta metodologia analítica que pogués ser d'utilitat tant en estudis clínics com amb animals d'experimentació sensible a diferents rangs de concentració, es van utilitzar mostres de voluntaris que havien consumit 40 g de cacau en una dosi i orines de rates les quals havien consumit una dieta amb un 4% de cacau i una altra dieta amb un 10% de cacau durant 2 setmanes (Figura 24).

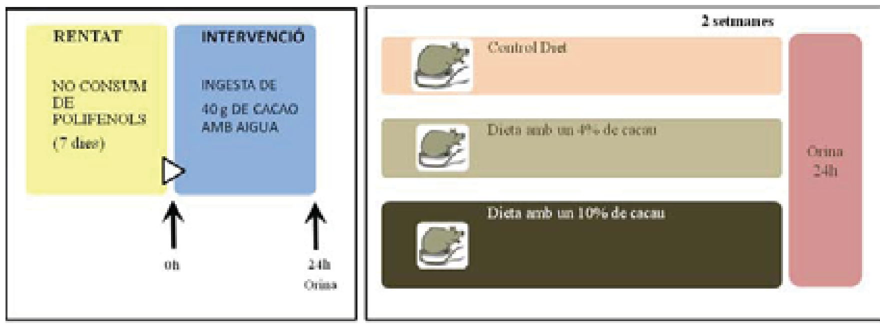

Figura 24: Esquemes dels estudis en humans i en rates

El mètode va ser validat amb una recuperació i exactitud d'entre 87-109% i 87.5-113.8%, respectivament, i amb una precisió menor del 15%.

La procianidina B2 es va detectar i va quantificar per primera vegada en orina humana i de rata després del consum de cacau. Es va observar un increment en àcids fenòlics i flavanols urinaris del 0.001-59.43 nmol/mg creatinina en humans i del 0.004–181.56 nmol/mg creatinina en rates.

Aquest mètode permet una bona estimació del consum de proantocianidines i flavanols que podria ser de gran utilitat per a desenvolupar en un futur recomanacions dietètiques.

Epicatechin, procyanidin and phenolic microbial metabolites after cocoa intake in humans and rats

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Keywords: Cocoa, epicatechin, procyanidin, microbial metabolites, HPLC-MS/MS

Abstract

Proanthocyanidins, flavonoids possessing cardiovascular protection, constitute a major fraction of flavonoid ingested. Although they are poorly absorbed, they are metabolized by the intestinal microbiota into various phenolic acids. An analytical method based on optimized 96-well plate solid-phase extraction and liquid chromatography tandem mass spectrometry for the analysis of 19 phenolic microbial metabolites, and monomeric and dimeric flavanols in urine samples was developed and validated. Human urine samples were obtained before and after ingestion of 40g of cocoa, and rat urines before and after 2 weeks of daily cocoa diets. The mean recovery and accuracy of the method was in the range of 87-109% and 87.5-113.8%, respectively, and the precision was lower than 15%. Procyanidin B2 has been detected and quantified for the first time in human and rat urine after cocoa consumption. Changes in human and rat urine levels of microbial phenolic acids and flavanols were in the range of 0.001-59.43nmol/mg creatinine and 0.004–181.56nmol/mg creatinine, respectively. This method allows an accurate estimation of proanthocyanidin consumption in order to determine its bioavailability and develop recommendations for the beneficial effects claimed by generally accepted scientific evidence.

Introduction

Polyphenols are among the most abundant antioxidant compounds in our diet and may play a key role in the prevention of cardiovascular and neurodegenerative diseases and cancer. These antioxidants are widely distributed in plants and are present in fruits, cocoa, legumes and, in fruit-derived products such as juices, wine, beer and cider. Health effects derived from polyphenol consumption depend on their bioavailability (absorption, distribution, metabolism and elimination), a factor which is also influenced by their chemical structure [1]. Among the flavonoids, isoflavones, which are limited to soya and derived products, present the best bioavailability. In contrast, oligomers and polymers of flavan-3-ol (proanthocyanidins), which are very abundant in our diet, are poorly absorbed [1]. These polyphenols reach the colon and are metabolized by the intestinal microbiota into various phenolic acids, mainly including phenylpropionic, phenylacetic and benzoic acid derivatives [2]. Recently, it has been reported that some of these metabolites may also present biological properties including antioxidant, anti-platelet aggregation [3] and antiproliferative activities [4].

Accurate estimation of polyphenol intake or exposure is of maximum importance in order to determine the bioavailability of these compounds and, then, to be able to calculate the polyphenol doses that could be related to certain health effects in epidemiological studies. Although normally based on food composition tables, which are often incomplete, polyphenol intake can also be estimated by measuring biomarkers of exposure in plasma or urine, such as phenolic microbial metabolites [5;6]. This is particularly important in the case of proanthocyanidins for which intake doses are very difficult to estimate due to their complexity and lack of adequate analytical methods. These facts, together with recent findings demonstrating that some microbial metabolites derived from proanthocyanidin consumption possess certain biological properties, prompt the need for analytical methods that allow a rapid and sensitive measuring of these compounds in biological samples generated in large epidemiological studies.

Tandem mass spectrometry coupled to liquid chromatography has been demonstrated to be highly suitable for the analysis of phenolic metabolites [7-9]. However, sample preparation is a very important step to reduce matrix effects and increase sensitivity when quantifying phenolic metabolites by LC-MS/MS [8]. Liquid-liquid extraction (LLE) is the most common technique used for the extraction of phenolic microbial metabolites in urine samples [10-12]. However, LLE involves multistep sample extraction and clean-up procedures that are time-consuming, and that use large amounts of solvent, which can result in analyte loss and large matrix interferences in LC-MS/MS. To improve these procedures, in the present work we have introduced the use of solid-phase extraction (SPE) in 96 well-plates for the extraction of phenolic microbial metabolites in order to increase sample throughout and minimize matrix effects [8]. In addition, a very short-run time and highly sensitive LC/MS-MS method has been developed for the simultaneous determination of epicatechin, procyanidin and phenolic microbial metabolites in urine samples. The analytical method has been validated and applied to both human and rat urines collected after ingestion of nutritional doses of cocoa, which is a very rich source of proanthocyanidins.

Experimental

Standards and Reagents

Phenylacetic acid (≥98% purity); 3-hydroxyphenylacetic acid (≥97% purity); 3,4-dihydroxyphenylacetic acid (homoprotocatechuic acid; 98% purity); 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid; 99% purity); 4-hydroxyphenylpropionic acid (phloretic acid; ≥98% purity); 3,4-dihydroxyphenylpropionic acid (hydrocaffeic acid) (≥98% purity); *p*-coumaric acid (≥98% purity); caffeic acid (≥95% purity); ferulic acid (≥98% purity); protocatechuic acid (>97% purity); 4-

hydroxybenzoic acid (≥98% purity); 3-hydroxybenzoic acids (≥98% purity); hippuric acid (98% purity); enterodiol (95% purity); enterolactone (95% purity); ethyl gallate (≥96% purity); epicatechin (≥98% purity), procyanidin B2 (≥90% purity), creatinine and β-glucuronidase/sulfatase (from *Helix pomatia*) were purchased from Sigma-Aldrich (St. Louis, MO). 4-hydroxyhippuric acid (>99% purity) was purchased from PhytoLab GmbH&Co.KG (Vestenbergsgreuth, Germany). Vanillic acid, 4-*O*-methylgallic acid and *m*-coumaric acid were purchased from Extrasynthèse (Genay, France). HPLC grade solvents methanol, acetonitrile, glacial acetic acid and formic acid were purchased from Scharlau (Barcelona, Spain). Hydrochloric acid was purchased from Panreac (Barcelona, Spain).

Subjects and Study Design

Twenty-one non-smoking healthy volunteers (9 women and 12 men) between 18 and 50 years old with a corporal mass index of 21.6 ± 2.1 were recruited [13]. None reported any history of heart disease, homeostatic disorders or other medical disease, nor received any medication or vitamin supplement. All gave written informed consent before their inclusion in the trial, and the Institutional Review Board of the Hospital Clínic of Barcelona (Spain) approved the study protocol.

Participants were instructed to abstain from polyphenol-rich foods for at least 48 h before and during the intervention day. After overnight fasting, they were provided with a single dose of 40g of cocoa powder with 250mL water. After 4 hours of the cocoa intake, a light meal of bread and cheese was provided. Urine samples were collected before and after 24h of the cocoa consumption and stored at –80ºC until analysis.

The cocoa powder used in the study contained 47 g/100g of carbohydrates (sucrose, 46 g/100 g; starch, 1 g/100g), 16 g/100g of fiber, 5.4 g/100 g of fat, and 14.1 g of protein. The phenolic composition (SD) of the cocoa powder was determined according to the methodolody of Andres-Lacueva et al [14] and Roura et al [15]: 23.1% of monomers with 0.71 (0.09) mg/g of (-)-epicatechin and 0.21(0.01) mg/g of (+)-catechin, 13.4% of dimers, among which 0.64 (0.06) mg/g of procyanidin B_2, 63.6% of 3-8mers [13;16] and flavonols including 33.87 µg/g isoquercitrin, 5.74 µg/g quercetin, 4.33 µg/g quercetin-3-glucuronide and 36.32 µg/g quercetin-3-arabinoside. The total polyphenolic content was 11.51 (0.95) mg catechin/g cocoa.

Animal Study Design

Dams with 15 day-old Wistar rat litters were obtained from Harlan (Barcelona, Spain). Rats were housed in cages of 10 pups per lactating mother in controlled conditions of temperature and humidity in a 12:12 light:dark cycle.

At day 21, pups were weaned and randomly distributed in three different groups (n=7 for each group) receiving the following diet: (1) 4.8 g cocoa powder/kg/day by oral gavage with free access to control chow and water, (2) chow containing 10% (w/w) cocoa and free access to water, and (3) control diet group [17]. After 2 weeks of dietary treatment, rats were maintained in metabolic cages for 24 h to collect urine output. Urine samples were frozen at -80 ºC for further analysis.

The phenolic composition (SD) of the cocoa powder and the chow containing 10% (w/w) cocoa was : 2.2 (0.1) and 0.34 (0.01) mg/g of (-)-epicatechin, respectively; 0.74 (0.05) and 0.10 (0.004) mg/g of (+)-catechin, respectively; 1.68 (0.11) and 0.23 (0.01) mg/g of procyanidin B2, respectively, 0.05 (0.001) and 0.02 (0.00) mg/g of isoquercetin, and 0.03 (0.00), 0.01 (0.00) of quercetin; and 21.85 (1.08) and 0.90 (0.54) mg catechin/g cocoa for total polyphenols [17].

The study was performed in accordance with the institutional guidelines for the care and use of laboratory animals and the experimental procedures were approved by the Ethical Committee for Animal Experimentation of the University of Barcelona (ref. 3131).

Enzymatic Hydrolyses

One milliliter of urine sample was spiked with 100µL of 10.1 nmol/mL ethyl gallate as internal standard (IS). Ethyl gallate was selected as the IS due to its absence in human nutrition and body fluids, its selectivity, recovery (93%) and precision (RSD= 8%). Samples were then hydrolyzed as previously described, although some modifications were applied [18]. Briefly, samples were acidified with 50 µL of 0.58 mol/L acetic acid to pH 4.9 and incubated with β-glucuronidase/sulfatase at 37ºC for 45 min. Straight afterwards, samples were acidified to pH 2 with 6 mol/L HCl.

Preparation of standard solutions

The different phenolic standards (*n*=19) and epicatechin and procyanidin B2 were dissolved in 80% methanol to prepare 800 mg/L stock solutions. A standard pool solution (16 mg/L of each analyte) was prepared in Milli-Q ultrapure water from the stock solution and stored at 4 °C. For calibration purposes, a wide range of standards (0.5-500 µg/L) with 6 different concentration levels, was prepared from the pool in synthetic urine. [19]

Solid-Phase Extraction

SPE was performed using Oasis® MCX (mixed-mode-cation-exchange/reversed phase sorbent) 96-well plates, Oasis® MAX (mixed-mode-anion-exchange/reversed phase sorbent) 96-well plates (Waters, Mildford, Massachusetts), a vacuum manifold and a vacuum source. Ninety-six-well plates were selected in order to optimize sampling time. Analyzing 96 different samples at the same time is of great utility in large-scale studies. Before selection of specified cartridges, optimization of extraction method was done with MCX and MAX taking into account fabricant recommendations (Waters Oasis®).

The MCX 96-well plate was conditioned with 1mL of methanol followed by 1 mL of 2% formic acid. One milliliter of the analyte standard solution in synthetic urine or hydrolyzed urine sample were then loaded onto the plate and washed with 1 mL of 2% formic acid. The plates were thoroughly dried by vacuum (5-15 in.Hg). Analytes were then eluted with methanol (1.5 mL) by gravity and finally by vacuum (max. 5 in.Hg).

The MAX 96-well plate was conditioned with 1mL of methanol followed by 1 mL of water. One milliliter of the analyte standard solution in synthetic urine or hydrolyzed urine sample were then loaded onto the plate and washed with 1 mL of 5% ammonium hydroxide. The plates were thoroughly dried by vacuum (5-15 in.Hg). Analytes were then eluted with methanol (1.5 mL) and with 1mL of 2% formic acid in methanol by gravity and finally by vacuum (max. 5 in.Hg).

Eluates from both cartridges were evaporated to dryness under a stream of nitrogen. Residues were reconstituted with 100µL of mobile phase.

LC-MS/MS

The analyses were carried out by a liquid chromatography tandem mass spectrometry. LC-DAD analyses were performed using a Perkin Elmer series 200 (Norwark CT) equipped with a quaternary pump and a refrigerated autosampler. An Applied Biosystems API 3000 Triple Quadrupole mass spectrometer (PE Sciex, Concord, Ontario, Canada) equipped with a Turbo IonSpray ionizing in negative mode was used. A Phenomenex Luna C_{18} analytical column [50 x 2.0 mm i.d., 5µm] (Torrance, CA) with mobile phase A (95% water, 5% acetonitrile and 0.1% formic acid) and B (100% acetonitrile and 0.1% formic acid) was used with the following linear gradient at a flow rate of 400 mL/min (%mobile phase B, time (min)): (4, 0), (40, 1), (100, 3), (100, 5), (6, 4) and (10, 4). Then, the column was washed and re-equilibrated for 6

min. The volume injected was 15µL. MS/MS parameters for phenolic acid compounds were optimized by infusion and flow injection analysis (FIA). Direct infusion experiments were performed for each analyte at a constant flow rate of 5 µL/min to optimize the following MS parameters: Capillary voltage, focusing potential, entrance potential, declustering potential, and collision energy (CE). Nebulizer, curtain, collision, and auxiliary (nitrogen) gas flow rates, as well as the auxiliary gas temperature were optimized using FIA experiments.

For quantification of phenolic acids, procyanidin and epicatechin, data were collected in the multiple reaction monitoring (MRM) mode which track the transition of parent and product ions specific for each compound. A dwell time of 100 ms was used for each injection. Due to its high concentration in urine, hippuric acid was analyzed by DAD at 240 nm and identified by comparing with the retention time and UV spectrum of the authentic standard.

Human and rat urine creatinine concentrations were measured by a colorimetric assay using picric acid [15].

Statistical analysis

SPSS Statistical Analysis System, Ver. 14.0 (SPSS) was used to perform the statistical analysis. Because the data were skewed (Kolmogorov and Levene tests), the Wilcoxon test for related samples (human urines) and the Mann-Whitney test for no related samples (rat urines) were used to compare changes in outcome variables in response to intervention period or intervention type. Significant increases between 0 and 24 h for human urine samples, and between the control group and the cocoa groups for rat urine samples were expressed as means (s.e.m). Statistical tests were 2-tailed, and the significance level was 0.05.

Results and discussion

Quality Parameters of the Method

To evaluate the method, the following criteria were used: selectivity, linearity, sensitivity, recovery, precision and accuracy.

LC-MS/MS Optimization. The optimized MS/MS parameters for the phenolic acids and for the flavanols in infusion and FIA analyses were as follows: capillary voltage, -3700V; focusing potential, -200V; entrance potential, -10V; declustering potential, -50V; nebulizer gas, 10 (arbitrary units); curtain gas, 12 (arbitrary units); collision gas, 5 (arbitrary units); auxiliary gas temperature, 400ºC; auxiliary gas flow rate, 6000 cm^3/min. The collision energy (V) and the MRM transition obtained for each phenolic compound were: 3,4-dihydroxyphenylpropionic acid (-16, 181/137); 4-hydroxyphenylpropionic acid (-16, 165/93); m and p-coumaric acids (-30, 163/119); caffeic acid (-21, 179/135); ferulic acid (-25, 193/134); 3,4-dihydroxyphenylacetic acid (-12, 167/123); 3-methoxy-4-hydroxyphenylacetic acid (-10, 181/137); 3-hydroxyphenylacetic acid (-12, 151/107); phenylacetic acid (-12, 135/91); protocatechuic acid (-20, 153/109); vanillic acid (-20, 167/152); 3 and 4-hydroxybenzoic acid (-16, 137/93); 4-*O*-methylgallic acid (-26, 167/108); 4-hydroxyhippuric acid (-20, 194/100); enterodiol (-30, 301/253); enterolactone (-26, 297/253); (-)-epicatechin (-25, 289/245), and procyanidin B2 (-25, 577/289).

Selectivity. Under the chromatographic and MS/MS conditions used for the assay, metabolites and standards were well resolved and no peaks at the mass transition and at the retention time of analytes were observed in synthetic urine.

Extraction Recovery, Linearity and Residuals. SPE was performed between MCX and MAX 96-well plates in their optimum conditions. Similar recoveries were obtained with both cartridges when standards

were considered. However, after loading urine or hydrolyzed urine samples recovery was a 20% higher with MCX than with MAX cartridges. This was due to the fact that eluted solution was observed cleaner after MCX cartridges than after MAX cartridges. MAX cartridges work by both exchange anionic and by phase reverse. This fact caused the elution of compounds of interest and also interfering compounds that cause signal suppression due to the matrix effect when was analyzed in LC-MS/MS. Unlike MAX, MCX eluted all studied phenolic compounds with methanol and therefore, the interfering compounds remained retained in the cartridges.

The total extraction recovery was defined as the detector response obtained from an amount of analyte added to and extracted from a standard in synthetic urine compared to the detector response obtained for the true concentration of that standard solution. The total recovery values, expressed as the mean percentage value (%, SD) were the followings: 3,4-dihydroxyphenylpropionic acid (94%, 9.0); 4-hydroxyphenylpropionic acid (104%, 7.0); *m*-coumaric acid (91%, 4.5); *p*-coumaric acid (94%, 1.8); caffeic acid (102%, 7.0); ferulic acid (92%, 4.8); 3,4-dihydroxyphenylacetic acid (109%, 4.0); 3-methoxy-4-hydroxyphenylacetic acid (93%, 8.0); 3-hydroxyphenylacetic acid (91%, 4.9); phenylacetic acid (95%,6.0); protocatechuic acid (96%, 14.7); vanillic acid (95%, 10.0); 4-hydroxybenzoic acid (97%, 4.7); 3-hydroxybenzoic acid (91%, 4.9); 4-hydroxyhippuric acid (86%, 3.9); hippuric acid (94%, 5.5); 4-*O*-methylgallic acid (89%, 4.0); enterodiol (97%, 9.5); enterolactone (89%, 3.3); (-)-epicatechin (87%, 3.0) and procyanidin B2 (10%, 8.5). Taking into account that procyanidin B2 met the criteria in its accuracy and precision, samples were quantified although a minor extraction recovery was obtained. Furthermore, in order to confirm the presence of procyanidin B2 in samples, the extraction recovery was improved and optimized with the elution solvents up to 80% (9.5).

Calibration curves were performed over a wide range of concentrations (0.5-500 μg/L). The 6-point calibrator concentrations showed a linear and reproducible curve for standards. Weighted ($1/x^2$) least-square regression analysis yielded equation regression lines, correlation coefficients and residual analysis [mean range (SD)] as seen in Table 1.

Detection and Quantification Limits. The limit of detection (LOD) was defined as the concentration of analyte that produced a signal-to-noise ratio of 3. The limit of quantification (LOQ) was defined as the minimum analyte concentration required to ensure precise quantitative measurements and was determined as the concentration of analyte that produced a signal-to-noise ratio of 10. The LODs (LOQs) varied between 0.030 μg/L (0.10 μg/L) for enterodiol, and 44.4 μg/L (148 μg/L) for phenylacetic acid, indicating a wide range of sensitivity between the different analytes (Table 1). The sensitivity of this method represents a significant improvement of between 6 and 100 times more in the major part of compounds when comparing to other LLE-LC/MS-MS published methods [10;11]. This will prove useful in nutritional studies when low levels must be evaluated.

Precision and Accuracy. Accuracy and precision (repeatability) were determined for the different analytes using three different concentration levels in the range of expected concentrations (low, medium and high, in function of the LOD of each particular analyte) and performing five determinations per concentration over a 5-day period. The precision of the method, expressed by the relative standard deviation (%RSD), met acceptance criteria since it was lower than 15% at each tested concentration level (Table 2) [20]. The accuracy of the method, expressed as [(mean observed concentration)/(added concentration)x100], was acceptable at each concentration level: 90.3-113.8% for the low concentration value, 89.5-112.1% for the medium concentration value, and 87.5-108.7% for the high concentration value (Table 2) [20].

The precision of the retention time of the different analytes was also evaluated (Table 1).

Analysis of Flavanols and Phenolic Acids in Human and Rat Urines

The validated method was applied to determine the concentration of phenolic acid microbial metabolites, as well as epicatechin and dimeric procyanidin metabolites in urine samples collected after the consumption of nutritional doses of cocoa in humans and rats, which is a very rich source of proanthocyanidins. To identify the flavanols and phenolic acid metabolites in urine samples MRM transition, MS spectra, retention time, and UV compared with authentic standards were used. Quantification was done from the peaks obtained in the MRM mass chromatograms.

Phenolic Acid Metabolites in Urine. Table 3 shows the changes in concentration of the different phenolic acid metabolites identified in human and rat urine samples after cocoa consumption.

In humans, with the exception of phenylacetic acid, an increase in the concentration of all the metabolites was registered after cocoa consumption. In particular, caffeic acid, ferulic acid, 3-hydroxyphenylacetic acid, vanillic acid, 3-hydroxybenzoic acid, 4-hydroxyhippuric acid, hippuric acid, (-)-epicatechin and procyanidin B2 showed a statistically significant (p<0.05) increase after 24h of cocoa consumption in human volunteers (Table 3). Interindividual differences were observed in this study as were also observed in similar phenolic bioavailability studies [21]. These results are in accordance with those reported in the study carried out by Rios et al [22] in which volunteers ingested a double dose of cocoa. However, contrary to the present work these authors did not find significant changes in the concentration of 3-hydroxyphenylacetic, 3-hydroxybenzoic, 4-hydroxyhippuric and hippuric acid after 24h of cocoa consumption. In another human feeding trial where six different polyphenol-rich beverages were compared [11], a significant increase (p<0.05) in (-)-epicatechin was found after 24h of cocoa beverage intake, which is also in agreement with the present results.

The metabolic profile described in this work is focused on the metabolites determined after cocoa intake. However, the method presented here is also available for other phenolic metabolites including enterolactone and enterodiol, which are produced by the microbial intestinal metabolism of lignans and for 4-*O*-methylgallic acid, described as a biomarker of wine or tea consumption and associated with the metabolism galloylated flavanols [5;23].

In rats, a significant increase in the concentration of the following compounds was observed after the intake of 4.8 g/kg/day of cocoa diet during 2 weeks when compared to the control diet (Table 3): 3,4-dihidroxyphenylpropionic acid, *m*-coumaric acid, 3-hydroxyphenylacetic acid, protocatechuic acid, vanillic acid and (-)-epicatechin. In addition to the above compounds, after consumption of the diet containing 10% (w/w) cocoa, a significant increase was also recorded for caffeic acid, 3-methoxy-4-hydroxyphenylacetic acid, 3-hydroxybenzoic acid, enterolactone and procyanidin B2 (Table 3). Finally, significant differences were also observed between the two different cocoa diet groups for *m*-coumaric acid, caffeic acid, 3-hydroxyphenylacetic acid, protocatechuic acid, vanillic acid, 3-hydroxybenzoic acid, enterolactone, (-)-epicatechin and procyanidin B2 in favor of the 10% w/w cocoa chow diet (Table 3). In general, from these results, it could be noted that major differences were registered for phenolic acids containing hydroxyl groups at C3 and C4, or at C3 position.

This is the first study related to the microbial metabolism in rats after the consumption of cocoa. Previously, Gonthier et al. 2003 [18;24], studied the excretion of phenolic acids in rats after the ingestion of wine polyphenolic extract or catechin [18] and after the consumption of diets containing catechin, or dimeric, trimeric and polymeric procyanidins [24].

The minor increased concentrations found in human urine in comparison to rat urine, as well as the difference in predominant phenolic acids observed between the two species, could be due to the different ingested dose and to the different microbial metabolism in the intestine of each species, human and rodent. The ingested dose of cocoa in humans was ~10-fold minor than the 4.8 g cocoa/kg/day diet in rats, and 20-fold minor than the 10% w/w cocoa chow diet.

Besides the above mentioned metabolites, the phenylvalerolactones 5-(3',4'-dihydroxyphenyl)-γ-valerolactone (DHPV) and (3-methoxy-4-hydroxyphenyl)-γ-valerolactone (MHPV) were also identified based on their mass spectra in the human and rat urine samples after cocoa consumption These epicatechin-derived microbial metabolites have been previously identified after tea [25] and wine powder consumption[18] but are detected for the first time in humans and rats after cocoa consumption. In order to identify these metabolites, hydrolyzed urine samples were injected in the product ion scan mode of deprotonated molecules ($[M-H]^-$) in negative mode. The MS spectra and the fragmentation pattern generated for DHPV (*m/z* 207) and MHPV (*m/z* 221) are shown in Figure 1. The fragment at *m/z* 163, previously described [25], as well as the generation of other characteristic fragment ions supports the identification of these compounds.

Procyanidin in Urine. Procyanidin B2 has been identified and quantified in human and rat urine after cocoa consumption. MRM chromatograms of procyanidin B2 (577/289) in human and rat urine are shown in Figure 2. It is important to highlight that other peaks in the same transition (577/289) could be observed. Tentatively, the peak at 6 min in human urine could be another procyanidin that due to unavailable of standard cannot be confirmed.. Rat urine chromatogram showed two major unidentified peaks that did not correspond to procyanidin B1 or fragments from trimeric compounds (procyanidin C1) due to closer retention times to procyanidin B2 [26]. Changes in concentration levels recorded in humans and rats after cocoa consumption are shown in Table 3. Dimeric procyanidins (B1 or B2) have been previously detected in human plasma and serum but this evidence is only limited to two studies [27;28]. In rats, procyanidin B2 was quantified in plasma and urine after oral administration of procyanidin B2 [29] and recently, dimeric and trimeric procyanidins have also been detected in rat plasma after the consumption of apple procyanidins [30].

Conclusions

The SPE-LC-MS/MS method developed in the present work allows the simultaneous determination of 19 microbial phenolic metabolites, as well as monomeric and dimeric flavanols in human and rat urine samples collected after cocoa consumption. This method mainly offers significant improvement in sensitivity and recovery leading its application to studies with a lot of number of samples. Procyanidin B2 has also been detected for the first time in human and rat urine after cocoa consumption. Furthermore, at a qualitative level, two poorly-described metabolites (DHPV and MHPV) derived from procyanidin, epicatechin and/or catechin metabolism have also been confirmed. Major advantages over previous reported methods also include the reduction of laboratory work in the sample preparation step by the use of 96-well SPE plates, and the sensitive measurement of a large number of metabolites in a very short run time, which makes it ideal for use in epidemiological studies. More studies about the effects of food consumption in microbial metabolism are required. Some of these described compounds could be accurate biomarkers of proanthocyanidin consumption, the major fraction of flavonoid intake.

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Table 1. Calibration curves, limits of detection (LOD) and quantification (LOQ), residual analysis and retention time of analytes of the SPE-HPLC-MS/MS method.

Analyte	Retention Time (%RSD)	Calibration curve; R^2	Residuals %mean (SD)	LOD (μg/L)	LOQ (μg/L)
C_6C_3					
3,4-dihydroxyphenylpropionic acid	2.53 (0.9)	y = 0.31x + 0.02; 0.991	95 (2.8)	1.67	5.57
4-hydroxyphenylpropionic acid	4.28 (5.2)	y= 5.69e-4x + 1.1e-4; 0.999	103 (6.8)	16.50	55.02
m-coumaric acid	4.87 (3.5)	y = 1.06x + 0.005; 0.953	97 (5.6)	0.26	0.87
p-coumaric acid	4.39 (3.9)	y = 0.42x + 0.002; 0.986	98 (12.8)	0.52	1.74
caffeic acid	3.11 (5.8)	y = 2.02x + 0.03; 0.999	102 (5.4)	0.05	0.18
ferulic acid	4.90 (1.3)	y = 0.06x + 0.002; 0.997	98 (9.8)	0.49	1.62
C_6C_2					
3,4-dihydroxyphenylacetic acid	1.43 (2.7)	y = 0.28x + 0.003; 0.998	100 (10.2)	0.71	2.37
3-methoxy-4-hydroxyphenylacetic acid	3.22 (8.1)	y = 0.02x + 0.003; 0.999	97 (7.9)	37.11	124.12
3-hydroxyphenylacetic	3.36 (3.7)	y = 0.07x + 0.001; 0.996	102 (7.0)	2.75	9.17
phenylacetic acid	5.00 (3.8)	y = 0.02x + 0.0001; 0.995	106 (8.3)	44.40	148.01
C_6C_1					
protocatechuic acid	1.21 (2.5)	y = 0.93x + 0.01; 0.988	100 (13.1)	0.63	2.10
vanillic acid	2.88 (11.2)	y = 7.00e-3x + 0.004; 0.999	95 (7.5)	5.12	17.10
4-hydroxybenzoic acid	1.86 (6.2)	y = 0.32x + 0.004; 0.993	98 (10.4)	1.68	5.60
3-hydroxybenzoic acid	3.19 (3.3)	y = 0.13x + 0.004; 0.998	103 (10.0)	9.09	30.30
4-hidroxyhippuric acid	1.11 (3.5)	y = 0.42x + 0.01; 0.993	99 (11.1)	0.18	0.59
hippuric acid	2.17 (2.2)	y = 3.67e-3x + 1.73; 0.999	98 (0.1)	0.22	0.72
4-*O*-methylgallic acid	5.64 (0.4)	y = 3.06x + 0.005; 0.996	98 (12.4)	0.03	0.11
Enterolignans					
enterodiol	5.48 (0.5)	y = 1.60x + 0.002; 0.993	98 (8.9)	0.03	0.10
enterolactone	5.87 (0.4)	y = 1.60x + 0.004; 0.988	100 (7.6)	0.06	0.20
Flavanols					
(-)-epicatechin	4.34 (2.0)	y=0.405x+2.69e-4; 0.996	96 (10.3)	0.49	1.64
procyanidin B2	4.17 (7.8)	y=3.88e-3x - 2.33e-4; 0.996	100.0 (8.5)	4.68	15.63

Table 2. Precision and Accuracy Data obtained from the LC-MS/MS of phenolic acids and flavanols in different days at three different concentration levels.

Analyte	Low concentration (µg/L)				Medium concentration (µg/L)				High concentration (µg/L)			
	Added	**Mean**	**Accuracy (%)**	**Precision (%RSD)**	**Added**	**Mean**	**Accuracy (%)**	**Precision (%RSD)**	**Added**	**Mean**	**Accuracy (%)**	**Precision (%RSD)**
C_6C_3												
3.4-dihydroxyphenylpropionic acid	5.0	4.8	96.0	14.4	50.0	56.1	112.1	10.9	500.0	448.4	89.7	11.9
4-hydroxyphenylpropionic acid	100.0	103.9	103.9	15.0	250.0	238.1	95.2	10.7	500.0	543.3	108.7	13.0
m-coumaric acid	0.5	0.5	100.4	3.6	10.0	9.4	94.0	5.5	100.0	102.9	103.0	6.5
p-coumaric acid	1.0	1.0	96.8	8.5	50.0	55.9	111.8	14.0	500.0	463.4	89.4	14.5
caffeic acid	0.5	0.5	95.8	7.5	50.0	53.3	106.6	13.2	500.0	478.2	95.6	4.4
ferulic acid	1.0	1.1	113.8	14.7	50.0	48.1	96.2	15.0	500.0	475.1	95.0	4.7
C_6C_2												
3.4-dihydroxyphenylacetic acid	1.0	1.0	95.9	2.2	10.0	9.65	95.5	8.3	250.0	242.6	97.0	11.4
3-methoxy-4-hydroxyphenylacetic acid	50.0	47.3	94.6	10.5	100.0	90.7	90.7	10.7	500.0	478.0	96.0	11.2
3-hydroxyphenylacetic	5.0	4.9	99.5	12.8	100.0	105.5	105.4	8.8	500.0	442.4	88.5	4.5
phenylacetic acid	50.0	56.2	112.4	4.1	250.0	278.9	111.6	2.9	500.0	499.0	99.8	4.8
C_6C_1												
protocatechuic acid	1.0	0.9	90.3	8.8	10.0	10.8	108.0	14.9	250.0	245.2	98.1	9.4
vanillic acid	10.0	9.6	96.4	5.3	50.0	47.3	94.6	13.0	250.0	234.3	93.7	9.5
4-hydroxybenzoic acid	5.0	5.0	100.0	3.6	50.0	55.3	110.4	14.3	250.0	237.1	94.8	10.0
3-hydroxybenzoic acid	10.0	9.4	94.0	8.4	100.0	102.4	102.4	6.2	500.0	457.4	91.5	12.4
4-hidroxyhippuric acid	0.5	0.5	98.0	9.9	10.0	11.2	112.1	6.6	250.0	213.6	85.4	7.2
hippuric acid	100.0	91.3	91.3	11.7	500.0	501.5	100.3	0.1	1000.0	995.0	99.5	6.9
4-*O*-methylgallic acid	0.5	0.5	98.7	4.8	10.0	9.0	89.8	0.1	250.0	232.8	93.1	13.8
Enterolignans												
Enterodiol	0.5	0.5	104.1	3.0	50.0	44.7	89.5	3.5	500.0	437.5	87.5	3.4
Enterolactone	0.5	0.5	97.9	5.3	10.0	9.9	99.2	3.4	100.0	97.9	97.9	4.0
Flavanols												
Epicatechin	1.0	0.9	93.5	12.6	100.0	92.5	92.6	10.3	500.0	498.4	99.7	6.9
Procyanidin B2	100.0	102.4	998.5	3.0	250.0	226.5	90.6	13.3	500.0	535.3	107.1	8.5

Table 3. Changes in concentration levels of microbial phenolic acids and flavanols in human and rat urine samples after cocoa consumption.

Analyte	Human urine Δ (s.e.m.) 0-24h nmol/mg creatinine	Rat urine Δ (s.e.m.) control-cocoa diet (4.8g/kg cocoa diet) nmol/mg creatinine	Rat urine Δ (s.e.m.) control-cocoa diet (10% w/w cocoa chow) nmol/mg creatinine
C_6C_3			
3,4-dihydroxyphenylpropionic acid	0.79 (2.4)	0.37 (0.2) [a]	0.93 (0.2) [a]
4-hydroxyphenylpropionic acid	< LOQ	n.d.	n.d.
m-coumaric acid	0.014 (0.007)	14.64 (2.1) [a]	46.13 (4.6) [a,b]
p-coumaric acid	0.057 (0.03)	0.17 (0.7)	1.73 (0.8)
caffeic acid	0.39 (0.13) [a]	0.04 (0.3)	1.26 (0.4) [a,b]
ferulic acid	10.52 (2.3) [a]	3.48 (2.5)	-1.94 (2.0)
C_6C_2			
3,4-dihydroxyphenylacetic acid	-0.02 (0.2)	0.94 (0.6)	0.40 (0.3)
3-methoxy-4-hydroxyphenylacetic acid	1.54 (1.4)	5.92 (4.6)	11.27 (2.7) [a]
3-hydroxyphenylacetic	6.53 (4.0) [a]	67.67 (23.6) [a]	181.56 (18.6) [a,b]
phenylacetic acid	-35.84 (19.8) [a]	36.70 (27.4)	20.52 (46.2)
C_6C_1			
protocatechuic acid	0.51 (1.4)	5.66 (1.8) [a]	18.84 (2.5) [a,b]
vanillic acid	1.14 (0.6) [a]	15.78 (6.6) [a]	50.57 (7.5) [a,b]
4-hydroxybenzoic acid	0.55 (1.2)	20.08 (13.8)	10.28 (14.0)
3-hydroxybenzoic acid	0.45 (0.2) [a]	0.73 (0.3)	7.20 (1.1) [a,b]
4-hidroxyhippuric acid	1.27 (0.7) [a]	-4.97 (8.1)	-0.79 (11.0)
hippuric acid	59.43 (20.1) [a]	62.45 (56.4)	75.34 (71.7)
4-*O*-methylgallic acid	n.d.	n.d.	n.d.
Enterolignans			
Enterodiol	0.001 (0.002)	0.004 (0.004)	0.004 (0.005)
Enterolactone	0.11 (0.2)	0.26 (0.3)	2.05 (0.8) [a,b]
Flavanols			
Epicatechin	0.53 (0.35) [a]	5.49 (2.0) [a]	36.62 (3.4) [a,b]
procyanidin B2	0.20 (0.2) [a]	0.49 (0.3)	3.10 (0.5) [a,b]

[a] Significant difference with respect to the control diet group, $P < 0.05$;
[b] Significant difference with respect to the 4.8g cocoa/kg/day diet group, $P < 0.05$
LOQ: limit of quantification; n.d.: not detected.

FIGURE CAPTIONS

Figure 1. Negative ion MS/MS spectra and proposed fragmentation pattern of 5-(3'4'-dihydroxyphenyl)-γ-valerolactone (A) and (3-methoxy-4-hydroxyphenyl)-γ-valerolactone (B) in human urine after cocoa consumption.

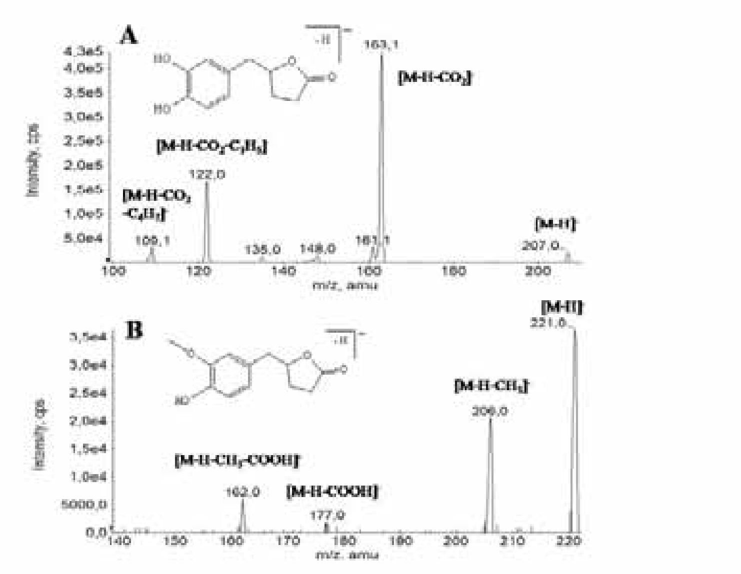
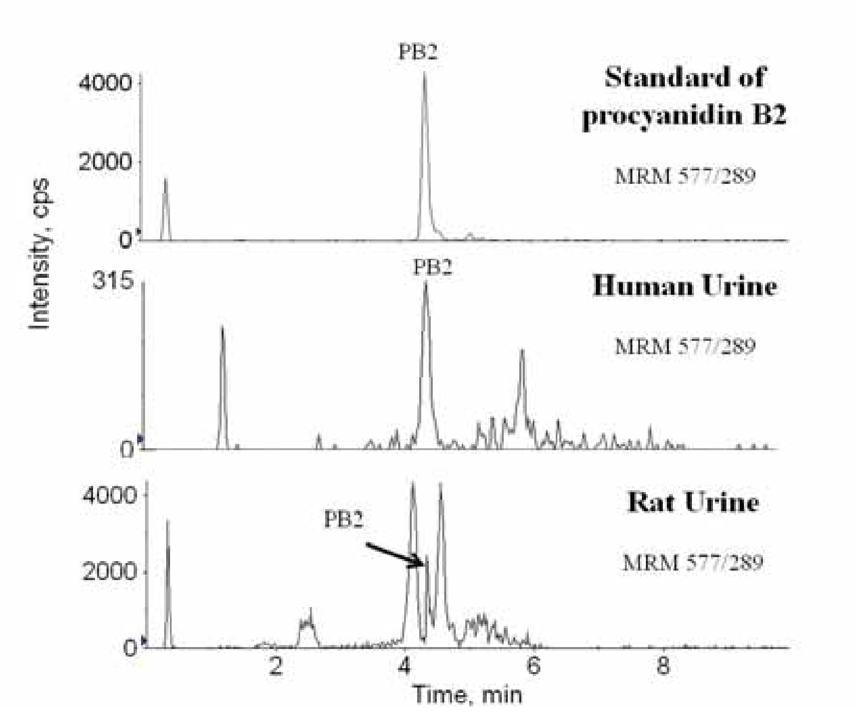


Figure 2. Multiple Reaction Monitoring chromatogram of the standard procyanidin B2 and its presence in Human Urine after intake of 40g cocoa, and in Rat Urine after the intake of the 10% (w/w) cocoa chow.



2.2 Identificació i quantificació dels metabòlits urinaris del cacau. Aplicació de la metodologia analítica en un estudi d'intervenció de cacau en rates

Publicació IV: Dietes enriquides en cacau augmenten l'activitat antioxidant i modulen la composició limfocitària en el timus de rates joves.

Emma Ramiro-Puig, Mireia Urpi-Sarda, Francisco J. Pérez-Cano, Àngels Franch, Cristina Castellote, Cristina Andrés-Lacueva, Maria Izquierdo-Pulido, and Margarida Castell. Cocoa-Enriched Diet Enhances Antioxidant Enzyme Activity and Modulates Lymphocyte Composition in Thymus from Young Rats. *Journal of Agricultural and Food Chemistry*. **2007**, 55 (16):6431-8

Resum:

El cacau és una important font de flavonoids, principalment (-)-epicatequina, (+)-catequina i procianidines. En aquest treball es va considerar l'efecte del consum regular de cacau en la capacitat antioxidant del plasma i d'òrgans limfoides i fetge en rates joves.

El disseny de l'estudi va consistir en tres grups amb deu rates Wistar deslletades en el qual cadascun va rebre: 1) una dieta control, 2) una dieta amb un 4% de cacau administrat en forma oral per *gavage*, 3) una dieta amb un 10% de cacau natural adjuntat en el pinso. Es va administrar aquesta dieta durant 3 setmanes, període que correspon a tota la seva infància (Figura 25).

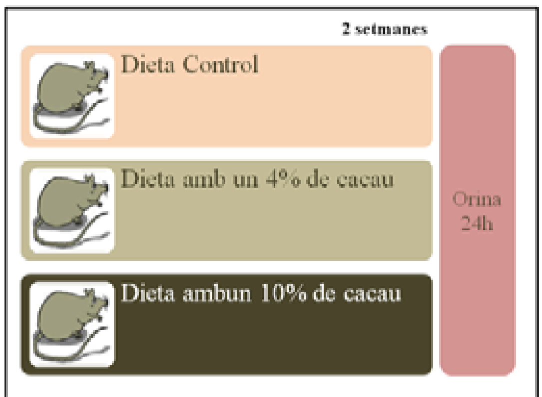


Figura 25: Esquema de l'estudi

Aquest treball es va realitzar en col·laboració amb la Dra. Emma Ramiro-Puig. El treball que forma part d'aquesta tesi doctoral va ser la valoració del consum i la biodisponibilitat dels flavonoide del cacau a través de la quantificació de metabòlits de l'epicatequina en orina per espectrometria de masses en tàndem. Es van identificar i van quantificar tres metabòlits glucuronidats de l'epicatequina, tres metabòlits glucuronidats de la metilepicatequina i l'epicatequina en les mostres d'orina després del consum d'ambdues dietes riques en cacau.

El consum de cacau incrementava la capacitat antioxidant total en tots els teixits i especialment en el timus. A més, les activitats superòxid dismutasa i catalasa van ser incrementades de manera dosi depenent. També es va estudiar si l'increment del sistema antioxidant podia influenciar la composició cel·lular i es va trobar un augment en el percentatge de timocits en estadis avançats de desenvolupament. En conclusió, una dieta en cacau augmenta les defenses antioxidants en el timus i influencia la diferenciació en timocits.

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Cocoa-Enriched Diet Enhances Antioxidant Enzyme Activity and Modulates Lymphocyte Composition in Thymus from Young Rats

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Cocoa is a rich source of flavonoids, mainly (−)-epicatechin, (+)-catechin, and procyanidins. This article reports the effect of continuous cocoa intake on antioxidant capacity in plasma and tissues, including lymphoid organs and liver, from young rats. Weaned Wistar rats received natural cocoa (4% or 10% food intake) for three weeks, corresponding to their infancy. Flavonoid absorption was confirmed through the quantification of epicatechin metabolites in urine. Total antioxidant capacity (TAC) and the activity of antioxidant enzymes, superoxide dismutase (SOD) and catalase, were examined. Cocoa intake enhanced TAC in all tissues especially in thymus. Moreover, thymus SOD and catalase activities were also dose-dependently increased by cocoa. It was also analyzed whether the enhanced antioxidant system in thymus could influence its cellular composition. An increase in the percentage of thymocytes in advanced development stage was found. In summary, cocoa diet enhances thymus antioxidant defenses and influences thymocyte differentiation.

KEYWORDS: **Catalase activity; SOD activity; cocoa; lymphocyte phenotype; thymocyte differentiation**

INTRODUCTION

The production of reactive oxygen species (ROS), including free radicals, is a well-established physiological process which is controlled by intrinsic antioxidant systems. The antioxidant defense comprises enzymatic and nonenzymatic mechanisms. Antioxidant enzymes, such as superoxide dismutase (SOD) and catalase, constitute a first line defense by catalyzing the conversion of specific ROS (superoxide anion ($O_2^{\bullet-}$) and H_2O_2, respectively) into less reactive or nonreactive products. Nonenzymatic antioxidants comprise an array of molecules, including albumin, glutathione, and uric acid among others, which act neutralizing nonspecifically highly reactive oxidants, such as hydroxyl radical ($HO^{\bullet}$), overproduced when specific intracellular defenses are overwhelmed (*1*). In certain situations, the intrinsic oxidant/antioxidant balance is shifted toward the production of reactive species and consequently oxidative stress is enhanced. An elevated oxidized state within a cell can be extremly harmful, resulting in radical generation that leads to lipid peroxidation, DNA cross-linking, and the formation of disulfide bonds in proteins (*2*). Oxidative stress is clearly involved in a wide range of pathological and physiological disorders such as cancer, cardiovascular disease, and aging (*3*, *4*). Therefore, improving antioxidant defenses could be a key strategy to prevent or reduce the risk of disease.

A great interest in developing functional foods has recently emerged to improve the state of health and well-being, or reduce disease risks (*5*). Cocoa is a rich source of antioxidants, mainly flavonoids such as (−)-epicatechin, (+)-catechin, and polymers derived from these monomers called procyanidins (*6*, *7*). In this regard, a serving size of certain cocoa-derived products provides more phenolic antioxidants than beverages and fruits such as tea and blueberries, traditionally considered high in antioxidants (*8*). In many countries, cocoa-derived products are very commonly consumed and in the European Union and US cocoa intake is estimated at ~2.6 and ~2.35 g/day of cocoa powder per capita, respectively (*8*). Besides considering cocoa as a dietary antioxidant source, it could also be viewed as a natural product with therapeutic properties.

In vitro studies have demonstrated the protective effect of cocoa and its flavonoids on different cellular models of oxidative stress (*9*−*11*). However, these effects cannot be directly extrapolated to humans given that bioavailability and metabolism should be taken into account. Cocoa flavonoids are stable during gastric transit (*12*), rapidly absorbed, and found in plasma after cocoa beverage consumption (*13*). Flavonoids are extensively metabolized to mainly glucuronides, sulfates, and O-methylated forms during transfer through the small intestine and then again

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in the liver. Nonabsorbed flavonoids reach the colon where gut microflora convert them into phenolic acids which are able to be absorbed and further metabolized in the liver (*14*). Therefore, flavonoids tested in *in vitro* studies are obviously not the same as the ones that reach cells in *in vivo* conditions.

Some studies have shown that cocoa also presents antioxidant properties *in vivo*. Cocoa intake enhances antioxidant capacity and decreases lipid oxidation products in plasma from healthy humans (*15*−*17*) and rats (*13*, *18*). Moreover, cocoa consumption also reduces lipid peroxidation in plasma from individuals with increased oxidative stress (*19*, *20*). Most of those studies were conducted in adult animals or humans after receiving a single cocoa dose. To date, the effects of continuous cocoa consumption on young rats whose immune system is still in maturation (*21*) remain to be explored.

In this article, we determined the antioxidant status in healthy young rats that were provided a continuous cocoa diet from weaning and during three weeks. The intestinal absorption and metabolism of cocoa flavonoids were assessed by quantifying their metabolites in urine. Then, we focused on the analysis of total antioxidant capacity (TAC) in plasma, lymphoid tissues (spleen and thymus), and liver. SOD and catalase activities in tissues were also evaluated as representative antioxidant enzymatic systems. Given the importance of redox status in lymphocyte maturation and the high antioxidant enzyme activity detected in the thymus, further assays were designed to establish changes in its cellular phenotype.

MATERIALS AND METHODS

Apparatus: LC−MS/MS. High-performance liquid chromatography (HPLC) was performed using a Perkin-Elmer series 200 (Norwalk, CT, USA) equipped with a quaternary pump, a refrigerated autosampler, and a diode-array detector. A triple quadrupole mass spectrometer API 3000 (Applied Biosystems, PE Sciex, Concord, Ontario, Canada) equipped with a Turbo IonSpray source operated in the negative-ion mode was used to obtain the MS and MS/MS data.

Reagents, Standards, and Diets. Reagents and standards were obtained from the following sources: methanol and acetonitrile (HPLC grade) from Scharlau (Barcelona, Spain); formic acid, (−)-epicatechin, (+)-catechin, quercetin, creatinine, β-glucuronidase and sulfatase (type H-2), 2-2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diamonium salt (ABTS), and bovine serum albumin (BSA) from Sigma-Aldrich-Fluka (St. Louis, MO); Trolox from Calbiochem (Darmstadt, Germany); taxifolin and procyanidin B2 from Extrasynthese (Genay, France); and isoquercetin from Promochem (Barcelona, Spain). Ultrapure water (Milli-Q) was obtained from Millipore System (Bedford, MA). RPMI 1640 media and fetal bovine serum (FBS) were purchased from PAA (Pashing, Austria). 2-Mercapto-ethanol (ME) was provided by Merck (Darmstadt, Germany).

Fluorescein isothiocyanate (FITC)-conjugated anti-rat CD3 (1F4) MAb, phycoerithrin (PE)-conjugated anti-rat TCR$\alpha\beta$ (R73) and CD4 (OX-35) MAb, and peridinin-chlorophyll-a protein (PerCP)-conjugated anti-rat CD8α (OX-8) MAb were purchased from BD Biosciences (Heidelberg, Germany). FITC-conjugated anti-rat CD90 (Thy-1) MAb was obtained from Caltag (Burligame, CA).

Natural Forastero cocoa (Nutrexpa, Barcelona, Spain) was used for this study. The AIN-93G formulation (*22*), which provides the nutrients required for optimal rat growth, was used as the control diet. The 10% cocoa diet was produced from modified AIN-93G containing 100 g of cocoa per kg. Diet compositions are detailed in **Table 1**.

Phenolic Content of Cocoa and Chow. The total phenolic content was determined by the Folin−Ciocalteu method and was expressed as (+)-catechin equivalents in mg/g (*23*). The determination and quantification of individual phenolic compounds in cocoa powder and chow (mg/g) were analyzed by HPLC as previously described (*24*).

Animals and Experimental Design. Dams with 15-day-old Wistar rat litters (50% male, 50% female) were obtained from Harlan (Barcelona, Spain). Rats were housed in cages of 10 pups per lactating mother in controlled conditions of temperature and humidity in a 12:12 light:dark cycle. At day 21, pups were weaned and randomly assigned to the following dietary groups.

Table 1. Composition of the Experimental Diets (g/kg)[a]

components	control chow (AIN-93G)	10% cocoa-enriched chow
casein	200	178
L-cystine	3	3
corn starch	397.486	381.486
maltodextrin	132	132
sucrose	100	100
soybean oil	70	59
cellulose	50	24.5
mineral mix (TD94046)	35	35
vitamin mix (TD94047)	10	10
choline bitartrate	2.5	2.5
TBHQ	0.014	0.014
natural cocoa powder		100
22% protein		
16% carbohydrate		
11% lipid		
25.5% cellulose		
total calories (kcal/kg diet)	3700	3700

[a] 10% cocoa-enriched diet was prepared from the AIN-93G control diet removing 72.8 g/kg (16 g/kg of corn starch, 11 g/kg of soybean oil, 25.5 g/kg of cellulose, and 22 g/kg of casein) and adding natural cocoa.

4% Cocoa-Enriched Diet Group (4%-Cocoa Group). Animals received daily 4.8 g of cocoa/kg of rat by oral gavage. According to the chow intake per day, this dose corresponded to ∼4% (g of cocoa/100 g of chow). Rats were given free access to control chow and water.

4%-Cocoa Control Diet Group. Animals received daily water (cocoa vehicle) by oral gavage. Rats were given free access to control chow and water.

10% Cocoa-Enriched Diet Group (10%-Cocoa Group). Animals were given free access to water and chow containing 10% (w/w) cocoa (**Table 1**).

10%-Cocoa Control Diet Group. Animals were given free access to water and control chow.

Studies were performed in accordance with the institutional guidelines for the care and use of laboratory animals, and experimental procedures were approved by the Ethical Committee for Animal Experimentation of the University of Barcelona (ref 3131).

Sample Obtaining. After 2 weeks of dietary treatment, rats were maintained in metabolic cages for 24 h to collect urine output. Urine was centrifuged (2000g, 15 min, room temperature (RT)), acidified by adding HCl (0.2 M in urine), and frozen at −80 °C for further flavonoid metabolite analysis. After 3 weeks of cocoa or control diet, 6-week-old rats were anesthetized to obtain blood by cardiac puncture and thymus, spleen, and liver were excised. Organs were rinsed with a phosphate buffered saline (PBS) solution (pH 7.4) and immediately frozen in liquid nitrogen and then stored at −80 °C until analysis. Blood samples were kept on ice and were centrifuged (3000g, 10 min, 4 °C) to obtain plasma which was stored at −80 °C until antioxidant capacity assays.

Urine Extraction Procedure. Urine was thawed at 4 °C and processed as previously described (*25*) with some modifications in solid-phase extraction (SPE) support. Briefly, 1 mL of urine was loaded onto a Waters Oasis HLB cartridges (60 mg) (Waters, Mildford, MA) that had been preconditioned with 1 mL of methanol and equilibrated with 2 mL of 1.5 mol/L formic acid in water. The cartridge was washed with 2 mL of 1.5 mol/L formic acid and with 2 mL of water/methanol (95:5; v:v). Elution was achieved with 2 mL of methanol with 0.1% formic acid. The eluate was evaporated to dryness and reconstituted with mobile phase to 100 μL. Percent recovery of spiked control urine was >71% (±5.3%). Urine creatinine was measured by a colorimetric assay using picric acid (*26*).

Liquid Chromatography/Tandem Mass Spectrometry (HPLC−MS/MS) Analyses. The chromatographic and spectrometric method was performed as previously described (*24*). Briefly, a Luna C_{18} column (50 × 2.0 mm i.d., 5 μm) (Phenomenex, Torrance, CA) was used with a gradient elution with 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B at 800 μL/min. The MS and MS/MS parameters were as previously described (*24*). Each sample was measured by HPLC−MS/MS in the multiple reaction monitoring (MRM) mode with the following transitions: (−)-epicatechin (289/245), glucuronided epicatechin (465/289), sulfated epicatechin (369/289), *O*-methyl epicatechin (303/288), *O*-methyl epicatechin glucuronide (479/289), sulfated *O*-methyl epicatechin (383/289), sulfated epicatechin glucuronide (545/289), sulfated *O*-methyl epicatechin glucuronide (559/289), procyanidin B2 (577/289), and taxifolin (303/285). Peaks were identified with a product ion scan at a cycle time of 1 s.

To confirm the conjugated forms an enzymatic hydrolysis was performed as described previously (*27*) with some modifications. A 1 mL urine sample was acidified to pH 5 with 50 μL of 0.58 mol/L acetic acid and incubated at 37 °C for 45 min under the presence of an *Helix pomatia* extract containing 1100 U of β-glucuronidase and 42 U of sulfatase (Sigma). After acidification to pH 2 with 40 μL of 6 mol/L HCl, the urine was loaded onto a preconditioned cartridge and treated as described above. The concentration of (−)-epicatechin metabolites was expressed as epicatechin equivalents (*25*, *28*).

Tissue Homogenates. Tissue samples were homogeneized in 5 mL of ice-cold buffer (50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA) per gram of tissue, using a Polytron (Kinematica, Switzerland). Then, samples were centrifuged (10000*g*, 15 min at 4 °C) and supernatants were stored at −80 °C until use (<1 month).

ABTS Assay. Total antioxidant capacity (TAC) in plasma and tissue samples was determined by the ABTS method described by Re et al. (*29*) with modifications. This assay is based on the sample ability, by means of its nonenzymatic antioxidants, to reduce $ABTS^{\bullet+}$ (blue-green color), resulting in a decolorization.

ABTS was dissolved in water at 7 mM, and immediately $K_2S_2O_8$ was added (2.45 mM final concentration) to convert ABTS into $ABTS^{\bullet+}$ radical cation. This solution was kept in continuous agitation and in darkness for 12−16 h before using it to allow $ABTS^{\bullet+}$ generation (blue-green color). Then, $ABTS^{\bullet+}$ was diluted with distilled water at a 1:50 ratio. The assay was performed in 96-well plates. Trolox, a hydrosoluble vitamin E derivative, was used as a standard. Diluted $ABTS^{\bullet+}$ (100 μL) was added to 100 μL of each sample or standard diluted in phosphate buffered saline solution (PBS) pH 7.4. Absorbance at 420 nm was monitored every 60 s throughout 360 s. The percentage of absorbance inhibition was calculated and plotted as a function of time and concentration. Sample values were interpoled into a trolox standard curve (*29*). Results were expressed as mM trolox for plasma samples and μmol/g for tissue homogenates.

Antioxidant Enzyme Activities. Superoxide dismutase and catalase activities in tissue samples were determined by means of colorimetric methods using commercial kits (Calbiochem, Darmstadt, Germany). Superoxide dismutase activity assay is based on the detection of superoxide radicals generated by xanthine oxidase addition. A tetrazolium salt was used as a chromogen, and absorbance values were interpoled into a SOD standard curve. Results were expressed as U/mg protein. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

Catalase activity was established by the sample ability to react with methanol in the presence of H_2O_2. Formaldehyde produced was measured spectrophotometrically by using purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole) as chromogen. Absorbance values were interpoled into a formaldehyde standard curve, and results were expressed as nmol of formaldehyde/min/mg of protein.

Protein content was quantified by the Bradford assay (*30*) with bovine serum albumin as standard.

Thymocyte Isolation. Thymocyte cell suspension was obtained by passing the thymus through a steel mesh (Cellector). Cell suspension was incubated on ice to remove tissue debris by sedimentation for 10 min. Thereafter, cells were centrifuged (500*g*, 5 min, 4 °C) and resuspended with RPMI media containing 10% FBS and 0.05 mM ME (complete media). Cell counting and viability were determined by fluorescence light microscopical analysis.

Table 2. Total Phenolic Content (Folin–Ciocalteu) and Polyphenols (HPLC) in Cocoa Powder and Chow Containing 10% Cocoa (mg/g)[a]

	cocoa powder	10% cocoa chow
total polyphenol	21.85 ± 1.08	0.90 ± 0.54
(+)-catechin	0.74 ± 0.05	0.10 ± 0.004
procyanidin B2	1.68 ± 0.11	0.23 ± 0.01
(−)-epicatechin	2.20 ± 0.10	0.34 ± 0.01
isoquercetin	0.05 ± 0.001	0.02 ± 0.00
quercetin	0.03 ± 0.00	0.01 ± 0.00

[a] Results were expressed as mean ± SD (*n* = 3).

Phenotype by Immunofluorescence Staining and Flow Cytometry Analysis. Thymocytes were stained with anti-rat MAb conjugated to FITC, PE, or PerCP: anti-TCRαβ (R73), anti-CD4 (OX-35), anti-CD8α (OX-8), anti-CD3 (1F4), and anti-CD90 (Thy-1). 2 × 10^5 cells were labeled with saturating concentrations of FITC-, PE-, and PerCP-MAb in PBS pH 7.2 containing 1% FBS and 0.09% NaN_3 (30 min, 4 °C, in darkness). A negative control staining using an isotype-matched MAb was included for each sample. After washing with PBS pH 7.2, cells were fixed with 0.5% p-formaldehyde and stored at 4 °C in darkness. Analyses were performed using a Coulter Epics XL2 Corporation cytometer (Miami, FL), and data was assessed by cytometer software (Summit V3.1, Cytomation, Inc). Results were expressed as percentage of positive cells in the lymphocyte population previously selected according to their forward (*FSC*) and side (*SSC*) scatter characteristics.

Statistical Analysis. Results from figures and tables were expressed as mean ± SEM, except for **Table 2** that was expressed as mean ± SD. Data of epicatechin and metabolites concentrations were skewed (Kolmogorov and Levene tests). The Wilcoxon test for related samples was carried out to compare changes between both cocoa intakes. Conventional one-way ANOVA was performed, considering diet groups as independent variables. When cocoa intake had a significant effect on the dependent variable, Bonferroni's test was applied. Significant differences were accepted when $P < 0.05$. After comparing the experimental groups, no statistical differences were seen between both control groups. Therefore, in order to simplify the interpretation of the results, the data from the two control groups (gavage and nongavage) were pooled in the graphs. SEM of control groups in graphs indicates the low dispersion for these groups. Significant differences marked in the graphs are owing to the comparison between treatment group and its corresponding control group. All data were analyzed using SPSS Statistical Analysis System, V. 11.5 (SPSS).

RESULTS

Polyphenols Content in Cocoa and Chow. Total and individual phenolic content in cocoa powder and 10% cocoa chow is shown in **Table 2**. Both cocoa powder and 10% cocoa chow contained substantial amounts of polyphenols, whereas the phenolic levels of control diet (AIN-93G) were under the detection limit. Therefore, in our study polyphenols were provided by cocoa.

Epicatechin Metabolites in the 24 h Rat Urine. The quantification of epicatechin metabolites is established as a biomarker of flavonoid absorption (*31*). The identification of metabolites in biological samples was based on 3 parameters: comparison of retention time of available standard, MRM metabolite transition and epicatechin transition [with higher DP in collision-induced dissociation (CID)/MS/MS conditions], and product ion spectra.

Epicatechin aglycone was identified in urine samples from cocoa fed rats (peak 1) (**Figure 1**). Three epicatechin glucuronides and three glucuronided *O*-methyl epicatechins were identified in the rat urine (**Figure 1**). Peaks **2**, **3**, and, **4** represent the glucuronided metabolites with MRM of 465/289 that were

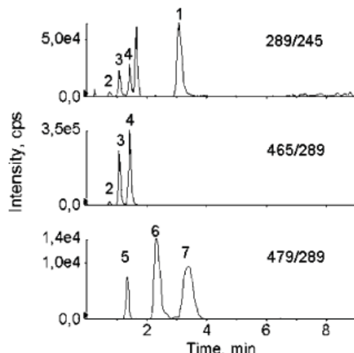

Figure 1. Representative multiple reaction monitoring chromatogram of one rat urine. Peaks: (**1**) (–)-epicatechin, (**2**, **3**, **4**) glucuronided epicatechin, (**5**, **6**, **7**) glucuronided *O*-methyl epicatechin.

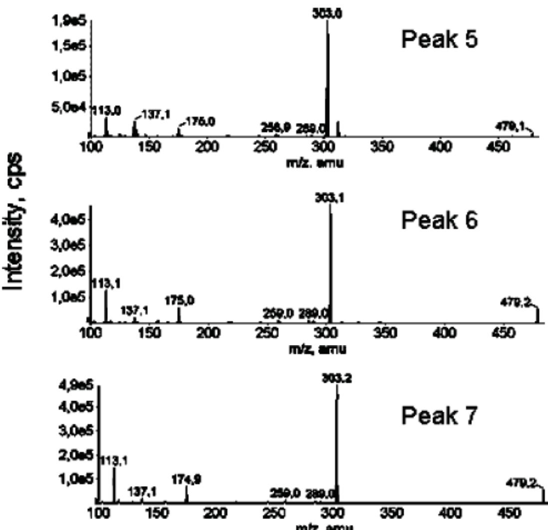

Figure 2. Product ion spectra of peaks **5**, **6**, and **7**. They confirm the three *O*-methyl epicatechin glucuronides in different positions.

confirmed with the epicatechin transition (289/245). Peaks **5**, **6**, and **7** with MRM of 479/289 correspond to glucuronided *O*-methyl epicatechin identified with the product ion scan of the deprotonated molecule (*m*/*z* 479). The product ion spectra of the three peaks showed the deprotonated molecule (*m*/*z* 479) and the ions *m*/*z* 303 corresponding to methyl epicatechin, *m*/*z* 137 corresponding to the classic epicatechin-related A-ring fragment, and *m*/*z* 175 and *m*/*z* 113 corresponding to the glucuronid acid (**Figure 2**). These confirming results agree with those of Schroeder et al. (*28*), who showed the spectra of authentic standards of various glucuronided methyl-epicatechin isomers.

The low concentration of other epicatechin metabolites did not allow us to confirm their presence by product ion scan or by MRM transitions (*32*).

Epicatechin and Epicatechin Metabolites in the Rat Urine. After 2 weeks of dietary supplementation with cocoa (gavage or chow), epicatechin and epicatechin metabolites were found in the urine of all rats, whereas no metabolites were detected in control rat urines. **Table 3** shows the amount of epicatechin and its metabolites in urine after diets with 4% or 10% cocoa. Cocoa metabolites found in urine were proportional to cocoa intake, but no significant differences were found between both groups ($P > 0.05$). A dose-dependent excretion and the high variability among different rats were described by Baba et al. (*33*).

Table 3. Mean (±SEM) of (–)-Epicatechin, Glucuronided Epicatechin, and Glucuronided *O*-Methyl Epicatechin (μmol/g Creatinine) from Rat Urine Excreted after Administration of 4% Cocoa in Gavage or Chow Containing 10% (w/w) Cocoa (n = 6–12)

	4%-cocoa group	10%-cocoa group
(–)-epicatechin	1.92 ± 0.93	4.00 ± 1.85
glucuronided epicatechin	14.26 ± 1.60	25.57 ± 18.14
O-methyl epicatechin glucuronide	0.53 ± 0.07	0.99 ± 0.62
total epicatechin metabolites	16.66 ± 2.20	30.55 ± 18.53

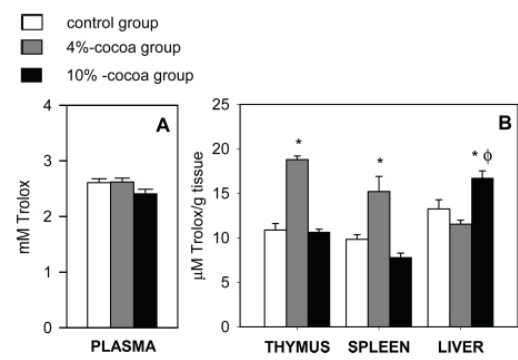

Figure 3. Plasma (**A**), thymus, spleen, and liver (**B**) total antioxidant capacity (TAC) in young rats after three weeks of cocoa intake. Each bar represents the mean ± SEM (n = 9–15). *$P < 0.05$ in 4%- or 10%-cocoa groups vs their respective control groups. $^{\phi}P < 0.05$ in 4%- vs 10%-cocoa group.

Table 4. Percentages of Thymocytes Expressing Low Levels and High Levels of TCRαβ, and the Coreceptors CD4 and/or CD8 on Their Surface in Young Rats after Three Weeks of 10% Cocoa Intake[a]

	control group (%)	10%-cocoa group (%)
TCRαβ^{high}	13.24 ± 2.81	13.88 ± 1.31
TCRαβ^{low}	61.94 ± 2.92	49.16 ± 3.18 *
total TCRαβ+	75.18 ± 2.80	63.05 ± 4.14 *
CD8+CD4+ (DP)	88.61 ± 0.22	82.19 ± 1.20 *
CD8–CD4– (DN)	1.90 ± 0.09	3.41 ± 0.19 *
total immature thymocytes (CD8+CD4+, CD8–CD4–)	90.52 ± 0.22	85.59 ± 1.07 *
CD8+CD4– (SP)	3.42 ± 0.59	4.67 ± 0.76
CD8–CD4+ (SP)	6.07 ± 0.71	9.75 ± 0.95 *
total mature thymocytes (CD8+CD4–, CD8–CD4+)	9.18 ± 0.45	14.41 ± 1.01*

[a] Values represent the mean ± SEM (n = 4). *$P < 0.05$ in 10%-cocoa group vs control group.

Effect of Cocoa Diet on Plasma and Tissue Total Antioxidant Capacity (TAC). TAC was measured in plasma, thymus, spleen, and liver by means of ABTS assay (**Figure 3**). Plasma TAC from both cocoa groups did not significantly differ from that of control rats (**Figure 3A**). However, tissue TAC was significantly improved: 4% cocoa diet enhanced thymus and spleen TAC by ~73 and 55%, respectively($P < 0.05$). Unexpectedly, 10% cocoa diet did not affect thymus and spleen

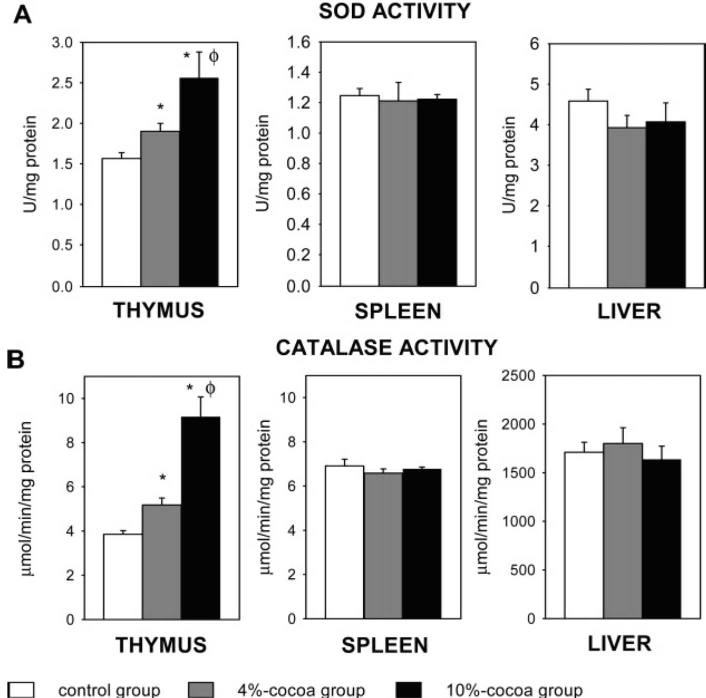

Figure 4. Thymus, spleen and liver superoxide dismutase (SOD) (**A**) and catalase (**B**) activities in young rats after three weeks of cocoa intake. Each bar represents the mean ± SEM ($n = 9–15$). *$P < 0.05$ in 4%- or 10%-cocoa groups vs their respective control groups. $^{\phi}P < 0.05$ in 4%- vs 10%-cocoa group.

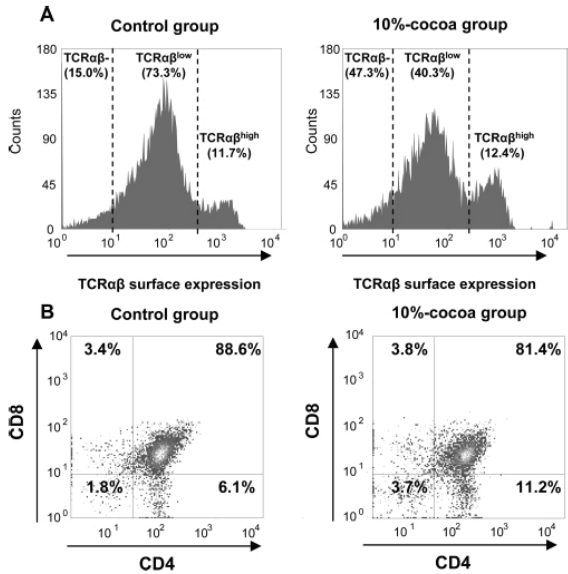

Figure 5. Histograms showing the distribution of thymocytes according to the TCR$\alpha\beta$ surface expression from a representative control rat and a 10% cocoa fed rat (**A**). Biparametric histograms showing the distribution of thymocytes according to the surface expression of coreceptors CD4 and CD8 from a representative control rat and a 10% cocoa fed rat (**B**).

TAC, but was capable of increasing liver TAC by ~26% ($P < 0.05$) (**Figure 3B**).

Effect of Cocoa Diet on Thymus, Spleen, and Liver Antioxidant Enzymes. SOD and catalase activities were measured in thymus, spleen, and liver after 3 weeks of treatment. Cocoa diets did not modify SOD and catalase activities in spleen and liver, but interestingly, this diet dose-dependently enhanced both enzyme activities in thymus, achieving an increase of 1.64-fold in SOD and 2.4-fold in catalase activities with respect to control groups ($P < 0.05$) (**Figure 4**).

Effect of Cocoa Diet on Thymocyte Phenotype. Thymocyte phenotype was analyzed in 10% cocoa fed rats and its corresponding control group. In both groups, 99% of thymocytes expressed CD90 and CD3 on their surface. Thymocyte differentiation is characterized by the expression of well-defined cell-surface markers, including CD4 and CD8, as well as T-cell receptor (TCR$\alpha\beta$). Thus, single positive (SP) cells (CD8+CD4− and CD8−CD4+) with high levels of TCR$\alpha\beta$ surface expression (TCR$\alpha\beta^{high}$) corresponded to the more mature thymocytes present in the thymus. 10% cocoa diet significantly decreased the proportion of T cells with low expression of TCR$\alpha\beta$ ($P < 0.05$ vs control group); however, the proportion of mature lymphocytes (TCR$\alpha\beta^{high}$) was not significantly affected (**Table 4** and **Figure 5A**). Moreover, when coreceptor was studied, a higher percentage of thymocytes in advanced development stage, specifically SP CD4+ cells ($P < 0.05$ vs control group), and a lower proportion of developing DP T cells (CD8+CD4+) were found in 10%-cocoa group ($P < 0.05$ vs control group) (**Table 4** and **Figure 5B**). However, 10% cocoa increased the proportion of double negative cells (DN or CD8−CD4−), which also may act as non-T cell precursors ($P < 0.05$) (**Table 4**).

DISCUSSION

Previous *in vitro* studies showed the inhibitory effects of cocoa flavonoids on ROS production from activated immune cells (*10*, *34*). This article reports for the first time the effect of continuous cocoa intake on lymphoid tissue antioxidant capacity

in young rats. The cocoa used in this study contained 2.2% (w/w) of total polyphenols, (−)-epicatechin being the major monomeric compound (~10% of total polyphenols). Weaned rats were fed with cocoa-enriched diets for three weeks, which corresponds to their infancy (puberty is estimated at 7−9 weeks in both sexes) (*35*). Two cocoa dosages were tested: 4% of food intake administered by oral gavage and 10% included in the chow. In both administration protocols, cocoa flavonoids were absorbed and metabolized by rats as demonstrated by the total amount of flavonoid metabolites excreted in urine that is well correlated with flavonoid absorption (*31*). Although bioavailability may be affected by the type of oral administration (gavage vs chow), our results showed a dose-dependent epicatechin absorption regardless of whether cocoa was given by oral gavage or was included in the diet.

Given that cocoa flavonoids were absorbed, antioxidant capacity was expected to be increased. However, no differences in plasma TAC were found among our groups, and this could be attributed to the short plasmatic half-lives of flavonoids. In this regard, a transitory increase of plasma TAC 10−45 min postgavage, correlating with the plamastic peak of epicatechin, was detected in rats (*13*, *18*). In our study, blood from 4%-cocoa fed rats was obtained ~20 h postgavage and 10%-cocoa fed rats were deprived of cocoa-enriched chow ~2 h before blood collection. Therefore, the gap between cocoa intake and blood collection could explain the lack of plasma TAC changes among groups.

Although plasma TAC was not modified by cocoa intake, tissue TAC were significantly enhanced. A hierarchy in reducing activity was observed: thymus > spleen > liver. This could be attributed to flavonoid accumulation in specific target tissues allowing a maintained enhancement of their antioxidant capacity. In this regard, it has been described that, after long-term consumption, quercetin, a flavonoid also present in cocoa, was accumulated mainly in lung > testes > kidney > thymus in a dose-dependent manner (*34*). The effects on thymus and spleen TAC were found not to be significantly dose-dependent. Thus, 4% cocoa diet produced a strong increase in both thymus and spleen TAC, whereas 10% cocoa diet only increased TAC in liver. One possible reason for this fact may be the activation of oxidative pathways in thymus and spleen as a cell compensatory mechanism triggered by high levels of antioxidants accumulated in those tissues (*4*). On the contrary, in the liver the metabolic rate of flavonoids would decrease their antioxidant capacity and therefore an oxidative compensatory effect would not occur. However, further studies must be conducted to clarify this hypothesis.

Apart from TAC, the activity of endogenous antioxidant enzymes was also analyzed. The cell lacks a specific system to remove hydroxyl radical ($HO^{\bullet}$), considered the most reactive oxygen species responsible for cell damage. Therefore, the scavenging of $O_2^{\bullet-}$ and H_2O_2 by SOD and catalase enzymes, respectively, is crucial to prevent $HO^{\bullet}$ generation. In the present study, thymus SOD and catalase activities were dose-dependently increased by cocoa intake, whereas spleen and liver were not affected. Cocoa antioxidants, especially flavonoids, seem to be responsible for the enzyme activity enhancement. In this regard, polyphenols can increase SOD and catalase activities by neutralizing their substrates ($O_2^{\bullet-}$ and H_2O_2, respectively) and by upregulating the expression of these enzymes (*37*). Both SOD and catalase activities increased only in the thymus that possibly due to a higher flavonoid accumulation than in other tissues as previously described (*36*). The higher amount of free epicatechin, proportional to the cocoa dose ingested, could reflect the major target concentration of free epicatechin in thymus (lipophilic tissue) in a dose-dependent manner due to its partition coefficient (*38*, *39*).

In addition, spleen and liver contain higher proportions of phagocytes producing ROS than thymus (macrophages in the red pulp of spleen and Kupffer cells in the liver). Therefore, the high activity of SOD and CAT in thymus could be in part due to a lower consumption of such enzymes in this tissue.

The influence of cocoa on thymus antioxidant activity led us to believe that cocoa could also affect lymphocyte composition as we previously found in spleen and gut-associated lymphoid tissue (GALT) (*40*). This study shows that 10% cocoa diet seems to accelerate the progress of immature thymocytes (DN and DP cells with $TCR\alpha\beta^{low}$ expression) toward more mature T cell stages (SP cells with $TCR\alpha\beta^{high}$). Thus, 10% cocoa diet may influence the microarchitecture and cell signaling, inducing changes in T cell development. It has been described that a maintained reducing environment of the cell stimulates a slight shift toward a mildly oxidizing environment that promotes cell differentiation and maturation (*4*). Therefore, a continuous antioxidant environment in thymus may promote a mild oxidant state that favors lymph maturation. On the other hand, DN cells, a subset whose proportion was also increased by cocoa diet, have multilineage potential, including B cell, T cell, myeloid cell, natural killer cells, and dendritic cells (*41*). In this sense, high cocoa intake may promote the differentiation of other immune cell subsets. During infancy the immune system is still in development and consequently the body is more susceptible to infection; therefore enhancing lymphocyte differentiation may be beneficial to prevent or reduce the risk of disease.

In summary, cocoa was effectively absorbed and metabolized by young rats after continuous intake, but did not affect plasma antioxidant activity. However, our results show the enhancement of antioxidant capacity in tissues including thymus, spleen, and liver. In the thymus, the richest cocoa diet produced a strong increase in the activity of antioxidant enzymes and may also enhance thymic maturation.

SAFETY

Rat urine samples were considered as potentially infectious. Guidelines for work with organic solvents and acids were respected. Universal precautions for the handling of chemicals and fluids were applied.

ACKNOWLEDGMENT

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2.3 Perfil metabòlic urinari i plasmàtic dels flavanols del cacau després d'un consum regular en humans amb risc cardiovascular

Publicació V: Estudi del perfil metabòlic urinari i plasmàtic dels flavanols del cacau en voluntaris amb risc de malaltia cardiovascular després d'una ingesta regular i dietètica de cacau soluble

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En procès de revisió

Resum:

El cacau és una font rica en flavonoids, principalment (-)-epicatequina, (+)-catequina i procianidines. El consum de cacau a llarg termini podria reduir els paràmetres inflamatoris relacionats amb la malaltia cardiovascular.

Quaranta-dos voluntaris amb alt risc cardiovascular van ser reclutats per a l'estudi. Per a la inclusió en l'estudi, es va requerir que els voluntaris tinguessin almenys 3 dels següents factors de risc cardiovascular: fumar, diabetis (glicèmia>126mg/dl), hipertensió (> 140/90 mmHg), colesterol LDL 160 mg/DL, colesterol≥ HDL 35≤ 25kg/m2),≥mg/DL, obesitat (índex de massa corporal història familiar de risc cardiovascular prematur. I es van rebutjar aquells que van presentar els següents criteris: presència d'algun esdeveniment cardiovascular, reaccions al·lèrgiques a algun component del cacau, malalties gastrointestinals, neurològiques, psiquiàtriques, endocrines o tumorals, virus de la immunodeficiència humana, alcoholisme crònic o addicció a les drogues. L'estudi, randomitzat creuat i controlat, va consistir en el consum durant un mes de 40g/dia de cacau "light" dissolts en 250 ml de llet desnatada, seguit per altre mes de consum control de 250 ml/dia de llet desnatada. Les mostres de plasma i orina es van obtenir abans de l'estudi i després de cada tractament (Figura 26).

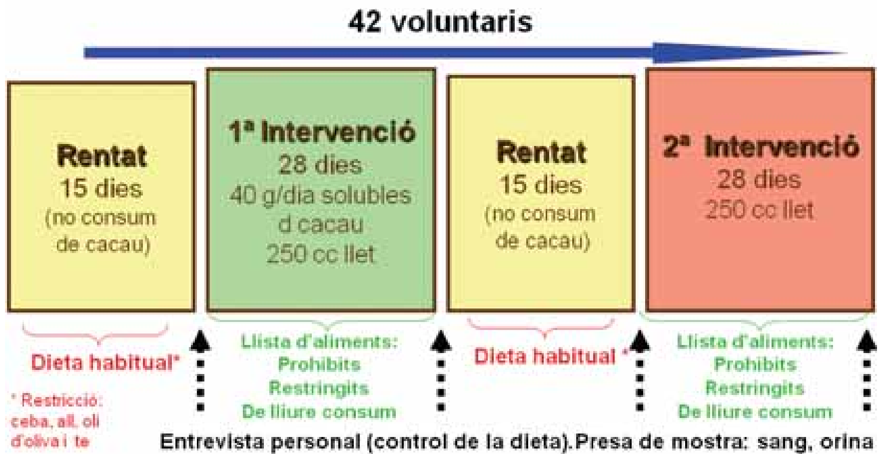

Figura 26: Esquema de l'estudi

Després del consum d'un mes de cacau es van observar increments significatius del metaboloma urinari i plasmàtic dels flavanols que provenien del cacau així com dels àcids fenòlics formats per la microbiota colònica.

Els principals metabòlitos de fase II que van incrementar després del consum d'un mes de cacau en orina van ser els glucurònids i sulfats de l'epicatequina, els glucurònids i sulfats de la metil-epicatequina, els glucurònids i sulfats de la dihidroxifenilvalerolactona i els glucurònids i sulfats de la metoxihidroxifenilvalerolactona.

També es va observar un augment urinari del perfil metabòlic colònic després del consum de cacau observant diferències significatives amb el tractament control amb llet per a l'àcid vanillic i l'àcid 3-hidroxifenilacètic.

A causa del consum regular de cacau i a les concentracions màximes descrites per a la epicatequina (tmax: 2h), no es van trobar metabòlits de fase II en el plasma dels voluntaris que havien consumit cacau. En canvi, es va observar un perfil metabòlic plasmàtic d'àcids fenòlics amb concentracions elevades de dihidroxifenilvalerolactones, i àcid 3-hidroxifenilacètic.

TARGETED PHENOLIC METABOLIC PROFILE AFTER REGULAR CONSUMPTION OF COCOA POWDER IN HUMAN SUBJECTS WITH HIGH CARDIOVASCULAR RISK FACTORS

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Abstract

Cocoa (*Theobroma cacau*) and its derived products represent a very rich source of dietary flavonoids and its consumption has been associated with numerous health benefits. Health effects derived from cocoa consumption depend on the bioavailability of cocoa polyphenols. A randomized, crossover and controlled clinical trial was designed in order to study the profile of conjugated and non-conjugated metabolites derived from phase II-enzymes and from the intestinal microbiota in both 24-h urine and fasting plasma after regular consumption of cocoa powder with skimmed milk in subjects presenting high risk of cardiovascular heart disease. Subjects (*n*=42) received two sachets of 20g of cocoa powder (40% cocoa) per day with 250 mL skim milk each or only 500 mL/day of skim milk for 4 weeks in a random order. The analysis of urine revealed the presence of (-)-epicatechin-*O*-glucuronide, (-)-epicatechin-sulfate, *O*-methyl-epicatechin-*O*-glucuronide, *O*-methyl-epicatechin-sulfate. In addition, glucuronide and sulfate derivatives of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone and 5-(3'-methoxy-4'-hydroxyphenyl)-γ-valerolactone were also detected. In the case of plasma, only glucuronide derivatives of both hydroxyphenylvalerolactones were identified. Microbial-derived phenolic metabolites included hydroxyphenylvalerolactones, hydroxyphenylpropionic acids, hydroxyphenylacetic acids, hydroxycinnamic acids, hydroxybenzoic acids and hydroxyhippuric acids. Regular consumption of cocoa resulted in a significant increase in (-)-epicatechin, vanillic acid, 3-hydroxyphenylacetic and particularly in 5-(3',4'-dihydroxyphenyl)-γ-valerolactone in urine, whereas only the two latter metabolites showed a significant increase in plasma, representing a 13% and 5% increment in total microbial metabolites, in urine and plasma respectively in comparison to the intake of milk alone. The results found herein indicate that 5-(3',4'-dihydroxyphenyl)-γ-valerolactone could be a good biomarker of regular consumption of flavanols.

Key words. Cocoa, epicatechin, 5-(3',4'-dihydroxyphenyl)- -valerolactone, gut microbiota.

INTRODUCTION

Cocoa (*Theobroma cacau*) and its derived products represent a very rich source of dietary flavonoids cocoa containing higher amounts of flavonoids per serving than tea or red wine (Lee et al. 2003). Flavanols are the most abundant flavonoids in cocoa. They comprise the monomeric flavanols (+)-catechin and (-)-epicatechin, and their oligomeric and polymeric forms (procyanidins). (-)-Epicatechin has been reported as the major monomeric flavanol in cocoa, representing ca. 35% of the total phenolic content (Wollgast and Anklam, 2000). In contrast to most fruits which contains the (+)-catechin enantiomer, as a consequence of processing, cocoa-derived products mainly contains the (-)-catechin enantiomer, which is less bioavailable (Donovan et al., 2006). Cocoa procyanidins consist primarily of (-)-epicatechin (Porter et al., 1991, Rigaud et al, 2003) up to the level of decamer (Hammerstone et al., 1999; Adamson et al., 1999). Oligomers (procyanidins B1, B2, B5 and C1) and polymers account for ≥90% of total polyphenols, and monomers for 5-10% (Wollgast and Anklam, 2000; Adamson et al., 1999).

Numerous cocoa human feeding trials have been performed in the last years (Ding et al., 2006; Cooper et al., 2008). Biomarkers significantly affected in these trials are related to: *i) antioxidant effects* (decrease in LDL oxidation and oxidative stress, increase in antioxidant status); *ii) anti-platelet effects* (inhibition of platelet activation and function, and improvement in insulin sensitivity); *iii) effects on lipid metabolism* (increase in HDL concentration); *iv) effects on vascular function* (increase in NO bioactivity, lower systolic and diastolic blood pressure, improvement in endothelial function).

Health effects derived from cocoa consumption depend on the bioavailability of cocoa polyphenols. Flavanol bioavailability is influenced by their degree of polymerization (Manach et al. 2004). Monomers are readily absorbed in the small intestine. As the result of phase II enzymes, (-)-epicatechin is converted into glucuronided and sulfated metabolites but also into methylated metabolites which could be also glucuronided and sulfated. The absorption of dimeric procyanidins in humans seems to be very limited. Only one study reports the detection of low amounts of procyanidin B2 in human plasma after the consumption of a flavanol-rich cocoa (Holt et al., 2002). Although it was first thought that cocoa procyanidins could be degraded into monomers in the gastrointestinal tract (Spencer et al., 2000), further studies have confirmed that this does not occur in vivo (Rios et al., 2002). Oligomers up to the level of pentamers have been recently detected in rat plasma after consumption of apple procyanidins (Shoji et al., 2006). However, it is well established that polymeric procyanidins are not absorbed in humans in their native form and reach the colon where they are degraded by the intestinal microbiota. Consumption of procyanidin-rich foods by healthy humans resulted in the production of hydroxyphenylvalerolactones which in turn could be degraded into a series of organic acids, including phenylvaleric, phenylpropionic, phenylacetic and benzoic acid derivatives that could be further absorbed and contribute to the health effects derived from their consumption (Li et al., 2000; Meng et al., 2002; Rios et al., 2003; Ward et al., 2004; Gao et al., 2006). In fact, it has been recently reported that these metabolites may also exert several biological activities, such as inhibition of platelet aggregation and activation function (Rechner et al., 2005), inhibition of IL-1, IL-6 and TNF-α synthesis in PBMC (Monagas et al., 2008), inhibition of cyclooxygenase-2 (COX-2) in HT-29 colon cancer cells (Karlsson et al., 2005), reduction of the synthesis of prostanoids in colon cells (Russell et al., 2006), antiproliferative activity in prostate and cancer cells (Gao et al., 2006) and, finally, influence cell proliferation, apoptosis and signalling pathways in human colon carcinoma cells (Glinghammar & Rafter, 2001).

To our knowledge there is only one study reporting describing microbial-derived metabolites in urine after consumption of a single dose (80g) of chocolate in healthy subjects (Rios et al, 2003). The aim of the present study was to determine the profile conjugated and non-conjugated metabolites derived from phase II enzymes, as well as from the intestinal microbiota in both urine and plasma after regular consumption (4 weeks) of cocoa powder with milk in subjects presenting high risk of cardiovascular heart disease.

MATERIALS AND METHODS

Subjects

A total 42 volunteers (19 men and 23 women, mean age of 69.7± 11.5 years) presenting high risk of CHD were included in the study. Subjects included in the study were required to present 3 or more of the following cardiovascular risk factors: tobacco smoker, diabetes mellitus (glycemic ≥ 126mg/dL), hypertension (BP≥ 140/90 mmHg), LDL-cholesterol ≥ 160 mg/dL, HDL-cholesterol ≤ 35 mg/dL, obesity (corporal mass index ≥ 25), family history of premature CHD. Exclusion criteria included: presence of previous cardiovascular event; allergic reactions to any cocoa components; gastrointestinal, neurological, psiquiatric, endrocrine or tumoral diseases; human immunodeficiency virus infection; chronic alcoholism or drug addiction. The institutional review board of the Hospital Clinic de Barcelona approved the study protocol and all participants gave written consent before participation in the study.

Study design

The study was a 4-week randomized, crossover and controlled clinical trial. After a 2-week lead-in diet, subjects received two sachets of 20g of cocoa powder (40% cocoa) per day (one for breakfast and another one after dinner) (total/day: 40g; 46 mg of epicatechin ingested) with 250 mL skim milk each (total/day: 500 mL) **(milk+cocoa intervention)** or only 500 mL/day of skim milk **(milk intervention)** for 4 weeks in a random order. The phenolic and nutritional composition of the cocoa powder (defatted and sugar free) used in the study is presented in **Table 1**. Total phenolic determination was performed by Folin-Ciocalteu reagent (Singleton and Rossi, 1965), and individualized phenolic compounds were determined by HPLC (Andrés-lacueva et al., 2000). The mean degree of flavanol polymerization was 8 as estimated by thyiolsis (Monagas et al., 2003). Fasting blood samples and a 24h-urine specimen were collected at baseline and after each intervention (milk+cocoa or milk). Samples were stored at -80 °C until analysis.

Standards and Reagents.

Phenylacetic acid (≥98% purity); 3-hydroxyphenylacetic acid (≥97% purity); 3,4-dihydroxyphenylacetic acid (homoprotocatechuic acid; 98% purity); 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid; 99% purity); 4-hydroxyphenylpropionic acid (phloretic acid; ≥98% purity); 3,4-dihydroxyphenylpropionic acid (dihydrocaffeic acid) (≥98% purity); *p*-coumaric acid (≥98% purity); caffeic acid (≥95% purity); ferulic acid (≥98% purity); protocatechuic acid (>97% purity); 4-hydroxybenzoic acid (≥98% purity); 3-hydroxybenzoic acids (≥98% purity); ethyl gallate (≥96% purity); (-)-epicatechin (≥98% purity); (+)-catechin (≥98% purity); procyanidin B2 (≥90% purity), creatinine and -glucuronidase/sulfatase (from *Helix pomatia*) were purchased from Sigma-Aldrich (St. Louis, MO). 4-hydroxyhippuric acid (>99% purity) was purchased from PhytoLab GmbH&Co.KG (Vestenbergsgreuth, Germany). Vanillic acid, 4-*O*-methylgallic acid and *m*-coumaric acid were purchased from Extrasynthèse (Genay, France). HPLC grade solvents methanol, acetonitrile, glacial acetic acid and formic acid were purchased from Scharlau (Barcelona, Spain). Hydrochloric acid was purchased from Panreac (Barcelona, Spain).

Sample preparation for determination of microbial metabolites

One milliliter of urine or 1 ml of plasma samples was spiked with 100µL of 10.1 nmol/mL ethyl gallate as internal standard (IS). Samples were then hydrolyzed as previously described (Urpi-Sarda et al. 2008). Briefly, samples were acidified with 50 µL of 0.58 mol/L acetic acid to pH 4.9 and incubated with β-glucuronidase/sulfatase at 37ºC for 45 min. Straight afterwards, samples were acidified to pH 2 with 6 mol/L HCl.

Solid-phase extraction of samples was performed using Oasis MCX 96-well plates (Waters, Mildford, Massachusetts), a vacuum manifold and a vacuum source (Urpi-Sarda et al. 2008). The plate was conditioned with 1mL of methanol followed by 1 mL of 2% formic acid. The hydrolyzed samples were

then loaded onto the plate, washed with 1 mL of 2% formic acid and were thoroughly dried by vacuum (5-15 in.Hg). Analytes were then eluted with methanol (0.5 mL x 3) by gravity and finally by vacuum (max. 5 in.Hg) and the eluates were evaporated to dryness under a stream of nitrogen. Residues were reconstituted with 100µL of mobile phase.

Sample preparation for determination of phase II metabolites of epicatechin and its conjugated ring-fission metabolites.

Solid-phase extraction of non-hydrolyzed samples was performed using Oasis HLB 96-well plates (Waters, Mildford, Massachusetts) as described by Roura et al. 2005. The plate was conditioned with 1mL of methanol followed by 1 mL of 1.5 mol/L formic acid. One milliliter of plasma with 20µl of *o*-phosphoric acid or 3 ml of urine samples were loaded onto the cartridge plate. Then, the cartridges were washed with 1 mL of 1.5 mol/L formic acid and 1 mL of 5% methanol in water. Analytes were eluted with 1.5 ml of 1 ml/l formic acid with methanol by gravity and finally by vacuum. The eluates were evaporated to dryness under a stream of nitrogen.

LC-MS/MS.

The analyses of hydrolyzed and non-hydrolyzed samples were carried out by liquid chromatography-tandem mass spectrometry. LC-DAD analyses were performed using an Agilent 1200 system equipped with a quaternary pump and a refrigerated autosampler plate (Waldbronn, Germany). An Applied Biosystems API 3000 Triple Quadrupole mass spectrometer (PE Sciex, Concord, Ontario, Canada) equipped with a Turbo IonSpray ionizing in negative mode was used. A Phenomenex Luna C_{18} analytical column [50 x 2.0 mm i.d., 5µm] (Torrance, CA) with mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) was used. The linear gradient for phenolic acid determination at a flow rate of 400 mL/min was (%mobile phase B, time (min)): (8, 0), (50, 4), (100, 5.2) and (100, 7)(Urpi-Sarda et al. 2008). And the linear gradient for epicatechin metabolites determination at a flow rate of 400 mL/min was (%mobile phase B, time (min)): (9, 0), (80, 6), (100, 6.5), (100, 8). In each case, the column was re-equilibrated for 6 min. The sample volume injected was 15µL. MS/MS parameters used were as follows: capillary voltage, -3700V; focusing potential, -200V; entrance potential, -10V; declustering potential, -50V; nebulizer gas, 10 (arbitrary units); curtain gas, 12 (arbitrary units); collision gas, 5 (arbitrary units); auxiliary gas temperature, 400ºC; auxiliary gas flow rate, 6000 cm^3/min. For quantification purposes data were collected in the multiple reaction monitoring (MRM) mode, tracking the transition of parent and product ions specific for each compound with a dwell time of 100 ms. The collision energy (V) and the MRM transition for different phenolic acids were: 3,4-dihydroxyphenylpropionic acid (-16, 181/137); 4-hydroxyphenylpropionic acid (-16, 165/93); *m*- and *p*-coumaric acids (-30, 163/119); caffeic acid (-21, 179/135); isoferulic acid (-25, 193/134); 3,4-dihydroxyphenylacetic acid (-12, 167/123); 3-methoxy-4-hydroxyphenylacetic acid (-10, 181/137); 3-hydroxyphenylacetic acid (-12, 151/107); phenylacetic acid (-12, 135/91); protocatechuic acid (-20, 153/109); vanillic acid (-20, 167/152); 3- and 4-hydroxybenzoic acids (-16, 137/93) , 4-hydroxyhippuric acid (-20, 194/100), 3-hydroxyhippuric acid (-20, 194/150), 5-(3',4'-dihydroxyphenyl)-γ-valerolactone (-20, 207/163), 5-(3'-methoxy-4'-hydroxyphenyl)- -valerolactone (-25, 221/162). MRM transitions for (-)-epicatechin and its phase II and ring-fission metabolites were as follows: epicatechin (289/245), epicatechin-*O*-glucuronide (465/289), *O*-methyl epicatechin-*O*-glucuronide (479/303), epicatechin sulfate (369/289), O-methyl epicatechin sulfate (383/303), 5-(3',4'-dihydroxyphenyl)-γ-valerolactone (207/163), 5-(3'-methoxy-4'-hydroxyphenyl)- -valerolactone (221/162), 5-(3',4'-dihydroxyphenyl)-γ-valerolactone glucuronide (383/207), 5-(3',4'-dihydroxyphenyl)-γ-valerolactone sulfate (287/207), 5-(3'-methoxy-4'-hydroxyphenyl)- -valerolactone glucuronide (397/221), 5-(3'-methoxy-4'-hydroxyphenyl)- -valerolactone sulfate (301/221).

Human urine creatinine concentration was measured by a colorimetric assay using picric acid (Roura et al. 2006).

Statistical analysis.

SPSS Statistical Analysis System, Ver. 14.0 (SPSS) was used to perform the statistical analysis. Descriptive statistics were use to obtain the mean, standard error and standard deviation of the different variables after each intervention Because the data were skewed (Kolmogorov and Levene tests), the Wilcoxon test for related samples was used to compare changes in outcome variables in response to the intervention trial. Statistical tests were 2-tailed, and the significance level was 0.05.

RESULTS

A total of 21 metabolites, flavanol metabolites [(-)-epicatechin (*m/z* 289/245)] including microbial-derived metabolites were identified by LC/MS-MS in 24h- hydrolyzed human urine collected at baseline and after each intervention **(Table 2)**. Microbial-derived phenolic metabolites included: *Hydroxyphenylvalerolactones* [5-(3',4'-dihydroxyphenyl)-γ-valerolactone (*m/z* 207/163)]; *hydroxyphenylpropionic acids* [3,4-dihydroxyphenylpropionic acid (*m/z* 181/137) and 4-hydroxyphenylpropionic acid (*m/z* 165/121)]; *hydroxyphenylacetic acids* [3,4-dihydroxyphenylacetic acid (*m/z* 167/123), 3-methoxy-4-hydroxyphenylacetic acid (*m/z* 181/137), 3-hydroxyphenylacetic acid (*m/z* 151/107), phenylacetic acid (*m/z* 135/91)]; *hydroxycinnamic acids* [*m*-coumaric acid (*m/z* 163/119), *p*-coumaric acid (*m/z* 163/119); caffeic acid (*m/z* 179/135) isoferulic acid (*m/z* 193/134); *hydroxybenzoic acids* [protocatechuic acid (m/z 153/109), vanillic acid (*m/z* 167/152), 4-hydroxybenzoic acid (*m/z* 137/93), 3-hydroxybenzoic acid (*m/z* 137/93)], and *hydroxyhippuric acids* [4-hydroxyhippuric acid (*m/z* 194/100), 3-hydroxyhippuric acid (*m/z* 194/150)]. The same metabolites were identified in plasma samples, with the exception of (-)-epicatechin, 3-methoxy-4-hydroxyphenylacetic acid, *m*-coumaric acid and 3-hydroxybenzoic acid that were not detected.

In order to study the profile of conjugation of (-)-epicatechin and its ring-fission metabolite 5-(3',4'-dihydroxyphenyl)-γ-valerolactone, non-hydrolyzed urine and plasma samples were also screened. The analysis of urine revealed the presence of the one (-)-epicatechin-*O*-glucuronide (*m/z*= 465/289), two (-)-epicatechin sulfate (*m/z*=369/289), three *O*-methyl epicatechin-*O*-glucuronide (*m/z*=479/303) and three *O*-methyl epicatechin sulfate (*m/z*=383/303). In addition, two glucuronide (*m/z*= 383/207) and two sulfate (*m/z*= 287/207) derivatives of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone, as well as one glucuronide (*m/z*= 397/221) and one sulfate (*m/z*= 301/221) derivatives of 5-(3'-methoxy-4'-hydroxyphenyl)-γ-valerolactone were also detected. In the case of plasma, two glucuronide derivatives of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone and one glucuronide derivative of 5-(3'-methoxy-4'-hydroxyphenyl)-γ-valerolactone were detected in non-hydrolyzed samples. In order to identify these metabolites, urine samples were hydrolyzed and injected in the product ion scan mode of deprotonated molecules ($[M-H]^-$) in negative mode. The spectra and the proposed fragmentation pattern generated for 5-(3',4'-dihydroxyphenyl)-γ-valerolactone (*m/z* 207) and 5-(3'-methoxy-4'-hydroxyphenyl)-γ-valerolactone (*m/z* 221) were the same as already described in Urpi-Sarda et al (2008). Although no commercial standards of these hydroxyvalerolactone are available, the fragment at *m/z* 163, previously described (Li et al., 2000), as well as the generation of other characteristic fragment ions supports the identification of these compounds.

A significant increase in the urinary levels of the different conjugates of (-)-epicatechin and hydroxyphenylvalerolactones was found in non-hydrolyzed 24h-urine samples after consumption of cocoa with milk (**Table 2)**. For both metabolites, the sulfate derivatives were the most abundant compounds, although the total sulfate derivatives of hydroxyvalerolactones [(5-(3',4'-dihydroxyphenyl)-γ-valerolactone sulfate + (3'-methoxy-4'-hydroxyphenyl)-γ-valerolactone sulfate] was present in a much higher concentration than the corresponding to the total sulfate derivatives of (-)-epicatechin [(-)-epicatechin sulfate + 3-*O*-methyl-epicatechin sulfate]. According to Unno et al. (2003), 5-(3',4'-

dihydroxyphenyl)-γ-valerolactone mainly exist in conjugated form in urine and the level of conjugation is higher than for (-)-epicatechin. As expected from the changes observed in their corresponding conjugated forms, (-)-epicatechin and 5-(3',4'-dihydroxyphenyl)-γ-valerolactone, together with 3-hydroxyphenylacetic acid and vanillic acid, showed a significant increase in hydrolyzed 24h-urine samples after the consumption of cocoa in comparison to the consumption of milk The % increase in comparison to the intake of milk was 316% for 5-(3',4'-dihydroxyphenyl)-γ-valerolactone, 182% for vanillic acid, 98% for 3-hydroxyphenylacetic acid and 12% for (-)-epicatechin. The urinary levels of the remaining metabolites kept practically constant after cocoa consumption **(Table 2)**. Regular consumption of 40g of cocoa powder with milk/day resulted in a urinary excretion of 288.32 nmol/mg creatinine of microbial-derived phenolic acids in 24h, which represents a 13% increment in comparison to the the intake of milk alone. The predominant metabolites in 24h urine samples after the consumption of cocoa were 5-(3',4'-dihydroxyphenyl)-g̃-valerolactone, hydroxyphenylacetic acids (3-methoxy-4-hydroxyphenylacetic acid; 3-hydroxyphenylacetic acid; phenylacetic acid) and hydroxybenzoic acids (protocatechuic acid, vanillic acid, 4-hydroxybenzoic acid, and 3- and 4-hydroxyhippuric acids).

Table 3 shows the levels of phenolic metabolites detected in fasting plasma after the consumption of cocoa in comparison to the intake of milk. In this case, only two of the four metabolites described above for urine, 5-(3',4'-dihydroxyphenyl)-γ-valerolactone and 3-hydroxyphenylacetic acid, presented a significant increase (149% and 64% increase, respectively) after the consumption of cocoa. The consumption of 40g of cocoa powder resulted in a total of 38.8 μmol/L of microbial-derived phenolic acids in fasting plasma, which corresponds to a 5% increase in comparison to the intake of milk alone. As expected from its pharmacokinetic ($T_{max} \approx 2h$) (Holt et al., 2002), (-)-epicatechin metabolites were absent from both hydrolyzed and non-hydrolyzed in fasting plasma samples. However, glucuronide derivatives of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone and (3'-methoxy-4'-hydroxyphenyl)-γ-valerolactone were detected in non-hydrolyzed plasma samples. Both derivatives increased after cocoa consumption but this change was only significant for 5-(3',4'-dihydroxyphenyl)- -valerolactone glucuronide. The predominant metabolites in fasting plasma after the consumption of cocoa were phenylacetic acid, protocatechuic acid, 4-hydroxybenzoic acid and vanillic acid. Although in comparison to urine, fewer metabolites were detected in plasma, changes observed in the metabolic profile seem to be consistent with those observed in urine. To our knowledge, this is the first report related to the plasmatic levels of microbial-derived phenolic metabolites after the consumption of cocoa in human subjects.

DISCUSSION

In the present study, both tissular and microbial-derived phenolic metabolites were identified and quantified in 24h-urine and fasting plasma samples after regular (4 weeks) consumption of cocoa powder with milk (in comparison to the intake of milk) in patients with high risk of cardiovascular heart disease.

Numerous *in vitro* and *in vivo* animal studies on the microbial degradation of monomeric flavanols and procyanidins using purified compounds, have allowed the partially elucidation of the pathway leading to the formation of these metabolites. In contrast to procyanidins, monomeric flavanols are directly absorbed in the small intestine where they are first conjugated and subsequently in the liver into methyl, glucuronide and sulphate derivative. These forms pass into the bile through enterohepatic circulation and may reach the colon to be further degraded by the intestinal microbiota together with the procyanidins which escape small intestine absorption. Although the exact degradation pathway for the microbial degradation of procyanidins has not been fully established, a first step consisting in the possible depolymerization of oligomeric forms into monomers has been proposed (Groenewoud and Hundt, 1984, 1986). The microbial degradation of cocoa flavanols starts with the reductive cleavage of the heterocyclic C-ring resulting in the formation of diphenylpropan-2-ols which are further lactonized to give hydroxyphenylvalerolactones, namely 5-(3',4'-dihydroxyphenyl)-γ-valerolactone and its methyl

derivative 5-(3'-methoxy-4'-hydroxyphenyl)-γ-valerolactone, and 5-(3'-hydroxyphenyl)-γ-valerolactone. Subsequent fission of the valerolactone ring leads to 3,4-dihydroxyphenylvaleric acid and 3-hydroxyphenylvaleric acid (Das and Griffiths, 1968; Groenewoud et al., 1984, 1986; Meselhy et al., 1997; Déprez et al., 2000; Li et al., 2000; Scheline et al., 1991; Gonthier et al., 2003 a,b). Further degradation of the side chain length of hydroxyphenylvaleric acids by the progressive loss of carbon atoms by β-oxidation results in hydroxyphenylpropionic and hydroxybenzoic acids (Meselhy et al., 1997). α-Oxidation of hydroxyphenylpropionic acid may also occur leading to phenylacetic acids (Scheline et al., 1991). In relation to the hydroxylation pattern, 3-hydroxylated phenolic acids (i.e., 3-hydroxyphenylacetic, 3-hydroxybenzoic acid) are likely to be produced via the selective dehydroxylation of the OH group of dihydroxylated compounds at C-4 (Meselhy et al., 1997). Similarly, 4-hydroxylated phenolic acids (i.e. 4-hydroxyphenylpropionic acid, 4-hydroxybenzoic acid) are the result of the dehydroxylation at C-3, although it seems to be only characteristic of the degradation of procyanidins and not of monomeric flavanols (Groenewoud and Hundt, 1984, 1986; Gonthier et al., 2003ab). These monohydroxylated phenolic acids could be further dehydroxylated resulting in non-hydroxylated compounds, such as benzoic acid. After absorption by enterohepatic recirculation via the bile duct, microbial-derived phenolic metabolites could be conjugated into their glucuronide and sulfate esters in the liver and kidney before excretion in the urine. Other reactions occurring in the liver and kidney include: conjugation of 3-hydroxybenzoic acid with glycine also leads to 3-hydroxyhippuric acid (Quick, 1931); dehydrogenation of 3- and 4-hydroxypropionic acids results in *m*- and *p*-coumaric acids, respectively, which could be further *p*-hydroxylated and methylated to ferulic acid (Gonthier et al., 2003a; Scheline et al., 1991); and methylation of protocateuchic acid or 3,4-dihydroxyphenylacetic acid leads to vanillic and homovanillic acid, respectively (Gonthier et al., 2003a).

The urinary and plasmatic profile of phenolic metabolites presented herein after regular cocoa consumption seems to be consistent with the characteristic metabolic pathway of both monomeric flavanols and procyanidins described above. In relation to monomeric flavanols, (-)-epicatechin, the most abundant monomer in cocoa, was absent from fasting plasma but presented a significant increase in 24h-urine samples after the consumption of cocoa. In accordance with previous studies, (+)-catechin could not be detected in urine samples after cocoa intake (Roura et al., 2005; Tomás-Barberán et al., 2007). The low bioavailabilty of (+)-catechin in cocoa products has been recently established (Donovan et al., 2006). In contrast to previous studies, procyanidin B2 was absent from both urine and plasma samples (Holt et al., 2002). The glucuronide, sulfate and methylglucuronide derivatives of (-)-epicatechin detected in non-hydrolyzed urine samples were most likely formed in the small intestine and liver (tissular metabolites) from the direct absorption of this compound in the small intestine. According to Gonthier et al. (2003a), no monomeric flavanols as a consequence of a possible depolimerization of procyanidins by the microbiota could be detected when a dimeric, trimeric or a polymeric diet were administered to rats. Gu et al., (2007) also failed to demonstrate that procyanidins could be depolymerized in the colon during administration of sorghum brand to rats. However, it could be stated that the microbial phenolic metabolites identified in plasma and urine derived from the degradation of both monomeric flavanols and procyanidins, since a fraction of (-)-epicatechin previously absorbed in the small intestine may reach the colon by enterohepatic circulation to be further degraded by the intestinal microbiota. Due the high degree mDP of the cocoa powder ingested (mDP=8), microbial-derived phenolic metabolites represented the largest proportion of total phenolic metabolites in urine whereas (-)-epicatechin represented only a minor proportion (0.041% of total metabolite excretion). This is consistent with previous reports for other plant-derived foods, such as wine (Gonthier et al., 2003) or sorghum bran (Gu et al., 2007), that contains a high polymerized polyphenol fraction, whereas the contrary has been observed for a diet rich in monomeric forms (Gonthier et al., 2003).

There are very few studies concerning the metabolic fate of monomeric flavanols and procyanidins in humans by the intestinal microbiota. Consumption of tea catechins by humans resulted in the formation hydroxyphenylvalerolactones in plasma and urine (Li et al., 2000; Meng et al 2002), in agreement with previous *in vivo* studies performed with rats (Watanabe, 1959) and with subsequent *in vitro* fermentation

studies performed with (-)-epicatechin-3-*O*-gallate, (-)-epicatechin and (+)-catechin (Meselhy et al., 1997), or with procyanidin dimer B3 (Groenewoud and Hundt, 1986). According to Meselhy et al (1997), renal excretion of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone occurred within the first 24h after incubation of (-)-epicatechin-3-*O*-gallate with human intestinal bacteria or after oral administration of (-)-epicatechin (Unno et al., 2003) and (-)-epicatechin-3-*O*-gallate (Takizawz et al., 2003) to rats. In humans, maximum concentration of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone has been reported to occurred at 3-6h for pure (-)-epicatechin or at 8-24h after green tea consumption (Li et al., 2000; Meng et al 2002). In agreement with these studies, 5-(3',4'-dihydroxyphenyl)-γ-valerolactone presented a major increase in 24h-urine samples after consumption of cocoa flavanols in comparison to the intake of milk. Consistent with previous reports, the sulfate derivatives of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone were the most abundant conjugate metabolites in urine after cocoa consumption (Li et al., 2001). In the case of (-)-epicatechin metabolites, sulfated metabolites have been also reported as the predominant metabolites in urine, whereas glucuronide derivatives were the most abundant ones in plasma (Roura et al., 2007; Tomás-Barberán et al., 2007). Peak plasma level of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone has been reported to be around 13h after tea intake in humans (Li et al., 2000; Meng et al., 2002). Therefore detection of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone in fasting plasma may be due to the intake of the second cocoa dose (evening dose). To our knowledge this is the first report of hydroxyphenylvalerolactones and their conjugate metabolites in human urine and plasma after the intake of cocoa polyphenols.

In addition to the positive changes observed for (-)-epicatechin and 5-(3',4'-dihydroxyphenyl)-γ-valerolactone, a significant increase in the urinary content of vanillic acid and 3-hydroxyphenylacetic acid, the α-oxidation degradation product of 3-hydroxyphenylpropionic acid or 3,4-dihydroxyphenylpropionic acid, was also found after cocoa consumption. Das et al (1971), first reported that the metabolic fate of (+)-catechin by humans resulted in the formation of 3-hydroxyphenylpropionic acid, as the main urinary metabolite. These results were previously confirmed in rats by Griffiths (1962) and later by the *in vitro* fermentation of tea catechins with human or rat colonic microbiota (Meselhy et al., 1997) or by the incubation of human microbiota with nonlabeled and ^{14}C-labeled purified proanthocyanidin polymers (Déprez et al., 2000). Maximum renal excretion of this metabolite occurred after 48h of incubation (Meselhy et al., 1997). In line with our results, Ward et al. (2004) reported an increased urinary excretion of 3-hydroxypropionic acid and 3-hydrophenylacetic acid after human consumption of grape seed polyphenols. Our results are also consistent with those of Rios et al. (2003) that reported a significant increase in the urinary excretion of 3-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, ferulic acid, vanillic acid, and 3-hydroxybenzoic acid in healthy humans after acute consumption of a flavanol-rich chocolate (80g, flavonol monomers: 146.4 mg). According to Rios et al. (2003), the increase in the levels of vanillic acid observed after cocoa consumption, which is also agreement with the results found in the present study, was most likely originated from the oxidation of vanillin added to the product as vanilla flavour (Table 1). With the exception of this metabolite, which showed a maximum excretion between during the first 3h after chocolate intake, the excretion of the remaining microbial metabolite increased from 6-48h after consumption and registered maximum levels from 24-48h (Rios et al. 2003), which could explain the fact that no additional significant compositional changes were observed in 24h-urine after cocoa consumption in the present study. Another aspect to be considered is that the mean age of volunteers included in this study was 69.7$\pm$ 11.5 years and that age, together with other factors such as stress, disease or diet, affects the human microbiota and therefore produces changes in the microbial metabolome, leading to large inter-individual variationin metabolite concentrations (Rowland et al., 1999). In addition, it is important to highlight that phenolic compounds themselves are able to modulate the microbiota (Tzounis et al. 2008). A change in the population of bacteria towards tannin-resistant Gram-negative species (i.e. *Enterobacteriaceae* and *Bacteriodes*) have been reported after 3 week admistration of condensed tannins to rats (Smith and Mackie, 2004). Therefore, the long-term ingestion of cocoa phenolics may have produced changes in the bacterial population which may help to explain differences in the urine metabolic

profile in comparison to the study of Rios et al. (2003). In this sense, Gu et al. (2007) reported that urinary excretion of 3-hydroxyphenylpropionic acid was high in rats fed a high amount of sorghum bran (20-40% of the diet) during 50 days, whereas the concentration of hydroxyphenylacetic acid was high when low amount of sorghum bran was administered but decreased when higher amount were administered.

In conclusion, monomeric flavanols in cocoa are mainly absorbed as glucuronide, sulphate, *O*-methylglucuronide and *O*-methylsulfate derivatives of (-)-epicatechin, whereas as the occurrence of glucuronide and sulfate derivatives of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone and its methyl ester [5-(3'-methoxy-4'-hydroxyphenyl)-γ-valerolactone] could be indicative of the absorption of both monomeric flavanols and procyanidins in cocoa. However, microbial-derived phenolic acids constitute the most abundant metabolites in both urine and plasma. Some of these phenolic acids have been recently proved to inhibit the secretion of pro-inflammatory cytokine involved in early stages of atherosclerosis from LPS-induced human PBMC (Monagas et al., 2008). As a consequence of the intake of cocoa, changes were observed in some microbial-derived metabolites in particular for 5-(3',4'-dihydroxyphenyl)-γ-valerolactone. This is the first report of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone after long-term feeding of cocoa polyphenols in human subjects. The results found herein indicate that this compound could be a good biomarker of the consumption of flavanols. One aspect that deserves further consideration is the effect of long-term feeding of food containing high polymerized proanthocyanidins in the human colonic microbiota population, posterior metabolism and its implication in gut and overall health.

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Table 1. Nutritional and phenolic composition of the cocoa powder used in the study.

Parameter	Value
Starch (%)	16.1
Carbohydrates (%)	65.7
Lactose (%)	26.6
Sucrose (%)	< 1.0
Fiber (%)	19.1
Fat (%)	5.3
Cholesterol (mg/100g)	4.9
Proteins (%)	17.1
Theobromine (%)	1.1
Caffeine (%)	0.09
Calcium (mg/100g)	712
Copper (ppm)	27.5
Iron (mg/100g)	43.9
Magnesium (mg/100g)	298
Phosphorus (mg/100g)	872
Potasium (mg/100g)	1230
Sodium (mg/100g)	182
Zinc (mg/100g)	4.0
Vitamin B1 (mg/100g)	0.27
Vitamin B2 (mg/100g)	0.45
Vitamin B3 (mg/100g)	1.8
Vitamin B5 (mg/100g)	1.5
Vitamin B6 (mg/100g)	<0.20
Vitamin B9 (µg/ 100g)	20.0

	Mean ± SD
Total polyphenols **(Folin Ciocalteu)** (mg catechin/g)	12.38 ± 0.69
Individualized polyphenols **(HPLC)** (mg/g)	
(+)-Catechin	0.2603 ± 0.007
(-)-Epicatechin	1.1519 ± 0.003
Procyanidin B2	0.9135 ± 0.015
Vanillin	0.9447 ± 0.123
Isoquercitrin	0.0557 ± 0.001
Quercitin	0.0054 ± 0.001
Quercetin-3-arabinoside	0.0175± 0.001
Quercetin-3-glucuronide	0.0025± 0.001

Table 2. Levels of microbial and flavanol metabolites at baseline and after each intervention in hydrolyzed 24h-urine samples.

	Milk		Milk+cocoa		
nmol/mg creatinine	**mean**	**SEM**	**mean**	**SEM**	***p*** [c]
Conjugated metabolites [a]					
Flavanols					
(-)-Epicatechin-*O*-glucuronides	0.21	0.08	0.36	0.07	0.002
(-)-Epicatechin sulfates	0.66	0.11	1.30	0.28	0.002
O-methyl epicatechin-*O*-glucuronides	0.15	0.04	0.34	0.08	<0.001
O-methyl epicatechin sulfates	1.57	0.45	10.45	2.49	<0.001
Hydroxyphenylvalerolactones					
5-(3',4'-dihydroxyphenyl)-γ-valerolactone glucuronides	4.52	1.07	17.39	5.65	<0.001
5-(3',4'-dihydroxyphenyl)-γ-valerolactone sulfates	244.28	53.05	775.75	192.50	<0.001
5-(3'-methoxy-4'-hydroxyphenyl)-γ-valerolactone glucuronides	0.91	0.14	1.87	0.77	0.010
5-(3'-methoxy-4'-hydroxyphenyl)-γ-valerolactone sulfates	5.60	2.62	16.78	5.91	<0.001
Non-conjugated metabolites [b]					
Flavanols					
Epicatechin	0.00	0.00	0.13	0.04	<0.001
Hydroxyphenylvalerolactones					
5-(3',4'-dihydroxyphenyl)-γ-valerolactone	3.22	1.21	20.43	4.75	<0.001
Hydroxyphenylpropionic acids					
3,4-dihydroxyphenylpropionic acid	2.14	0.41	1.80	0.44	0.337
4-hydroxyphenylpropionic acid	3.19	1.21	2.52	1.05	0.178
Hydroxyphenylacetic acids					
3,4-dihydroxyphenylacetic acid	0.51	0.08	0.70	0.13	0.230
3-methoxy-4-hydroxyphenylacetic acid	18.22	3.39	20.81	4.94	0.866
3-hydroxyphenylacetic acid	7.32	1.53	18.97	4.14	0.000
phenylacetic acid	24.33	3.86	28.38	5.40	0.456
Hydroxycinnamic acids					
m-coumaric acid	0.31	0.08	0.37	0.19	0.683
p-coumaric acid	0.20	0.06	0.19	0.45	0.876
caffeic acid	0.82	0.03	0.03	0.01	0.234
isoferulic acid	2.51	0.58	2.27	0.54	0.515
Hydroxybenzoic acids					
protocatechuic acid	16.28	2.31	19.70	3.70	0.517
vanillic acid	9.28	1.73	34.34	8.50	<0.001
4-hydroxybenzoic acid	20.65	3.89	23.17	5.47	0.683
3-hydroxybenzoic acid	0.80	0.24	1.14	0.45	0.402
Hydroxyhippuric acids					
4-hydroxyhippuric acid	35.60	6.32	37.48	6.79	0.755
3-hydroxyhippuric acid	96.69	22.24	86.41	19.55	0.239

[a]Non-hydrolyzed samples

[b]Hydrolyzed samples

[c]Wilcoxon signed ranks test for 2 related samples

SEM (standard error), SD (standard deviation)

Table 3. Levels of microbial and flavanol metabolites after each intervention in fasting plasma samples.

nmol/L	Milk mean	Milk SEM	Milk+cocoa mean	Milk+cocoa SEM	p [c]
Conjugated metabolites [a]					
5-(3',4'-dihydroxyphenyl)-γ-valerolactone glucuronides	28.19	9.71	153.05	47.98	0.005
5-(3'-methoxy-4'-hydroxyphenyl)-γ-valerolactone glucuronides	28.87	2.87	36.77	6.12	0.163
Non-conjugated metabolites [b]					
Flavanols					
Epicatechin	nd		nd		
Hydroxyphenylvalerolactones					
5-(3',4'-dihydroxyphenyl)-γ-valerolactone	110.97	39.00	476.29	93.91	0.004
Hydroxyphenylpropionic acids					
3,4-dihydroxyphenylpropionic acid	189.36	37.07	197.41	37.28	0.751
4-hydroxyphenylpropionic acid	158.06	36.88	234.71	76.21	0.159
Hydroxyphenylacetic acids					
3,4-dihydroxyphenylacetic acid	101.68	22.33	105.56	21.04	0.808
3-methoxy-4-hydroxyphenylacetic acid	nd		nd		
3-hydroxyphenylacetic	59.48	17.81	122.26	24.39	0.005
phenylacetic acid	19209.57	1275.02	20319.71	1196.02	0.732
Hydroxycinnamic acids					
m-coumaric acid	nd		nd		
p-coumaric acid	29.68	4.19	31.94	4.90	0.316
caffeic acid	85.44	12.33	84.41	11.24	0.909
isoferulic acid	216.48	23.09	207.67	20.62	0.849
Hydroxybenzoic acids					
protocatechuic acid	10094.06	1572.75	10524.48	1614.13	0.889
vanillic acid	2799.00	344.05	2710.52	306.51	0.713
4-hydroxybenzoic acid	9372.63	462.70	9732.32	497.81	0.751
3-hydroxybenzoic acid	nd		nd		
Hydroxyhippuric acids					
4-hydroxyhippuric acid	92.61	12.71	113.62	17.73	0.469
3-hydroxyhippuric acid	300.50	95.74	480.03	182.93	0.209

[a]Non-hydrolyzed samples
[b]Hydrolyzed samples
[c]Wilcoxon signed ranks test for 2 related samples
SEM (standard error), SD (standard deviation)
nd, not detected

3. ISOFLAVONES

Els estudis realitzats amb isoflavones s'han realitzat en el *Institute Nacional de la Recherche Agronomique* (INRA) de Clermont-Ferrand (Francia) sota la direcció de la Dra. Claudine Manach i gràcies a 3 ajudes per a estades breus dintre del programa de mobilitat que ofereixen les beques FPI del Ministerio de Ciencia e Innovación (MICINN) (EST20060540824, EST20070659550, EST2008001501) amb la finalitat d'optar a la menció de doctor europeu.

3.1 Determinació del perfil metabòlic d'isoflavones en pròstata de voluntaris amb hiperplàsia benigna de pròstata

Publicació VI: Identificació de glucurònids d'isoflavones en pròstata humana

Laurent Guy, Nicolas Védrine, Mireia Urpi-Sarda, Angel Gil-Izquierdo, Nawaf Al-Maharik, Jean-Paul Boiteux, Augustin Scalbert, Christian Remesy, Nigel P. Botting, Claudine Manach. Orally administered isoflavones are present as glucuronides in the human prostate. *Nutrition and Cancer*. **2008**, 60 (4): 461-8

Resum:

El coneixement de la biodisponibilitat i el metabolisme de les isoflavones en teixit de pròstata és necessari per a investigar els mecanismes d'acció en la prevenció de càncer de pròstata.

Per a determinar els nivells d'isoflavones que poden arribar a la pròstata, dotze homes amb hiperplàsia benigna de pròstata van rebre suplements d'extracte de soja (3 càpsules Evestrel®, amb un total de 112.5 mg/dia d'equivalent d'isoflavones) durant 3 dies abans de la cirurgia (Figura 27). Es va extreure la sang i els teixits de pròstata dels voluntaris i es van identificar els metabòlits glucuronidats de les isoflavones per espectrometria de masses en tàndem i per comparança als estàndards sintetitzats químicament.

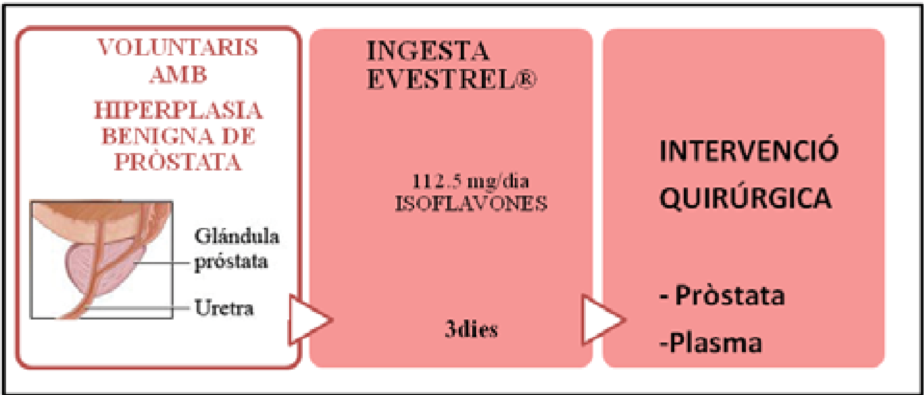

Figura 27: Esquema de l'estudi

Els principals metabòlits oposats van ser els mateixos en teixit prostàtic que en plasma, dos mono-glucurònids de daidzeina i dos mono-glucurònids de genisteina. Les concentracions de les isoflavones totals mesurades en pròstata van ser de 1.05 ±0.62 nmol/g teixit (rang 0.30-2.23) al temps de presa de mostra que va ser a les 12h després de l'últim suplement. En aquest punt, les concentracions trobades en pròstata van ser menors que en plasma per a tots els voluntaris: 0.47 nmol/g vs 0.66 µmol/l per a la daidzeína i 0.58 nmol/g vs 0.78 µmol/l per a la genisteina.

Per tant, una vegada coneguts els metabòlits que arriben intactes al teixit prostàtic, el seu mecanisme d'acció s'hauria d'investigar a través d'estudis cel·lulars *in vitro* utilitzant concentracions fisiològiques semblants a les trobades *in vivo* (concentracions intracel·lulars per sota de 5 nmol/g) i a més, utilitzant metabòlits mono-glucuronidats a nivell intracel·lular.

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Orally Administered Isoflavones Are Present as Glucuronides in the Human Prostate

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Better knowledge of the bioavailability and metabolism of isoflavones in prostate tissue is needed to further investigate their mechanisms of action in the context of prostate cancer prevention. A total of 12 men with benign prostatic hyperplasia received soy
25 **extract supplementation (3 Evestrel® capsules, providing a total of 112.5 mg isoflavones aglycone eq/day) for 3 days before prostate surgery. Blood and prostate tissues were sampled and metabolites were identified using electrospray ionization liquid chromatography tandem mass spectrometry (ESI-LC-MS/MS) and chemically**
30 **synthesized standards of glucuronidated isoflavones. The main metabolites were the same in prostate tissue and in plasma, namely, 2 monoglucuronides of daidzein and 2 monoglucuronides of genistein. Concentrations of total isoflavones measured in prostate reached 1.05 ± 0.62 nmol/g tissue (range 0.30–2.23) at the time**
35 **of sampling, for example, 12 h after the last isoflavone supplementation. At that time point, prostate concentrations were lower than plasma concentrations in all volunteers: 0.47 nmol/g vs. 0.66 μM for daidzein and 0.58 nmol/g vs. 0.78 μM for genistein. Isoflavone mechanisms of action should thus be investigated in in vitro cell studies using physiological conditions, for example, intracellular** 40 **concentrations below 5 nmol/g and no intracellular deconjugation of the monoglucuronide metabolites.**

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INTRODUCTION

Prostate cancer is the most frequent cancer and 1 of the Q1 leading causes of death for men in Western countries (1,2). In 45 fact, it is so frequent in aged men that it might be regarded as a normal aging-related phenomenon. The development of the disease spreads over decades, making it particularly open to modulation by environmental factors such as nutrition. The traditional Asian diet is believed to slow down the progression 50 of latent prostate intraepithelial neoplasia toward clinically ap- Q2 parent tumors, at least in Asian populations (3). Soy and its exclusive micronutrients, isoflavones, have been recognized by worldwide experts as potential chemopreventive agents and are

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actively studied. However, existing data supporting the protective effects of isoflavones are still limited, although studies have shown that mortality from prostate cancer is lowest in areas of high intake of soy and isoflavones, especially in Asia (e.g., (4)). Increased risk of prostate cancer for Asian men who migrated to Western countries or adopted a Westernized lifestyle, highlighted the key role of environmental factors beyond genetic factors (5–7). A recent meta-analysis compiling 2 cohort studies and 6 case-control studies on Western and Asian populations estimated that soy food consumption can be related to a 30% reduction in prostate cancer risk (8). Studies on animal models have further supported the protective role of isoflavones. Isoflavones have been reported to reduce both incidence and tumor sizes in more than 40 studies carried out with a variety of rodent models (3,9). In addition, isoflavones have inhibited proliferation of all cultured cell lines classically used to study prostate cancer (10,11).

These epidemiological and experimental data suggest a protective role for soy isoflavones in the prevention of prostate cancer; nevertheless, they do not provide direct evidence for their preventive effects. Some clinical trials have investigated the effect of isoflavone intake on some prostate cancer related endpoints such as prostate-specific antigen (PSA) level (12,13). However, these studies were only designed to evaluate the pharmacological effects of isoflavones, using short-term exposures to high doses of isoflavones, on men with already diagnosed prostate cancer. Demonstrating the preventive effects of long-life exposure to isoflavone-rich food on healthy men is much more challenging and cannot be realized with such trials. The complexity of the human diet, possible interactions with other nutrients, variability in interindividual responses to the diet due to genetic polymorphisms and diverse microbiota, multiplicity of possible biological targets, and the low amplitude of the chronic effects expected from nutrition are some of the factors that make the demonstration of isoflavone-preventing effects difficult. In addition, validated biomarkers for early stages of prostate cancer development are not available to date. One possible way to progress in the understanding of the role of isoflavones in prostate cancer prevention is to clarify their molecular mechanisms of action in animal and cellular models. A number of studies have provided clear evidence that isoflavones are active compounds. They have been shown to bind estrogen receptors (ER)α and ERβ and to induce agonist or antagonist responses depending on tissues and conditions (14). Many other molecular mechanisms have been proposed such as modulation of steroid biosynthesis, transport, and metabolism; modulation of growth factors signaling pathways (epidermal growth factor, insulin-like growth factor-1, nuclear factor-κ B, Akt) and inhibition of tyrosine kinase activities; modulation of xenobiotic metabolism; regulation of genes involved in the cell cycle; induction of apoptosis; reduction of oxidative stress; and inhibition of angiogenesis (10,15). However, the issue of bioavailability has not been taken into account in the design and interpretation of mechanistic studies. Cell cultures have been generally exposed to concentrations far exceeding the concentrations that may be achieved in the body.

The aim of our study was to look for the presence of isoflavone metabolites in the human prostate after ingestion of an isoflavone-rich supplement to compare the concentrations achieved in the prostate tissue to the plasma concentrations and to determine the nature of the metabolites present in plasma and prostate tissue. The underlying objective is to provide the information needed to design more relevant in vitro studies to investigate the mechanisms of action of dietary isoflavones involved in the prevention of prostate cancer.

MATERIALS AND METHODS

Materials

Standards of daidzein, genistein, daidzin, genistin, and equol were purchased from Extrasynthese (Genay, France). A standard of glycitein was purchased from Interchim (Montluçon, France). Standards of daidzein 7-glucuronide and genistein 7-glucuronide were chemically synthesized according to the method previously published (16). β-glucuronidase/sulfatase from *Helix pomatia* was obtained from Sigma.

Isoflavone Content of the Evestrel® Supplement

Evestrel capsules were provided by Théramex (CITY, Monaco). The isoflavone composition of the Evestrel extract was analyzed as follows. A total of 20 mg extract powder were homogenized in 10 ml methanol/water (70/30, vol/vol) using a Polytron mixer. Concentrations of isoflavone aglycones and glycosides were determined by high-performance liquid chromatography (HPLC)-CoulArray as described following before and after acid hydrolysis. Acid hydrolysis was carried out by addition of 0.25 volume 6M HCl followed by incubation for 2 h at 90°C. All glycosylated forms of isoflavones are hydrolyzed into aglycones by this procedure.

HPLC analysis was performed using a system consisting of 2 pumps (Model 580, ESA, Chelmsford, MA) for high-pressure gradient, a temperature-controlled autosampler (Gilson, Villiers-le-Bel, France), a 150 × 2.1 mm SymmetryShield RP18-5μ column (Waters, Milford, MA) and an 8-channel CoulArray detector (model 5600, Eurosep, Cergy, France). Mobile phases consisted of a 30 mmol/l NaH_3PO_4 buffer (pH 3) containing 5% acetonitrile (A) and 40% acetonitrile (B). Separation was achieved using a gradient elution (flow = 0.4 ml/min): 0–25 min, linear gradient from 100% A to 100% B; 25–29 min, 100% B. Potentials were set at 200, 320, 490, 550, 600, 680, 750, and 800 mV (Pd as reference).

Each capsule contained a total of 37.5 mg isoflavones, including 65.7% genistein, 31.7% daidzein, and 2.5% glycitein in aglycone equivalents. Daidzein and daidzin represented 24.9% and 75.1% (in aglycone eq.) of the total daidzein forms. Genistein and genistin represented 22.2% and 54.9% (in aglycone eq.) of the total genistein forms, the rest corresponding

probably to malonylated and acetylated glycosides also hydrolyzed into aglycones by our procedure. Glycitein represented 29% of the total glycitein forms.

Subjects

Sixteen men with benign prostatic hyperplasia (BPH) scheduled for surgery were included consecutively in the study. All the patients presented severe symptoms related to BPH. For all of them, conservative medical treatment had failed to improve their urinary symptoms. Preoperative evaluation included physical examination with digital rectal examination, free uroflowmetry, measurement of post void residual volume, blood analysis, coagulation parameters, PSA determination, and measurement of prostate volume using ultrasound. Due to the volume of the prostate, surgical procedure was a transurethral resection in 15 cases and an open prostatectomy for 1 patient.

Characteristics of the patients (mean ± SD) were weight 80 ± 5 kg (range 69–130), body mass index 27 ± 2 kg/m^2 (range 22.4–44.9), and 67 ± 2 years old (range 54–77). Patients had normal PSA level (<4 ng/ml). Exclusion criteria included prostate cancer, diabetes, kidney or liver failure, antibiotic treatment within 3 months as well as a strict vegetarian or vegan diet or a high isoflavone consumption through soy-based food or supplement intake evaluated by a diet history questionnaire.

Study Design

Volunteers were randomly allocated to the group of 12 men who received soy extract supplementation for 3 days before prostate resection or to the control group of 4 men who did not receive any supplementation before the surgical treatment.

During the whole experimental period and the 3 days before, the volunteers were asked to maintain their normal food intake and to avoid isoflavone consumption. They were given a list of prohibited food items. All soy-based foods (tofu, tempeh, miso, natto, soymilk, soybeans, soy cheese, soy-based yogurts, and desserts) as well as cereal bars, breakfast cereals, foods for vegetarians, nuggets, burgers, and meal substitutes were prohibited.

The isoflavone supplementation consisted of 3 Evestrel capsules per day, providing a total of 112.5 mg total isoflavones (aglycone eq.). Volunteers were asked to take the 3 capsules at dinner the 3 days before the procedure.

Blood was sampled at the beginning of the surgical operation; 5 to 10 g of the inner prostatic tissue were collected in the transitional zone and immediately clamped in liquid nitrogen. The whole operation lasted about 60 min.

For each volunteer, a pool of prostate chips (about 1 g) was ground in liquid nitrogen to obtain homogenized powder that was stored at −80°C until analyses. The remaining chips were also stored at −80°C.

The protocol was approved by the regional Biomedical Research Ethics Committee (CCPPRB, Clermont-Ferrand, France), and all participants gave their written informed consent.

Sample Treatment

For quantitative analysis, plasma samples were acidified to pH 4.9 with 10 mmol/l acetic acid and incubated for 18 h in the presence of 1,000 units β-glucuronidase and 45 units sulfatase (from *Helix pomatia*, Sigma G0876). Samples were then treated with 4 vol methanol/HCl 200 mM and centrifuged 5 min at 12,500 *g*. For qualitative analysis, plasma samples were directly extracted with 4 vol methanol/HCl 200 mM and centrifuged 5 min at 12,500 *g*. Supernatants were analyzed by LC-MS/MS as described following.

For quantitative analysis in prostate tissue, 150 mg prostate tissue powder were homogenized in 9 vol of cooled methanol/water (70/30, vol/vol) using a Polytron mixer (Kinematica, Lucerne, Switzerland). After centrifugation (3,500 rpm, 10 min, 4°C), and keeping the supernatant, the pellet was extracted again with 4 vol of cooled methanol/water (70/30, vol/vol). Pooled supernatants were evaporated to dryness and then reconstituted in 1 vol sodium acetate buffer 0.1 M pH 4.9 before incubation with β-glucuronidase/sulfatase (*Helix pomatia*) at 37°C for 18 h (2,000 units β-glucuronidase and 90 units sulfatase). After incubation, isoflavones were extracted with 4 vol methanol/HCl 200 mM. We performed 5 replicates of extraction and analysis for each sample.

For determination of the nature of tissue metabolites, prostate chips rather than prostate tissue powder were used because of possible metabolite hydrolysis during grinding. Prostate chips (150 mg) were extracted with 4 vol methanol/H_20 (70/30) containing 200 mM HCl using the Polytron mixer. After centrifugation at 3,500 rpm, 4°C for 10 min, supernatants were analyzed by LC-MS/MS.

As aglycones and conjugated derivatives, such as glucuronides, have quite different polarity and stability, giving different behavior during extraction and analysis, it was considered not relevant to use an aglycone as internal standard because a high proportion of conjugated forms of isoflavones could be present in our plasma and tissues samples. The extraction recovery and the efficiency of hydrolysis were checked using control plasma and prostate tissue supplemented with known concentrations (2 and 5 μmol/l in duplicate) of aglycones and chemically synthesized standards of isoflavones glucuronides and were treated the same way as the samples. The recovery rate ranged from 85% to 103% for aglycones and glucuronides in plasma and from 88% to 106% in prostate tissue. Glucuronides were not detectable after hydrolysis with β-glucuronidase/sulfatase in the conditions described previously.

LC-MS/MS Analysis of Isoflavone Metabolites

Liquid chromatography (LC) analyses were performed using a Hewlett-Packard 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump and an autosampler. An Applied Biosystems API 2000 triple quadrupole mass spectrometer (PE Sciex, Concord, Ontario, Canada), equipped with a Turbo IonSpray source ionizing in

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the positive mode at 500°C, was used to obtain the mass spectrometry (MS) and MS/MS data. SymmetryShield™ RP18 column (Waters, Milford, MA), 2.1 × 150 mm id, 5 μm, was used for chromatographic separation (injection volume = 20 μl). Gradient elution was carried out with 0.5% acetic acid in 20% acetonitrile as mobile Phase A and 0.5% acetic acid in 80% acetonitrile as mobile Phase B: 0–20 min from 100% A to 100% B at a constant flow rate of 400 μl/min. The column was reequilibrated for 10 min.

Parameters for the detection of aglycones and metabolites were the following: capillary voltage 5,500 V, collision gas 5 (arbitrary units), and curtain gas 20 (arbitrary units). Declustering potential, focusing potential, entrance potential, and collision energy were optimized with infusion experiments of daidzein (51, 400, 15, and 55, respectively), genistein (50, 400, 12, and 60, respectively), daidzein-7-glucuronide (30, 375, 5, and 25, respectively), genistein-7-glucuronide (20, 200, 10, and 30, respectively), equol (19, 370, 9, and 17, respectively), and glycitein (30, 375, 10, and 50, respectively).

To determine the nature of isoflavone metabolites present in nonhydrolyzed prostate and plasma, MS data were collected in multiple reaction monitoring (MRM) mode by monitoring specific transitions of parent and product ions for each metabolite (Table 1). Dihydrodaidzein, dihydrogenistein, dihydroglycitein, and O-desmethylangolensin were checked using selected-ion monitoring (Table 1). For keeping the sensitivity in LC-MS/MS, we monitored 5 transitions for each analysis in the MRM mode, with a dwell time of 400 ms.

For quantitative analyses in hydrolyzed prostate and plasma samples, the MRM mode was used with a dwell time of 500 ms, monitoring 4 transitions for each analysis: daidzein (255/91), genistein (271/91), equol (243/123), and glycitein (285/270). Calibration curves were prepared in human plasma by spiking control pools with known concentrations of genistein, daidzein, glycitein, and equol (0; 0,08; 0,16; 0,32; 0,64; 1,28; and 2,56 μmol/l). The limit of detection was estimated as 12 nmol/l for daidzein and genistein, 19 nmol/l for glycitein, and 2.1 μmol/l for equol. The limit of quantification was estimated as 41 nmol/l for daidzein and genistein, 65 nmol/l for glycitein, and 7.1 μmol/l for equol.

HPLC-CoulArray Analysis of Equol

Because the limit of detection was high for equol in our LC-MS/MS conditions, equol was analyzed using CoulArray detection. Mobile phases consisted of a 30 mmol/l NaH_3PO_4 buffer (pH 3) containing 20% acetonitrile (A) and 40% acetonitrile (B). Separation on a 150 × 2.1 mm SymmetryShield RP18-5 μ column (Waters) using a gradient elution (flow = 0.4 ml/min): 0–15 min, linear gradient from 100% A to 100% B; 15–19 min, 100% B; 19.01–25 min, 100% A. Potentials were set at 200, 320, 490, 550, 600, 680, 750, and 800 mV (Pd reference) on the HPLC-CoulArray system described previously. The detection limit was 8 nmol/l.

Statistics

Values are given as mean ± SD. The significance of differences was determined using the paired *t*-test (INSTAT, GraphPad Software, San Diego, CA). A P value < 0.05 was considered significant.

RESULTS

After daily supplementation with 112.5 mg isoflavones (aglycone eq, composed of 31.7% daidzein eq, 65.7% genistein eq, and 2.5% glycitein eq) for 3 days, plasma concentrations of total isoflavones measured by LC-ESI-MS/MS ranged from 0.40 to 2.54 μM, with a mean value of 1.45 ± 0.77 μM, whereas concentrations measured in prostate tissues ranged from 0.30 to 2.23 nmol/g tissue, with a mean value of 1.05 ± 0.62 nmol/g tissue (Fig. 1). Significant interindividual variability was observed regarding total concentrations of isoflavones, but prostate concentrations were lower than plasma concentrations for all volunteers.

The main aglycones recovered after β-glucuronidase/sulfatase hydrolysis were daidzein and genistein, with low concentrations of glycitein. The genistein/daidzein ratio was 1.3 in prostate (0.58/0.47 nmol/g) as well as in plasma samples (0.78/0.66 μmol/l).

As measurements were only made at 1 time point, it is not possible to compare the absorption/elimination efficiency for genistein and daidzein.

Equol, the well-known microbial metabolite of daidzein produced by only 30% of the Western population, was detected in 2 volunteers only. Equol concentrations in prostate samples from equol producers were 0.36 and 0.12 nmol/g vs. 0.95 and 0.33 μmol/l in the corresponding plasma samples.

The nature of prostate and plasma metabolites was determined using ESI-LC-MS/MS and chemically synthesized standards of glucuronidated conjugates of isoflavones. The major metabolites were the same in prostate tissue and in plasma, namely, 2 monoglucuronides of daidzein and 2 monoglucuronides of genistein (Fig. 2). Daidzein 7-*O*-glucuronide and genistein 7-*O*-glucuronide were identified by comparison with the chemically synthesized standards. We assume that the other major metabolites detected with the MS transitions 431/255 and 447/271 are 4′-*O*-glucuronides of daidzein and genistein, respectively, which are the other isoflavone glucuronides that have already been reported in human plasma. The relative proportion of 7-*O*-glucuronides and 4′-*O*-glucuronides in plasma and prostate samples varied between the volunteers.

Table 1 summarizes the MRM transitions that have been searched in nonhydrolyzed plasma and prostate samples and the metabolites that were detected in addition to the monoglucuronides of daidzein and genistein. Daidzein and genistein aglycones were detected in prostate tissue of only 5 volunteers among the 12. Their concentration represented less than 10% of the total aglycone concentrations measured after β-glucuronidase/sulfatase hydrolysis of the prostate samples.

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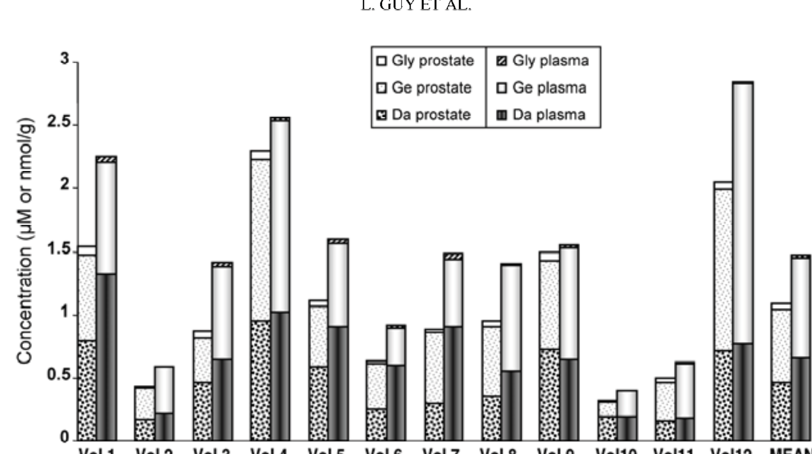
TABLE 1
Isoflavone Metabolites Detected in Nonhydrolyzed Plasma and Prostate of 12 Volunteers Who Received 112.5 mg Isoflavones/Day for 3 Days[a]

Metabolite	MRM Transitions	Nonhydrolyzed Plasma	Nonhydrolyzed Prostate
Daidzein-glucuronide	431/255	++	++
Genistein-glucuronide	447/271	++	++
Daidzein	255/91	+/−	+/−
Genistein	271/91	+/−	+/−
Daidzein-diglucuronide	607/255	tr	tr
Genistein-diglucuronide	623/271	tr	tr
Daidzein-sulfate	335/255	tr	tr
Genistein-sulfate	351/271	tr	tr
Glycitein-glucuronide	461/285	tr	tr
Glycitein	285/270	Nd	Nd
Equol	243/123	Nd	Nd
Equol-glucuronide	419/243	Nd	Nd
Dihydrodaidzein-glucuronide	433/257	Nd	Nd
Dihydrogenistein-glucuronide	449/273	Nd	Nd
Dihydroglycitein-glucuronide	463/287	Nd	Nd
Glycitein-sulfate	365/285	Nd	Nd
Equol-sulfate	323/243	Nd	Nd
Daidzein-sulfoglucuronide	511/255	Nd	Nd
Genistein-sulfoglucuronide	527/271	Nd	Nd
Glycitein-sulfoglucuronide	540/285	Nd	Nd
Equol-sulfoglucuronide	499/243	Nd	Nd
Glycitein-diglucuronide	637/285	Nd	Nd
Equol-diglucuronide	595/243	Nd	Nd
Daidzein-disulfate	415/255	Nd	Nd
Genistein-disulfate	431/271	Nd	Nd
Glycitein-disulfate	445/285	Nd	Nd
Equol-disulfate	403/243	Nd	Nd
Daidzin	417/255	Nd	Nd
Genistin	433/271	Nd	Nd
Glycitin	447/285	Nd	Nd
Daidzein-glycine	312/255	Nd	Nd
Genistein-glycine	328/271	Nd	Nd
Glycitein-glycine	342/285	Nd	Nd
Equol-glycine	300/243	Nd	Nd
Dihydrodaidzein	257	Nd	Nd
Dihydrogenistein	273	Nd	Nd
Dihydroglycitein	287	Nd	Nd
O-desmethylangolensin	259	Nd	Nd
Daidzein-glutathione	560/255	Nd	Nd
Genistein-glutathione	576/271	Nd	Nd
Q7 Glycitein-glutathione	590/285	Nd	Nd
Equol-glutathione	548/243	Nd	Nd

[a] Abbreviations are as follows: MRM, multiple reaction monitoring; Nd, not determined, tr, trace; +/−, present in some but not all samples.

Very low concentrations of diglucuronides and sulfates of daidzein and genistein were also detected for a few volunteers but in trace amounts not quantifiable in our conditions.

Sulfoglucuronides, glycosides, disulfates, glycine or glutathione conjugates, dihydrodaidzein, dihydrogenistein, dihydroglycitein, and O-desmethylangolensin were not detected in any plasma or prostate sample. 37



Q8 Q9 FIG. 1. Isoflavone concentrations measured by liquid chromatography electrospray ionization tandem mass spectrometry after β-glucuronidase/sulfatase hydrolysis in plasma and prostate samples from 12 volunteers supplemented for 3 days with 112.5 mg/d total isoflavones (aglycone eq). Gly, glycitein; Ge, genistein; Da, daidzein.

DISCUSSION

375 The chosen daily dose (112.5 mg total isoflavones, aglycone eq) was quite high but achievable with natural food sources. It is equivalent to the highest nutritional intake reported for Asian rural populations and can be achieved occasionally with 2–3 servings of soy-based foods (17). This dose was chosen 380 to ensure sufficient levels in prostate, allowing quantitative and qualitative analysis of the metabolites.

Using this dose, isoflavone concentrations reached 1.05 ± 0.62 nmol/g in the prostate tissue and 1.45 ± 0.77 μmol/l in plasma when measured 12 h after the last intake of isoflavones. 385 The time of sampling was imposed by anesthesia constraints. Considering that $t_{max} = 4$–7 h and $t_{1/2} = 5$–8 h for isoflavones in plasma, concentrations measured at this stage should still represent around 50% of peak plasma concentrations (18). The kinetics of penetration and elimination of isoflavones in tissues 390 is not known. Assuming that it follows the plasma pharmacokinetics, twice these concentrations may have been reached in the prostate tissue of the volunteers at earlier time points.

Two previous studies have measured isoflavone concentrations in human prostate or prostatic fluid without prior 395 supplementation. First, Morton et al. (19) determined the daidzein concentration in the prostatic fluid of men living in Hong-Kong, in Portugal, or in the United Kingdom. The basal level of daidzein was 6-fold to 15-fold higher for men living in Hong Kong (0.275 μmol/l) than for men living in Europe. Pro- 400 static fluid was more concentrated than plasma (0.275 μmol/l vs. 0.123 μmol/l, respectively). Hong et al. (20) determined the concentration of isoflavones in plasma and prostate tissues of 15 Korean men who had benign prostatic hyperplasia. Hong et al.'s results were consistent with ours, with isoflavone concen- 405 trations in prostate being about half the plasma concentrations: 0.167 nmol/g vs. 0.382 μmol/l for daidzein and 0.242 nmol/g vs. 0.695 μmol/l for genistein, respectively. Similar concentrations were measured in volunteers with normal prostate. Two additional studies have focused on Western men receiving isoflavone supplementation. In one, 45 healthy American men were classi- 410 fied into low or high isoflavones consumers and supplemented with a soy beverage for 1 wk (42–60 mg isoflavones/day) (21). Daidzein was 4 times more concentrated in the prostatic fluid than in plasma: 1.88 vs. 0.44 μmol/l for low consumers and 2.78 vs. 0.54 μmol/l for high consumers. In contrast, genistein did 415 not concentrate in the prostatic fluid and was present at lower concentration (0.4–0.48 μmol/l). In the last study, Rannikko et al. (22) supplemented men with prostate cancer with a high dose of red clover phytoestrogens. After 2 wk, genistein as well as daidzein concentrations were twice as high in prostate tis- 420 sue than in plasma: 1.38 and 1.07 vs. 0.66 and 0.49 μmol/l, respectively.

Compilation of these limited data indicates that concentrations as high as 1–2.5 nmol/g of each isoflavone may be achieved in human prostate or prostatic fluid after short-term supplemen- 425 tation with high nutritional doses of isoflavones and that in Asian men, basal levels of total isoflavones are about 0.4 nmol/g.

Hedlund et al. (21) as well as Morton et al. (19) have reported significantly higher daidzein and equol concentrations in prostatic fluid from high isoflavone consumers compared to 430 low consumers. This raises the question of a possible accumulation of isoflavones in prostate or prostatic fluid with regular soy intake. For daidzein, concentrations achieved remain modest, suggesting that elevated concentrations may reflect the last intakes of isoflavones in preceding days rather than a real storage 435 in tissue. The situation may be different for equol for which striking prostatic fluid/plasma concentration ratios have been

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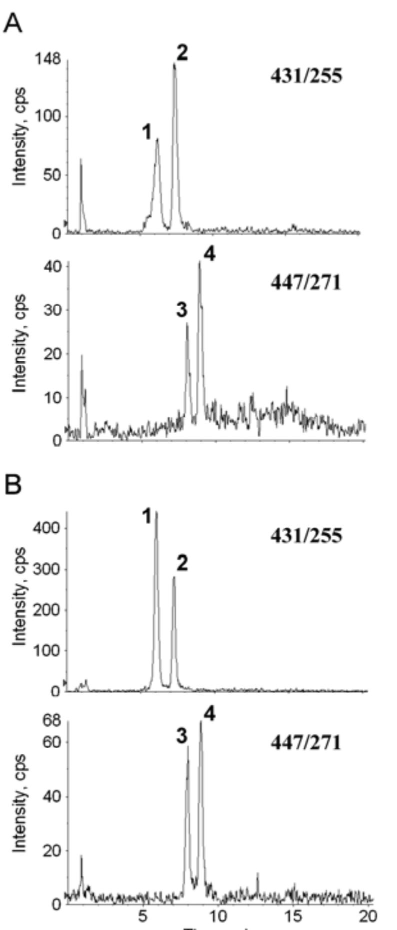

FIG. 2. Nature of the major isoflavone metabolites detected by liquid chromatography electrospray ionization tandem mass spectrometry in plasma (A) and prostate (B) samples from 12 volunteers supplemented for 3 days with 112.5 mg/day total isoflavones (aglycone eq). Compounds 1 and 3 were identified as daidzein 7-*O*-glucuronide and genistein 7-*O*-glucuronide by comparison with chemically synthesized standards. Compounds 2 and 4 were tentatively identified as daidzein 4′-*O*-glucuronide and genistein 4′-*O*-glucuronide on the basis of ion transitions and retention times. cps, counts per second. Q10

observed in high isoflavone consumers but not in low isoflavone consumers. The equol concentration was 40-fold higher in the prostatic fluid of men from Hong Kong than in their plasma (19) and 22-fold higher in the prostatic fluid of high isoflavone consumers than in their plasma in the study of Hedlund et al. (21). However, this observation relies on a very small number of volunteers. We did not observe higher concentrations of equol in prostate compared to plasma in this study. More data are needed on a larger number of volunteers from various populations.

Due to extensive intestinal and hepatic metabolism, isoflavone glycosides and aglycones present in foods are recovered in human plasma primarily as monoglucuronidated metabolites along with low amounts of aglycones and sulfate esters (23–25). Specific uptake of some metabolites by the cells as well as intracellular metabolism, especially possible deglucuronidation, may lead to differences in the nature and concentration of isoflavone metabolites present in plasma and in inner tissues such as prostate.

Our study shows for the first time that daidzein and genistein are mainly recovered as glucuronides in prostate tissue. We only detected very low concentrations of aglycones in some prostate samples and observed that artifactual hydrolysis of glucuronides into aglycones easily occurred during sample preparation. Extraction had to be realized rapidly in ice. We looked for the presence of various other metabolites that may be present due to classical conjugation reactions known for xenobiotics (26). Trace amounts of diglucuronides and sulfates were detected for a few volunteers. Other metabolites were not detected. However, their presence in low amount can not be ruled out because we were not able to optimize extraction and detection conditions for these compounds due to the lack of pure standards. It is worth noting that tumoral cells may contain more aglycones than normal cells because glucuronidase activity has been reported to be markedly higher in tumors (27).

Data on the nature of tissue metabolites is crucial because the biological properties of conjugated metabolites may differ markedly from those of aglycones. For instance, the affinity of isoflavone glucuronides for estrogen receptors has been reported to be 10 to 40 lower than that of the aglycones (28). Sulfation of isoflavones was also shown to decrease their antioxidant activity and their effect on platelet aggregation, inflammation, cell adhesion, and chemotaxis (29,30). Q1

Most in vitro studies that have investigated the mechanisms of action of isoflavones have used concentrations as high as 50 to 100 μmol/l isoflavones provided as aglycones or glycosides. Such concentrations have never been achieved in the human body, even with pharmacological doses. Peak plasma concentrations of isoflavone metabolites generally range between 0.4 and 4 μmol/l, with a mean value of 2 μmol/l, after consumption of 50 mg aglycone equivalent dose of isoflavone, which is about the mean intake in Asian countries (18). The highest isoflavone concentrations reported so far were detected in the plasma of volunteers challenged with a pharmaceutical dose of genistein to test its potential genotoxic effects (300 mg/day for 28 days, then 600 mg/day for 56 days) (31). A high interindividual variability was observed in the study, and maximum plasma concentrations ranged between 4 and 27 μmol/l genistein among the 20 volunteers. The results suggest that plasma concentrations higher than 10 μmol/l can only be achieved in a low proportion of individuals and after intake of pharmacological doses at least sixfold higher than nutritional doses.

Our study shows that the isoflavone concentration in prostate remains below 5 nmol/g after a high-dose supplementation.

In vitro studies using concentrations exceeding these physiological concentrations may provide erroneous information on isoflavone mechanisms of action and contribute to delays in our understanding of their complex health effects. Future studies on isoflavone activities in prostate cancer prevention will thus have to use conditions of isoflavone exposure that lead to intracellular isoflavone concentrations below 5 nmol/g. Furthermore, glucuronide metabolites will have to be tested in addition to the classical aglycones under conditions in which they are not hydrolyzed into aglycones inside the cultured cells.

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3.2 Distribució tissular d'isoflavones en ovelles després d'una ingesta dietètica

Publicació VII: Distribució tissular de les isoflavones en ovelles després del consum de trèvol vermell.

Mireia Urpi-Sarda, Christine Morand, Catherine Besson, Guillaume Kraft, Didier Víala, Augustin Scalbert, Jean-Michel Besle, Claudine Manach. Tissue distribution of isoflavones in ewes after consumption of red clover silage. *Archives of Biochemistry and Biophysics*. **2008**, 476 (2):205-10.

Resum:

Als anys 50 es va observar que les isoflavones podien provocar la síndrome d'infertilitat en ovelles que pasturaven amb trèvol. Altres efectes d'aquests fitoestrògens s'han documentat posteriorment.

L'objectiu d'aquest estudi va ser determinar la distribució dels metabólits de les isoflavones en teixits per a buscar un enllaç amb el seu impacte fisiològic

Per a aquest fi, i a causa de la dificultat per a obtenir teixits o suficient quantitat de teixit en altres espècies, dues ovelles femelles van ser alimentades amb un 50% de trèvol vermell (varietat *Pawera*) durant un mes amb una ingesta diària de 157.6 mg/kg d'isoflavones totals. Aquestes només van consumir les isoflavones en forma d'aglicona a causa de l'estat de fermentació de l'ensitjat (Figura 28).

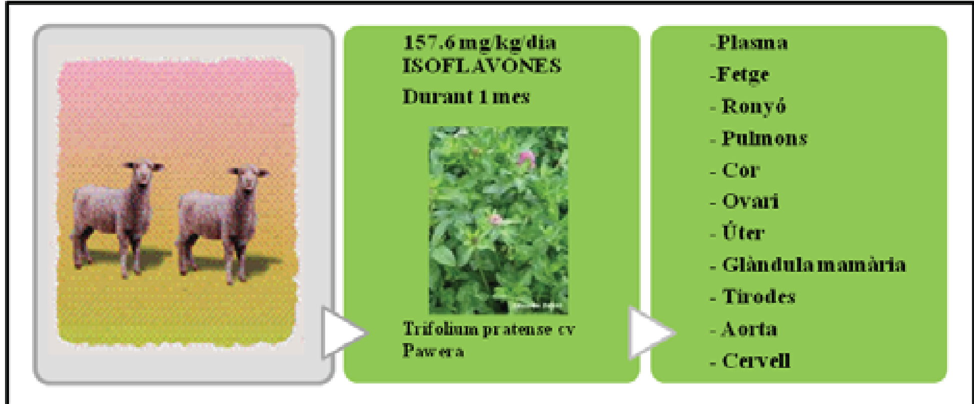

Figura 28: Esquema de l'estudi

Es van analitzar els següents teixits per a metabòlits i aglicones d'isoflavones per HPLC-coularray i LC-MS/MS: sang, fetge, ronyó, pulmó, cor, múscul, ovaris, úter, glàndula mamària, glàndules suprarenals, timus, aorta, tiroides, glàndula pituïtària, cerebel, lòbuls olfactius i hemisferis cerebrals.

Els majors compostos recuperats en teixits van ser l'equol i la daidzeina presents com glucurònids. Les concentracions en el ronyó van ser 10 vegades majors que en altres teixits. La penetració de les isoflavones en cervell va ser bastant limitat. Els òrgans reproductius van tenir concentracions més elevades d'isoflavones que el múscul, cor o timus.

La distribució de les isoflavones en teixits d'ovella és desigual, per tant, pot reflectir l'impacte específic en alguns teixits diana.

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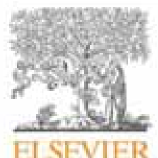

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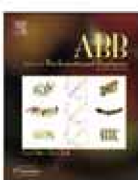

Tissue distribution of isoflavones in ewes after consumption of red clover silage

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ABSTRACT

When discovered in the 50's, isoflavones were suspected to provoke infertility syndrome in sheep grazing on clover. Many others effects of these phytoestrogens have been documented afterwards. To determine the distribution of isoflavone metabolites in ewe tissues and look for a link with their physiological impact, two ewes were fed a diet containing 50% red clover silage (variety Pawera) for one month with a daily intake of 157.6 mg/kg bw of total isoflavones. Only aglycones were fed due to the fermentation stage of the silage. At the sacrifice, isoflavone metabolites and aglycones were analyzed in blood, liver, kidney, lung, heart, muscle, ovaries, uterus, mammary glands, suprarenal glands, thymus, aorta, thyroid, pituitary gland, cerebellum, olfactory lobes, and brain hemispheres using HPLC-Coularray and LC–MS–MS. The major compounds recovered in tissues were equol and daidzein, present as glucuronides. Kidney concentrations were 10-fold higher than in other tissues. Penetration in brain was very limited. Reproductive organs contained higher concentrations of isoflavones than heart, muscle, or thymus. Distribution of isoflavones in ewe tissues is unequal and may reflect specific impact in some target tissues.

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Isoflavones, a class of phytoestrogens, are plant substances structurally similar to steroidal estrogens. In the 1940 s, a syndrome with temporary to permanent infertility effects, called "clover disease", affected Australian sheep foraging upon subterranean clover [1]. The isoflavones formononetin, biochanin A, daidzein, and genistein were isolated from clover and Davis and Hill showed that the problems related to sheep reproduction may be more or less severe in relation to the formononetin content of clover cultivars [2]. Subterranean (*Trifolium subterraneum*) and Red Clover (*Trifolium pratense*) were among the richest cultivars in formononetin [3].

In humans, the major source of isoflavones is soy and soy products. Human health benefits of isoflavone consumption have been linked to their estrogenic properties [4,5]. Soy consumption has been shown to affect many physiologic, endocrinologic, and metabolic processes that may have consequences on human health [6]. Most of these effects have been attributed to specific isoflavones and/or their metabolites, such as equol which is a microbial metabolite of daidzein with estrogenic properties [7]. To assess the potential risks and benefits of dietary soy isoflavones and the mechanisms of action by which health effects can occur, it is essential to have a more complete understanding of their bioavailability. Plasma pharmacokinetics of isoflavones has been widely studied therefore the nature and concentrations of metabolites that can be achieved in blood after isoflavone consumption are quite well documented, in animal models but also in humans. Depending on the dose and the dietary source of isoflavones, their elimination from the body through the bile and urine occurs with a mean elimination half-life of 8–11 h for plasma [8,9]. However, descriptive data are still very limited on the presence of isoflavone metabolites in tissues. A crucial question is to assess whether specific organs are able to concentrate and store some isoflavone metabolites. The determination of the achievable levels of isoflavone metabolites in target organs is useful information to determine their mechanisms of action. Some biological activities, such as inhibition of topoisomerase II or direct scavenging of free radicals have only been observed with high concentrations of isoflavones (>20 μM) whereas others, such as those linked to the binding to estrogen receptors, can occur when isoflavones are present in the nanomolar range [10–13].

To provide new descriptive data on isoflavone tissue distribution, we conducted a feeding study in ewes and determined the tissue concentrations of isoflavones and their metabolites after 1-month exposure to a diet containing 50% red clover silage and providing 157.6 mg/kg bw per day of isoflavones. A wide range of tissues were examined: kidney, liver, ovary, uterus, mammary gland, brain (cerebellum, hemisphere, olfactory lobe, and pituitary gland), aorta, suprarenal glands, thyroid, lung, thymus, heart, and muscle, some of them such as thyroid, pituitary gland or aorta being hardly analyzable in small animals such as rodents.

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Materials and methods

Chemicals

Standards of daidzein, equol, formononetin biochanin A and genistein, the purities of which were at least 95%, were purchased from Extrasynthese (Genay, France); β-Glucuronidase/sulfatase from *Helix pomatia* was obtained from Sigma (L'Isle d'Abeau Chesnes, France); Standards of daidzein-7-glucuronide and genistein-7-glucuronide were chemically synthesized and kindly provided by Nigel P. Botting from the University of St. Andrews (UK), according to the method previously published [14].

Animals and diets

Two 3-year-old lactating ewes (A and B, Race Lacaune) were fed *ad libitum* during one month with a diet based on red clover (*Trifolium pratense*, cv *Pawera*) silage. The daily amount of red clover silage ingested was, for A and B, 810 and 860 g dry matter, respectively (average 830 g), accounting for 49.7% and 51.3%, respectively (average 50.5%), of the total diet. In addition they received the same amount of hay, barley and sunflower oilmeal (120, 470, and 230 g/d dry matter, respectively). The red clover silage was prepared from plants mowed at the beginning of the flowering stage (second cutting of spring 2003), wilted during one day, chopped and ensiled with a regular addition of formic acid (0.5% fresh matter). The final composition of the red clover silage is indicated in Table 1. The silage provided 12.3 mg/g isoflavones, which corresponds to a daily intake of 157.6 mg/kg bw total isoflavones.

The corresponding human dose can be calculated using the equation: human dose = animal dose × $(Wanimal/Whuman)^{(1-b)}$, with an allometric component $b = 0.67$ as recommended in the US-FDA Guidance for Industry "Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers" (July 2005, http://www.fda.gov/cder/Guidance/5541fnl.htm). For a human weight of 70 kg, the corresponding human dose is 153.6 mg/d total isoflavones.

At the end of the experiment, the animals A and B weighed 62.4 and 67.2 kg, respectively. Just before sacrifice, blood samples were collected through a catheter in the jugular vein and plasma samples were immediately separated by centrifugation in heparinized tubes. Animals were knocked out and immediately bleeded. Liver, kidney, lung, heart, muscle, fat, ovaries, uterus, mammary glands, suprarenal glands, thymus, aorta, thyroid, pituitary gland, cerebellum, olfactory lobe, and brain hemispheres were sampled and immediately freeze-clamped and stored at −80 °C.

This study was carried out in accordance with French recommendations and with the guidelines of the Animal Care and Use Committee of the Institut National de la Recherche Agronomique (INRA) on the use of experimental animals, including animal welfare and appropriate conditions.

Determination of red clover silage composition

Two aliquot samples of silage were collected at the middle of each week during the experiment .They were frozen, freeze dried and ground to pass through a 1 mm mesh sieve. A previous report [15] showed that the isoflavone glycosides were totally hydrolyzed after ensiling, therefore only the analysis of free aglycones was carried out. In brief, the ground sample (100 mg) was extracted twice with 12 ml ethanol/water solution (80/20 V/V) at room temperature (20 °C) in a centrifugation tube with magnetic stirring for 30 min and centrifuged each time at 1500*g* for 10 min. The supernatants were pooled and concentrated under vacuum with a rotary evaporator to yield a 2 mL ethanol/water (80/20 V/V) fraction, which was then filtered through an acetate cellulose membrane (minisart Sartorius). This ethanol–water fraction was analyzed by HPLC with a photodiode array detector (200–400 nm, Kontron). The sample was injected (20 μL) and eluted at ambient temperature at a rate of 0.9 mL min^{-1} through a Superspher column® 60 R.P.8 (Merck, 125 × 4 mm, 5 μm). The mobile phases used were A (H_2O/H_3PO_4 85%, 100/0.3 v/v) and B (Acetonitrile/H_2O/H_3PO_4 85% 80/20/0.3 v/v/v), and a linear gradient was performed in 56 min from 0% to 80% B.

Table 1
Isoflavone contents (g/kg dry weight (SD)) in the red clover silage

	Structure	Red clover silage (g/kg dry matter)
Formononetin	R_1: H; R_2: CH_3	6.39 ± 0.60
Bichainin A	R_1: OH; R_2: CH_3	5.08 ± 1.42
Genistein	R_1: OH; R_2: H	0.59 ± 0.06
Daidzein	R_1: H; R_2: H	0.24 ± 0.03
Total		12.3 ± 2.11

Extraction procedure

About 150 mg frozen tissue were weighed in an Eppendorf tube with a little steel ball and homogenized twice with 9 volumes of methanol/water (70/30) (v/v) using a Mixer Mill (MM 300 Retsch, QIAGEN, VWR International A/S) at 20 Hz during 2 min. Pooled supernatants were evaporated to dryness and dissolved in 1 volume of sodium acetate buffer 0.1 M pH 4.9, and incubated for 18 h at 37 °C with β-glucuronidase/sulfatase (*Helix pomatia*, 2000 U β-glucuronidase and 90 U sulfatase). After incubation, isoflavones were extracted with 4 volumes methanol/HCl 200 mM (v/v). Five replicates of extraction and analysis were performed for each sample. Our method markedly differs from the enzymatic hydrolysis method reported by Gu et al. [16] to underestimate the tissue concentrations of isoflavone conjugates. Plasma samples were acidified to pH 4.9 with 10 mmol/L acetic acid before incubation for 18 h with β-glucuronidase/sulfatase (1000 and 45 U, respectively). The isoflavones were then extracted with 4 volumes methanol/HCl 200 mmol/L (v/v) and centrifuged 5 min at 12500*g*. Triplicates were performed for each plasma sample. The coefficient of variation between replicates was <15% in plasma and tissues.

The fraction of equol and daidzein present as aglycones was determined in tissue samples by comparison of the concentrations measured with and without β-glucuronidase/sulfatase hydrolysis.

As aglycones and conjugated derivatives, such as glucuronides, have quite different polarity and stability, giving different behaviour during extraction and analysis, it was considered not relevant to use an aglycone as internal standard because a high proportion of conjugated forms of isoflavones could be present in our plasma and tissues samples. The extraction recovery and the efficiency of hydrolysis were checked using control rat plasma and liver tissue (obtained from rat fed a semi-synthetic isoflavone-free diet), supplemented with known concentrations (2 and 5 μmol/L, in duplicate) of aglycones and chemically synthesized standards of isoflavones glucuronides, and treated the same way as the samples. The recovery rate ranged from 89% to 105% for aglycones and glucuronides in plasma and from 82% to 103% in liver. Glucuronides were not detectable after hydrolysis with β-glucuronidase/sulfatase in the conditions described above.

Analytical procedure

HPLC-CoulArray

The isoflavones from tissues were analyzed by HPLC with multi-electrode coulometric detection. The interest and reliability of this detection method for the determination of isoflavones has been previously described [17,18]. The system is composed of two pumps (Model 580, ESA, Chelmsford, USA) for high pressure gradient, a temperature-controlled autosampler (Gilson, Villiers-le-Bel, France), a thermostatic chamber, a 150 × 2.1 mm SymmetryShield RP18-5 μ column (Waters) and a eight-channel CoulArray detector (model 5600, Eurosep, Cergy, France).

Mobile phases consisted of a 30 mmol/L NaH_3PO_4 buffer (pH 3) containing 20% acetonitrile (phase A) and 40% acetonitrile (phase B). Separation was achieved using a gradient elution (flow = 0.4 ml/min, 40 °C): 0–15 min: linear gradient from 100% A to 100% B, 15–19 min: 100% B, 19.01–25 min: 100% A. Potentials were set at 200; 280; 450; 550; 600; 650; 700; 750 mV (Pd as reference). Injection volume was 20 μl.

Calibration curves have been prepared in control rat plasma by supplementation with known concentrations of isoflavones: 0, 1, 2, 5, and 10 μmol/l. Quality control samples (duplicates of two different concentrations in plasma treated exactly the same way as the samples: same hydrolysis and extraction procedure) were added in each batch of analyses to control the accuracy of the quantification. The accuracy was good, with differences between the measured value and the actual value always <9%.

LC–MS/MS

Analyses were performed using a Hewlett-Packard 1100 HPLC system (Waldbronn, Germany) equipped with a quaternary pump and an autosampler. An Applied Biosystems API 2000 triple quadrupole mass spectrometer (Applied Biosystems, PE Sciex, Ontario, Canada), equipped with a Turbo IonSpray source ionizing in the positive and negative mode at 500 °C, was used to obtain the mass spectrum of each identified metabolite.

Optimum parameters for detection of the aglycones and metabolites were the following: capillary voltage 5500 V, collision gas 5 (arbitrary units) and, curtain gas 20 (arbitrary units). Declustering potential, focusing potential, entrance potential, and collision energy were optimized with infusion experiments of available standards and were the followings: daidzein (31, 300, 10, and 50, respectively), genistein (50, 400, 12, and 60, respectively), daidzein-7-glucuronide (30, 375, 5, and 25, respectively), genistein-7-glucuronide (20, 200, 10, and 30, respectively), equol (19,

370, 9, and 17, respectively), formononetin (75, 200, 5, and 35, respectively), Biochanin A (75, 200, 4, and 35, respectively). SymmetryShield™ RP18 column (Waters, Milford, MA, USA), 2.1 × 150 mm i.d., 5 μm, was used for chromatographic separation. The injection volume was 20 μL, and the flow rate was 400 μL/min. Mobile phase A was 15% acetonitrile with 0.5% acetic acid and phase B was 80% acetonitrile with 0.5% acetic acid. The separation was achieved using a linear gradient from 0% to 100% B between 0 and 20 min.

To determine the nature of isoflavone metabolites present in hydrolyzed and non-hydrolyzed tissues, MS data were collected in multiple reaction monitoring (MRM) mode by monitoring specific transitions of parent and product ions for each metabolite. The MRM mode was used with a dwell time of 300 ms, monitoring the following transitions: daidzein (255/91), genistein (271/91), equol (243/123), daidzein-glucuronide (431/255), genistein-glucuronide (447/271), equol-glucuronide (419/243), formononetin (269/253) and biochanin A (285/152).

Results

Isoflavone intake from red clover silage

Isoflavone contents in the red clover silage are shown in Table 1. We tested some samples and confirmed that the initial glycosides have been totally hydrolyzed during the fermentation stage of the silage. The main isoflavones were formononetin and biochanin A, as already reported for red clover [19]. Ewes ingested about 12.8 g/kg/day red clover silage, providing in average 81.8 mg/kg bw formononetin, 64.8 mg/kg bw biochanin A, 7.6 mg/kg bw genistein, and 2.9 mg/kg bw daidzein.

Nature of metabolites in tissues

Plasma and tissues samples were first analyzed without any hydrolysis to identify the physiological forms present. Mono-glucuronides of daidzein (peak 1 and 2) and equol (peak 3 and 4) were the major forms present in plasma as well as in tissues (Fig. 1). The daidzein-glucuronide with the lower retention time (peak 1) was identified as the 7-*O*-glucuronide of daidzein, by comparison with the chemically synthesized standard. The second glucuronide may correspond to the 4′-*O*-glucuronide of daidzein, but the standard was not available. Accordingly, the major compounds recovered in tissues after hydrolysis by the β-glucuronidase/sulfatase were equol and daidzein.

Although they were the major isoflavones provided by the red clover silage, genistein, formononetin and biochanin A, either glucuronidated or not, were not recovered in plasma or tissues.

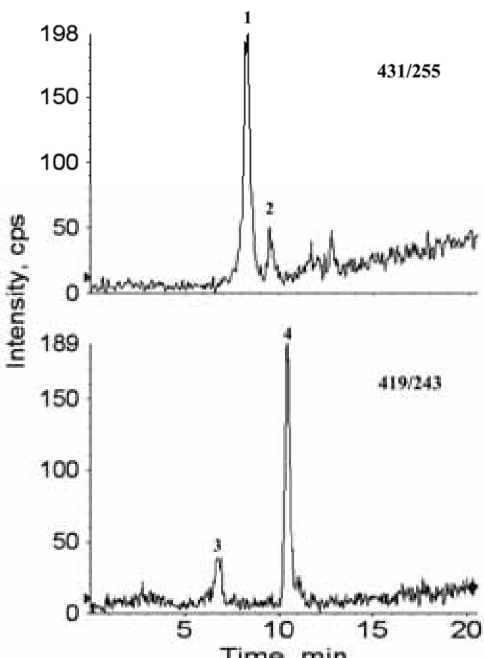

Fig. 1. LC-MS-MS chromatogram of ewe kidney sample. (A) Multiple reaction monitoring of daidzein-glucuronide (431/255). (B) Multiple reaction monitoring of equol-glucuronide (419/243).

Concentrations of isoflavone metabolites in tissues

Plasma levels of equol and daidzein were 18.28 ± 3.0 and 8.55 ± 0.5 μmol/L (mean ± SD), respectively.

Analysis of tissues from ewes fed a red clover silage diet for one month revealed an unequal tissue distribution of daidzein and equol (Fig. 2). However, the distribution of isoflavones over the different tissues was similar for the two animals.

The highest concentration of isoflavones was found in the kidneys of both ewes with a mean concentration of 181.06 nmol/g tissue for equol and 71.23 nmol/g tissue for daidzein. The liver was the second organ with highest levels of isoflavones, 26.41 and 18.24 nmol/g tissue for daidzein and equol, respectively. The liver is the only tissue that showed higher concentration of daidzein than equol. The equol/daidzein ratio in other tissues varied between 2 and 5. Ovary and aorta have quite high levels of isoflavones with a mean of 14.96 and 15.17 nmol/g, respectively, for equol and 5.56 and 4.69 nmol/g, respectively, for daidzein. Both ewes have intermediate levels of isoflavones in suprarenal glands, uterus and, thyroid, with a mean for equol of 10.35, 10.30, and 8.55 nmol/g, respectively, and a mean for daidzein of 5.20, 3.45, and 1.84 nmol/g, respectively. We found less than 10 nmol/g total isoflavones in mammary glands, pituitary gland, lungs, thymus, heart, and muscle. The lowest concentrations were found in the brain. The different parts of the brain, hemisphere, cerebellum, and olfactory lobe have an equol concentration (mean) of 0.73, 1.70, and 2.69 nmol/g tissue, respectively, and daidzein concentration of 0.36, 0.34, and 0.69 nmol/g tissue, respectively.

We compared hydrolyzed and non-hydrolyzed tissues to determine the fraction of equol and daidzein present as aglycones. Daidzein was not recovered in the aglycone form in any non-hydrolyzed tissue, although equol was found in non-hydrolyzed ovaries (8.0 ± 3.9%), mammary glands (1.9 ± 1.9%) and aorta (2.6 ± 0.8%).

Discussion

This study shows for the first time that isoflavones and their metabolites are widely distributed in ewe tissues after long-term oral exposure to red clover silage, a rich source for isoflavones in animal nutrition.

Formononetin and biochanin A are the two major isoflavones present in the red clover silage, but were not recovered in ewe tissues. This result is consistent with previous studies showing that these methylated isoflavones are extensively metabolized in the rumen [20]. Biochanin A is known to be demethylated into genistein and further degraded via ring cleavage into 4-ethylphenol and 4-hydroxyphenyl-2-propionic acid [20]. Formononetin is mainly demethylated into daidzein which can be converted into equol by hydrogenation and ring cleavage [7]. These compounds can be further metabolized to phenolic compounds similar to genistein. In humans, genistein and daidzein have been shown to be the major metabolites present in plasma after ingestion of biochanin A and formononetin [21,22]. In all the tissues examined in the study, equol and daidzein, mainly present as glucuronides, were the only metabolites recovered. The absence of genistein in plasma and tissue is consistent with the extensive bacterial metabolism of

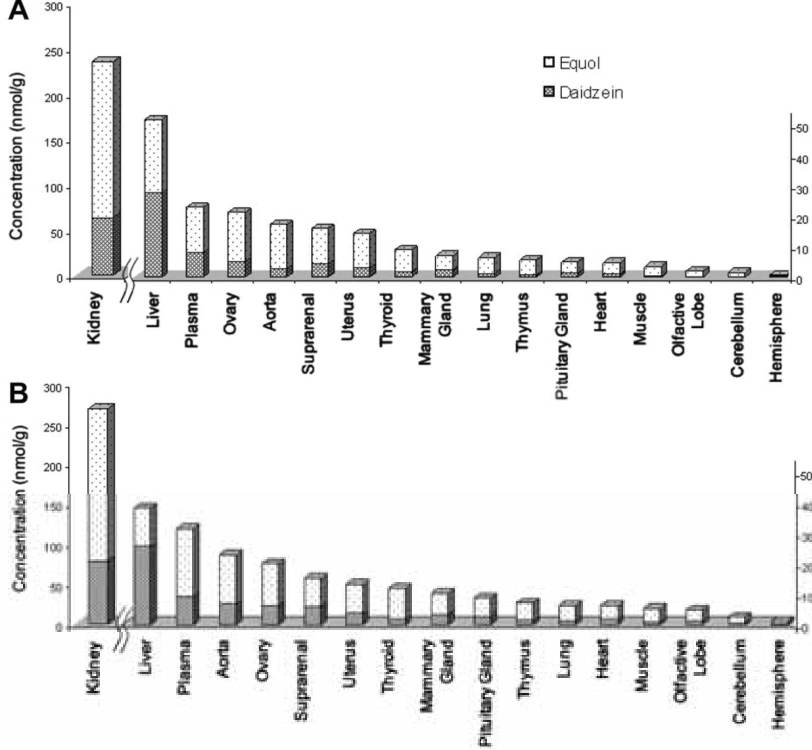

Fig. 2. Distribution of equol and daidzein in the ewe tissues. (A: Ewe 1, B: Ewe 2).

this aglycone which has already been documented. With 24 h incubations of biochanin A in bovine rumen fluid, Dickinson et al. previously observed its degradation to unknown compounds instead of genistein [23]. Bowey et al. showed that microbial degradation was much more extensive for genistein than for daidzein [24]. Consistently, Maubach et al. [25] reported that after ingestion of soy-based food supplements containing 66% of genistein, 24% of daidzein and 10% of glycitein, daidzein and equol largely predominated over genistein in the human breast tissue and serum. In the present study, the extensive microbial degradation of genistein in ewe rumen was not directly confirmed since the bacterial metabolites such as *p*-ethylphenol or 4-hydroxyphenyl-2-propionic acid, were not checked by the analytic methods used. The determination of all metabolites should be investigated using labeled compounds, but the cost of such intervention in large animals like ewes is prohibitive. Furthermore, we considered that the metabolism of isoflavones by bacteria is expected to be very different in ewes, which are ruminants, and in rodents or in humans for which the microbial metabolism occurs in the large intestine. Our primary objective was to examine the tissue distribution of the major isoflavone metabolites recovered in various animal species and humans, namely daidzein, genistein, equol, and their conjugates [26].

With the exception of the liver, equol was more abundant than daidzein in all the tissues. It has also been reported to be the major metabolite in serum of rats and monkeys after soy intake [26]. In addition, equol remains in plasma for a longer period of time than do genistein and daidzein, as shown in humans [27,28]. Altogether these results support the need to investigate the biological effects of equol to understand the impact of isoflavones.

Our study also shows that daidzein and equol were not present as aglycones in tissues, or only in trace amounts, but were recovered as glucuronidated derivatives. Other authors found a higher proportion of isoflavone aglycones in rat tissues [29]. The conjugation process may differ between species [26], but must be taken into account because it has been clearly shown that metabolites such as glucuronides or sulfates may exert biological activities significantly different from those of aglycones [30,31].

The overall tissue distribution of daidzein and equol metabolites in ewes shows that no accumulation seems to occur in any specific organs, except in the kidney. Concentrations in kidneys were one order of magnitude higher than concentrations in other tissues. Such striking difference between kidney and other tissues has not been observed in rodents so far, even if kidney was always a tissue where high concentrations of isoflavones were recovered. The high expression of conjugative enzymes and transporters in the kidney, in favor of the excretion of glucuronidated and sulfated metabolites, may be responsible for the accumulation of conjugated isoflavones in this organ. We cannot rule out the possibility that urine interfere as a cofounder, since we have no information on the urine content of our kidney samples. Another hypothesis to consider is that isoflavones may accumulate in the ewe kidney due to pH partition and “ion trapping”. Feeding animals with a diet presenting a high Dietary Cation Anion Difference (DCAD), such as red clover silage, is expected to result in an alcalinisation of the urine pH, as demonstrated in sheep [32]. At pH 8, isoflavones and their conjugated metabolites mainly exist as anionic forms, which are not able to diffuse across cell membranes. Ionised isoflavones may thus be trapped in the high pH compartment of kidney tubular lumen. The high concentration of isoflavones observed in kidney supports their protective effects observed in a variety of experimental and human types of chronic renal disease [33]. Independently from a possible effect of isoflavones on chronic renal disease, the impact of isoflavones on the renal function certainly deserves further investigation.

The relatively high amounts of isoflavones observed in the liver can be related to the role of this organ in the metabolism of xenobiotics, which implies a good uptake capacity of compounds such as isoflavones. Previous studies with rats also showed major amounts of isoflavones in liver tissues after soy extract or genistein ingestion [29,34].

Ovaries, uterus and mammary glands were found to contain concentrations of equol and daidzein compatible with a biological activity. Estrogen-responsive tissues such as mammary glands, uterus, ovaries, and pituitary glands have been considered to be putative targets of isoflavones due to their phytoestrogenic properties conferred by their structural similarity with steroidal estrogens [35]. In ewes, this may contribute to explain the clover disease, syndrome with temporary or permanent infertility. Woclawek-Potocka et al. recently demonstrated that phytoestrogen metabolites (equol and *p*-ethylphenol) stimulated testosterone and luteolytic prostaglandin F-2 alpha synthesis in bovine steroidogenic corpus luteum cells, which may explain in part the reproductive disorders observed in ruminants fed isoflavone-rich diets [36]. A modification of serum hormone levels and an increase of the menstrual cycle length have been observed in some studies in women supplemented with soy isoflavones, however, the results are still quite inconsistent [37–40]. In humans, isoflavones are also considered to exert preventive effects towards hormone dependent cancers, especially breast cancer [41].

The high concentration found in aorta may be related to studies that reported beneficial impact of isoflavones on the endothelial function in humans [42]. In humans, consumption of soy isolate protein or pure genistein for 1–12 months has been shown to significantly improve the endothelial function measured by flow-mediated dilation (FMD) of the brachial artery [43,44]. Equol was also reported to increase gene expression of endothelial nitric oxide synthase (eNOS) in aortic vein endothelial cells *in vitro* and cause acute endothelium- and nitric oxide (NO)-dependent relaxation of aortic rings [45].

Our results also showed the presence of equol in thyroid. Previously, Doerge et al. reported a dose-dependent increase of the intra-thyroidal concentration of genistein in Sprague–Dawley rats fed diets containing 5, 100, and 500 ppm genistein [46]. Thyroid is another organ for which a potential impact of isoflavones has been suggested. Soy-based products can adversely affect thyroid function in animal models and in humans and have been considered as goitrogenic, especially in a context of iodine deficiency [46]. Ingestion of red clover silage has been shown to stimulate the secretion of thyroid hormones and ERα immuno-reactivity of thyroid glands in ovariectomized ewes [47].

The very limited accumulation of daidzein and equol in brain tissues is striking by comparison with all other tissues examined and probably reflects a poor penetration of isoflavones into this organ due to the blood–brain barrier. However, soy isoflavones have been shown to exert positive effects on the cognitive function in females, possibly by mimicking the actions and functions of estrogens on brain [48]. The underlying cellular mechanisms are not yet clear, however, an estrogen receptor-mediated pathway is a plausible mechanism since it is compatible with the low concentrations recovered in brain. We did observe differences between the various parts of the brain collected. It cannot be ruled out that local accumulation of isoflavone metabolites may occur in specific locus in the brain. Furthermore, some non detected metabolites may be present, such as the lipophilic fatty acid monoesters of genistein described by Kaamanen et al. [49] after *in vitro* incubation of labeled genistein in human plasma.

A low concentration of isoflavone metabolites was found in the muscle, which means that sheep meat cannot be considered as a potential source of isoflavones compared to soy-based foods. The muscle could supply around 10 μg of daidzein and 80 μg of equol for 100 g of meat; therefore this could not be considered as an appreciable source for isoflavones.

As in most tissue distribution studies, we measured isoflavone concentrations in the whole tissues, comprising cells, interstitial fluid, and blood. Residual blood may represent a volume fraction up to 10–25% for a limited number of highly irrigated tissues, namely spleen, lung, kidney, liver, and heart [50]. In these tissues, a significant error may thus occur if the blood and cellular concentrations widely differ. Consequently, it cannot be ruled out that isoflavone concentrations may have been slightly underestimated in kidney and liver and overestimated in lung and heart in our study.

Several studies have investigated the isoflavone distribution in other species. Chang et al. [29] observed a significant dose-dependent increase in genistein concentration in brain, liver, mammary, ovary, prostate, testis, thyroid, and uterus of male and female Sprague–Dawley rats fed a diet supplemented with 5, 100, and 500 mg/g with genistein aglycone. The authors also described a very limited penetration of genistein in the brain, as well as a high concentration of genistein in the liver, especially in female rats. In contrast to our study they found that high proportion of genistein was recovered in tissues in the form of aglycone. Gu et al. [34] analyzed the isoflavone tissue distribution in male and female Sprague–Dawley rats after a 4-day supplementation with soy protein. Highest levels of isoflavones were found in the kidney. The concentrations of daidzein and genistein were also high in prostate and pituitary gland. Low concentrations (0.28–0.42 nmol/g tissue) were found in mammary glands and uterus. Genistein concentration was again very low in the brain. After an oral dose of [^{14}C]-genistein (4 mg/kg), the tissue distribution of radioactivity in female and male rats showed higher levels in liver and in reproductive organs such as vagina, uterus, ovary, and prostate compared with other peripheral organs at the 2 and 7 h sampling points [51]. Interestingly, this study showed considerable differences in metabolite profiles between genders.

One important limit of such studies, including the present study, is that the tissue concentrations are only measured at one or a few time points and thus do not reflect the variations over time. In the case of ewes or rodents, the food intake is spread over a long period during the day so that variations of plasma and tissue concentrations are expected to be quite limited. However, for humans the isoflavone intake occurs sporadically, at irregular intervals, which may lead to more pronounced peaks of concentrations but a more transient presence of isoflavones in plasma.

Tissue distribution is governed by complex physiological and biochemical processes which are partly dependent on the compound-specific properties such as lipophilicity, size, affinity for plasma, and intracellular proteins. In the case of estradiol, tissue ER[1] content and binding affinity, in addition to vascular permeability, tissue–blood partition coefficients, and extrahepatic metabolism were shown to be important determinants for its disposition [52]. The role of these parameters in the tissue distribution of isoflavone metabolites thus definitely deserves further investigation.

In conclusion, this study demonstrated that long-term exposure to red clover silage in ewes results in an unequal distribution of isoflavones in the ewe tissues. The maximum levels were found in kidney following by decreasing concentrations in liver, ovary, aorta, suprarenal gland, uterus, thyroid, mammary gland, thymus, pituitary gland, lung, heart, and muscle. Concentrations were very low in brain. Such descriptive data regarding the concentration and nature of the metabolites in target tissues provide insights into the potential cellular and molecular mechanisms of action of isoflavones, assuming that for example an antioxidant activity through

[1] *Abbreviation used:* ER, Estrogen receptor.

direct scavenging of free radicals is unlikely to occur at the low concentrations present in the brain, whereas, activities through the binding to nuclear receptors are still achievable at such concentrations.

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DISCUSSIÓ GLOBAL

V. DISCUSSIÓ GLOBAL

La primera part del treball d'investigació de la tesi doctoral comprèn la posta a punt de mètodes d'extracció optimitzats i específics de compostos polifenòlics en diferents tipus de mostres biològiques per a la seva posterior detecció per espectrometria de masses. Es van estudiar i van comparar els tres tipus de mètodes d'extracció més utilitzats per a determinar compostos polifenòlics i els seus metabòlits en aquest tipus de mostres: la precipitació de proteïnes (PPT) (Morand et al. 2001), l'extracció líquid-líquid (LLE) (Del et al. 2003) i l'extracció en fase sòlida (SPE) (Walle et al. 2004). Aquesta metodologia es va validar i es va posar a punt amb mostres biològiques que provenien d'estudis clínics d'intervenció després d'un consum moderat i puntual de vi negre a causa de el seu elevat contingut en resveratrol en comparació d'altres aliments (Ragab et al. 2006; Hurst et al. 2008).

La recuperació del resveratrol en una matriu de LDL depèn del mètode de preparació de la mostra i del detector utilitzat. Quan la quantificació es va realitzar amb un detector ultraviolat/visible, es va observar que el mètode d'extracció amb millor recuperació va ser la PPT amb metanol acidulat, amb un valor de 112± 3%, seguit de la SPE (87 ±1%) i l'extracció LLE (65 ± 6%). No obstant això, per a incrementar la sensibilitat i la selectivitat del mètode per a la determinació de resveratrol, es va utilitzar l'espectrometria de masses (triple quadripol, API 3000) prèviament optimitzant els paràmetres necessaris a través de la infusió i del FIA (*flow injection analysis*), així com la fase mòbil i columna. Els resultats de recuperació més favorables corresponien a la SPE (87 ± 6%) respecte als resultats per PPT (57±2%) observant un gran efecte matriu en la ionització del resveratrol quan s'utilitzava la PPT i mostrant valors superiors de ràtio senyal/soroll quan s'utilitzava la SPE. Finalment es va validar el mètode seguint les recomanacions de la FDA (U.S.Department of Health and Human Services 2001) obtenint paràmetres inferiors al 15% per a l'exactitud i la precisió, linealitat i anàlisi de residuals del 100.1 ± 15.3% en el rang de concentracions estudiat (0.44 - 438.60 pmol/mL). Els límits de detecció i quantificació en LDL van anar de 0.15 pmol/mL i 0.44 pmol/mL, respectivament, i la recuperació del mètode del 87 ± 4%.

Aquest mètode va ser aplicat a les mostres de LDL que provenien de voluntaris després d'un consum moderat i puntual de vi negre on es va realitzar un estudi dirigit a determinar els metabòlits del metabolisme en fase II del resveratrol.

El següent pas va ser adaptar la metodologia analítica per a poder ser aplicada a estudis clínics i epidemiològics amb un gran nombre de mostres. Així mateix, ampliar el perfil metabòlic del resveratrol i determinar els compostos sulfatats després d'augmentar la sensibilitat i la selectivitat del mètode. Es va optimitzar el mètode d'extracció en SPE utilitzant plaques de 96 pous, permetent analitzar 96 mostres en 6 hores. Addicionalment es va reduir el temps d'anàlisi cromatogràfic a 10 minuts i finalment es van optimitzar les condicions cromatogràfiques per a la identificació i determinació dels metabòlits sulfatats del resveratrol. Aquest mètode es va validar per al resveratrol i el piceid (glucòsid del resveratrol) en mostres biològiques obtenint linealitat, exactitud i precisió inferiors al 15% en els rangs estudiats (4.4-3289.5 nmol/L per al resveratrol i 12.8-1923.1 nmol/L per al piceid). La seva aplicació per a diferents tipus de mostres biològiques van mostrar límits de detecció variables per al resveratrol i el piceid sent de 0.2 nmol/L i 1.2 nmol/L en matriu de LDL, de, 4.0 nmol/L i 8.4 nmol/L per a matriu d'orina i 0.3 i 1.9 nmol/L per a matriu d'orina sintètica. Addicionalment es va avaluar l'estabilitat d'aquests compostos a curt i llarg termini mostrant que el resveratrol, el piceid i els glucurònids del resveratrol eren estables en les nostres condicions d'emmagatzematge (-80ºC) i durant tot el processat de la mostra i detecció per espectrometria de masses. Quan es va valorar l'estabilitat a llarg termini en condicions de -80ºC i durant un període de 5 anys, es va observar un òptim coeficient de variació (10.8%).

Aquesta metodologia es va aplicar a estudis clínics en els quals els voluntaris van consumir una quantitat moderada de vi negre, amb l'objectiu d'identificar i quantificar el perfil metabòlic del resveratrol a causa del metabolisme en fase II. Per primera vegada es va determinar resveratrol lliure i metabòlits glucuronidats i sulfatats en LDL de 11 voluntaris a les 24h d'una ingesta dietètica de 250 mL de vi negre, sent el trans-resveratrol-3-*O*-glucurònid el metabòlit majoritari trobat en LDL. Aquest és el primer i únic estudi *in vivo* on s'han identificat el resveratrol i els seus metabòlits units a la LDL. La majoria d'estudis han observat l'efecte del vi negre sobre l'oxidació de la LDL *in vitro* (Frankel et al. 1993) o en estudis *in vivo* on han mostrat que el consum de vi negre

incrementava l'activitat antioxidant (Nigdikar et al. 1998). També hi ha treballs que confirmen la capacitat d'altres compostos fenòlics d'unir-se a les LDL, com els metabòlits del tirosol i de l'hidroxitirosol (de la Torre-Carbot et al. 2006) al consumir oli d'oliva verge.

Prèviament, altres estudis havien identificat alguns dels metabòlits del resveratrol com el trans-resveratrol-3-*O*-glucurònid, el trans-resveratrol-3-sulfat o disulfats del resveratrol en orina i teixits de rates (Yu et al. 2002; Wang et al. 2005; Wenzel et al. 2005) o en orina de voluntaris (Walle et al. 2004). Per primer cop s'ha identificat tant els isòmers 3 i 4' com les formes *cis* i *trans* dels diferents metabòlits de resveratrol.

La posta a punt de la metodologia analítica per a determinar el resveratrol i els seus metabòlits en mostres biològiques provinents d'estudis després d'un consum moderat de vi, va proporcionar les competències necessàries per a desenvolupar mètodes analítics per a l'anàlisi de diferents classes de compostos polifenòlics a partir de la ingesta de productes rics en diversos compostos polifenòlics, com els flavanols del cacau.

El cacau és un aliment ric en flavanols on els seus monòmers estan presents en un 5-10% i els oligòmers (procianidines) i els polímers es troben en >90% (Andres-Lacueva et al. 2008). Les procianidines constitueixen la fracció majoritària dels flavonoids de la dieta i al seu torn estan relacionades amb la protecció cardiovascular. Les procianidines, al ser flavanols dimèrics amb un elevat pes molecular, s'absorbeixen poc en l'intestí i per tant, arriben al còlon on la microbiota intestinal les degradarà a àcids fenòlics que podran ser absorbits (Gonthier et al. 2003b).

Disposar de mètodes sensibles, selectius i reproduïbles per a determinar els compostos fenòlics de la microbiota intestinal després del consum d'aliments rics en polifenols i principalment procianidines, ens proporcionarà les eines necessàries per a conèixer quins són els compostos potencialment responsables dels efectes beneficiosos d'aquests aliments en la salut. A més, aquesta determinació ajudarà a estimar el consum de procianidines i així poder avançar en el camp de les recomanacions dietètiques depenent del seu benefici nutricional i a la generació de noves i acceptades evidències científiques.

Per tant, per a avaluar l'efecte de la microbiota intestinal després del consum d'aliments rics en procianidines, s'ha desenvolupat i validat un mètode analític per espectrometria

de masses prèvia extracció en fase sòlida en placa de 96 pous per a determinar els metabòlits de la microbiota intestinal que provenen de la ingesta de cacau. El primer pas va ser l'optimització de l'extracció en fase sòlida per a determinar els àcids fenòlics i flavanols que poden provenir del consum de procianidines. Per a aquesta fi es va optimitzar el mètode d'extracció per a mostres biològiques provant diferents tipus de cartutxos Oasis® per a obtenir la major sensibilitat i menor efecte matriu en la detecció per espectrometria de masses. Els cartutxos d'intercanvi catiònic amb fase reversa van donar els millors resultats de recuperació per als àcids fenòlics i aglicones de flavanols.

Després de l'optimització dels compostos per infusió i FIA en l'espectròmetre de masses triple quadripol, la metodologia va ser validada per a 19 metabòlits fenòlics microbians així com per a flavanols en mostres d'orina. La recuperació i exactitud del mètode es troben en un rang de 87-109% i 87.5-113.8%, respectivament, amb una precisió menor del 15% en tots els casos complint amb els criteris de la FDA (2001) per a tots els compostos estudiats.. Aquest mètode suposa un increment significatiu en sensibilitat d'un major nombre de compostos, amb menor despesa econòmic, major respecte pel medi ambient i menor temps d'anàlisi respecte a metodologies anteriors ja descrites (Gonthier et al. 2003c; Ito et al. 2005).

El mètode validat es va aplicar a diferents estudis tant clínics amb voluntaris com amb animals d'experimentació, considerant tant un consum puntual com un consum regular de cacau.

El primer estudi que es va realitzar va ser amb 21 voluntaris que van prendre una ingesta puntual de cacau (40g). Aquest estudi mostra l'aplicabilitat del mètode validat en orines d'aquests voluntaris així com l'increment significatiu d'alguns àcids fenòlics després del consum de cacau: àcid cafeic, àcid ferúlic, àcid 3-hidroxifenilacetic, àcid vanillic, àcid 3-hidroxibenzoic, àcid 4-hidroxihipúric i àcid hipúric, així com epicatequina i procianidina B2. Com s'ha comentat anteriorment, les procianidines s'absorbeixen poc en l'intestí encara que algun alguns autors ho havien observat ja prèviament per a la procianidina B1 i/o B2, dues d'elles en humans (Sano et al. 2003; Holt et al. 2002a) i els altres dos en rates (Baba et al. 2002a; Shoji et al. 2006). Els resultats obtinguts en aquesta tesi doctoral concorden amb un estudi previ de Rios et al (Rios et al. 2003) on els voluntaris van consumir 80g de cacau. La nostra metodologia

també va permetre la identificació per espectrometria de masses d'altres compostos de degradació de la epicatequina com són la 5-(3',4'-dihidroxifenil)-γ-valerolactona i la (3-methoxy-4-hydroxyphenyl)-γ-valerolactona només descrits prèviament després del consum de te (Li et al. 2000).

El segon estudi de cacau realitzat va ser amb rates que van consumir o una dieta amb un 4% de cacau o amb un 10% de cacau durant 2 setmanes. Els resultats obtinguts van ser coherents amb l'estudi dels voluntaris humans encara que a causa de la major ingesta de cacau administrada a aquestes rates (entre 10 i 20 vegades major), es van observar concentracions més elevades d'alguns àcids fenòlics predominant els compostos monohidroxilats en posició 3' o dihidroxilats en posició 3' i 4'. Prèviament Gonthier et al (Gonthier et al. 2003b; Gonthier et al. 2003a) havia estudiat el metabolisme microbià de dietes riques amb catequines, procianidines, trímers o polímers per separat o d'una dieta suplementada amb extracte de vi negre que contenia procianidines. Van observar que després d'un consum de catequina els principals metabòlits van ser l'àcid 3-hidroxifenilpropiònic, l'àcid 3-hidroxibenzoic i el 3-hidroxihipúric encara que després del consum de vi negre els majoritaris van ser els àcids hipúric, *p*-cumàric, vaníllic i 3 i 4-hidroxibenzoics.

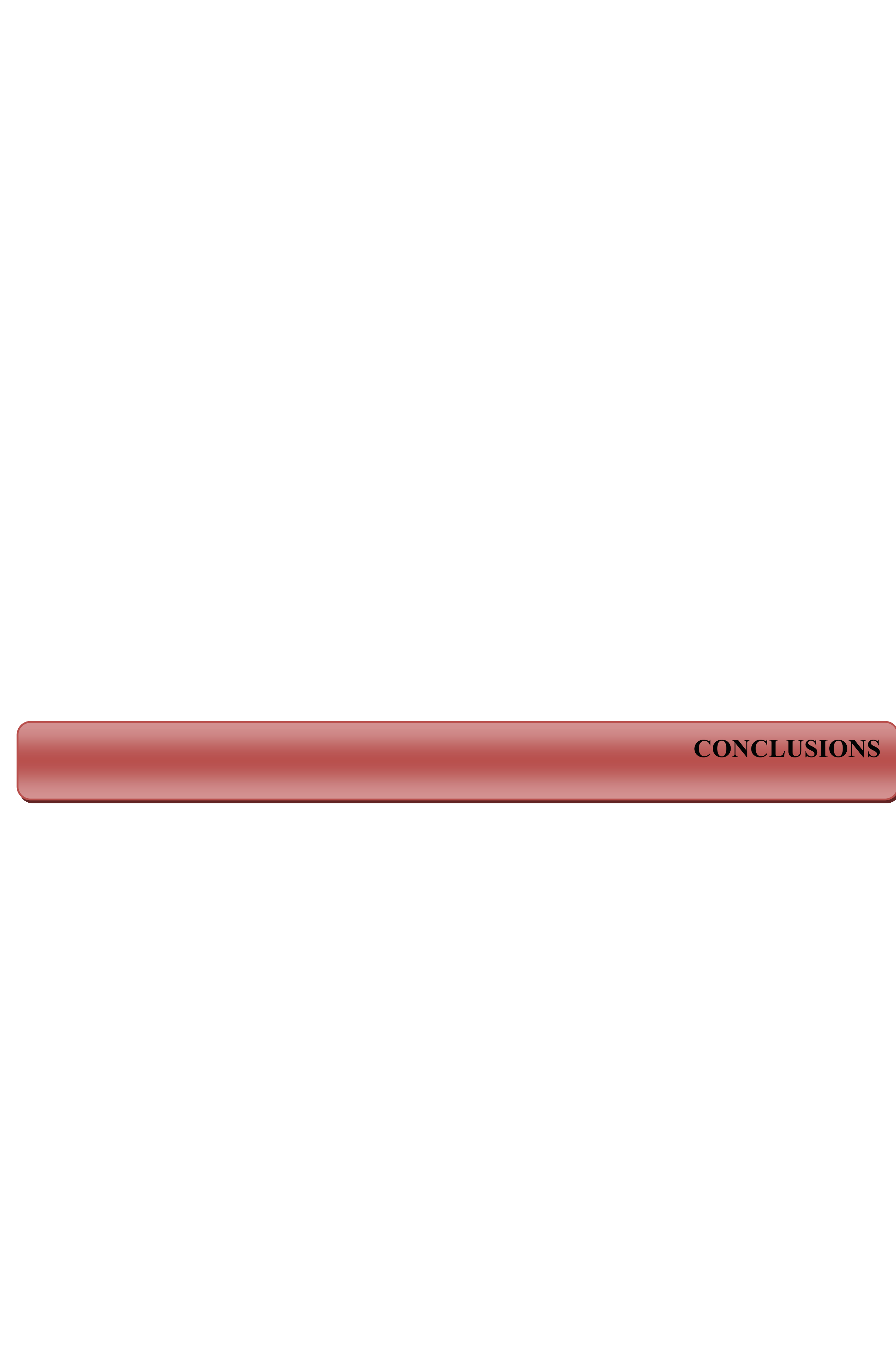
Aquesta metodologia juntament amb la metodologia desenvolupada per Roura et al (Roura et al. 2008) van permetre avaluar el perfil metabòlic urinari i plasmàtic després del consum regular de 40g/dia de cacau durant un mes en voluntaris de >50 anys amb elevat risc de malaltia cardiovascular. Entre ambdues metodologies, es van determinar tant els metabòlits de fase II de l'epicatequina com els metabòlits microbians. En plasma, es van observar increments significatius després del consum de cacau en els compostos 5-(3',4'-dihidroxifenil)-γ-valerolactona i 3-hidroxifenilacètic, i en orina, van ser també observades diferències significatives per a l'àcid vaníllic i l'epicatequina a més d'observar metabòlits de fase II glucuronidats i sulfatats de l'epicatequina i de la metilepicatequina, Aquest estudi ha considerat el major nombre de metabòlits descrits que conformen el perfil metabòlic de l'epicatequina provinent del consum regular de cacau. Prèviament Rios et al (Rios et al. 2003) van observar l'increment urinari a les 24h de la ingesta de sis àcids fenòlics després del consum de 80g de xocolata: 3-hidroxifenilpropiònic, ferúlic, 3,4-dihidroxifenilacètic, 3-hidroxifenilacètic, vaníllic i 3-hidroxibenzoic, dues dels quals, l'àcid vaníllic i l'àcid 3-hidroxifenilacétic, coincidents

amb els resultats observats en el treball d'aquesta tesi doctoral després d'un mes de consum regular de cacau.

Finalment es van realitzar dos estudis de biodisponibilitat tissular d'isoflavones, un amb voluntaris amb hiperplàsia benigna de pròstata i l'altre amb ovelles, ambdós estudis després d'un consum dietètic d'isoflavones. En aquests dos estudis es va utilitzar una extracció per precipitació de proteïnes prèviament a la seva detecció per espectrometria de masses o per detecció electroquímica amb CoulArray. En el cas de les isoflavones, a causa de la seva elevada absorció, la precipitació de les proteïnes amb metanol no va suposar minvaments significatius de sensibilitat que dificultessin o impedissin la seva identificació i posterior quantificació (Manach et al. 2004), i així es van mantenir els avantatges d'aquesta metodologia d'extracció, com són la seva rapidesa i estalvi econòmic respecte a la SPE. En l'estudi amb ovelles que van consumir regularment trèvol vermell durant un mes i es va observar una distribució tissular irregular dels metabòlits d'isoflavones en teixits. Els principals metabòlits que es van trobar van ser l'equol i la daidzeina i els seus derivats glucuronidats. La genisteina no es va poder identificar en aquests teixits a causa de una degradació microbiana extensiva ja descrita, que va donar lloc a compostos desconeguts (Dickinson et al. 1988; Bowey et al. 2003). Entre els diferents teixits considerats, el ronyó va ser l'òrgan amb major acumulació d'isoflavones podent ésser a causa de la interferència directa de l'orina o a l'acumulació en rumiants a conseqüència del pH alcalí (Las et al. 2007). Es van trobar quantitats biològiques i actives en els òrgans de reproducció que podrien estar relacionades amb la presència de receptors estrogènics en aquests òrgans on les isoflavones actuarien com fitoestrògens (Hewitt and Korach 2003). En l'encèfal es va observar una limitada acumulació degut probablement a la poca penetració a conseqüència de la barrera hematoencefàlica encara que estan descrits determinats efectes positius d'aquests compostos relacionats amb la funció cognitiva en femelles (Lee et al. 2005).

La distribució tissular en pròstata i plasma de les isoflavones també es va estudiar en voluntaris amb hiperplàsia benigna de pròstata que prèviament havien consumit tres comprimits d'un extracte d'isoflavones durant els 3 dies previs a la intervenció quirúrgica. Es van determinar dos metabòlits glucuronidats de la daidzeina i dos metabòlits glucuronidats de la genisteina en pròstata i plasma d'aquests voluntaris. Els nostres resultats van ser consistents amb Hong et al (Hong et al. 2002) qui havien

identificat prèviament la daidzeina i la genisteina en pròstata i plasma de voluntaris amb hiperplàsia benigna.



CONCLUSIONS

VI. CONCLUSIONS

D'aquest treball de tesi doctoral podem obtenir les següents conclusions:

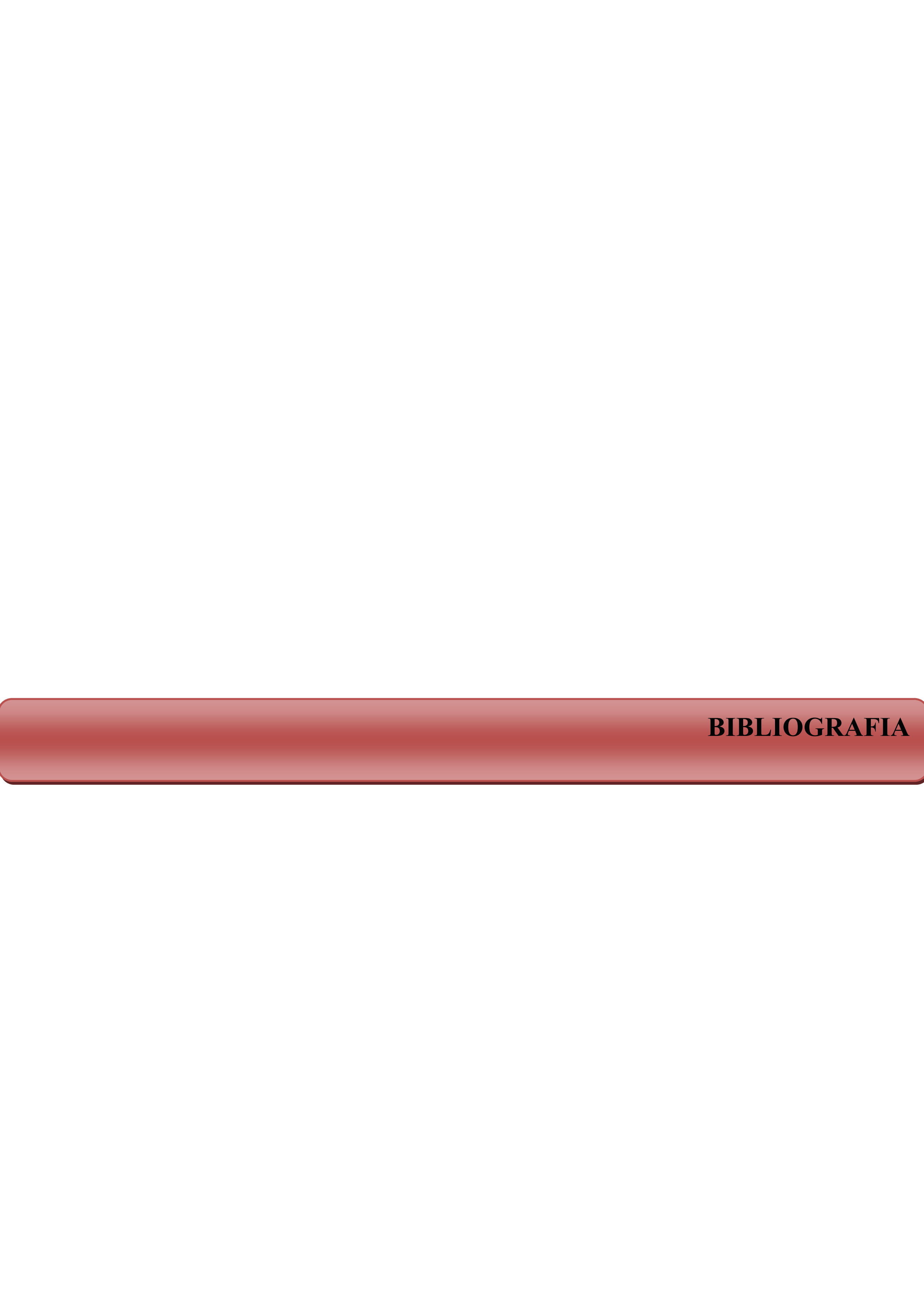
1. S'ha validat un mètode sensible, específic, ràpid i reproduïble per a determinar i quantificar el perfil metabòlic del resveratrol, en diversos teixits biològics. Aquest mètode permet determinar tant els metabòlits glucuronidats, sulfatats, com aquells originats a partir de la microbiota intestinal, com el dihidroresveratrol.

2. S'han identificat i quantificat per primera vegada el resveratrol i els seus metabòlits glucuronidats i sulfatats en mostres de LDL de voluntaris a les 24h d'un consum puntual i moderat de vi negre. A més, s'ha determinat el perfil metabòlic del resveratrol amb els seus metabòlits glucuronidats i sulfatats en mostres d'orina de voluntaris amb un consum puntual i moderat de 250 ml de vi negre.

3. S'ha validat un mètode sensible, ràpid, reproduïble, que permet l'anàlisi simultània d'un gran nombre de mostres per a determinar els àcids fenòlics que provenen de la microbiota intestinal després de la ingesta de cacau, a més de permetre identificar i quantificar l'epicatequina i la procianidina B2. Aquest mètode ha permès identificar altres compostos microbians que provenen de la degradació de l'epicatequina, com la 5-(3',4'- dihidroxifenil)-γ-valerolactona i la (3-metoxi-4-hidroxifenil)-γ-valerolactona.

4. Aplicant la metodologia validada s'ha determinat un ampli espectre del metaboloma polifenòlic urinari de voluntaris o rates després del consum puntual i regular de cacau.

5. S'ha demostrat una distribució irregular de les isoflavones en teixits d'ovella amb una major acumulació en ronyó seguit del fetge, plasma, òrgans de reproducció, timus, cor, múscul i encèfal. A més s'han determinat metabòlits glucuronidats de daidzeina i genisteina en pròstata de voluntaris amb hiperplàsia benigna de pròstata.



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ANNEX

VIII ANNEX

1. ALTRES PUBLICACIONS EN REVISTES

En aquest annex s'inclouen les publicacions en les que també he col·laborat.

1.1 Evaluació diagnòstica dels metabòlits del resveratrol en orina com a biomarcador del consum de vi.

Clinical Chemistry 52:7
1373–1380 (2006)

Endocrinology and
Metabolism

Diagnostic Performance of Urinary Resveratrol Metabolites as a Biomarker of Moderate Wine Consumption

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Background: Nutritional biomarkers may be better measures of dietary exposure than self-reported dietary data. We evaluated resveratrol metabolites, potential biomarkers of wine consumption, in humans after moderate consumption of sparkling, white, or red wines.
Methods: We performed 2 randomized, crossover trials and a cohort study. In the first study, 10 healthy men consumed 30 g of ethanol/day as sparkling wine or gin for 28 days. In the second trial, 10 healthy women consumed 20 g of ethanol/day as white or red wine for 28 days. We also evaluated 52 participants in a study on the effects of a Mediterranean diet on primary prevention of cardiovascular disease (the PREDIMED Study). We used liquid chromatography–tandem mass spectrometry to analyze urinary total resveratrol metabolites (TRMs) and predictive values and ROC curve analyses to assess the diagnostic accuracy.
Results: We observed significant increases in TRMs [72.4 (95% confidence interval, 48.5–96.2; P = 0.005), 211.5 (166.6–256.3; P = 0.005), and 560.5 nmol/g creatinine (244.9–876.1; P = 0.005)] after consumption of sparkling, white, or red wine, respectively, but no changes after the washout or gin periods. In the cohort study, the reported daily dose of wine consumption correlated directly with TRMs (r = 0.654; P <0.001). Using a cutoff of 90 nmol/g, we were able to use TRMs to differentiate wine consumers from abstainers with a sensitivity of 72% (60%–84%); and a specificity of 94% (87%–100%).
Conclusions: Resveratrol metabolites in urine may be useful biomarkers of wine intake in epidemiologic and intervention studies.
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Epidemiologic studies have shown a negative correlation between moderate wine consumption and cardiovascular disease *(1)*. In addition to ethanol, wine contains several minor compounds, such as polyphenols, that contribute to the differences observed between wine and distillates *(2, 3)*. To date, no studies have been performed to determine biomarkers of wine consumption. Resveratrol (3,5,4′-trihydroxystilbene) and piceid (resveratrol-3-*O*-β-glucoside) are phenolic compounds present mainly in grapes and wine *(4)*, and these compounds may have a role in the prevention of cancer, cardiovascular disease *(1)*, and neurodegenerative diseases *(5)*. In addition, they may be useful as biomarkers of wine consumption.

Biomarkers for epidemiologic and clinical assays have 3 distinct advantages over dietary data obtained by food frequency questionnaires (FFQs)[5] *(6, 7)*. One advantage is that biochemical markers of the intake of some nutrients are more precise than dietary assessment. Another advantage is that dietary data obtained by FFQ are often inadequate because of insufficient reporting of food com-

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[5] Nonstandard abbreviations: FFQ, food frequency questionnaire; LC-MS/MS, liquid chromatography–mass spectrometry; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval; and TRMs, total resveratrol metabolites.

position. The third advantage is that biomarker analysis provides a more proximal measure of specific nutrient intake than do FFQ data because it is an integrated measure of the bioavailability and metabolism of the component.

Recent advances in analytical techniques have improved the effectiveness and expanded the possibilities of biomarker analyses. Tandem mass spectrometry increases the sensitivity and selectivity of measurement of the metabolites of some nutrients *(8, 9)*. Resveratrol metabolites could be the best nutritional biomarkers for wine consumption because *trans*-resveratrol-3-*O*-glucuronide has been reported to be the main resveratrol metabolite in human blood *(10)*, urine *(11)*, LDL *(12)*, and target organs *(13)*. Other phenolic metabolites previously used as biomarkers of food consumption include 4′-*O*-methylgallic acid (the main gallic acid metabolite) for tea *(14)*, isoferulic acid for coffee *(14)*, and isoflavonoids for soy *(15)*.

The aim of this study was to determine the concentrations of resveratrol metabolites in blood and urine in 2 different studies after 4 weeks of wine consumption and to evaluate their usefulness as potential biomarkers of wine intake in intervention studies. In addition, we analyzed baseline data from a cohort included in a large intervention study to assess the diagnostic performance of this biomarker in real-life conditions.

Material and Methods

STUDY PARTICIPANTS

Clinical trials. The 2 intervention studies were open, prospective, randomized, crossover, single-blinded clinical trials.

The sparkling wine study (January to June 2005) included 10 healthy men [mean (SD) age, 28.2 (7.3) years; body mass index, 25.2 (1.3) kg/m^2], and the wine study (September to December 2004) included 10 healthy women [mean (SD) age, 38.1 (9.2) years; body mass index, 24.1 (4.0) kg/m^2]. All participants in both studies were healthy, and none reported any prior relevant disease.

COHORT STUDY

The PREDIMED (PREvención con DIeta MEDiterránea) Study is a large, parallel group, multicenter, controlled, randomized 4-year clinical trial aimed at assessing the effects of the Mediterranean diet on the primary prevention of cardiovascular disease (http://www.predimed.org). In the present study, we analyzed the baseline data of 52 consecutively admitted trial participants (30 men and 22 women admitted April to July 2005). Exclusion and inclusion criteria have been described previously by Estruch et al. *(16)*. Twenty-nine participants (55.8%) reported a mean (SD) daily intake of 118.3 (112.3) mL of wine. Seven (13.5%) reported intermittent drinking, mostly during weekends, consuming a mean of 98.0 (28.7) mL of wine per week, and 16 participants (30.7%) did not drink. All but 2 (93%) of the daily drinkers reported to preferentially consume red wine, although 24% also reported drinking lower amounts of white wine and sparkling wine. The Institutional Review Board of the Hospital Clinic of Barcelona approved the 3 study protocols, and written informed consent was obtained from each participant.

STUDY DESIGN

Clinical trials. Both studies were carried out over a 16-week period. During the first 4 weeks, the participants did not drink any alcoholic beverages (first washout period). During the next 4 weeks, they underwent the first intervention, after which they underwent a second 4-week washout period. During the final 4 weeks, the participants underwent the second intervention.

In the sparkling wine study, the interventions consisted of the intake of 30 g of ethanol/day as sparkling wine (300 mL/day) or as gin (100 mL/day) in a random order during dinner. In the wine study, the volunteers consumed 20 g of ethanol/day as red wine (200 mL/day) or white wine (200 mL/day), also in a random order during dinner.

In both studies, diet was monitored before and after each intervention period by use of a 3-day food-and-drink recall questionnaire, which had been validated previously in our country *(17)*. We converted the reported consumption into nutritional data with the Professional Diet Balancer software (Cardinal Health Systems, Inc.). The clinical investigators and laboratory technicians did not know the sequence of the intervention.

Reports from the participants and the number of empty bottles returned showed adherence. We did not observe significant differences between nutrient intake, anthropometric variables, and energy expended in physical activity before and after the evaluated interventions.

Urine and serum samples were collected the morning after the interventions and washout periods after overnight fasting and were coded with random numbers and stored at −80 °C until analyses, which were performed with no knowledge of the clinical data.

Cohort study. At baseline, participants completed a 137-item validated FFQ *(18)* and the validated Spanish version *(19)* of the Minnesota Leisure Time Physical questionnaire. Data collected included information on drinking habits, such as amount, frequency, and type of alcohol intake. We took samples of fasting blood and morning urine from all participants. Energy and nutrient intakes were calculated from Spanish food composition tables *(20)*. Urine samples were coded and stored at −80 °C until analyses. The clinical investigators and laboratory technicians were blinded to clinical data.

Reported daily consumption of the key food items and nutrients, as well as estimated energy expenditure from physical activity, were similar in the participants who drank wine daily, those who drank intermittently, and those who did not drink any kind of wine.

measurement of total resveratrol in beverages by hplc with a diode array detector

We concentrated 5 mL of sparkling wine, white wine, or gin, under reduced pressure and protected against exposure to ultraviolet light, to a final volume of 2 mL. Wines were injected directly into the HPLC according to the previously described method *(21)*. Results are reported as milligrams of total resveratrol consumed per day.

quantification of resveratrol metabolites from human samples

We used liquid chromatography–tandem mass spectrometry (LC-MS/MS) as described elsewhere *(12)* to analyze resveratrol metabolites extracted from urine and serum samples by solid-phase extraction. Briefly, urine samples (5 mL) were loaded on Oasis HLB cartridges (60 mg; Waters) that had been equilibrated. The cartridges were washed, and resveratrol metabolites were eluted with acidified methanol solution and ethyl acetate. The organic extract was evaporated under N_2. The samples were redissolved with 100 μL of the mobile phase used for the LC initial conditions with taxifolin as internal standard and then analyzed in the LC-MS/MS system.

We identified and quantified resveratrol metabolites in urine and serum with an LC system (Perkin-Elmer s200) coupled to a triple-quadrupole mass spectrometer (API 3000; Perkin-Elmer Sciex) as described elsewhere *(12)*. The intra- and interassay CVs for *trans*-resveratrol were 2.4% and 4.8%, respectively, and the analyses were performed in duplicate. All results for urinary resveratrol metabolites were corrected for urinary creatinine and are reported as nanomoles per gram of creatinine in the morning urine *(11, 12)*. Urinary creatinine was assayed with the standard Jaffe (alkaline picrate) kinetic method *(22)*. Serum (500 μL) was treated with 20 μL of *ortho*-phosphoric acid, vortex-mixed for 1 min, and processed by the same procedure.

statistical analysis

We used the standard statistical methods of the SPSS Statistical Analysis System, Ver. 11.5 (SPSS). Descriptive statistics with the mean (SD) were used for the baseline characteristics of the participants. Because the data were skewed (Kolmogorov and Levene tests), we used the Wilcoxon test for related samples to compare changes in outcome variables in response to each intervention period in both clinical trials. To exclude the presence of a carryover effect, we compared the observed outcome variables before both intervention periods. To compare groups in the cohort study, we used the 2-tailed *t*-test and ANOVA when indicated. We used Pearson correlations to examine associations between wine consumption and urinary excretion of resveratrol metabolites. To assess the accuracy of urinary resveratrol metabolite measurement for differentiating between wine consumers and nonconsumers, we calculated the sensitivity, specificity, positive (PPV) and negative predictive values (NPV), the likelihood ratio, and the ROC curve for the 2 randomized, crossover trials and the cohort study. With ROC curve analysis, we calculated a cutoff point that provided optimized sensitivity and specificity for the identification of wine consumers. Within- and between-group differences are expressed as means and 95% confidence intervals (CIs). All statistical tests were 2-tailed, and the significance level was 0.05.

Results

resveratrol concentrations in beverages

The amount of total resveratrol consumed per day in the clinical trials was 0.357, 0.398, and 2.56 mg for sparkling, white, and red wine, respectively. The content of resveratrol in gin was below the detection limits.

clinical trials

Sparkling wine study. After 28 days of dietary supplementation with 300 mL/day of sparkling wine, *cis*- and *trans*-resveratrol-3-*O*-glucuronides were found in the urine of all participants, whereas only very low concentrations of these metabolites were detected in the urine after the washout periods and the gin period. The mean concentrations of resveratrol metabolites in urine before and after each intervention are shown in Fig. 1. The amount of total resveratrol metabolites (TRMs) identified in this study increased by 72.4 nmol/g (95% CI, 48.5–96.2 nmol/g; $P = 0.005$) after sparkling wine consumption, whereas the concentration of these metabolites did not vary significantly after the gin period (Fig. 1). The order of interventions did not affect the results. No positive results were obtained when urine was checked for resveratrol aglycone, piceid, and sulfoconjugates. Serum concentrations of resveratrol and its metabolites were below the limits of detection in all participants evaluated.

White and red wine study. After 28 days of dietary supplementation with white or red wine (200 mL/day), *trans*- and *cis*-resveratrol-3-*O*-glucuronide were found in the urine of all participants, whereas only very low concentrations of these metabolites were detected in urine after the washout periods (Fig. 2). According to the TRM results, both metabolites increased by 211.5 nmol/g (95% CI, 166.6–256.3 nmol/g; $P = 0.005$) after white wine consumption and by 560.5 nmol/g (244.9–876.1 nmol/g; $P = 0.005$) after red wine intake. The differences between the changes observed after white and red wine intake significantly favored red wine [349.6 nmol/g (86.8–612.3 nmol/g); $P = 0.005$]. For all participants, no free resveratrol, piceid, or sulfoconjugates were detected in the urine, and resveratrol and its metabolites were below the limits of detection in serum.

both clinical trials

We used ROC curves to assess the effectiveness of urinary resveratrol metabolite measurement as a biomarker for wine intake. The optimal cutoff point was 90 nmol/g,

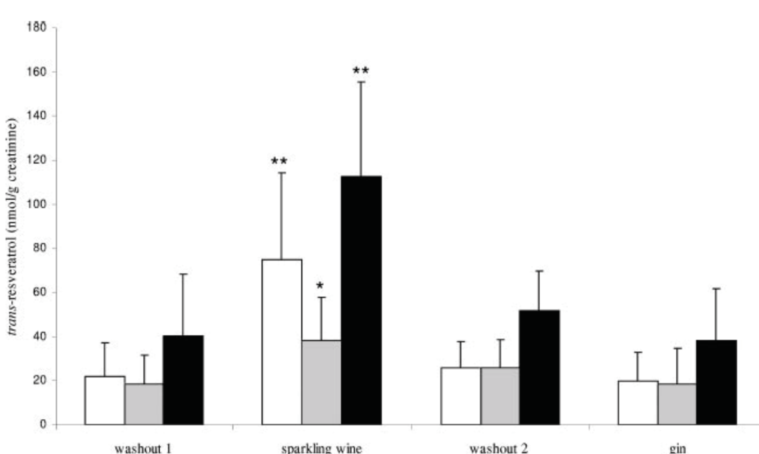

Fig. 1. Concentrations of urinary resveratrol glucuronides after washout period 1, sparkling wine intervention, washout period 2, and gin intervention.

Results are expressed as nmol of *trans*-resveratrol/g creatinine. Values are the means (SD; *error bars*) of duplicate measurement of samples from 10 volunteers. □, *trans*-resveratrol-3-glucuronide; ▨, *cis*-resveratrol-3-glucuronide; ■, total resveratrol glucuronides. Significant differences: *, P <0.05; **, P = 0.005 for difference from results obtained during the previous washout period (Wilcoxon test).

which allowed differentiation of the washout and gin periods from the wine periods (Fig. 3): area under the curve = 0.985 (95% CI, 0.928–0.999); sensitivity = 93% (88%–99%); specificity = 98% (94%–100%); likelihood ratio = 46.7 (35.8–57.6); PPV = 95.6% (91.1%–100%); NPV = 85.3% (77.5%–93.1%).

COHORT STUDY

Participants who reported wine consumption had significantly higher urinary concentrations of *trans*- and *cis*-resveratrol-3-*O*-glucuronide than those who did not consume wine (Fig. 4). The mean (SD) urinary TRM concentration was 282.7 (305.2) nmol/g for participants who reported moderate daily wine consumption, a value that differed significantly from that measured in those who did not consume wine [mean difference, 242.2 nmol/g (95% CI, 125.0–359.3 nmol/g); P = 0.001] and those who consumed wine intermittently [171.4 nmol/g (44.5–298.2 nmol/g); P = 0.01; Fig. 3]. Mean (SD) urinary TRM concentrations were 111.3 (69.1) nmol/g for participants who reported intermittent wine consumption, a value that differed from the concentration observed in abstainers [70.8 nmol/g (6.4–135.2 nmol/g); P = 0.035]. The reported daily wine consumption correlated directly with urinary concentrations of resveratrol glucuronides (r = 0.654; P <0.001).

The cutoff of 90 nmol/g enabled differentiation of moderate wine drinkers from those who did not drink wine with an area under the ROC curve (Fig. 5) of 0.863 (95% CI, 0.739–0.942), a sensitivity of 72% (60%–84%), a specificity of 94% (87%–100%), a PPV of 96.4% (91.3%–100%), an NPV of 59.1% (45.7%–72.5%), and a likelihood ratio of 11.6 (2.9–20.3). The percentage of false negatives was higher in those who consumed wine intermittently than in those who consumed it daily (43% and 24%, respectively); consequently, the sensitivity was higher in those who consumed moderate amounts of wine daily (76%) than in those who consumed wine intermittently (57%).

In all participants, no free resveratrol, piceid, or sulfoconjugates were detected in the urine, and resveratrol and its metabolites were below the limit of detection in the serum.

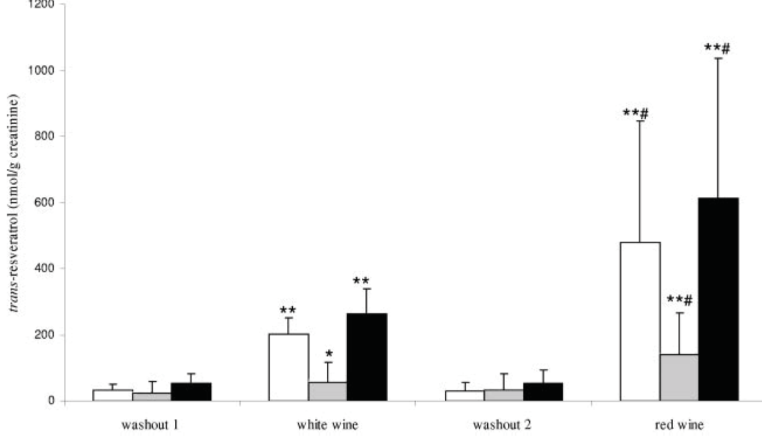

Fig. 2. Concentrations of urinary resveratrol glucuronides after washout period 1, white wine intervention, washout period 2, and red wine intervention.

Results are expressed as nmol of *trans*-resveratrol/g creatinine. Values are the means (SD; *error bars*) from duplicate measurements of samples from 10 volunteers. □, *trans*-resveratrol-3-glucuronide; ▨, *cis*-resveratrol-3-glucuronide; ■, total resveratrol glucuronides. Significant differences: *, P <0.05; **, P = 0.005 for differences from results obtained during the previous washout period (Wilcoxon test); #, P = 0.005 for difference from values after white wine intake (Wilcoxon test).

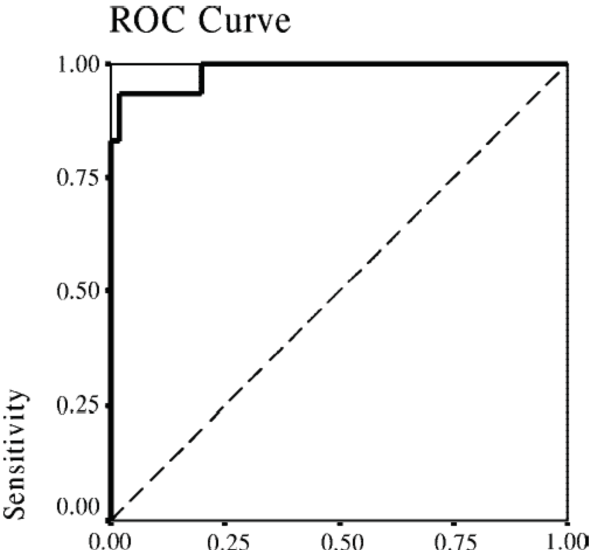

Fig. 3. ROC curve of urinary TRM concentrations for wine consumption periods vs washout and gin consumption periods in the clinical studies.

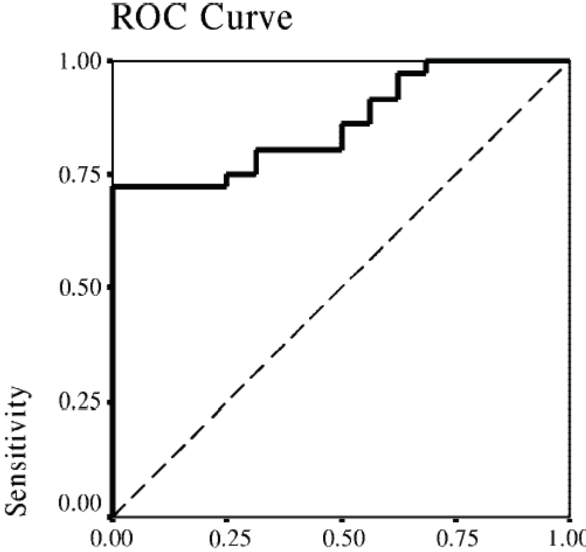

Fig. 5. ROC curve of urinary TRM for discrimination of wine consumers from non–wine consumers in the PREDIMED Study.

Discussion

An ideal biomarker should be specific, have an adequate half-life, and provide good correlation between the measured value and exposure *(7)*. The results of the current study indicate that resveratrol metabolites fulfill the criteria to be considered as a biomarker of wine intake.

The 2 intervention clinical trials, which included men and women, allowed assessment of a urinary concentration of resveratrol metabolites of 90 nmol/g as a cutoff to differentiate wine drinkers from non–wine drinkers: this cutoff had a sensitivity and specificity >90% and a PPV >95%. The usefulness of this biomarker was then tested in a cohort of 52 consecutively admitted participants in a large-scale feeding clinical trial, the PREDIMED Study. In real-life conditions, this biomarker had a sensitivity of 73%, a specificity of 93%, and a PPV of 96%. However, the NPV was 60% because of a high percentage of false negatives among intermittent drinkers. Thus, urinary concentrations of resveratrol metabolites are particularly useful as biomarkers of wine intake for moderate and regular drinkers, just as other phenolic compounds have been shown to be useful biomarkers for the intake of fruits, vegetables, tea, or coffee *(14, 23)*. We selected resveratrol as a marker of wine intake because it is a characteristic polyphenol of grape and wine products. Although a few other foods contain resveratrol *(24–27)*, the quantities in those foods are much lower than those observed in grape and wine products *(4, 21, 28)*.

Resveratrol metabolites were detected in morning urine after moderate and regular wine intake. Resveratrol metabolism has been investigated extensively in preclinical studies using animals *(11, 29, 30)*, but few studies have been performed in humans *(10, 12, 30–33)*. In the current trials, resveratrol intake ranged from 0.0040 mg/kg (0.35 mg of total resveratrol) for those who drank sparkling wine to 0.041 mg/kg (2.56 mg of total resveratrol) for those who consumed red wine; these values are

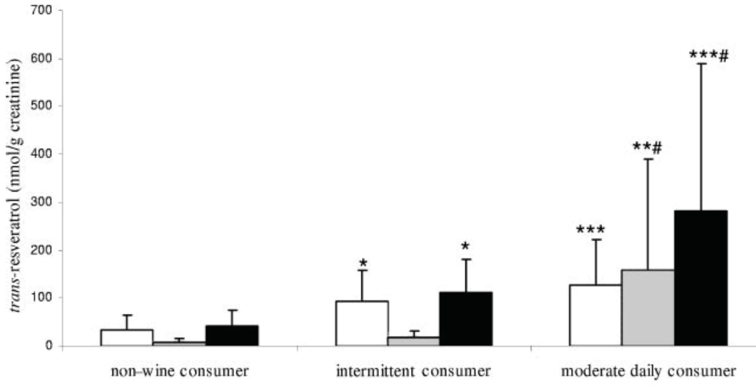

Fig. 4. Concentrations of urinary resveratrol glucuronides among non–wine consumers, intermittent consumers, and moderate daily consumers.

Results are expressed as nmol of *trans*-resveratrol/g creatinine. Values are the means (SD; *error bars*) from 52 participants. □, *trans*-resveratrol-3-glucuronide; ▨, *cis*-resveratrol-3-glucuronide; ■, sum of the resveratrol glucuronides. Significant differences: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for difference from results obtained for samples from non–wine consumers (unpaired *t*-test). #, $P < 0.01$ for difference from values for intermittent wine consumers (unpaired *t*-test).

similar those reported in the literature *(10, 30)*. These amounts cover the usual range of resveratrol intake from wine products *(34, 35)*. To achieve high amounts of resveratrol, supplements must be taken *(36)*. In the current trials, we were able to identify resveratrol metabolites in urine ~10 h after moderate intake of sparkling wine. Meng et al. *(30)* did not detect any resveratrol metabolites in the urine of volunteers after a comparable single dose of resveratrol, but the authors were able to quantify resveratrol metabolites in urine samples after a higher single dose (0.014 mg/kg). Because the accumulation of a metabolite increases after several days of ingestion *(6, 37)*, our results suggest that urine analysis may be useful for determining regular wine intake. The metabolically active compounds could enter the urine when their concentrations in plasma increase and exceed the relevant renal threshold *(38)*. Taking into account the bioactivity of wine phenols and comparing a single dose vs regular and moderate intake, Fisher and Hollenberg *(39)* observed an increased vascular response indicated by endothelial nitric oxide release over time. Furthermore, inclusion of resveratrol intake in a complex meal could increase or decrease the bioavailability of polyphenols *(40)*.

We also observed interindividual variability in our intervention studies (Figs. 1 and 2). Similar results have been described previously for resveratrol in LDL particles *(12)*, anthocyanins *(41)*, isoflavones *(42)*, and olive oil phenols such as tyrosol and hydroxytyrosol *(43)*. Despite this variability among individuals, however, we observed a highly significant correlation between wine intake and urinary concentrations of the metabolites. Higher concentrations of resveratrol metabolites were found after higher resveratrol intake (when red wine was consumed). Likewise, a lower concentration of resveratrol metabolites was found after lower resveratrol intake (sparkling or white wine). After the washout periods as well as after gin intervention, very low concentrations of resveratrol glucuronide were detected in urine, possibly from previous intake of food with very low resveratrol content or interference from compounds with a similar structure, such as estrogens. Similar results have been observed after washout periods in studies investigating quercetin *(44)* and other polyphenols *(45)*, as well as for hydroxytyrosol, a metabolite of dopamine, in an study of olive oil *(46)*. Mean (SD) TRM concentrations did not differ significantly in response to various periods of no wine intake in our studies [43.4 (23.5), 51.5 (36.1), and 40.5 (33.6) nmol/g for sparkling, white, and red wine, respectively] as part of the larger PREDIMED Study.

Urinary resveratrol glucuronide is the main metabolite of resveratrol in humans (~97%) *(30–32)* and rodents (>90%) *(29, 30)*, except in the study by Walle et al. (40%) *(33)*. In studies of low resveratrol intake, only the glucuronidate form was detected *(30)*. In the current studies, 2 monoglucuronides, *trans*- and *cis*-resveratrol-3-*O*-glucuronide, were identified after moderate wine consumption, findings similar to those of previous studies *(11, 47)*. Resveratrol sulfates were not detected in morning urine after wine intake. However, further investigation is needed in this regard. The sulfate form has been reported in human urine after administration of high resveratrol doses *(33)*. In other studies, the free form of resveratrol was not detected in human urine after moderate wine consumption but was found after high doses were consumed *(31, 32)*.

In the present study, resveratrol metabolites were also measured in serum. In these samples, however, no resveratrol metabolites were detected an average of 10 h after the consumption of wine. In previous studies, resveratrol has been detected in plasma after a minimum resveratrol intake of 0.357 mg/kg in a single dose *(31, 32)*. Furthermore, the half-life of resveratrol in human plasma is 2 h, with the highest concentrations being recorded at 30 min *(31, 32)*. Thus, plasma concentrations of resveratrol are not a useful marker for regular intake because plasma concentrations increase only after very recent intake.

In summary, we identified resveratrol metabolites in human urine after moderate wine intake, suggesting that these metabolites can be used as a biomarker of moderate wine intake in regular drinkers. This biomarker can also be used to exclude moderate wine drinking in abstainers but may be less effective in intermittent drinkers. Therefore, resveratrol metabolites may be used as a measure of compliance in interventional studies as well as an objective measure of wine consumption in epidemiologic studies.

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1.2 Contingut de flavanols i flavonols en productes de cacau: influència del processat d'elaboració

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Flavanol and Flavonol Contents of Cocoa Powder Products: Influence of the Manufacturing Process

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Major brands of cocoa powder products present in the Spanish market were analyzed for monomeric flavanols [(+)-catechin and (−)-epicatechin] and flavonols [quercetin-3-glucuronide, quercetin-3-glucoside (isoquercitrin), quercetin-3-arabinoside, and quercetin]. In addition, the influence of the manufacturing process of cocoa powder products, in particular, the alkalinization treatment (*Dutching*), on the original content of these flavonoids has been studied. (−)-Epicatechin was in the range of 116.02–730.26 μg/g, whereas (+)-catechin was in the range of 81.40–447.62 μg/g in the commercial cocoa products studied. Among flavonols, quercetin-3-arabinoside and isoquercitrin were the major flavonols in the cocoa powder products studied, ranging from 2.10 to 40.33 μg/g and from 3.97 to 42.74 μg/g, respectively, followed by quercetin-3-glucuronide (0.13–9.88 μg/g) and quercetin aglycone (0.28–3.25 μg/g). To our knowledge, these results are the first quantitative data in relation to the content of individualized flavonol derivatives in commercial cocoa powder products. The alkalinization treatment resulted in 60% loss of the mean total flavonoid content. Among flavanols, (−)-epicatechin presented a larger decline (67%, as a mean percentage difference) than (+)-catechin (38%), probably because of its epimerization into (−)-catechin, a less bioavailable form of catechin. A decline was also confirmed for di-, tri-, and tetrameric procyanidins. In the case of flavonols, quercetin presented the highest loss (86%), whereas quercetin-3-glucuronide, quercetin-3-arabinoside, and isoquercitrin showed a similar decrease (58, 62, and 61%, respectively). It is concluded that the large decrease found in the flavonoid content of natural cocoa powder, together with the observed change in the monomeric flavanol profile that results from the alkalinization treatment, could affect the antioxidant properties and the polyphenol biovailability of cocoa powder products.

KEYWORDS: Cocoa powder; catechin; epicatechin; flavonols; quercetin; alkalinization

INTRODUCTION

Cocoa (*Theobroma cacao*) and its derived products represent a very rich source of dietary flavonoids, and its consumption has increased 2.0 millions tons per year from 1960 to 2004 (*1*). Spain is the country that 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. Among the Spanish population, children between 7 and 14 years old are the largest consumers (Family Food Panel, Spain 2005–2006, Taylor Nelson Sofres). As recently reviewed by Ding et al. (*2*), outcomes from chocolate and cocoa human feeding trials are associated with a decrease in low-density lipoprotein (LDL) oxidation, oxidative stress, platelet activation, platelet function, and a increase in high-density lipoprotein (HDL) concentration, antioxidant status, and NO bioactivity, together with an improvement in endothelial function. A lower systolic and diastolic blood pressure and an improvement in insulin sensitivity are other potential health benefits reported from cocoa consumption (*2*).

According to Lee et al. (*3*), cocoa contains a higher content of flavonoids per serving than teas or red wine. Flavonoids present in cocoa include flavanols, anthocyanins, flavonols, and flavones (*4–9*). Flavanols, the most abundant flavonoids in cocoa, comprise the monomeric flavanols, (+)-catechin and (−)-epicatechin, and their oligomeric and polymeric forms (procyanidins). (−)-Epicatechin has been reported as the major monomeric flavanol in cocoa, representing ca. 35% of the total phenolic content (*4*). Cocoa is composed of a complex series of procyanidins consisting primarily of (−)-epicatechin (*10, 11*). Procyanidins with a degree of polymerization (DP) up to decamer have been identified and quantified by normal-phase high-performance liquid chromatography (HPLC)/mass spectrometry (MS) (*12, 13*). Oligomers (procyanidins B1, B2, B5,

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and C1) and polymers account for ≥90% of total polyphenols, and monomers account for 5–10% (*4, 13*). Anthocyanins identified in the cocoa bean include the −3-arabinoside and −3-galactoside derivatives of cyanidin and represent ca. 4% of the total polyphenol content of the cocoa bean; however, they could be hydrolyzed during the fermentation process of cocoa (*4, 14, 15*). The flavonol glycosides, quercetin-3-*O*-arabinoside and quercetin-3-*O*-glucoside (isoquercitrin), and quercetin aglycone have been identified in cocoa (*5, 16–18*). More recently, quercetin-3-*O*-galactoside (hyperoside) and quercetin-3-*O*-glucuronide and the flavones, apigenin, apigenin-8-*C*-glucoside (vitexin), apigenin-6-*C*-glucoside (isovitexin), luteolin, and luteolin-7-*O*-glucoside, have been identified for the first time in cocoa (*5, 18*). To our knowledge the concentration of flavonols and flavones in cocoa and its derived products has not been extensively reported (*6, 7*). Currently, the physiological effects derived from cocoa consumption have been ascribed only to flavanols (*2*). However, quercetin possesses higher free-radical scavenging properties than (+)-catechin (*19*) and has been shown to be one of the most effective flavonoids for the preservation of endogenous α-tocopherol in LDL cholesterol (*20*). In addition, quercetin and its metabolites isorhamnetin and tamarixetin produce vasodilation by means of endothelium-dependent and -independent mechanisms (*7*).

As occurs in other plant-derived foods, the phenolic content of cocoa-derived products is largely dependent upon the cultivar, origin, agricultural practices, and postharvest practices and processing (*4, 8*). Polyphenols in the cocoa beans are stored in the cotyledons. Once fermented and dried, the nib of the cocoa bean is roasted and ground, resulting in the cocoa liquor, which is the basis for chocolate manufacture. Cocoa powder is made by removing part of the cocoa butter from the cocoa liquor. Alkalinization (or *Dutching*) of the nibs, liquor, or powder can also be applied to change the color of the product, in particular, for the production of cocoa powder products (*21*). All of these steps, particularly fermentation and alkalinization, are assumed to lead to considerable losses of cocoa polyphenol, but scientific data in relation to this issue are still limited (*4, 22–24*).

This fact together with the necessity of more data related to the cocoa phenolic profile, in particular, of other antioxidant phenols, such as flavonols, have prompted us to determine the content of monomeric flavanols and novel flavonols in major brands of cocoa powder products present in the Spanish market and to study the influence of the manufacturing process of cocoa powder products, especially the alkalinization treatment, on the original phenolic content of cocoa.

MATERIALS AND METHODS

Standards. (+)-Catechin and quercetin were purchased from Sigma Chemical Co. (St. Louis, MO); (−)-epicatechin (>90% purity) was purchased from Fluka (Neu-Ulm, Switzerland); and quercetin-3-glucoside (isoquercitrin) and quercetin were purchased from Extrasynthése (Genay, France). Standard solutions were freshly prepared daily under dimmed light and stored in the refrigerator (4 °C) in amber glass bottles. Standards were filtered through Waters 4 mm PTFE 0.45 μm filter before injection on the column.

Samples. A total of 11 different cocoa powder products available in the Spanish market were analyzed. Different production batches of some of these products were also studied ($n = 6$ for cocoa 1, 2, and 3). To evaluate the effect of the manufacturing process of cocoa powder products on the phenolic composition of cocoa, 10 different batches of natural cocoa powder (10–12% fat) obtained from processed (fermented, dried, roasted, ground, and defatted) cocoa beans (Forastero variety of different origins) were studied. The different batches of natural cocoa powder were first submitted to alkalinization up to pH 7.2, and then both types of cocoa powders (natural and alkalinized) were manufactured into different cocoa powder products (drink mixes) by the addition of different ingredients, such as sugar or artificial sweeteners, salt, lecithin, calcium phosphate, wheat flour, fiber, and flavoring agents. One cocoa powder product derived from natural cocoa powder, cocoa product A (20–22% cocoa), and two different products made from alkalinized cocoa powder, cocoa product B (40–42% cocoa) and cocoa product C (16–18% cocoa), were analyzed.

Sample Preparation. Phenolic compounds from natural cocoa or cocoa powder products were extracted as described by Andrés-Lacueva et al. (*18*). Approximately 0.5 g of each sample was mixed with deionized water (5 mL at 100 °C) and methanol (20 mL). The extract was shaken in a vortex for 1 min at room temperature and centrifuged for 10 min at 1800*g*. It was then concentrated under vacuum below 40 °C to a final volume of 4 mL, avoiding UV light exposure. Finally, the samples were filtered through a Waters 4 mm PTFE 0.45 μm filter, before injection on the column.

LC−DAD Analysis of Flavonoids. A Hewlett-Packard series 1050 (Palo Alto, CA) liquid chromatography system equipped with a 1050 M diode array detector (DAD) and an automatic injector coupled to a Chemstation HP Rev. Asterix. 05.02 was used. Separation (100 μL) was performed on a reversed-phase Nucleosil 120 C_{18} (250 × 4 mm, 5 μm) column at 40 °C. A gradient consisting of solvent A [96.6:3.4 water/glacial acetic acid (v/v)] and solvent B [20:80 solvent A/acetonitrile (v/v)] was applied at a flow rate of 1.5 mL/min as described by Andrés-Lacueva et al. (*18*): 0–2% B linear from 0 to 5 min, 2–4% B linear from 5 to 10 min, 4% B isocratic from 10 to 12 min, 4–8% B linear from 12 to 14 min, 8% B isocratic from 14 to 18 min, 8–20% B from 18 to 22 min, 20–25% from 22 to 26 min, 25–35% from 26 to 30 min, 35–60% from 30 to 35 min, followed by washing (solvent B) and re-equilibration of the column. DAD detection was carried out at 280 and 365 nm. The identification of flavonoids was performed by LC−MS, as described below. Flavanols and flavonols were quantified at 280 and 365 nm, respectively, using external standard calibration curves. For the quantification of quercetin-3-glucuronide and quercetin-3-arabinoside, the calibration curves of quercetin and isoquercetrin were used, respectively.

LC−MS Identification of Flavonoids. Identification of flavonoids was performed by LC−MS as described by Andrés-Lacueva et al. (*18*). An Alliance 2690 module from Waters (Milford, MA) chromatography system equipped with an automatic injector and a VG Platform II quadrupole mass spectrometer (Micromass, Manchester, U.K.) with an atmospheric pressure chemical ionization (APCI) interface was used. Separation was performed on a reversed-phase Phenomenex Luna C_{18} analytical column (50 × 2.0 mm i.d., 5 μm) (Torrance, CA). The solvent gradient described above was applied at a flow rate of 0.4 mL/min. The working conditions for the APCI were as follows: drying gas (N_2) was heated to 150 °C and introduced into the capillary region at a flow rate of 200 L/h. The capillary was heated to 400 °C, and the corona voltage was held at −3.0 kV. The extraction voltage was −40 V. Full-scan data acquisition was performed scanning from *m/z* 140 to 900 in centroid mode and using a cycle time of 2.0 s and an interscan time of 0.2 s. Selected ion monitoring (SIM) was used with a dwell time of 300 ms for monitoring the following ions: (−)-epicatechin and (+)-catechin (*m/z* 289), procyanidins (*m/z* 577), trimers (*m/z* 865), and tetramers (*m/z* 1153).

RESULTS AND DISCUSSION

Flavanol and Flavonol Contents of Commercial Cocoa Powder Products. (+)-Catechin, (−)-epicatechin, quercetin-3-glucuronide, quercetin-3-glucoside (isoquercitrin), quercetin-3-arabinoside, and quercetin were quantified in the different cocoa powder products (**Figure** and **Table 1**). The identification of these flavonoids has been performed by LC−MS (*18*) and further confirmed by LC−MS/MS as described by Sánchez-Rabaneda et al. (*5*).

In general, flavanols were presented in a higher concentration than flavonols in all of the products studied (**Table 1**). As expected, (−)-epicatechin was the most abundant monomeric flavanol in cocoa powder products (*7, 22, 24*). Values ranged

Table 1. Concentration (μg/g) of Flavanols and Flavonols in Commercial Cocoa Powder Products[a]

Product #	(+)-Catechin	(−)-Epicatechin	Q-3-glucuronide	Isoquercitrin	Q-3-arabinoside	Quercetin
1						
Batch#1	157.61 (10.53)	322.00 (12.08)	0.91 (0.11)	9.74 (0.32)	13.00 (0.08)	0.57 (0.03)
Batch#2	174.96 (0.19)	335.28 (4.09)	1.27 (0.01)	10.92 (0.11)	9.72 (0.28)	1.58 (0.02)
Batch#3	166.45 (8.33)	344.30 (7.13)	1.48 (0.00)	11.07 (0.82)	12.26 (0.60)	2.03 (0.30)
Batch#4	237.71 (19.73)	413.58 (38.93)	2.06 (0.36)	14.88 (1.18)	15.95 (1.26)	2.87 (0.14)
Batch#5	183.45 (15.30)	331.03 (12.30)	2.38 (0.03)	13.57 (1.04)	13.22 (1.68)	2.97 (0.21)
Batch#6	184.59 (3.57)	427.40 (23.08)	1.30 (0.14)	13.44 (1.30)	14.83 (1.04)	2.46 (0.20)
Mean	184.13 (9.61)	362.26 (16.27)	1.57 (0.11)	12.27 (0.79)	13.16 (0.82)	2.08 (0.15)
2						
Batch#1	265.17 (11.53)	264.72 (26.14)	1.38 (0.01)	9.79 (0.26)	13.52 (0.95)	1.37 (0.04)
Batch#2	206.43 (16.12)	191.26 (17.42)	0.69 (0.08)	6.26 (0.67)	5.46 (0.07)	0.51 (0.03)
Batch#3	229.61 (21.21)	245.97 (25.63)	2.60 (0.16)	7.20 (0.34)	7.58 (1.06)	0.91 (0.06)
Batch#4	267.98 (1.16)	326.71 (23.86)	2.18 (0.18)	12.79 (1.17)	14.45 (1.31)	3.24 (0.44)
Batch#5	359.66 (7.79)	395.80 (19.37)	1.69 (0.10)	13.08 (0.74)	6.12 (0.43)	1.14 (0.18)
Batch#6	447.62 (22.16)	618.50 (52.94)	1.34 (0.11)	9.13 (1.15)	12.61 (0.88)	1.21 (0.16)
Mean	296.08 (13.33)	340.49 (27.56)	1.65 (0.11)	9.71 (0.72)	9.96 (0.78)	1.40 (0.15)
3						
Batch#1	124.75 (5.22)	168.45 (5.11)	1.27 (0.09)	5.42 (0.25)	5.96 (0.42)	0.28 (0.02)
Batch#2	81.40 (2.75)	116.02 (3.03)	0.13 (0.01)	3.97 (0.20)	4.36 (0.31)	0.62 (0.10)
Batch#3	150.57 (7.59)	166.48 (6.92)	0.29 (0.01)	5.22 (0.21)	6.11 (0.60)	0.56 (0.07)
Batch#4	178.56 (5.96)	280.85 (12.84)	0.55 (0.02)	6.58 (0.18)	7.91 (0.26)	0.70 (0.01)
Batch#5	171.35 (3.23)	250.57 (13.93)	1.03 (0.07)	7.72 (0.41)	9.28 (0.65)	0.68 (0.06)
Batch#6	115.91 (0.51)	212.59 (0.27)	0.86 (0.06)	6.53 (0.56)	7.43 (0.04)	0.62 (0.03)
Mean	137.09 (4.21)	199.16 (7.02)	0.69 (0.04)	5.91 (0.30)	6.84 (0.38)	0.58 (0.05)
4						
Batch#1	254.56 (15.99)	276.32 (28.70)	1.59 (0.04)	11.37 (0.64)	11.69 (1.69)	2.60 (0.24)
Batch#2	158.26 (4.17)	260.11 (23.88)	0.80 (0.11)	8.41 (0.72)	8.67 (0.26)	1.46 (0.15)
Batch#3	241.17 (16.35)	408.71 (29.75)	0.82 (0.06)	6.51 (0.41)	11.80 (0.34)	2.38 (0.17)
Batch#4	278.07 (14.15)	400.44 (31.88)	0.97 (0.07)	8.86 (1.58)	10.46 (0.73)	1.87 (0.10)
Batch#5	192.14 (5.95)	360.59 (24.93)	1.71 (0.20)	11.98 (1.06)	13.77 (0.07)	1.96 (0.23)
Batch#6	173.36 (18.45)	306.13 (2.48)	0.60 (0.05)	9.63 (0.58)	10.40 (0.50)	1.64 (0.14)
Mean	216.26 (12.51)	335.38 (23.60)	1.08 (0.09)	9.46 (0.83)	11.13 (0.60)	1.98 (0.17)
5	229.38 (24.87)	365.80 (29.41)	2.27 (0.18)	15.06 (1.02)	16.34 (0.22)	2.96 (0.50)
6	120.75 (12.21)	313.99 (30.31)	8.89 (0.62)	27.33 (0.86)	25.68 (1.80)	1.04 (0.10)
7	295.79 (18.54)	437.99 (29.14)	9.88 (0.69)	13.39 (1.23)	18.81 (1.32)	1.04 (0.07)
8	226.78 (23.74)	730.26 (60.72)	6.66 (1.17)	24.90 (2.31)	40.33 (1.36)	1.49 (0.10)
9	281.46 (19.70)	319.06 (32.31)	3.92 (0.01)	26.61 (2.88)	20.90 (1.46)	0.64 (0.05)
10	297.53 (23.41)	529.49 (34.67)	5.94 (0.47)	42.74 (3.12)	31.51 (2.21)	1.12 (0.09)
11	137.99 (10.99)	44.84 (4.30)	2.15 (0.37)	8.95 (0.15)	2.10 (0.15)	1.39 (0.08)

[a] Mean (n = 2); standard deviation (SD).

from 116.02 to 730.26 μg/g for (−)-epicatechin (mean = 327.91 μg/g), and from 81.40 to 447.62 μg/g for (+)-catechin (mean = 212.61 μg/g) (**Table 1**). The total monomeric content ranged from 182.84 to 1066.13 μg/g (mean = 540.52 μg/g). A range of 180–320 μg/g (mean = 262.0 μg/g, n = 15) has been reported for the (−)-epicatechin content of cocoa powder products (*25*). In another study, (−)-epicatechin ranged from 1580 to 2580 μg/g and (+)-catechin ranged from 610 to 900 μg/g for natural cocoa powders (n = 3) (*24*). However, lower values were found for alkalinized cocoa powders [180–380 μg/g for (−)-epicatechin and 230–350 μg/g for (+)-catechin (n = 2)] (*24*). Recently, Tomas-Barberan et al. (*26*) have reported (+)-catechin values of 6460 and 2020 μg/g and (−)-epicatechin values of 25650 and 3300 μg/g for a polyphenol-rich (n = 1) and a conventional (n = 3) cocoa powder, respectively.

Quercetin-3-arabinoside and isoquercitrin were the major flavonols in the cocoa powder products studied, and both were presented in very similar content [2.10–40.33 μg/g for quercetin-3-arabinoside and 3.97–42.74 μg/g for isoquercetrin] (**Table 1**). Quercetin-3-glucuronide ranged from 0.13 to 9.88 μg/g, whereas quercetin aglycone was found in very little amount (0.20–3.25 μg/g) (**Table 1**). The content of total flavonols quantified ranged from 9.08 to 81.31 μg/g. In general, products 6–10 were characterized by a higher concentration of quercetin derivatives than the remaining ones (products 1, 2, 3, 4, 5, and 11) (**Table 1**). To our knowledge, these results are the first quantitative data in relation to the content of individualized flavonol derivatives in commercial cocoa powder products. If the different quercetin derivatives were expressed in terms of quercetin equivalents in milligrams per 100 g, which is the form currently used in nutrient databases, the cocoa powder products analyzed herein would provide a content ranging from 0.42 to 6.54 mg/100 g of quercetin equivalents. On a weight basis, cocoa powder products would provide an amount of quercetin close to that of broccoli (frozen, chopped, and unprepared) (0.91–3.52 mg/100 g, n = 3), apples (Golden Delicious, with peel, raw, *Malus domestica*) (1.57–4.40 mg/100 g, n = 10), or grapes (red, raw, *Vitis vinifera*) (0.00–3.98 mg/100 g, n = 6) (*27*). However, the amount of quercetin per serving provided by cocoa powder products (0.083–1.3 mg for a 20 g serving) is much lower than that of the aforementioned food sources [broccoli (1.82–7.04 mg for a 200 g serving), apples (with skin) (3.14–8.80 mg in a 200 g serving), and red grapes (0.00–7.96 mg in a 200 g serving)].

Finally, the variability in the total monomeric content between the different commercial products was equivalent to 37% (expressed as the coefficient of variation, %) (**Table 1**). In the case of total flavonols, the intervariability was equal to 60%. This high variability in the flavonoid content between products could be attributed to many factors, such as cultivar type, geographical origin, environmental factors, agricultural and postharvesting practices, and processing (*4*). Neimenak et al. (*8*) concluded that the content of (−)-epicatechin, (+)-catechin, cyanidin-3-galactoside, and cyanidin-3-arabinoside in fresh and

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Table 2. Concentration (μg/g) of Flavanols and Flavonols in Natural and Alkalinized Cocoa, and their Derived Cocoa Powder Products (A, B and C)[a]

	batch number										
	1	2	3	4	5	6	7	8	9	10	mean
(+)-catechin											
natural cocoa powder	613.63 (63.80)	668.90 (46.82)	557.40 (27.70)	454.60 (5.10)	729.90 (0.30)	702.34 (6.20)	711.00 (0.10)	658.45 (7.30)	534.70 (0.30)	505.72 (42.90)	613.66 (20.05)
cocoa product A	177.20 (13.20)	155.30 (10.70)	175.10 (14.90)	171.20 (18.50)	155.00 (8.70)	153.20 (15.60)	133.85 (5.70)	136.74 (10.30)	151.30 (1.80)	126.06 (14.20)	153.49 (11.36)
alkalinized cocoa powder	319.88 (5.10)	381.26 (51.50)	407.14 (37.70)	412.40 (45.70)	406.24 (4.30)	337.56 (36.90)	389.64 (57.60)	378.46 (39.80)	392.10 (18.10)	400.70 (2.10)	382.54 (29.88)
cocoa product B	257.70 (26.50)	274.70 (5.90)	222.99 (13.20)	251.24 (12.10)	246.07 (9.10)	253.30 (7.80)	191.00 (8.90)	349.50 (10.00)	269.50 (11.00)	315.23 (26.20)	263.12 (13.06)
cocoa product C	80.04 (0.70)	138.05 (10.20)	129.55 (2.30)	112.00 (0.20)	188.30 (1.90)	122.50 (0.10)	158.00 (8.40)	113.23 (1.30)	129.30 (2.20)	173.47 (17.50)	134.44 (4.48)
(+)-epicatechin											
natural cocoa powder	2102.00 (58.00)	2070.00 (144.90)	1814.00 (201.00)	1531.00 (8.00)	2159.00 (5.00)	2198.00 (73.00)	1749.29 (263.00)	1530.00 (37.00)	2021.00 (61.00)	1911.68 (103.00)	1908.60 (95.39)
cocoa product A	391.20 (50.20)	458.80 (2.50)	601.40 (45.50)	356.80 (2.30)	270.90 (14.60)	492.20 (5.40)	304.70 (5.90)	310.14 (21.20)	343.86 (4.60)	294.09 (30.50)	382.41 (18.27)
alkalinized cocoa powder	490.43 (0.90)	693.50 (0.50)	669.56 (90.80)	720.13 (74.00)	684.03 (4.30)	500.38 (21.40)	680.00 (47.60)	672.20 (0.40)	561.90 (27.60)	560.00 (1.30)	623.21 (26.88)
cocoa product B	298.80 (18.00)	256.80 (3.80)	220.04 (7.50)	243.86 (7.20)	261.98 (3.60)	226.65 (3.60)	238.30 (13.20)	116.30 (2.90)	269.20 (15.10)	260.91 (7.90)	239.28 (8.28)
cocoa product C	61.77 (2.31)	93.16 (4.54)	101.94 (2.80)	79.49 (0.05)	36.91 (0.03)	76.84 (6.81)	82.75 (0.07)	76.89 (1.76)	73.42 (2.20)	134.43 (2.10)	81.76 (2.27)
q-3-glucuronide											
natural cocoa powder	14.01 (0.86)	14.34 (1.88)	13.04 (0.73)	11.76 (0.70)	13.68 (0.69)	15.12 (1.30)	9.38 (1.31)	13.08 (1.08)	12.12 (0.48)	10.68 (0.65)	12.72 (0.97)
cocoa product A	1.06 (0.03)	2.03 (0.10)	1.69 (0.20)	3.08 (0.31)	1.54 (0.01)	2.13 (0.12)	1.73 (0.12)	1.69 (0.07)	1.91 (0.08)	1.70 (0.20)	1.85 (0.13)
alkalinized cocoa powder	3.76 (0.47)	5.02 (0.05)	5.46 (0.63)	5.85 (0.91)	6.58 (0.21)	4.41 (0.61)	6.01 (0.78)	5.66 (0.02)	5.42 (0.59)	5.37 (0.11)	5.35 (0.43)
cocoa product B	2.61 (0.16)	2.95 (0.03)	2.02 (0.08)	2.42 (0.32)	2.84 (0.08)	2.59 (0.09)	2.02 (0.16)	1.04 (0.02)	2.08 (0.39)	3.40 (0.37)	2.40 (0.17)
cocoa product C	0.08 (0.00)	0.41 (0.08)	0.38 (0.04)	0.29 (0.00)	0.28 (0.02)	0.22 (0.01)	0.07 (0.00)	0.13 (0.01)	0.02 (0.00)	0.22 (0.01)	0.21 (0.02)
Isoquercitrin											
natural cocoa powder	45.19 (2.96)	46.64 (7.23)	43.73 (1.30)	38.12 (0.96)	42.24 (2.28)	45.68 (2.79)	33.34 (4.73)	41.33 (1.57)	33.42 (4.77)	33.70 (0.47)	40.34 (2.91)
cocoa product A	15.74 (0.06)	9.41 (0.22)	10.10 (0.62)	14.32 (0.50)	7.64 (0.31)	7.51 (0.02)	9.97 (0.04)	6.38 (0.94)	7.19 (0.63)	6.58 (0.55)	9.48 (0.39)
alkalinized cocoa powder	11.15 (0.37)	12.98 (0.91)	15.00 (1.78)	13.24 (1.84)	17.71 (0.69)	13.63 (1.97)	17.34 (2.12)	15.36 (0.26)	19.58 (1.05)	19.74 (0.12)	15.57 (1.11)
cocoa product B	8.75 (0.52)	10.46 (0.63)	8.08 (0.05)	9.36 (0.24)	8.84 (0.21)	7.88 (0.17)	7.84 (0.18)	7.90 (0.43)	12.41 (0.85)	15.54 (0.12)	9.71 (0.34)
cocoa product C	1.98 (0.08)	3.35 (0.08)	3.19 (0.10)	2.35 (0.02)	2.63 (0.18)	2.39 (0.05)	2.29 (0.05)	2.52 (0.05)	2.09 (0.05)	2.74 (0.29)	2.55 (0.10)
q-3-arabinoside											
natural cocoa powder	80.63 (4.81)	83.70 (12.30)	84.22 (0.21)	69.10 (5.80)	70.78 (5.57)	92.12 (0.29)	52.58 (0.12)	75.51 (0.10)	53.62 (1.28)	68.07 (3.44)	73.03 (3.39)
cocoa product A	21.26 (0.57)	16.30 (1.15)	17.51 (1.28)	13.97 (0.16)	19.78 (2.39)	13.03 (0.98)	14.05 (0.76)	12.06 (1.66)	13.45 (0.62)	12.40 (1.70)	15.38 (1.13)
alkalinized cocoa powder	23.10 (0.21)	27.70 (0.16)	27.88 (0.21)	33.30 (0.39)	32.30 (1.17)	24.48 (3.65)	30.40 (3.24)	25.27 (0.08)	33.80 (2.59)	22.02 (0.74)	28.03 (1.24)
cocoa product B	13.30 (0.97)	18.34 (0.42)	13.12 (0.73)	16.95 (0.16)	15.39 (0.48)	13.65 (0.39)	13.01 (1.19)	13.53 (1.82)	17.34 (1.14)	22.30 (0.80)	15.69 (0.81)
cocoa product C	3.52 (0.03)	5.64 (0.03)	5.78 (0.22)	4.60 (0.02)	4.33 (0.25)	4.67 (0.30)	4.36 (0.04)	4.78 (0.02)	3.43 (0.06)	3.93 (0.16)	4.50 (0.11)
quercetin											
natural cocoa powder	4.75 (0.21)	5.32 (0.78)	5.26 (0.29)	5.4 (0.51)	5.99 (0.47)	5.26 (0.51)	3.69 (0.36)	4.77 (0.04)	3.20 (0.26)	4.10 (0.33)	4.77 (0.38)
cocoa product A	0.67 (0.00)	0.94 (0.05)	1.02 (0.07)	0.75 (0.06)	[b]	[b]	0.41 (0.03)	0.47 (0.07)	0.52 (0.06)	0.41 (0.02)	0.65 (0.04)
alkalinized cocoa powder	0.49 (0.02)	0.59 (0.00)	0.66 (0.00)	0.60 (0.07)	1.45 (0.01)	0.45 (0.00)	0.22 (0.00)	0.31 (0.00)	1.15 (0.03)	0.80 (0.02)	0.67 (0.02)
cocoa product B	0.16 (0.00)	0.25 (0.02)	0.13 (0.00)	0.24 (0.04)	0.23 (0.01)	0.20 (0.03)	0.13 (0.02)	0.09 (0.00)	[b]	0.59 (0.05)	0.22 (0.02)
cocoa product C	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]	[b]
TOTAL											
natural cocoa powder	2860.20 (130.64)	2888.90 (213.92)	2517.65 (231.23)	2109.99 (21.07)	3021.58 (14.31)	3058.52 (84.09)	2559.27 (269.62)	2323.14 (47.09)	2658.05 (68.09)	2533.94 (150.79)	2653.13 (123.08)
cocoa product A	607.13 (64.06)	642.78 (14.72)	806.82 (62.57)	560.12 (21.82)	454.86 (26.01)	668.08 (22.13)	464.70 (12.55)	467.48 (34.25)	518.22 (7.79)	441.24 (47.17)	563.27 (31.32)
alkalinized cocoa powder	848.81 (7.08)	1121.05 (53.12)	1125.69 (131.12)	1185.51 (122.91)	1148.32 (10.68)	880.91 (64.53)	1123.62 (111.35)	1097.26 (40.56)	1013.95 (49.95)	1008.63 (4.38)	1055.37 (59.57)
cocoa product B	581.32 (46.15)	563.50 (10.80)	466.37 (21.56)	524.08 (20.06)	535.36 (13.49)	504.28 (12.08)	452.30 (23.54)	488.36 (15.17)	570.53 (28.48)	617.97 (35.44)	530.41 (22.68)
cocoa product C	147.40 (3.12)	240.60 (14.92)	240.83 (5.46)	198.72 (0.30)	232.45 (2.39)	206.62 (7.27)	247.48 (8.57)	197.56 (3.14)	208.27 (4.50)	314.79 (20.06)	223.47 (6.97)

[a] Mean ($n = 2$); (SD); [b] under limit of quantification.

a)

(+)-Catechin (-)-Epicatechin

b)

	R_1	R_2	R_3
Quercetin	OH	H	H
Quercetin-3-glucoside (isoquercitrin)	OH	H	glc
Quercetin-3-arabinoside	OH	H	arb
Quercetin-3-glucuronide	OH	H	gluc

glc= glucosa; arb= arabinoside; gluc= glucuronide acid

Figure 1. Chemical structure of the flavonoid compounds analyzed: (**a**) monomeric flavanols and (**b**) flavonols.

fermented-like beans was genotype-dependent. A large variation in the (−)-epicatechin content of cocoa has also been reported to occur after fermentation (*22, 28*).

Changes in Cocoa Flavanol and Flavonol Contents during the Manufacturing Process of Cocoa Powder Products. After fermentation and roasting at the site of cultivation, the cocoa nib was ground, resulting in the cocoa liquor. Part of the cocoa butter was then removed from the cocoa liquor, resulting in a cocoa powder containing 10–12% fat (natural cocoa powder). A fraction of this natural cocoa powder (10 different batches) was submitted to alkalinization and both natural and alkalinized cocoa powders were finally manufactured into different cocoa powder products as described under the Materials and Methods. Although originally performed to make the powder not to agglomerate or sink to the bottom, when it was added to milk or water-based drinks, the alkalinization process (or *Dutching*) is nowadays mainly applied to modify the flavor and color of cocoa powders.

The flavanol and flavonol contents in natural and alkalinized cocoa powders and in their derived cocoa powder products are summarized in **Table 2**. Natural cocoa presented a mean content of total flavonoids equivalent to 2653.13 μg/g (−)-epicatechin, representing the highest proportion. Batches of natural cocoa powder presented a coefficient of variation (CV) between 13% (for (−)-epicatechin) and 18% (for quercetin). A similar level of variability was registered between the different batches of alkalinized cocoa powder, with the exception of quercetin, which presented a 59% CV (**Table 2**).

A decrease in the concentration of all flavonoids studied was registered as a consequence of the alkalinization treatment, resulting in a 60% loss of the mean total flavonoid content (**Table 2**). Among flavanols, (−)-epicatechin presented a larger decline (67%, as a mean percentage difference) than (+)-catechin (38%), resulting in a change of the original monomeric flavanol profile. Because (−)-epicatechin possesses a higher absorption than (+)-catechin, the alkalinization treatment could affect the bioavailability of flavanols from cocoa products (*29*). A possible epimerization of (−)-epicatechin into (−)-catechin, which could explain the higher decline of the former after alkalinization, has been suggested to occur during chocolate manufacturing (*9*), although it has not been attributed to any concrete step of the production process. These authors also found that (−)-catechin, not naturally present in cocoa, was more abundant than (+)-catechin in 68 commercially available chocolate samples. This is another fact that could further affect the bioavailability of flavanols from cocoa products, because recently, it has been reported that (+)-catechin is more bioavailable than (−)-catechin (*30*).

To evaluate the effect of the alkalinization treatment on cocoa procyanidins, selected ion monitoring (SIM) experiments in negative mode $[M - H]^-$ were performed at *m/z* 577 for dimers, *m/z* 865 for trimers, and *m/z* 1153 for tetramers (**Figure 2**). According to literature data (*9*), the most abundant mass peak at each *m/z* corresponded to dimer B2 (epicatechin-4β-8-epicatechin) at Rt = 2.30 min, trimer C1 (epicatechin-4β-8-epicatechin-4β-8-epicatechin) at Rt = 3.50 min, and tetramer D (epicatechin-4β-8-epicatechin-4β-8-epicatechin-4β-8-epicatechin) at Rt = 3.80 min. SIM was also performed at *m/z* 289 to confirm the results obtained above for monomeric flavanols, (+)-catechin (Rt = 1.80 min) and (−)-epicatechin (Rt = 2.85 min). The percentage differences between natural and alkalinized cocoa powders for each peak were calculated using the SIM mass chromatogram areas. Losses registered as a consequence of the alkalinization process were 36% (as a mean) for (+)-catechin, 67% for (−)-epicatechin, 69% for dimer B2, 67% for trimer C1, and 31% for tetramer D. Using reverse- and normal-phase HPLC, Gu et al. (*24*) have also reported lower level of flavanols (catechins and procyanidins) in alkalinized cocoa powders in comparison to natural powders. However, currently, no concrete figures have been found in the literature concerning the exact influence of the alkalinization treatment on the cocoa flavanol composition.

In the case of flavonols, quercetin presented the highest loss (86%) after alkalinization, whereas quercetin-3-glucuronide, quercetin-3-arabinoside, and isoquercitrin showed a similar decrease (58, 62, and 61%, respectively) (**Table 2**). In general, changes occurring as the result the alkalinization treatment could be attributed to the oxidation of phenolic compounds under basic pH conditions, leading to brown pigments that are polymerized to different degrees. In particular, secondary reactions involving *o*-quinones previously formed during the fermentation stage by polyphenol oxidase (PPO) (*31, 32*) are probably involved in further reactions responsible for the browning developed during alkalinization. According to Bonheví and Coll (*25*), the chem-

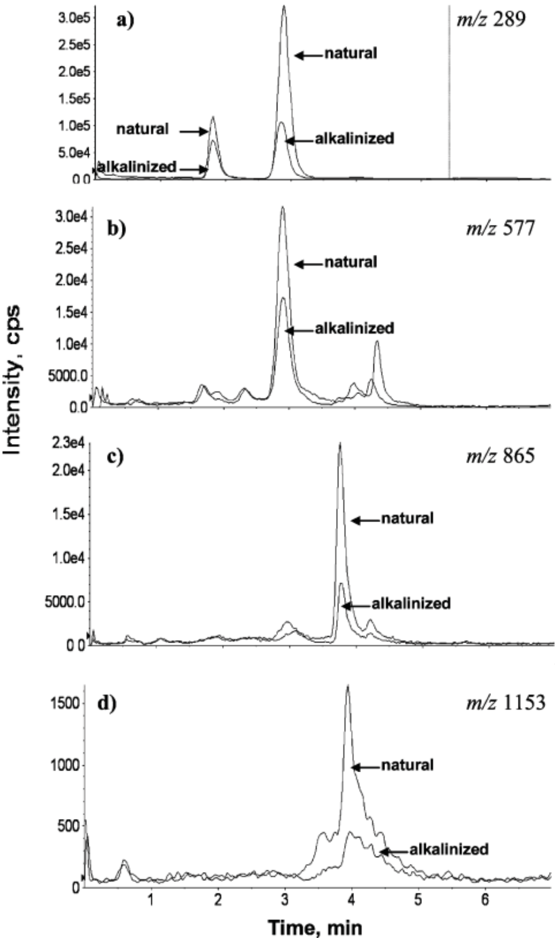

Figure 2. SIM chromatograms of natural and alkalinized cocoa powders at (**a**) *m/z* 289 (monomers), (**b**) *m/z* 577 (dimers), (**c**) *m/z* 865 (trimers), and (**d**) *m/z* 1153 (tetramers).

istry of the alkalinization process determines the biochemical characterization and browning behavior of cocoa beans.

Considering the cocoa powder products (products A, B, and C), as expected, the flavonoid concentration varied according to the formulation of each product (**Table 2**). Products A and B presented very similar mean content of total flavanoids (563.27 and 530.41 μg/g, respectively). However, in the case of product A, this figure only represented ca. 20% of that of the original raw material (i.e., natural cocoa powder), whereas in the case of alkalinized product B, it represented ca. 50% of the original alkalinized cocoa powder. This means that, because of its lower phenolic content, a higher amount of alkalinized cocoa powder in product B was needed to provide the same flavonoid content as in product A. Nevertheless, the percentage distribution of some flavonoids differed between both products. For example, the (+)-catechin/(−)-epicatechin/quercetin percentage distribution was 27:68:0.1% in product A, whereas in product B, it was equal to 50:45:0.05%. Alkalinized product C presented the lowest content of all flavanols and flavonols quantified (mean total flavonoids: 223.47 μg/g; 20% of the alkalinized raw material), with the quercetin levels being under the limit of quantification (*2*) (**Table 2**). However, this later product is a special formulation in which the nutritional quality of the product is balanced by the addition of cocoa fiber.

Finally, the variability in the total content of flavonoids between the different production batches of each cocoa product was relatively low, 21, 19, and 16% CV, for cocoa powder products A, B, and C, respectively, indicating a well-standardized manufacturing process (**Table 2**). Isoquercetrin and quercetin were the compounds that presented the highest variation in products A and B, whereas for product C, this was found for (−)-epicatechin and quercetin-3-glucuronide.

CONCLUSIONS

This paper provides for the first time quantitative data of individualized flavonol derivatives in cocoa powder products in a wide range of commercial products available in the Spanish market. Together with the monomeric flavanol content also given, this data is very useful for the calculation of daily flavonoid intake and its correlation with disease incidence or early markers in epidemiologic and clinical studies. The results found herein in relation to the alkalinization process indicate that the dramatically decrease found in the flavonoid content of natural cocoa powder together with the observed change in the monomeric flavanol profile negatively affect the content of antioxidant polyphenols of cocoa and probably their bioavailability. Considering that cocoa powder products have a lower level of saturated fats than chocolate bars, it seems necessary to establish a compromise between color and phenolic content, especially for cocoa powder products derived from alkalinized cocoa powder, which is a more expensive raw material but markedly reduced in polyphenol content.

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1.3 Els àcids fenòlics dihidroxilats que provenen del metabolisme microbià de polifenols inhibeixen la síntesi de citoquines a partir de cèl·lules mononuclears humanes

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Short Communication

Dihydroxylated phenolic acids derived from microbial metabolism reduce lipopolysaccharide-stimulated cytokine secretion by human peripheral blood mononuclear cells

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Oligomers and polymers of flavan-3-ols (proanthocyanidins) are very abundant in the Mediterranean diet, but are poorly absorbed. However, when these polyphenols reach the colon, they are metabolised by the intestinal microbiota into various phenolic acids, including phenylpropionic, phenylacetic and benzoic acid derivatives. Since the biological properties of these metabolites are not completely known, in the present study, we investigated the effect of the following microbial phenolic metabolites: 3,4-dihydroxyphenylpropionic acid (3,4-DHPPA), 3-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylacetic acid (3,4-DHPAA), 3-hydroxyphenylacetic acid, 4-hydroxybenzoic acid and 4-hydroxyhippuric acid (4-HHA), on modulation of the production of the main pro-inflammatory cytokines (TNF-α, IL-1β and IL-6). The production of these cytokines by lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells (PBMC) pre-treated with the phenolic metabolites was studied in six healthy volunteers. With the exception of 4-HHA for TNF-α secretion, only the dihydroxylated compounds, 3,4-DHPPA and 3,4-DHPAA, significantly inhibited the secretion of these pro-inflammatory cytokines in LPS-stimulated PBMC. Mean inhibition of the secretion of TNF-α by 3,4-DHPPA and 3,4-DHPAA was 84·9 and 86·4 %, respectively. The concentrations of IL-6 in the culture supernatant were reduced by 88·8 and 92·3 % with 3,4-DHPPA and 3,4-DHPAA pre-treatment, respectively. Finally, inhibition was slightly higher for IL-1β, 93·1 % by 3,4-DHPPA and 97·9 % by 3,4-DHPAA. These results indicate that dihydroxylated phenolic acids derived from microbial metabolism present marked anti-inflammatory properties, providing additional information about the health benefits of dietary polyphenols and their potential value as therapeutic agents.

TNF-α: IL-1β: IL-6: Atherosclerosis: Phenolic acids: Microbial metabolism

Polyphenols are among the most abundant antioxidant compounds of the Mediterranean diet and may play a key role in the prevention of cardiovascular and neurodegenerative diseases, and cancer(1). Health effects derived from polyphenol consumption depend on their bioavailability, a factor that greatly varies from one compound to another(2). Among polyphenols, the oligomers and polymers of flavan-3-ols, also called proanthocyanidins, are not absorbed or degraded into monomers during their transit through the stomach(2,3). However, when they reach the colon, they are metabolised by the intestinal microbiota into various phenolic acids, including phenylpropionic, phenylacetic and benzoic acid derivatives(4). Hydroxycinnamic acid esters and polyphenols linked to rhamnose are also degraded into phenolic acids by the microbiota(2). Recently, it has been reported that these metabolites may also exert several biological activities, such

Abbreviations: 3,4-DHPAA, 3,4-dihydroxyphenylacetic acid; 3,4-DHPPA, 3,4-dihydroxyphenylpropionic acid; 3-HPAA, 3-hydroxyphenylacetic acid; 3-HPPA, 3-hydroxyphenylpropionic acid; 4-HBA, 4-hydroxybenzoic acid; 4-HHA, 4-hydroxyhippuric acid; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cells.

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as the inhibition of platelet aggregation and activation function[5], inhibition of cyclo-oxygenase-2 in HT-29 colon cancer cells[6], reduction in the synthesis of prostanoids in colon cells[7], antiproliferative activity in prostate and cancer cells[8] and, finally, influence cell proliferation, apoptosis and signalling pathways in human colon carcinoma cells[9].

Atherosclerosis is now considered to be a low-grade chronic inflammatory process resulting from the interactions between plasma lipoproteins, cellular components (monocyte/macrophages, T lymphocytes, endothelial cells and smooth muscle cells) and the extracellular matrix of arterial wall[10]. Pro-inflammatory cytokines are involved in all phases of the atherosclerotic process: they stimulate chemokines and adhesion molecules, leading to early recruitment of monocytes and lymphocytes in the arterial intima, and later exert potential noxious effects promoting weakening of plaques that are more prone to rupture[10]. However, data concerning the effect of phenolic compounds on the production of the inflammatory mediators from mononuclear cells are scarce and contradictory. Some researchers[11] reported that polyphenols from cocoa may reduce the expression of IL-2 mRNA in human lymphocytes, and others[12] found differential effects of isolated cocoa procyanidin fractions (monomer to decamers) on the expression and secretion of IL-1β from peripheral blood mononuclear cells (PBMC). In the same way, cocoa polyphenols produced an increase in TNF-α secretion[13] or even a down-regulation of IL-2 secretion and IL-2 receptor surface expression on a lymphoid cell line[14], whereas in another study, polyphenols from olive oil had no effect on the secretion of TNF-α, IL-1β or IL-6 in the human whole blood[15].

Considering the lack of information regarding the anti-inflammatory properties of microbial-derived phenolic acids, the aim of the present study was to investigate the effect of some microbial phenolic metabolites on the modulation of the production of the most representative pro-inflammatory cytokines, i.e. TNF-α, IL-1β and IL-6, in lipopolysaccharide (LPS)-stimulated PBMC from healthy human volunteers.

Methods

Six healthy volunteers (two men and four women) with an average age of 29·3 (SD 2·1) years (range 27–33 years), weight of 62·8 (SD 18·5) kg (range 50–100 kg), height of 1·7 (SD 0·1) m (range 1·6–1·9 m) and BMI of 21·5 (SD 3·4) (range 17·9–27·7) participated in the study. None of them reported a history of heart disease, homeostatic disorder or any other medical disease. None were receiving any medication or taking any vitamin supplements. A 24 h food recall questionnaire was used to assess their habitual nutrient intake. This information was converted into dietary data using the Professional Diet Balancer software (Cardinal Health Systems, Inc., Edina, MN, USA) Their habitual diet (mean of six volunteers) included an intake of Q3 2339·82 calories/d: 117·41 g/d protein; 257·35 g/d carbohydrates; 24·99 g/d dietary fibre; 5·27 g/d soluble fibre; 76·43 g/d total sugar (14·93 g/d monosaccharides, 31·27 g/d disaccharides); 92·48 g/d of total fat (26·03 g/d saturated fat, 43·30 g/d monounsaturated fat, 15·93 g/d polyunsaturated fat, 1·22 g/d *trans*-fatty acids, 221·87 mg/d cholesterol); 863·77 RE/d vitamin A; 2·33 mg/d vitamin B1; 2·39 mg/d vitamin B2; 40·79 mg/d vitamin B3; 2·49 mg/d vitamin B6; 7·77 μg/d vitamin B12; 84·91 mg/d vitamin C; 4·89 μg/d vitamin D; 13·08 mg/d vitamin E; 144·47 mg/d estimated polyphenol intake; 181·65 mg/d phytoesterols.

Peripheral blood from the volunteers was collected and PBMC were isolated by density gradient centrifugation over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden)[16]. Harvested cells were washed with PBS 10 × buffer (Roche Diagnostics GmbH, Mannheim, Germany) and then counted in a haemocytometer chamber. Cell viability was estimated with trypan blue. PBMC were resuspended in RPMI-1640 (Biowhittaker, Verviers, Belgium) containing fetal bovine serum (10 %) and gentamicin (0·05 mg/ml) (RPMI-10 % fetal) up to a concentration of 1×10^6 viable cells/ml.

For each of the following phenolic acids, a 3 μM solution was prepared in RPMI-10 % fetal: 3,4-dihydroxyphenylpropionic acid (3,4-DHPPA); 3-hydroxyphenylpropionic acid (3-HPPA); 3,4-dihydroxyphenylacetic acid ((3,4-DHPAA); 3-hydroxyphenylacetic acid (3-HPAA); 4-hydroxybenzoic acid (4-HBA; Sigma-Aldrich, St Louis, MO, USA); 4-hydroxyhippuric acid (4-HHA; PhytoLab GmbH & Co. KG, Vestenbergsgreuth, Germany).

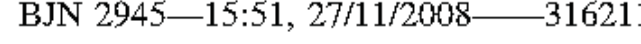
PBMC were cultured with the different phenolic acid solutions in the presence of LPS (Sigma-Aldrich). Five hundred microlitres of the 1×10^6 cells/ml suspension (5×10^5 cells, total number of cells) were pre-treated (16 h at 37°C, 5 % CO_2) with 250 μl of the 3 μM phenolic acid solution (1 μM, final concentration with cells) in 24-well plates. After the pre-treatment period, the cell viability was estimated with trypan blue and was higher than 95 %. LPS (1 μg/ml) was then added to the culture followed by incubation for 72 h at 37°C. Unstimulated and LPS-stimulated polyphenol-free cells were also cultured under the same conditions. Experiments were performed in duplicate. After the incubation period, the cultures were centrifuged and the supernatant collected and stored at −80°C until analysis. Pro-inflammatory cytokines IL-1β, IL-6 and TNF-α were determined in the culture supernatants by ELISA (Bender Med Systems GmbH, Vienna, Austria). Detection limits were as follows: 0·7 pg/ml for IL-1β; 0·92 ng/ml for IL-6; 1·65 pg/ml for TNF-α.

For the statistical treatment of the data, *t* test for paired samples were performed using the PC software package SPSS version 14.0 (SPSS Inc., Chicago, IL, USA). Differences between values were expressed as the percentage of enhancement or inhibition. All statistical tests were two-tailed, and the significance level was 0·05.

Results

The effects of tested phenolic acids on the secretion of TNF-α, IL-1β and IL-6 from the PBMC of healthy subjects are shown in Fig. 1 and Table 1. Stimulation of PBMC with LPS significantly increased the levels of the three pro-inflammatory cytokines up to 295·48 pg/ml (min = 48·34, max = 773·45) for TNF-α, 128·38 pg/ml (min = 44·24, max = 209·91) for IL-1β and 386·58 pg/ml (min = 331·36, max = 475·57) for IL-6.

3,4-DHPPA, 3,4-DHPAA and 4-HHA significantly reduced ($P<0·01$ for 3,4-DHPPA and 3,4-DHPAA, $P<0·05$ for 4-HHA). TNF-α secretion in LPS-stimulated PBMC by 84·9, 86·4 and 30·4 %, respectively (Table 1). Contrarily, 4-HBA significantly increased TNF-α secretion by 9·9 %. No significant



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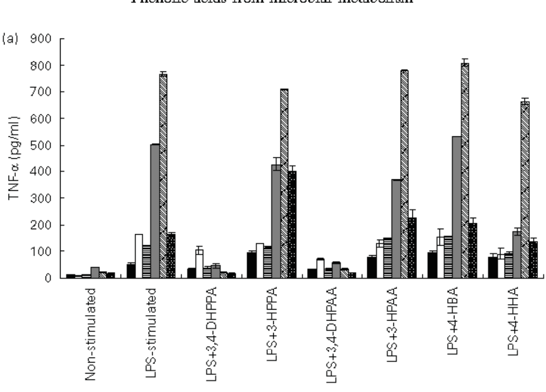

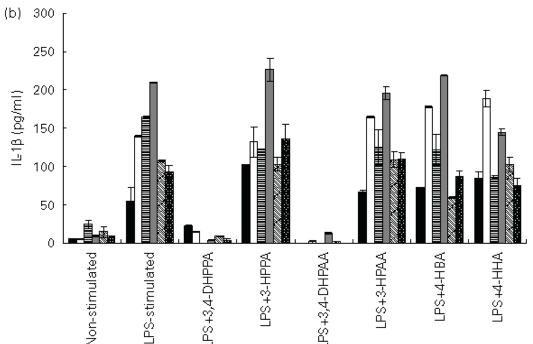

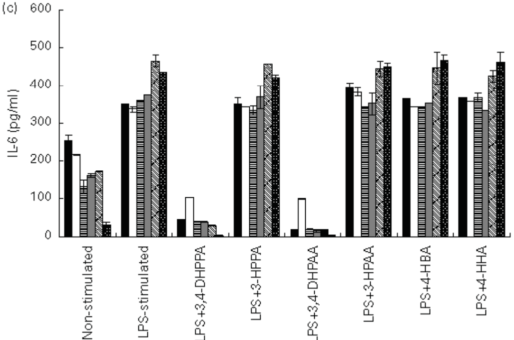

Fig. 1. Effect of phenolic acids (1 μM) derived from microbial metabolism on the secretion of (a) TNF-α, (b) IL-1β and (c) IL-6, after stimulation with lipopolysaccharide in peripheral blood mononuclear cells from six subjects (■, 1; □, 2; ▤, 3; ▦, 4; ▧, 5; ▩, 6).

changes in TNF-α secretion were recorded after the addition of the remaining tested phenolic acids, 3-HPPA and 3-HPAA. In the case of IL-1β, only 3,4-DHPPA and 3,4-DHPAC significantly ($P<0.001$) reduced the secretion of this cytokine in LPS-stimulated PBMC by 93·1 and 97·9 %, respectively (Fig. 1; Table 1). Similarly, a significant reduction ($P<0.001$) in IL-6 secretion from LPS-stimulated PBMC was also observed after treatment with 3,4-DHPPA and 3,4-DHPAA, resulting in

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Table 1. Summary results of the effect of phenolic acids (1 μM) derived from microbial metabolism on the secretion of TNF-α, IL-1β and IL-6 after stimulation with lipopolysaccharide (LPS) in peripheral blood mononuclear cells from six healthy subjects

(Mean values with their standard deviations)

n 6	Minimum	Maximum	Mean	SD	Enhancement or inhibition (%)	*P**
TNF-α (pg/ml)						
Non-stimulated	5·73	40·55	18·23	11·51		
LPS-stimulated	48·34	773·45	295·48	265·97		
LPS + 3,4-DHPPA	16·55	128·15	44·51	35·66	84·9	0·009
LPS + 3-HPPA	87·07	709·68	313·14	232·76	+6·0	0·588
LPS + 3,4-DHPAA	17·95	72·51	40·06	18·35	86·4	0·007
LPS + 3-HPAA	76·07	781·47	288·48	249·69	2·4	0·728
LPS + 4-HBA	88·51	819·10	324·87	270·58	+9·9	0·001
LPS + 4-HHA	69·86	672·04	205·67	216·82	30·4	0·026
IL-1β (pg/ml)						
Non-stimulated	4·36	28·12	11·39	7·41		
LPS-stimulated	44·24	209·91	128·38	52·60		
LPS + 3,4-DHPPA	0·00	22·28	8·85	7·94	93·1	0·000
LPS + 3-HPPA	97·49	236·79	138·57	46·73	+10·5	0·192
LPS + 3,4-DHPAA	0·00	12·00	2·72	4·65	97·9	0·000
LPS + 3-HPAA	65·05	202·09	128·54	44·20	+0·1	0·982
LPS + 4-HBA	59·15	218·71	122·92	60·68	4·3	0·583
LPS + 4-HHA	68·54	195·74	113·70	42·28	11·4	0·323
IL-6 (pg/ml)						
Non-stimulated	27·51	264·91	161·22	73·60		
LPS-stimulated	331·36	475·57	386·58	49·38		
LPS + 3,4-DHPPA	2·94	102·68	43·29	31·14	88·8	0·000
LPS + 3-HPPA	324·35	456·42	378·79	48·03	2·0	0·138
LPS + 3,4-DHPAA	2·94	99·42	29·59	33·18	92·3	0·000
LPS + 3-HPAA	331·36	458·06	393·83	44·24	+1·9	0·482
LPS + 4-HBA	343·01	476·96	386·05	54·08	0·1	0·946
LPS + 4-HHA	333·34	480·36	385·20	46·68	0·4	0·880

3,4-DHPPA, 3,4-dihydroxyphenylpropionic acid; 3-HPPA, 3-hydroxyphenylpropionic acid; 3,4-DHPAA, 3,4-dihydroxyphenylacetic acid; 3-HPAA, 3-hydroxyphenylacetic acid; 4-HBA, 4-hydroxybenzoic acid; 4-HHA, 4-hydroxyhippuric acid.
* Significant differences between LPS-stimulated cells and those in the presence of phenolic acids were determined by the *t* test.

an inhibition of 88·8 and 92·3 %, respectively. However, no significant changes were found in IL-1β and IL-6 levels after the addition of the remaining tested phenolic acids.

Discussion

In the present study, we observed that dihydroxylated phenolic acids derived from microbial metabolism presented marked *in vitro* anti-inflammatory properties, reducing the secretion of TNF-α, IL-1β and IL-6 in LPS-stimulated PBMC from healthy subjects. Six different phenolic acids (3,4-DHPPA, 3-HPPA, 3,4-DHPAA, 3-HPAA, 4-HBA and 4-HHA) derived from the microbial metabolism of polyphenols, in particular from monomeric flavanols and proanthocyanidins, were tested at a concentration level (1 μM) within the range (0·1–10 μM) found in plasma samples after the intake of a polyphenol-rich meal and recommended for *in vitro* studies[3]. Tested compounds were chosen on the basis of their structural features (i.e. hydroxylation pattern and side-chain length of the functional group) and on their abundance in biological fluids after the ingestion of polyphenol-rich foods. To our knowledge, the effects of microbial-derived phenolic acids on the production and release of pro-inflammatory cytokines from human PBMC have not been published previously.

Previous studies based on the consumption of catechin by human subjects resulted in an increase in 3-HPPA[17]. Consumption of catechins and proanthocyanidins from chocolate by human subjects resulted in an increased urinary excretion of 3-HPPA, 3,4-DHPAA, 3-HPAA and 3-HBA[18]. More recently, Ward *et al.* [19] described that 3-HPPA was the main metabolite in urine when the human subjects were supplemented with grape-seed polyphenols. Studies on laboratory animals have shown similar results. Main urinary metabolites formed from rats fed a catechin diet were 3-HPPA, 3-HBA and 3-HPA[20]. Besides these metabolites, 3-HPAA and 4-HBA were detected in the urine of rats fed wine polyphenols[20]. *In vitro* experiments also confirmed that 3-HPPA was the most abundant metabolite produced from proanthocyanidin polymers[4] by human colonic microflora, whereas 3,4-DHPAA was the main metabolite from rutin, which was further dehydroxylated to 3-HPAA[21]. This compound was also found to be the major end product of the colonic metabolism of chlorogenic acid by human faecal microbiota *in vitro* [22]. The yield of microbial metabolites could be high, in particular for the polyphenols that are poorly absorbed in the small intestine. For example, for chlorogenic and caffeic acids, it represented 57·4 and 28·1 % of the total intake, respectively[22]. However, in order for these metabolites to be effective at a physiological level, they need to be absorbed and reach target tissues. In fact, once produced by the microbiota, some colonic metabolites could be further absorbed and reach the liver and the kidney where they could be methylated, hydroxylated or conjugated with glycine[20,23]. Recently, the absorption mechanism of some of these colonic metabolites is beginning to be elucidated. Metabolites such as ferulic, *p*-coumaric, *m*-coumaric and 3-HPPA are absorbed by the

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monocarboxylic acid transporter, whereas caffeic acid and 3,4-DHPPA permeate across Caco-2 cells via the paracellular pathway[24 26]. Using immunohistochemical tests, Kawai *et al.* [27] have recently confirmed that polyphenol metabolites could penetrate the tissues. Quercetin-3-glucuronide, a major metabolite of quercetin, was permeable in LPS-stimulated macrophages, and was converted into the more active aglycone, a part of which was further converted into the methylated form. These data suggest that microbial phenolic metabolites could also undergo a similar pathway in injured cells.

Atherosclerosis is now considered as an inflammatory disease[28]. Recent epidemiological and clinical studies have shown that the Mediterranean diet or its main components, rich in polyphenols, are associated with a lower inflammatory status[29]. However, in epidemiological and even in clinical studies, it is difficult to control the effects of the diet consumed and physical activity performed[30]. Thus, *in vitro* studies allow us to obtain additional information in relation to the direct effect of some compounds (i.e. polyphenol metabolites) in biochemical pathways related to cardiovascular health, such as the production of pro-inflammatory cytokines that participate in the first stages of atherosclerosis. LPS is a bacterial protein and is used as a method to challenge immune cells to produce cytokines, including the inflammatory cytokines. Some of the inflammatory cytokines that are produced are those that have been associated with chronic inflammation and atherosclerosis risk.

The results found in the present study indicate that the effects of the tested phenolic acids on cytokine secretion by PBMC were structure dependent. With the exception of the effects of 4-HHA on TNF-α secretion, only the dihydroxylated phenolic acids, 3,4-DHPPA and 3,4-DHPAA, caused a statistically significant decrease in the secreted levels of the three different cytokines from LPS-stimulated PBMC (Table 1). The standard deviation of the data reflects large inter-individual difference in cytokine secretion among the volunteers. In addition, the degree of inhibition was found to be influenced by the cytokine family. The inhibition of IL-1β by both compounds was slightly higher than that for TNF-α and IL-6 (Table 1). Monohydroxylated phenolic acids (3-HPPA, 3-HPAA, 4-HBA and 4-HHA) did not produce significant changes in cytokine secretion with the exception of 4-HHA on TNF-α secretion, which produced a significant increase in this cytokine.

The present results on TNF-α, IL-1β and IL-6 secretion are in agreement with other studies performed with other polyphenols and cell types. Quercetin inhibited the expression of IL-8 and MCP-1 in TNF-α-stimulated synovial cells[31]. Small oligomeric procyanidin fractions (monomer to tetramer) isolated from cocoa reduced the secretion of IL-1 from PHA-stimulated PBMC, whereas polymers (pentamer to decamer) produced an increase in the secreted levels[12]. However, the same fractions promoted the secretion of TNF-α[13]. Dimeric flavanols isolated from pine bark also enhanced TNF-α levels in stimulated macrophages, while monomers strongly inhibited its secretion[32]. Ramiro *et al.* [33] found that epicatechin, isoquercitrin and cocoa extracts decrease the secretion of TNF-α by macrophages in a dose-dependent manner. Also in this line, it has also been reported that several flavones and flavanols inhibited TNF-α secretion by LPS-stimulated macrophages[34].

The superior effect of dihydroxylated phenolic acids in comparison with the monohydroxylated ones on the inhibition of pro-inflammatory cytokines has also been reported for other tested biological properties. Thus, 3,4-DHPAA showed more potent cytotoxicity against tumour cell lines than 4-hydroxyphenylacetic acid[8]. 3,4-DHPPA was also among the phenolic acids inhibiting the expression of P-selectin in resting platelets[5]. According to Russell *et al.* [35], dihydroxylated phenolic acids present a better antioxidant capacity than monohydroxylated ones due to their stabilisation into quinones.

The mechanism associated with the inhibitory or stimulatory activities of polyphenols on cytokine production may result from transcriptional and post-transcriptional events[14]. In fact, NF-κB, a transcription factor responsible for the activation of a series of cytokines, including TNF-α and IL-1β, is redox sensitive, and it is well known that the antioxidants such as flavonoids can inhibit its activation[32]. Other authors have recently suggested that, besides their antioxidant effects, polyphenols could also function as signalling molecules[36].

In this sense, Tedgui & Mallat[10] have suggested that future therapeutic approaches to treat atherosclerosis may include agents that block pro-inflammatory cytokine signalling or the transcription of inflammatory-mediating molecules, among others. The results found in the present study demonstrate that due to their down-regulating effect on the production of pro-inflammatory cytokines TNF-α, IL-1β and IL-6, polyphenols such as dihydroxylated phenolic acids derived from microbial metabolism could be among the new generation of therapeutic agents for the management of immunoinflammatory diseases such as atherosclerosis.

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1.4 La utilització dels metabolitos del resveratrol com biomarcadors del consum de vi en voluntaris amb una dieta lliure: l'estudi Predimed

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Resveratrol metabolites in urine as biomarker of wine intake in free-living subjects: the PREDIMED Study

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Running title: Resveratrol as biomarker of wine intake
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ABSTRACT

Several clinical and epidemiological studies have shown that moderate wine consumption may exert a protective effect against oxidative stress involved in several diseases, such as cardiovascular and neurodegenerative disorders. However, the epidemiological assessment of wine consumption has usually been obtained using self-reported questionnaires with less reliable information to assess total intake more accurately than nutritional biomarkers. A reliable biomarker for wine consumption is, therefore, needed. To validate urinary resveratrol metabolites (RMs) as a biomarker of wine consumption in a large cohort of free-living subjects. 1,000 consecutive subjects entering a substudy of the PREDIMED trial (PREvención con DIeta MEDiterránea) were evaluated. Data were collected in a validated semiquantitative food frequency questionnaire. RMs were measured in morning urine by LC-MS/MS. Urinary RMs values correlated directly with reported daily amounts of wine consumed (r = 0.895; *P* <0.001). One drink of wine per week can be detected. Using a cut-off of 411.4nmol/g creatinine, urinary RMs could discriminate wine consumers from non wine consumers with a sensitivity of 93.3% (95% confidence interval, CI 91.5-94.7%), and a specificity of 92.1% (CI 90.2-93.7%). Urinary RMs fulfill the criteria to be considered as nutritional biomarker of wine consumption in a large sample of free-living subjects. This biomarker would provide an additional tool to investigate more precisely the relationship between wine consumption and health benefits.

INTRODUCTION

Results of several epidemiological studies have supported the healthy effects of the Mediterranean food pattern (1). Wine is one of the most representative foods of this pattern and it has been reported that wine intake may explain why Southern European countries have a low prevalence of coronary heart disease (CHD), despite exhibiting relatively high prevalence of cardiovascular risk factors. Thus, for example, the French population consumes large amounts of saturated fat, but the incidence of CHD is low, fact known as the French Paradox (1, 2). Other potential benefits attributed to moderate wine consumption in epidemiological studies include a reduced risk of many diseases induced by oxidative stress, such as ischemic stroke (3), hypertension (4), diabetes (5), dementia (6) and several causes of mortality (7). However, epidemiological studies have yielded widely variable results (4-6), probably due to the fact that it is very difficult to accurately assess dietary habits (including alcoholic beverage consumption) and physical activity in these studies. In other words, there are many uncertainties associated with the dietary assessment methods currently used in epidemiological studies (8). Biomarkers gather several advantages over dietary self-reported data, since they reflect a more objective assessment of nutrient intake (9-11). Additionally, the appropriate use of biomarkers in epidemiologic and clinical studies requires their validity to first be verified in samples of free-living populations. Therefore, for a better insight into the health effects of moderate wine drinking, reliable biological markers for wine intake are needed.

The exact mechanisms of the beneficial effects of moderate wine consumption are still uncertain: part of these effects have been attributed to ethanol itself and part to substances present in wine, such as polyphenol and, more recently, stilbenes. Wine, especially red wine, is the richest identified dietary source of stilbenes, the most characteristic of which is resveratrol. The beneficial effects of resveratrol (12, 13) against cardiovascular complications induced by oxidative stress may be due to the regulation of nitric oxide synthases, thioredoxin-1, heme oxygenase 1, vascular endothelial growth factr, manganese superoxide dismutase and caveolin-1 (14, 15). The amounts of resveratrol and piceid (resveratrol glucoside) in international red wine are 4-fold and more than 100-fold higher than in grape and must as well as other resveratrol dietary sources (peanuts, pistachios and berries) respectively (16, 17). In the EPIC-Spanish cohort, the most important source of total resveratrol (sum of *cis*- and *trans*-resveratrol and piceid) was by far wine (>98%), with an estimated daily intake of 933μg (16). Following a recent study in which we reported that urinary excretion of resveratrol metabolites (RMs) may be used as a potential biomarker of wine consumption in clinical trials (18), in the current work we assessed the validity of this biomarker of wine consumption, measured using a validated food frequency questionnaire (FFQ) in a large cohort of free-living subjects.

SUBJECTS AND METHODS

Subjects

The present study is a cross-sectional assessment of the first 1,000 consecutively admitted participants recruited from October 2003 to July 2005 in a substudy of the PREDIMED (PREvención con DIeta MEDiterránea) Trial (www.predimed.org). This is a large, parallel group, multicenter, controlled, randomized 4-year clinical trial designed to evaluate the effects of the Mediterranean diet on the primary prevention of cardiovascular disease. Full details of the study protocol have been published elsewhere (19). The Institutional Review Board of all participant centers approved the study protocol, and the study has been registered in the Current Controlled Trials, London (ISRCTN 35739639).
Eligible participants are community-dwelling men, 55 to 80 years of age, and women, 60 to 80 years of age; without prior CHD; and with type 2 diabetes or at least three or more of the following CHD risk factors: current smoking, hypertension (blood pressure>140/90 mmHg, or treatment with antihypertensive drugs), LDL cholesterol ≥160 mg/dL (or treatment with hypolipidemic drugs), low HDL cholesterol (≤40 mg/dL), body mass index (BMI) ≥25 kg/m^2, or family history of premature CHD.

Dietary assessment

The baseline examination included administration of a validated 137-item FFQ (20). Data reported included information on drinking habits, such as amount, frequency, and type of alcohol intake. Energy and nutrients, and resveratrol and piceid intakes were calculated from Spanish food tables (21) and compiled Spanish food composition data (16), respectively.

Samples and analytical methods

Morning urine samples were collected from all participants. Urine samples were coded and stored at –80ºC until analyses. The clinical investigators and laboratory technicians were blinded to clinical data. RMs in urine samples were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (22, 23). Briefly, 1 mL of urine with the internal standard was loaded onto a previously equilibrated Oasis® HLB solid-phase extraction 96-wells plate (30 mg; Waters). Urinary RMs were eluted with acidified methanol solution and ethyl acetate. After evaporation, the samples were reconstituted with 100µL of the mobile phase and then analyzed in the LC (Perkin-Elmer s200) coupled to a triple-quadrupole mass spectrometer (API 3000; Perkin-Elmer Sciex) as described previously by Urpi-Sarda *et al* (22). Due to the validated method acceptance criteria for within and between-day precision, accuracy and extraction recovery, one sample for subject was analyzed. The overall time consuming per sample was around 14 minutes, considering clean up by solid-phase extraction, optimized run time by liquid chromatography and mass spectrometry detection (22). Total urinary RMs were calculated as the sum of individual metabolites (*trans*-resveratrol-3-*O*-glucuronide, *cis*-resveratrol-4'-*O*-glucuronide, *cis*-resveratrol-3-O-glucuronide, *trans*-resveratrol-4'-*O*-sulfate, *trans*-resveratrol-3-*O*-sulfate, *cis*-resveratrol-4'-*O*-sufate and *cis*-resveratrol-3-*O*-glucuronide). All results for urinary RMs were corrected for creatinine and were reported as nanomols per gram of creatinine in the morning urine (18). Urinary creatinine was measured by the standard Jaffe (alkaline picrate) kinetic method (24, 25).

Statistical analysis

Descriptive statistics with the mean (SD) were used for the baseline characteristics of the participants. Chi-square tests and analyses of variance (ANOVA) were used to compare qualitative traits and means of quantitative variables, respectively, between wine consumption groups. As urinary resveratrol data was skewed (Kolmogorov and Levene tests), and the natural logarithm of the variable could not be normalized, the median (interquartile range) was used to describe this variable, and comparisons between groups of wine consumption were performed using non-parametric tests (Kruskal-Wallis and Mann-Whitney with Bonferroni adjustment). Spearman's rank correlation was calculated to estimate the association between urinary RMs excretion and dietary wine intake or dietary resveratrol and piceid consumption. Using ROC curve analysis, a cut-off point providing optimized sensitivity, specificity, positive (PPV) and negative predictive values (NPV) for the identification of wine consumers were calculated. ROC curve provide a pure index of diagnostic accuracy by demonstrating the limits of the ability of our test to discriminate between wine and non-wine consumers over the complete spectrum of wine consumers at normal conditions. All statistical test were 2-tailed, and the significance level was $P<0.05$. Statistical analysis was performed using the SPSS 14.0.

RESULTS

We analyzed the baseline data of 1,000 high cardiovascular-risk participants (479 men and 521 women, mean (SD) aged 66.6 (6.2)). The mean (SD) daily alcohol intake of the evaluated subjects was 10.86 (16.33) g/day, mainly due to wine consumption. 45.8% of participants were moderately daily consumers of wine [182.1 (151.9) mL/d], 15.1% drank intermittently (less than 3 glasses a week) [12.16 (6.21) mL/d] and the remaining 39.1% did not drink any kind of wine. Among the wine drinkers, the majority preferentially consumed red wine (76.7%), and only a few white wine (11.9 %) and rosé wine (11.4%). The participants who reported to drink only beer and/or spirits (4.8%) were included in the group of non

wine consumers. **Table 1** shows the baseline characteristics of the subjects included in the study. Participants in the moderate daily wine group were somewhat younger, and showed a significantly higher percentage of males and current smokers, as well as a significant lower prevalence of diabetes mellitus than their counterparts. In addition, daily wine consumers also reported a significantly higher education level. Similar demographic characteristics have been already observed in other epidemiological studies (7, 26, 27) when wine consumption pattern was considered.

The median (interquartile range) urinary RMs amounts were 120.7 (205.5), 599.8 (381.3) and 1401.3 (1242.6) nmol/g for participants who reported non wine and intermittent and moderate daily wine consumption, respectively (**Figure 1**). Compared with the participants classified as moderate daily wine consumers, urinary RMs concentrations were significantly lower for the intermittent wine consumers (P<0.001) and for the non wine consumers (P<0.001). Significant differences were also observed when intermittent and non wine consumers were compared (P<0.001). A highly significant correlation was observed between reported wine consumption by participants and urinary RMs concentrations (r = 0.895; P <0.001). The estimated resveratrol and piceid consumption from validated FFQ also correlated directly with the urinary RMs concentration (r = 0.890; P <0.001). Significant differences between non-wine consumers and wine consumers of 1 glass/week were observed (P<0.001). The applied methodology allows detecting 1 drink of wine a week.

According to ROC curve analysis, the optimal cutoff point for urinary RMs was 411.38nmol/g, which allowed differentiation of non-wine consumers from wine consumers with an area under the ROC curve (**Figure 2**) of 0.983 (95% CI, 0.973-0.990), a sensitivity of 93.3% (CI 91.5-94.7%), a specificity of 92.1% (CI 90.2-93.7%), a positive predictive value (PPV) of 94.8% (CI 93.2-96.1%), and a negative predictive value (NPV) of 89.8% (CI 87.8-91.6%).

DISCUSSION

We report the first data demonstrating the usefulness of resveratrol as a nutritional marker of moderate wine consumption in a large sample of free-living subjects, as previously suggested in two controlled clinical trials and in a small cohort study (18). Spencer *et al.* (11) established the optimal criteria of potential compounds to serve as useful nutritional biomarker: i) robust methodology; ii) sensitivity; iii) specificity; iv) bioavailability, characteristics also confirmed by van Damm and Hu (28), who also emphasized that a good biomarker needs to be assessed in real conditions (free living population) and in large studies to evaluate several real dietary possibilities at the same time. According to our results RMs fulfil these criteria. It is noteworthy that the validated LC-MS/MS methodology used in this study allowed us to improve the measurement of resveratrol metabolome profile in biological samples. Parameters such as selectivity, sensitivity, recovery, linearity, precision and stability were maximally optimized (22). Moreover, the solid phase extraction adaptation into 96 wells and 10 min of chromatographic time allowed us to analyze a large number of samples, as it is necessary in epidemiological studies.

In two previous controlled, crossover and randomized clinical trials, we also observed an association between resveratrol dietary intake and concentrations of urinary RMs (18). In the current study, we showed that RMs concentration in urine correlated significantly with calculated resveratrol intake obtained from FFQ in large free-living populations (r: 0·890, P< 0.001). Moreover, urinary RMs also correlated highly significantly with reported wine consumption (r: 0·895, P< 0.001), which indicates the usefulness of such determination as a biomarker of wine consumption.

However, other issues should be also taken into account. The best specific dietary biomarker would only be modified by one food. Since this situation hardly ever occurs, the major food sources of a potential biomarker should be analyzed. In the case of resveratrol, it can be found in wines, mainly in red wines, but also in grape, must, peanuts, pistachios and berries (16). The amounts of resveratrol and piceid in red wine are 4-fold, 120-fold, and 105-fold higher than in grape, peanuts or pistachios, and berries,

respectively (16). Other important aspect to assess the specificity of a nutritional biomarker may be the variability in nutrient composition of a same food. It is well-known that resveratrol content in wines may vary up to 10-fold mainly due to grape variety, being Pinor noir the variety with the highest content (**Table 2**). However, considering wines from the same variety, not significant differences were observed among European, North and South American, and Australian wines in resveratrol content (17, 29). Thus, the resveratrol content in Spanish wines is in the same range as reported for international wines. In our study, resveratrol and piceid sources were wine (96.4%), grape and must (3.6%), and other foods (<0.1%), being wine the most important dietary source of resveratrol (16). This may indicate that this nutritional biomarker could be applied to other international populations with similar wine consumption pattern.

In the current study, specificity and sensitivity were also evaluated with a ROC curve that was used to distinguish between non-wine and wine consumers in the PREDIMED large free-living population. All diagnostic parameters were higher than in our previous two controlled clinical trials and the small cohort, probably due to the large number of participants included in the present cohort and the different technique used to determine RMs in urine. Moreover, the improved methodology carried out in this study considered a high number of urinary RMs (22).

Resveratrol bioavailability in humans has been investigated previously in several clinical trials (18, 22, 30-36). Pharmacokinetic parameters of resveratrol have been studied by Boocock *et al* (30) and they reported that RMs remained in plasma at least 12-24h after intake. In fact, Walle *et al* still detected radioactivity in plasma at 72h after oral or intravenous ^{14}C-labeled resveratrol dose (36). As expected, ^{14}C-labeled resveratrol was also observed in target tissues, mainly in the digestive tract, in intermediate amounts in liver and kidney and in low levels in brain, heart, lung and testis (37). In humans, resveratrol was found in LDL after a moderate wine consumption (23). High interindividual differences have always been described when phenolic bioavailability has been considered (38). This variability has also been observed for resveratrol concentrations in human studies analyzing urines (18, 22, 33, 36), plasma (33, 35) and LDL (23). In order to minimize this effect, high number of participants needs to be considered.

Urine sampling is particularly useful for polyphenols with relatively short half-lives, where plasma measurements may fail to represent accurate intake (11). Several studies have indicated that urinary levels of isoflavones, 4-*O*-methylgallic acid, and isoferulic acid were proportional to the amount of soy (r=0.52), tea (r=0.57) and coffee (r=0.26) consumed respectively (39, 40). Practical and logistic considerations to collect spot urines would be interesting as diagnostic tool for large epidemiologic studies.

The main limitation of this study could be the use of a FFQ, despite being validated, is not the optimal technique (gold standard) to estimate dietary and drinking habits. However, FFQs are useful to know the usual consumption of foods. Furthermore, the intake of wine and other alcoholic drinks is one of the food groups with less variability throughout the seasons (41). In a previous PREDIMED cohort (158 subjects), there were not statistical differences between the estimations of alcoholic drinks by FFQ (143.4 g/d) or by 3-day dietary records (145.1 g/d) (unpublished data). For this reason, we can consider this FFQ as a useful questionnaire to assess wine intake. Another limitation is the restricted potential usefulness of this biomarker for Spanish population mainly consuming Spanish wines. This could limit the generalizability of our finding to other populations consuming different types of wines. However, similar amounts of resveratrol have been described in wines from different origin (European, North and South American, and Australian) (17, 29). Another limitation could be that this population is specific due to the origin (Spain), old and with high cardiovascular risk. This could represent diversity in habits and wine quantities consumed compared to other populations. The proportions of non-wine consumers, intermittent and moderate daily wine consumers and also the quantities of wine consumed will be diverse in other populations. However the optimum cut-off point was calculated with a wide range of wine consumers and it should be very similar in other populations.

On the other hand, the strengths of the present study lay in its large-scale sampling of volunteers in free-living conditions. Moreover, a highly sensitive and specific method was used to measure the resveratrol metabolome in urine, providing highly reliable data.

In conclusion, our data support the use of RMs in morning urine as a specific and accurate biomarker of moderate wine consumption in a large free-living population. This biomarker would constitute an additional, more objective and reliable tool to investigate the relationship between wine consumption and health benefits.

Acknowledgments

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TABLE 1. Baseline characteristics of the 1,000 participants in the Predimed study examined.

Characteristic	Non wine consumers (*n*=391)	Intermittent wine consumers (*n*=151)	Daily wine consumers (*n*=458)	***P***
Mean (SD) age, *y*	68.1 (6.1)	66.1 (6.2)	65.5 (5.9)	<0.001
Men, *n (%)*	170 (43.8)	72 (47.7)	238 (52.0)	0.014
Mean (SD) BMI, kg/m^2	29.6 (3.5)	29.2 (3.4)	29.1 (3.0)	0.152
Current smokers, *n (%)*	34 (8.7)	22 (14.6)	103 (22.5)	<0.001
Type 2 diabetes mellitus, *n(%)*	214 (54.7)	64 (42.4)	192 (39.7)	<0.001
Hypertension, *n (%)*	318 (81.3)	124 (82.1)	349 (76.2)	0.069
Dyslipidemia, *n (%)*	237 (60.6)	97 (64.2)	280 (61.1)	0.914
Education level, *n (%)*				
Primary school	353 (90.2)	108 (71.5)	308 (67.2)	<0.001
First-degree high school	28 (7.2)	29 (19.2)	90 (19.7)	<0.001
High school or university degree	10 (2.6)	14 (9.3)	60 (13.1)	<0.001

TABLE 2. *trans*-Resveratrol content in red wines classified by grape variety and origin (mg/kg) (17, 42-49).

	EUA	Canadian	Australian	South American	Italian	French	Spanish	Greek	Central European
Cabernet Sauvignon	1.2 (0.7)	2.9 (1.1)	2.5 (0.9)	1.9 (1.3)	3.2 (1.3)	3.2 (1.8)	1.4 (0.4)	1.3 (0.3)	3.0 (1.8)
Merlot	1.5 (1.2)	2.5 (3.6)	1.0	2.5 (1.8)	3.4 (2.3)	3.1 (2.1)	3.5 (2.7)		3.8 (2.8)
Pinot noir	5.1 (2.6)	3.3 (1.9)	13.4	2.9 (1.6)	4.8 (1.4)	4.4 (1.73)	5.0 (4.2)		3.3 (2.0)

FIGURE LEGENDS

FIGURE 1. Box plots of urinary resveratrol metabolite concentrations among non-wine consumers (*n*=391), intermittent wine consumers (*n*=151), and moderate daily wine consumers (*n*=458).

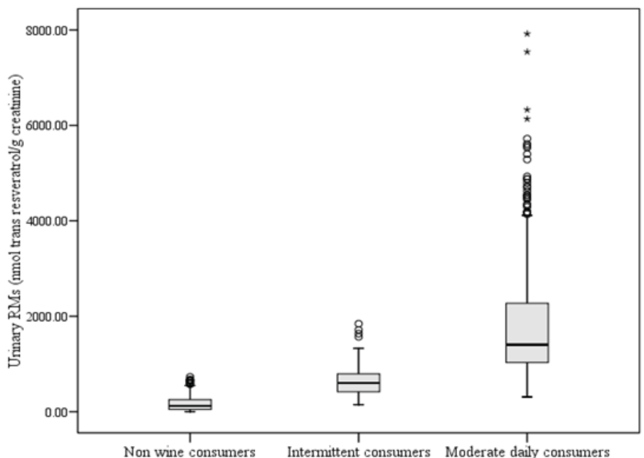
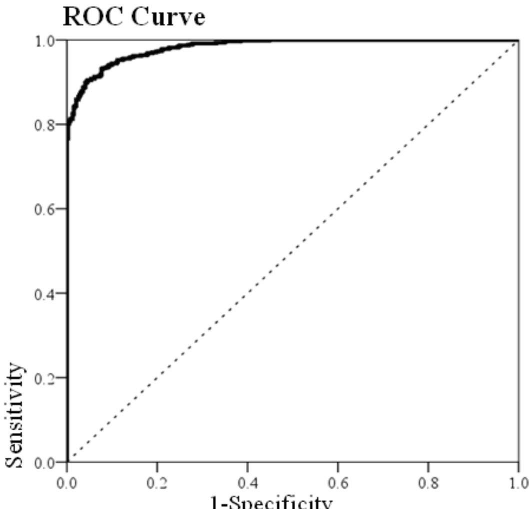


FIGURE 2. ROC curve of urine resveratrol metabolites for discrimination of wine consumers from non wine consumers in the PREDIMED Study.



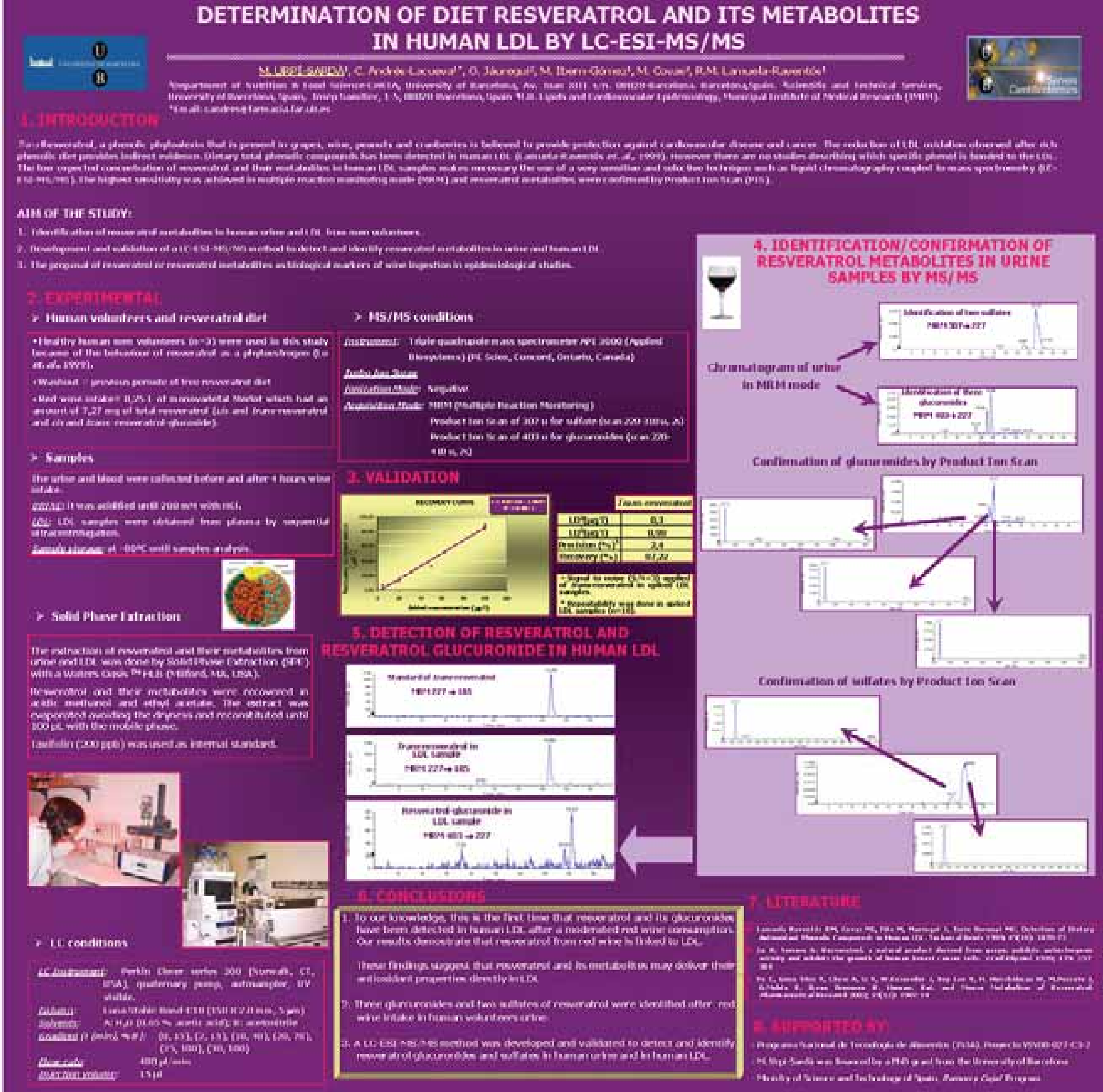
2. COMUNICACIONS A CONGRESSOS

Comunicació 1: Poster

Urpi-Sarda, M.; Andres-Lacueva, C.; Jauregui, O.; Ibern-Gomez, M.; Covas, M.; Lamuela-Raventos, RM.

Determination of diet resveratrol and its metabolites in human LDL by LC-ESI-MS/MS

1st International Conference on Polyphenols and Health, Vichy, França (2003)

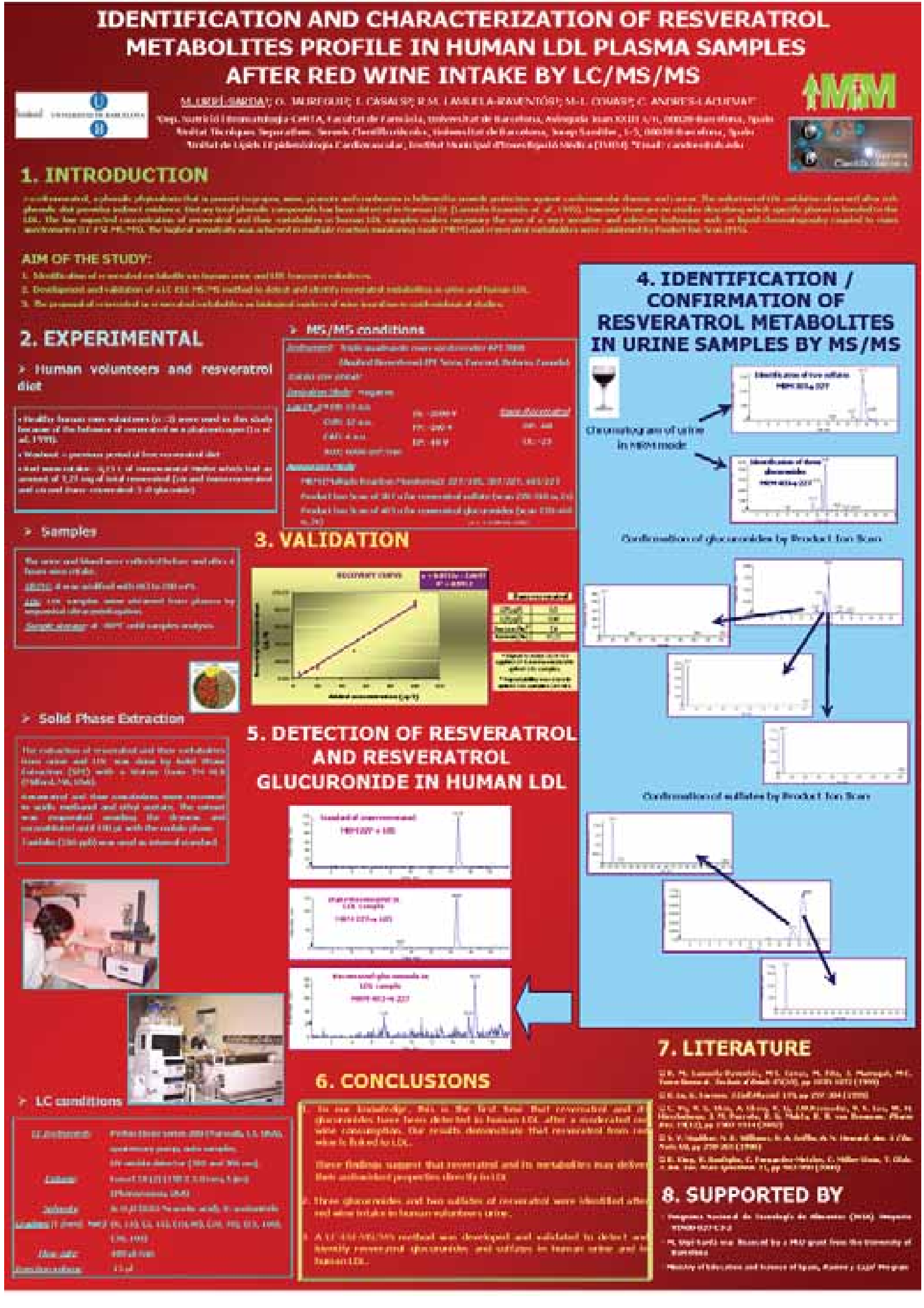


Comunicació 2: Poster

Urpí-Sardà, M.; Andrés-Lacueva, C.; Jáuregui, O.; Casls, I.; Covas, M.; Lamuela-Raventós, R.M.

Identification and characterization of resveratrol metabolites profile in human LDL plasma samples after red wine intake by LC/MS/MS

II Reunión Nacional de Espectrometría de Masas, Barcelona, Espanya (2004)

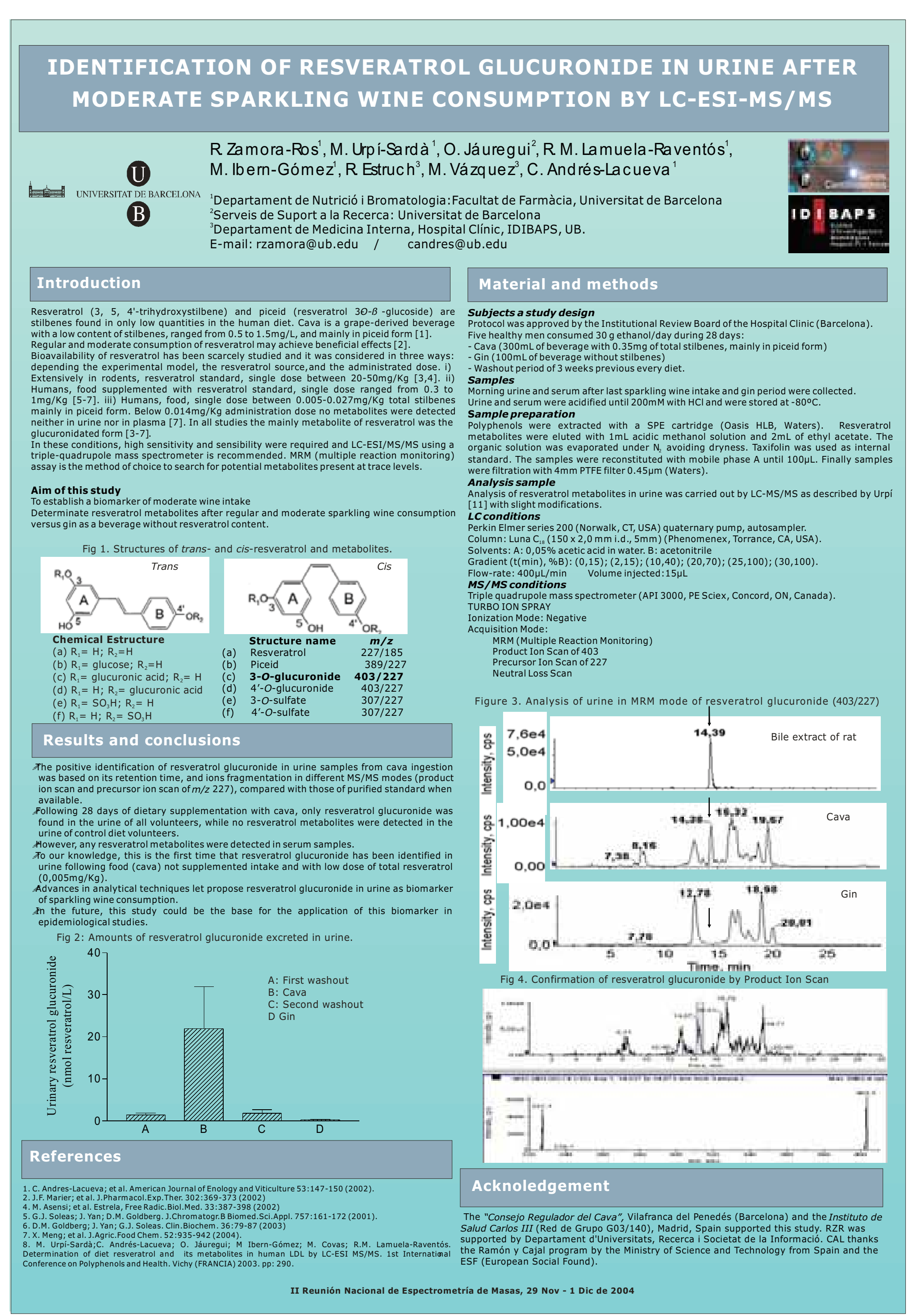


Comunicació 3: Poster

Zamora-Ros, R.; Urpí-Sardà, M.; Jauregui, O.; Lamuela-Raventós, R.M.; Ibern-Gómez, M.; Estruch, R.; Vázquez, M.; Andrés-Lacueva, C.

Identification of resveratrol glucuronide in urine after moderate sparkling wine consumption by LC-ESI-MS/MS

II Reunión Nacional de Espectrometría de Masas, Barcelona, Espanya (2004)



Comunicació 4: Comunicació oral

Andrés-Lacueva, C.; Urpí-Sardà, M.; Canals, R.; Gutiérrez, S.; Buxaderas, S.; de la Torre, C.

Compuestos Fenólicos como Marcadores Varietales en Cavas: Cinamatos y Resveratrol. Prospectiva: Influencia de la Maceración Pelicular

XXII Congreso Internacional del Cava, Sant Sadurní d'Anoia, Espanya (2004)

COMPUESTOS FENÓLICOS COMO MARCADORES VARIETALES EN CAVAS: CINAMATOS Y RESVERATROL.

PROSPECTIVA: INFLUENCIA DE LA MACERACION PELICULAR.

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Los fenoles no sólo presentan importancia en el color de los vinos y cavas sino que además, en estudios previos realizados por nuestro grupo de investigación ya se demostró el papel caracterizador varietal de la fracción fenólica en mostos y vinos blancos (de la Presa Owens *et* al., 1995; Romero-Pérez *et* al., 1996). De esta fracción fenólica, el contenido en fenoles pertenecientes al grupo de los hidroxicinamatos, y también la relación ácido *trans*-cutárico/ ácido *trans*-caftárico caracterizan vino y mosto. Además, el contenido en estilbenos (resveratrol y piceido, glucósido del resveratrol) se utiliza como marcador varietal en vinos blancos.

El propósito de este trabajo ha sido estudiar el perfil fenólico en 159 Cavas varietales con el fin de averiguar si los marcadores varietales de mostos y vinos blancos, anteriormente comentados, continúan siéndolo para los vinos espumosos cava (Andres-Lacueva et al., 2002).

Los Cavas analizados son monovarietales elaborados a partir de las variedades Macabeo, Xarel·lo, Parellada y Chardonnay, pertenecientes a diferentes bodegas de la comarca del Penedés y de distintas casas comerciales. El perfil fenólico de las muestras se ha determinado por cromatografía líquida de alta eficacia (C.L.A.E) y detección UV-visible con detector de fotodiodos, siguiendo el método descrito por Ibern-Gómez, *et* al., (2000).

Este es el primer estudio en el que se ha cuantificado el *trans*- y *cis*- piceido en vinos espumosos. Además, el contenido en estilbenos, hidroxicinamatos y la relación ácido *trans*-cutárico/ ácido *trans*-caftárico ha sido determinante para clasificar los Cavas de acuerdo con la variedad. La variedad Xarel·lo, al aplicar el análisis de los componentes, principales, destaca significativamente frente a la variedad Chardonnay por su mayor concentración en *cis*- y *trans*-piceido y en *cis*-resveratrol, y levemente en *trans*-resveratrol. Respecto a los fenoles pertenecientes al grupo de los hidroxicinamatos, la variedad Xarel·lo se distingue de las restantes por presentar una menor concentración en ácido *trans*-caftárico, ácido *trans*-cutárico y en el total de caftárico (ácidos *trans*- y *cis*-caftárico + ácido 2-S-glutationilcaftárico + ácido *trans*-cafeico), además de un alto contenido en ácido 2-S-glutationilcaftárico, en ácido *trans*-ferúlico y una elevada relación ácido *trans*-cutárico/ ácido *trans*-caftárico. Por lo tanto, el contenido en estilbenos, compuestos hidroxicinámicos y la relación ácido *trans*-cutárico/ácido *trans*-caftárico establecidos como marcadores taxonómicos de las variedades de uva para mostos y vinos blancos, mantiene el mismo patrón en Cavas, tras una segunda fermentación en botella cerrada.

Visto el papel caracterizador varietal de estos compuestos fenólicos a lo largo de la vinificación para la obtención de cava, nos planteamos realizar un estudio sobre la influencia de la maceración pelicular en vinos blancos de la variedad Xarel·lo del cual presentaremos los resultados preliminares. Este estudio se está realizando en la actualidad gracias a un proyecto de investigación financiado por la Obra Social de Caixa Penedès.

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- De la Presa-Owens, C; Lamuela-Raventós, R.M.; Buxaderas, S.; de la Torre-Boronat, M.C. Characterization of Macabeo, Xarel.lo and Parellada White Wines

- Ibern-Gomez, M.; Andrés-Lacueva, C.; Lamuela-Raventós, R.M.; Buxaderas,S.; Singleton,V.L.; de la Torre-Boronat, MC. Browning of Cava (Sparkling Wine) during Aging in Contact with Lees, due to the Phenolic Composition. Am. J. Enol. Vitic. (2000) (51) 29-36

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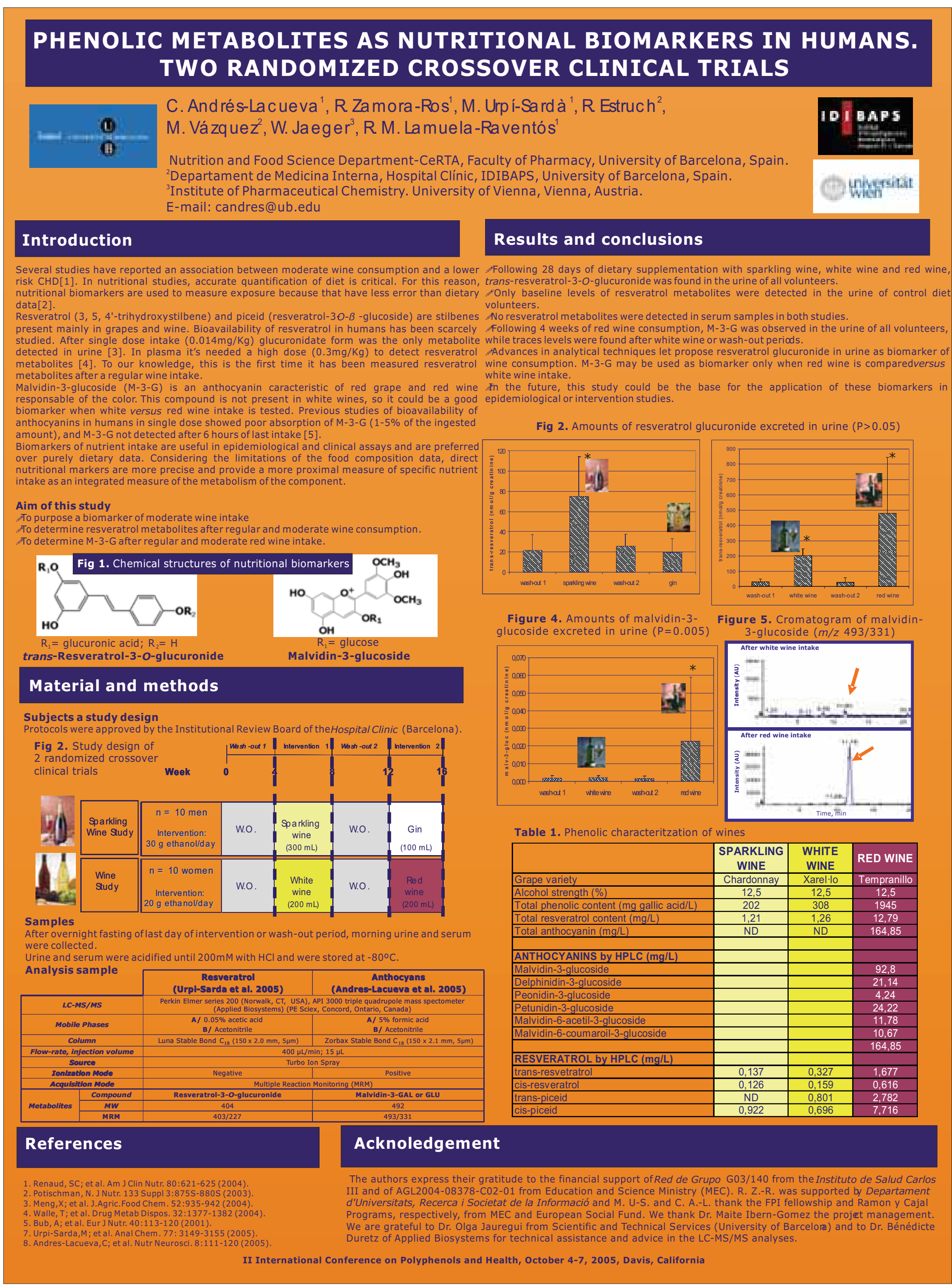
Comunicació 5: Comunicació oral

Andrés-Lacueva, C.; Zamora-Ros, R.; Urpí-Sardà, M.; Estruch, R.; Vázquez-Agell, M.; Jaeger, W.; Lamuela-Raventós, R.M.

Phenolic metabolites as nutritional biomarkers in humans. Two randomized controlled clinical trials

II International Conference on Polyphenols and Health, Davis, Estats Units (2005)



Comunicació 6: Comunicació oral

Zamora-Ros, R.; Urpí-Sardà, M.; Lamuela-Raventós, R.M.; Estruch, R.; Vazquez-Agell, M.; Serrano-Martinez, M.; Jaeger, W.; Andrés-Lacueva, C.

Urinary Resveratrol Metabolites as a Biomarker of Moderate Wine Consumption

11th Congress of the European Society for Biomedical Research on Alcoholism, Berlin, Alemania (2007)

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ABSTRACTS ESBRA 2007

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a similar magnitude as abstinence. Low-dose consumption on a controlled drinking situation should be considered on those chronic alcohol abusers who are not able to achieve complete abstinence.

Presentation S28-3

SCIENTIFIC EVIDENCE OF THE BENEFICIAL EFFECTS OF MODERATE ALCOHOL CONSUMPTION ON HEALTH

Estruch R, Sacanella E, Vazquez-Agell M, Mena MP, Monagas M, Fernandez-Solä J (Spain)

Aims. Numerous epidemiologic studies have found an association between moderate alcohol consumption and a reduced risk of coronary heart disease and ischemic stroke.

On the other hand, other types of evidence are related to the biologic plausibility of this hypothesis. Since atherosclerosis seems to be an inflammatory disease, the aims of the clinical trials performed were to evaluate the effects of red and white wines compared to those of an alcoholic beverage with low polyphenol content, on inflammatory biomarkers related to atherosclerosis.

Methods. Two randomized, crossover trials studies were performed. In study 1, 20 men consumed 30 g ethanol/day as cava (sparkling wine) or gin over 28 days, after a 15-day washout period. In study 2, 35 women consumed 20 g ethanol/day as white or red wine for 28 days. Serum and urine samples were collected after each 28-day period in both studies. Adhesion molecules involved in lymphocyte and monocyte—endothelium interactions were determined on the cell surface, and adhesions of human monocytes to endothelial cells were also measured in basal and stimulated conditions.

Results. Serum levels of C-reactive protein, intercellular adhesion molecule-1, CD40L, and interleUkin-6 decreased after either alcoholic beverage ($P < 0.01$; all). However, red wine and cava showed higher anti-inflammatory properties probably due to their polyphenolic content.

Conclusions. Although there is increasing evidence on the beneficial effects of moderate alcohol consumption on the health, without data from large randomized clinical trials, it is unclear how a physician can be in a position to advise his or her patients.

Presentation S28-4

URINARY RESVERATROL METABOLITES AS A BIOMARKER OF MODERATE WINE CONSUMPTION

Zamora-Ros R, Urpi M, Sarda Rosa M, Lamuela-Raventos Estruch, R, Vazquez-Agell M, Serrano-Martinez M, Jaeger W, Andres-Lacueva C (Spain and USA)

Aims. Background: Nutritional biomarkers may be better measures of dietary exposure than self-reported dietary data. We evaluated resveratrol metabolites, potential biomarkers of wine consumption, in humans after moderate consumption of sparkling, white, or red wines.

Methods. We performed 2 randomized, crossover trials and a cohort study. In the first study, 10 healthy men consumed 30 g of ethanol/day as sparkling wine or gin for 28 days. In the second trial, 10 healthy women consumed 20 g of ethanol/day as white or red wine for 28 days. We also evaluated 52 participants in a study on the effects of a Mediterranean diet on primary prevention of cardiovascular disease (the PREDIMED Study). We used liquid chromatography-tandem mass spectrometry to analyze urinary total resveratrol metabolites (TRMs) and predictive values and ROC curve analyses to assess the diagnostic accuracy.

Results. We observed significant increases in TRMs [72.4 (95% confidence interval, 48.5–96.2; P = 0.005), 211.5 (166.6–256.3; P = 0.005), and 560.5 nmol/g creatinine (244.9–876.1; P = 0.005)] after consumption of sparkling, white, or red wine, respectively, but no changes after the washout or gin periods. In the cohort study, the reported daily dose of wine consumption correlated directly with TRMs (r = 0.654; P < 0.001). Using a cutoff of 90 nmol/g, we were able to use TRMs to differentiate wine consumers from abstainers with a sensitivity of 72% (60%–84%); and a specificity of 94% (87%–100%).

Conclusions. Resveratrol metabolites in urine may be useful biomarkers of wine intake in epidemiologic and intervention studies.

SYMPOSIUM 29 WEDNESDAY SEPT. 26TH 9.00 AM–10.30 AM; ROOM: LECTURE HALL 1

GABA-B receptor: a new target for treating alcohol dependence?

Chairpersons: Leite-Morris KA (USA), Colombo G (Italy)

Presentation S29-1

ACTIVATION OF THE GABA(B) RECEPTOR IN ANIMAL MODELS OF ALCOHOL DEPENDENCE

Colombo G, Maccioni P, Orrù A, Lobina C, Agabio R, Addolorato G, Gessa GL, Carai MAM (Italy)

Aims. The present paper summarizes the different lines of experimental evidence featuring the suppressing effect of the GABA(B) receptor full agonists, baclofen and CGP44532, and positive allosteric modulators, CGP7930 and GS39783, on different alcohol-motivated behaviors.

Methods. These studies have been conducted testing different procedures of alcohol intake and alcohol self-administration in Sardinian alcohol-preferring (sP) rats, one of the few rat lines selectively bred worldwide for high alcohol preference and consumption.

Results. Administration of non-sedative doses of baclofen, CGP44532, CGP7930, and GS39783 to sP rats have been found to suppress: (a) acquisition and maintenance of alcohol drinking behavior under the standard 2-bottle 'alcohol vs water' choice regimen; (b) relapse-like drinking after a period of alcohol abstinence (the so-called 'alcohol deprivation effect'); (c) the increase in alcohol intake induced by the acute administration of opioids and cannabinoids; (d) oral self-administration of alcohol in rats trained to lever-press for alcohol on an FR4 schedule of reinforcement; (e) the motivational properties of alcohol, measured by the progressive ratio schedule of reinforcement and the extinction responding procedure in rats previously trained to self-administer alcohol on an FR4 schedule; (f) reinstatement (induced by the presentation of non-contigent, alcohol-associated stimuli) of alcohol-seeking behavior in rats trained to self-administer alcohol (another experimental model of alcohol relapse). Finally, acute administration of baclofen has been found to suppress the severity of different signs of alcohol withdrawal syndrome, including tremors and seizures, in Wistar rats made physically dependent on alcohol.

Conclusions. Taken together, these data suggest the involvement of the GABA(B) receptor in the neural substrate(s) controlling alcohol intake and relapse-like drinking, mediating alcohol's reinforcing and motivational properties, and underlying alcohol withdrawal syndrome. Of interest, subsequent preliminary clinical studies with baclofen have extended to human alcohol-dependent patients the majority of the above observations (Addolorato *et al*., this meeting).

Presentation S29-2

GABA(B) RECEPTOR ACTIVATION MODULATES NEUROTRANSMITTER LEVELS DURING ALCOHOL SEEKING

Leite-Morris KA (USA)

Aims. Dopamine neurons projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and other forebrain regions are implicated in the motivational behaviors related to ethanol seeking and intake. This presentation will discuss the findings that direct activation of GABA (B) receptors in the VTA modulate mesoaccumbens circuits and alter dopamine responses in the NAc that underlie the motivation of appetitive behaviors.

Methods. An animal model combining in vivo microdialysis with alcohol self-administration was designed to collect brain dialysate concurrently while subjects were lever pressing (extinction sessions). Long Evans rats were trained to perform a fixed number of lever presses (RR20) for a 20 minute presentation of 2% sucrose or 10% ethanol. Subjects were surgically implanted with guide cannula into the VTA for microinjections and NAc for in vivo microdialysis.

Following administration of intra-VTA artificial cerebral spinal fluid (aCSF), or the GABA (B) receptor agonist baclofen (0.25, 0.5, 1.0 ug) and/or the antagonist CGP 35348 (10 ug), single extinction sessions were performed each week. Brain dialysate was collected at 5 minute intervals prior to and during extinction trials.

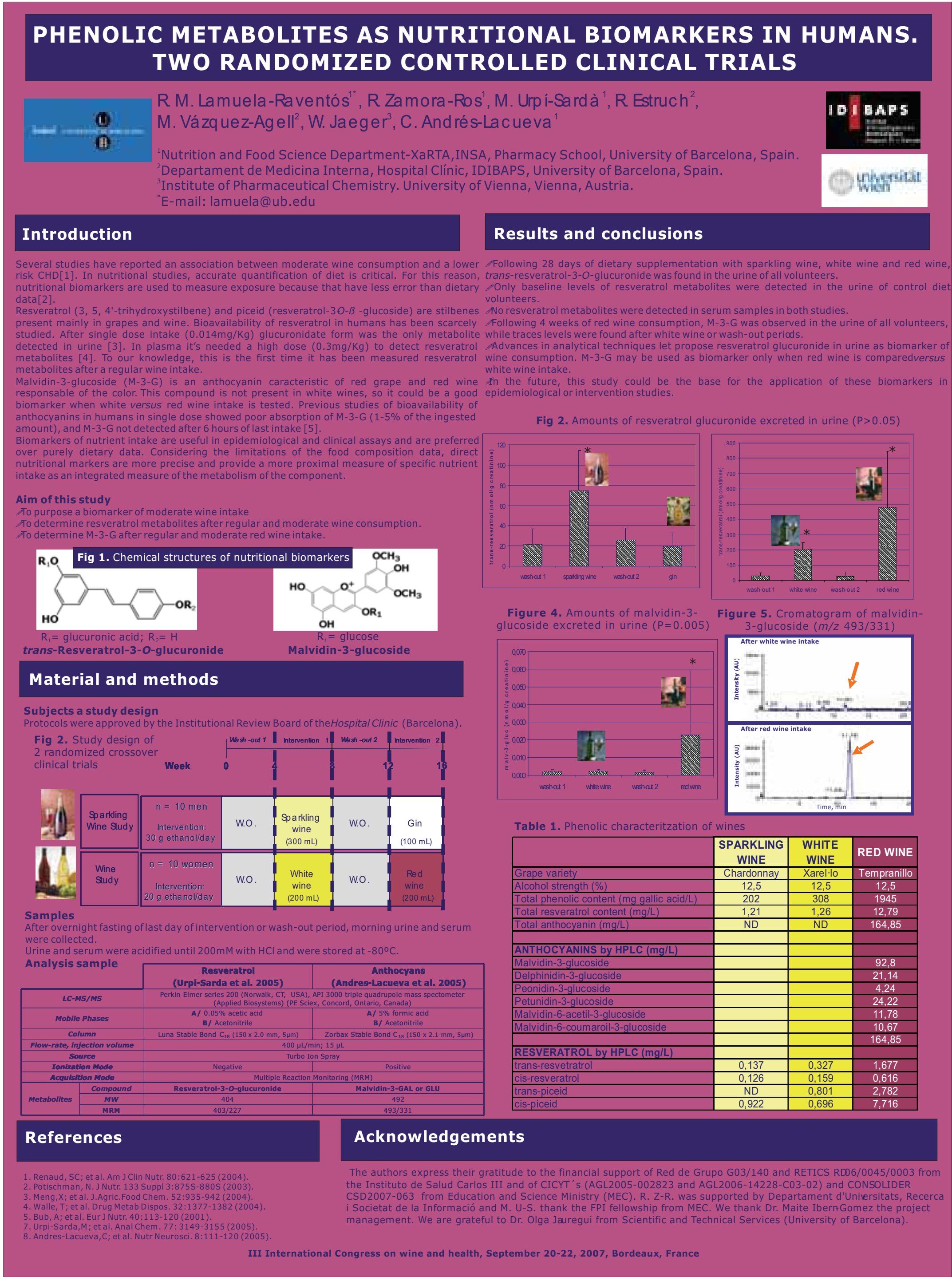
Results. Intra-VTA administration of baclofen (versus aCSF) resulted in a significant dose-dependent inhibition of ethanol seeking (lever pressing)

Comunicació 7: Poster

Lamuela-Raventós, R.M.; Zamora-Ros, R.; Urpí-Sardà, M.; Estruch, R.; Vázquez-Agell, M.; Jaeger, W.; Andrés-Lacueva, C.

Phenolic metabolites as nutritional biomarkers in humans. Two randomized controlled clinical trials.

III International Congress on wine and health, Bordeaux, França (2007)

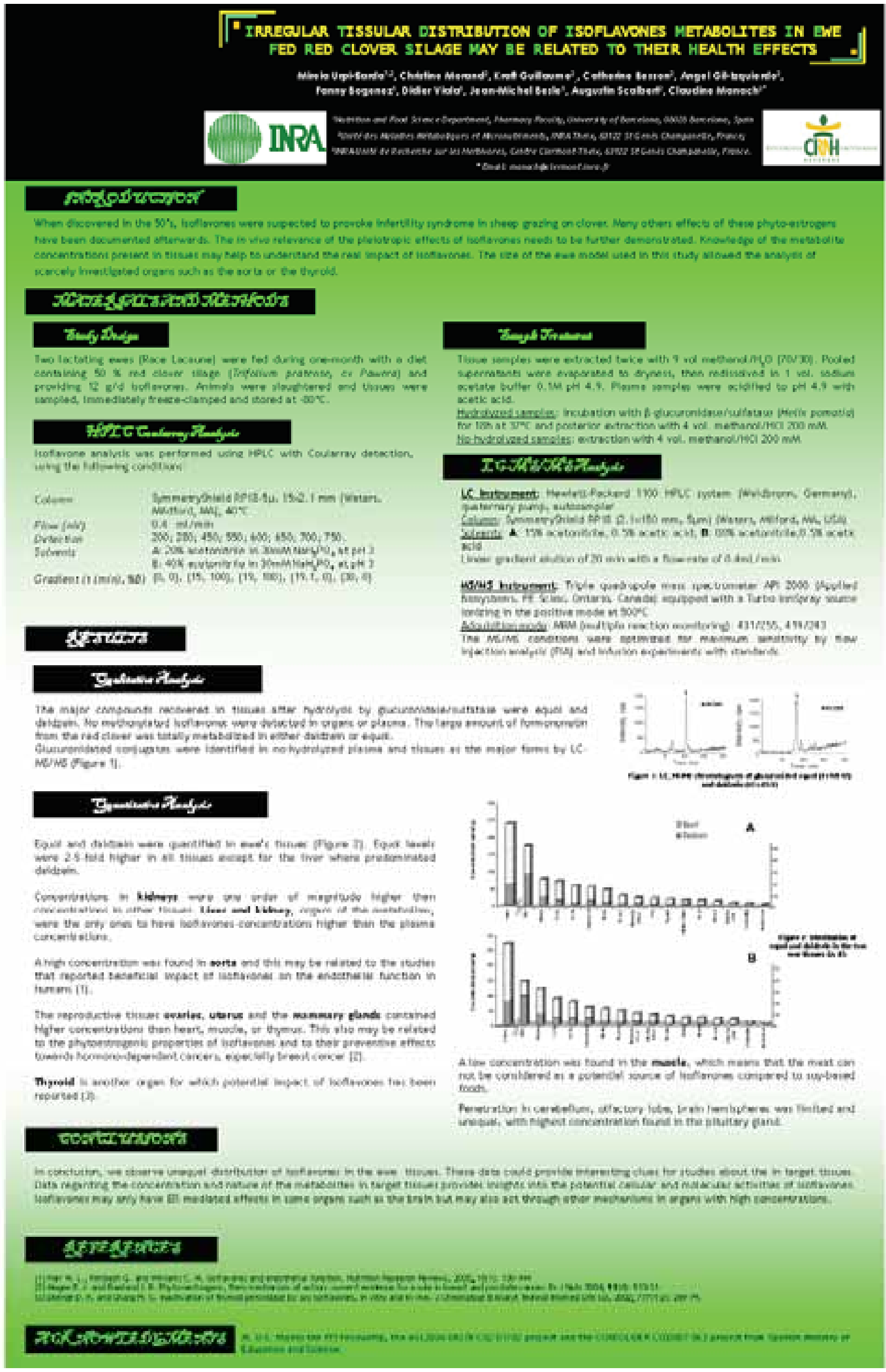


Comunicació 8: Poster

Urpi-Sarda M., Morand C., Guillaume K., Besson C., Gil-Izquierdo A., Bogenez F., Viala D., Besle J-M., Scalbert A., Manach C.

Irregular Tissular Distribution of Isoflavones Metabolites in Ewe Fed Red Clover Silage May Be Related to Their Health Effects

3rd International Conference on Polyphenols and Health, Kyoto, Japó (2007)

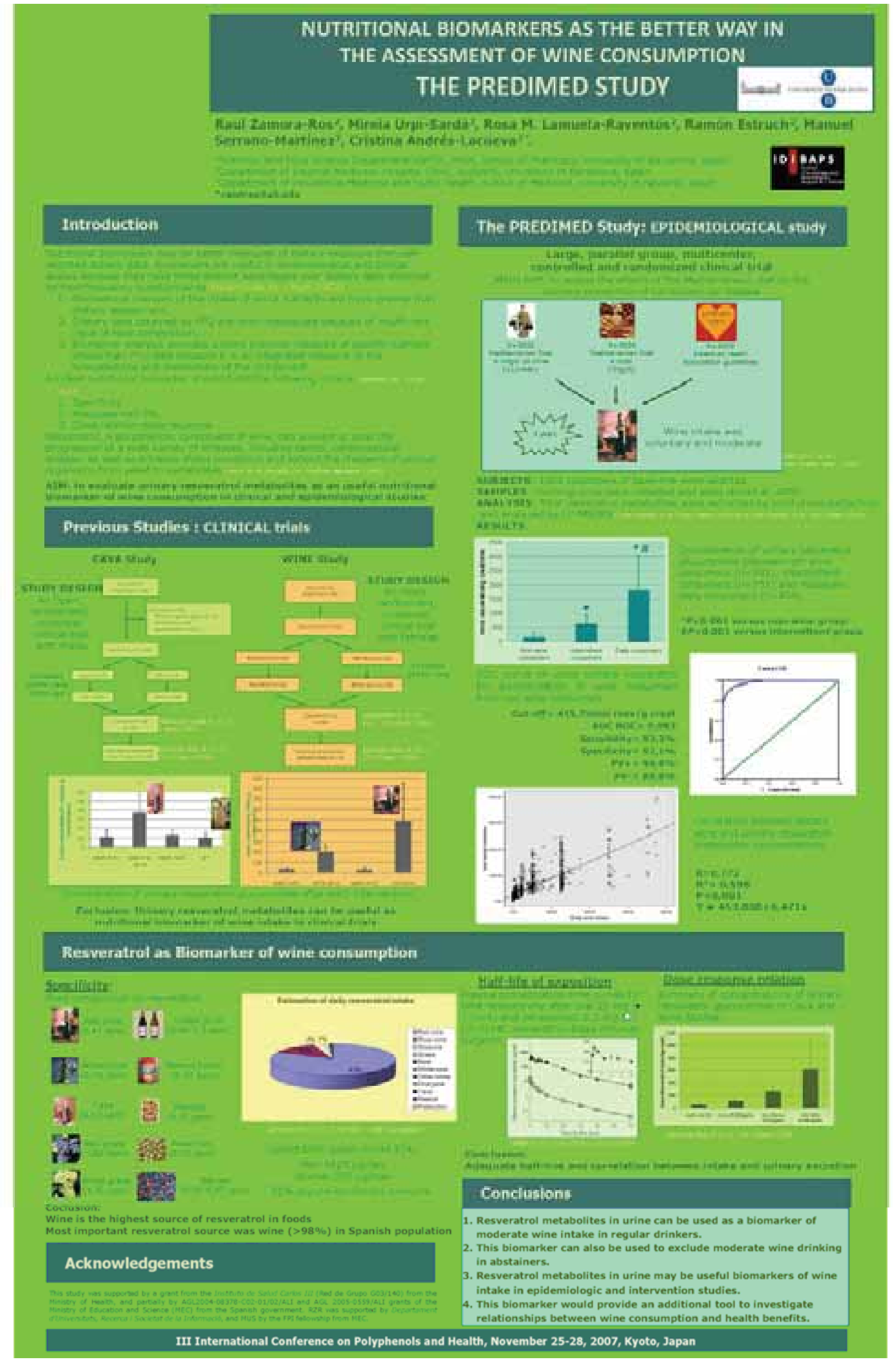


Comunicació 9: Poster

Zamora-Ros, R.; Urpí-Sardà, M.; Lamuela-Raventós, R.M.; Estruch, R.; Serrano-Martínez,M.; Andrés-Lacueva, C.

Nutritional Biomarkers as the Better Way in the Assessment of Wine Consumption the Predimed Study

3rd International Conference on Polyphenols and Health, Kyoto, Japó (2007)

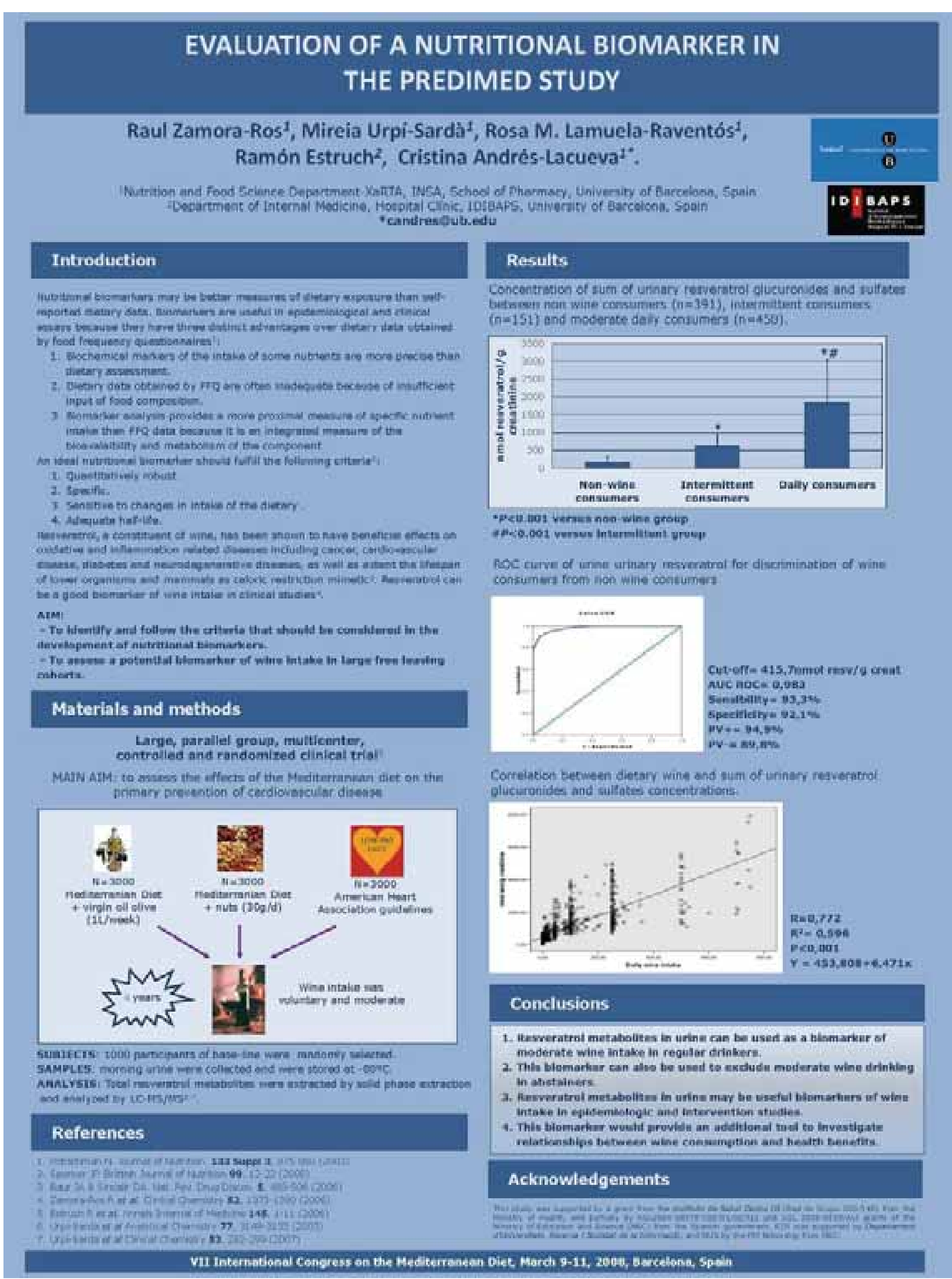


Comunicació 10: Poster

Zamora-Ros, R.; <u>Urpí-Sardà, M</u>.; Lamuela-Raventós, R.M.; Estruch, R.; Andrés-Lacueva, C.

Evaluation of a nutritional biomarker in the PREDIMED Study.

VII International Congress on the Mediterranean Diet, Barcelona, Espanya (2008)

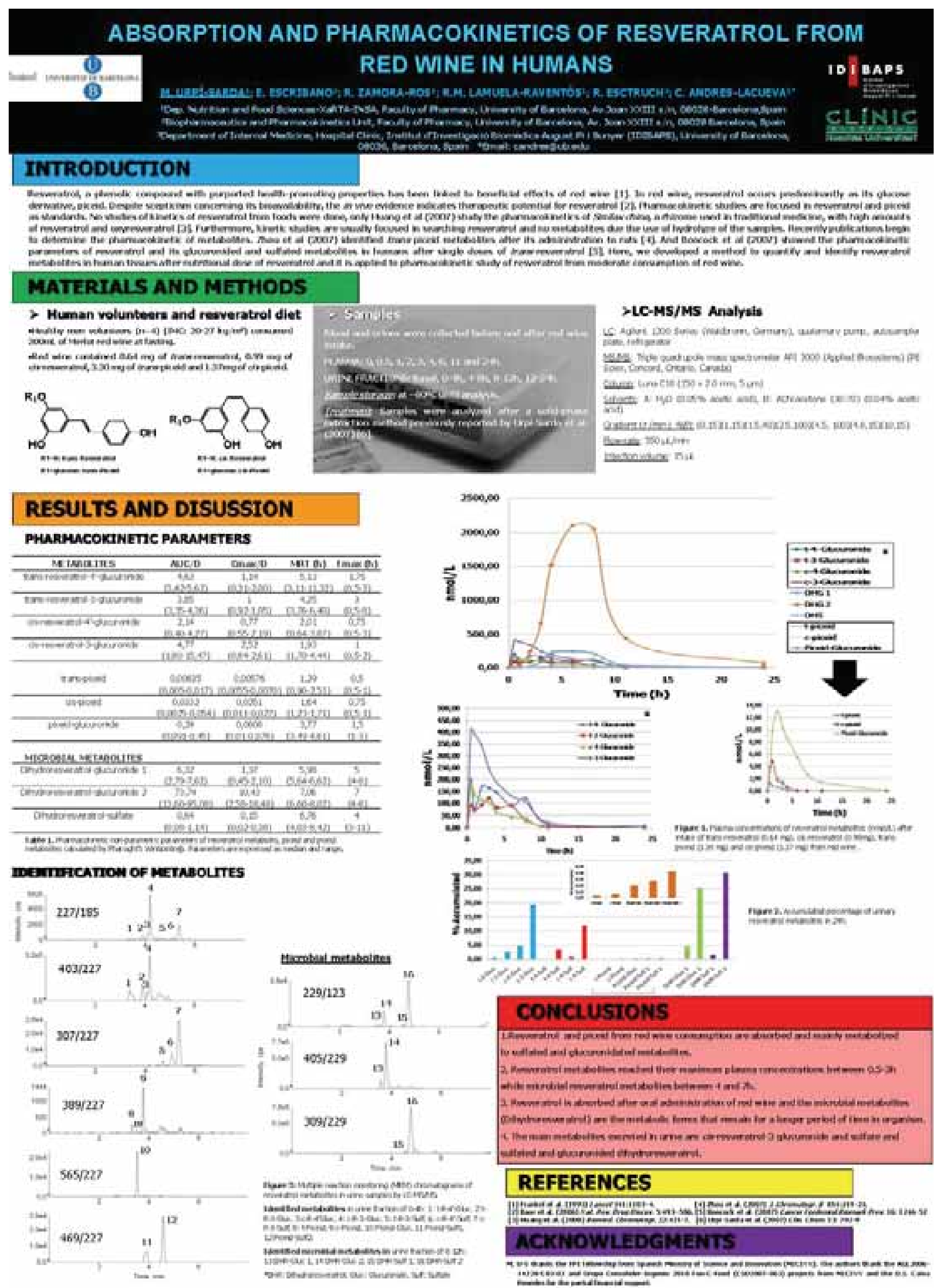


Comunicació 11: Poster

Urpi-Sarda, M.; Zamora-Ros, R.; Lamuela-Raventos, R.S.; Estruch, R.; Andres-Lacueva, C.

Absorption and pharmacokinetics of resveratrol from red wine in humans

XXIV th International Conference on Polyphenols, Salamanca, Espanya (2008)



Comunicació 12: Poster

Monagas, M.; Urpi-Sarda, M.; Khan, N.; Estruch, R.; M. Lamuela-Raventos, R.; Andres-Lacueva, C.

Application of a SPE-HPLC-MS/MS method for the simultaneos determination of microbial phenolic metabolites, and monomeric and dimeric flavan-3-ol metabolites in urine samples

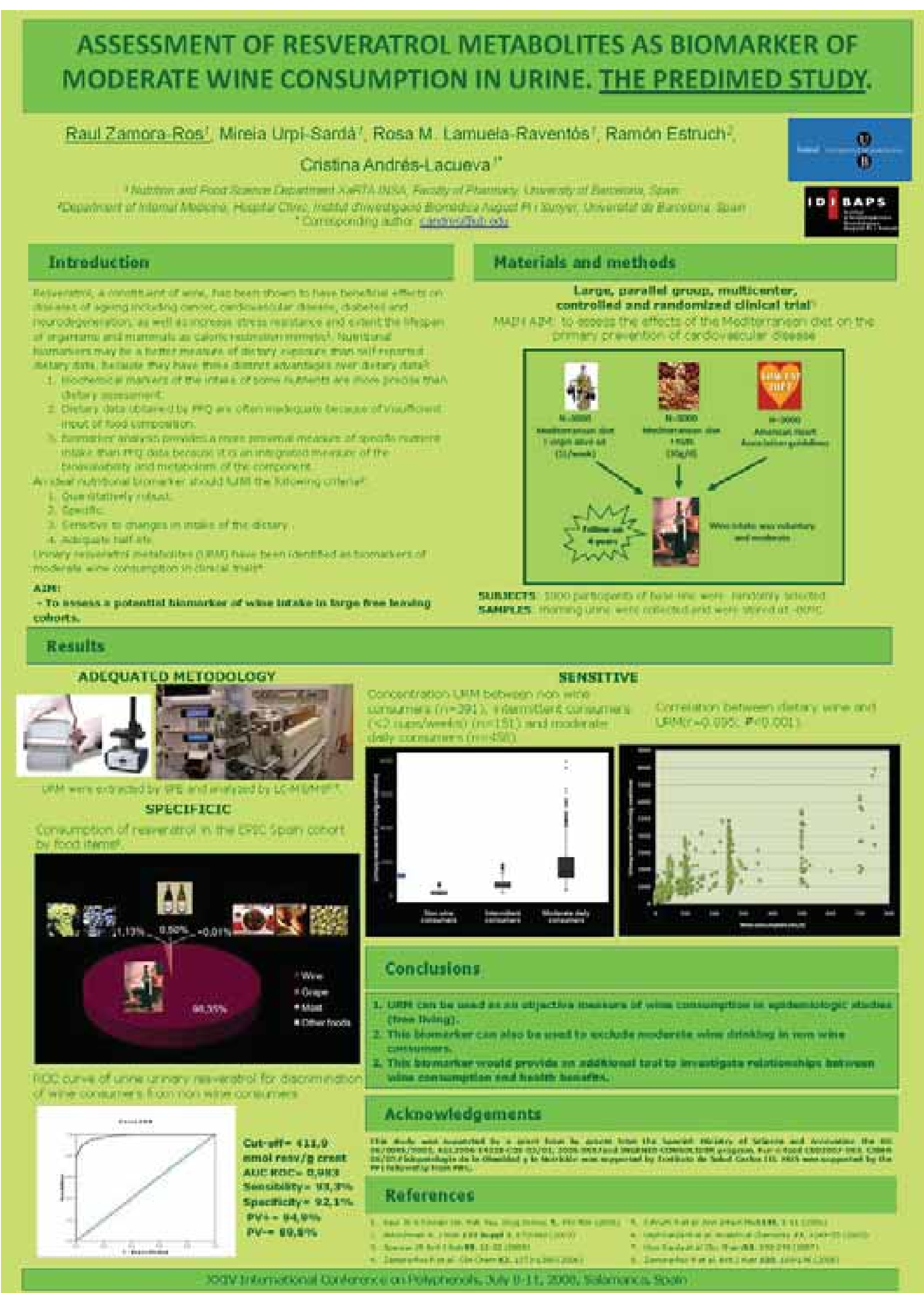
XXIV th International Conference on Polyphenols, Salamanca, Espanya (2008)

Comunicació 13: Poster

Zamora-Ros, R.; Urpi-Sarda, M.; Lamuela-Raventos, R.M.; Estruch, R.; Andres-Lacueva, C.

Assessment of resveratrol metabolites as biomarker of moderate wine consumption in urine. The predimed study

XXIV th International Conference on Polyphenols, Salamanca, Espanya (2008)



Comunicació 14: Poster

Chiva-Blanch, G.; Zamora-Ros, R.; Urpi-Sarda, M.; Medina-Remon, A.; Estruch, R.; Andres-Lacueva, C.

Determination of total polyphenols and resveratrol in Beer

XXIV th International Conference on Polyphenols, Salamanca, Espanya (2008)

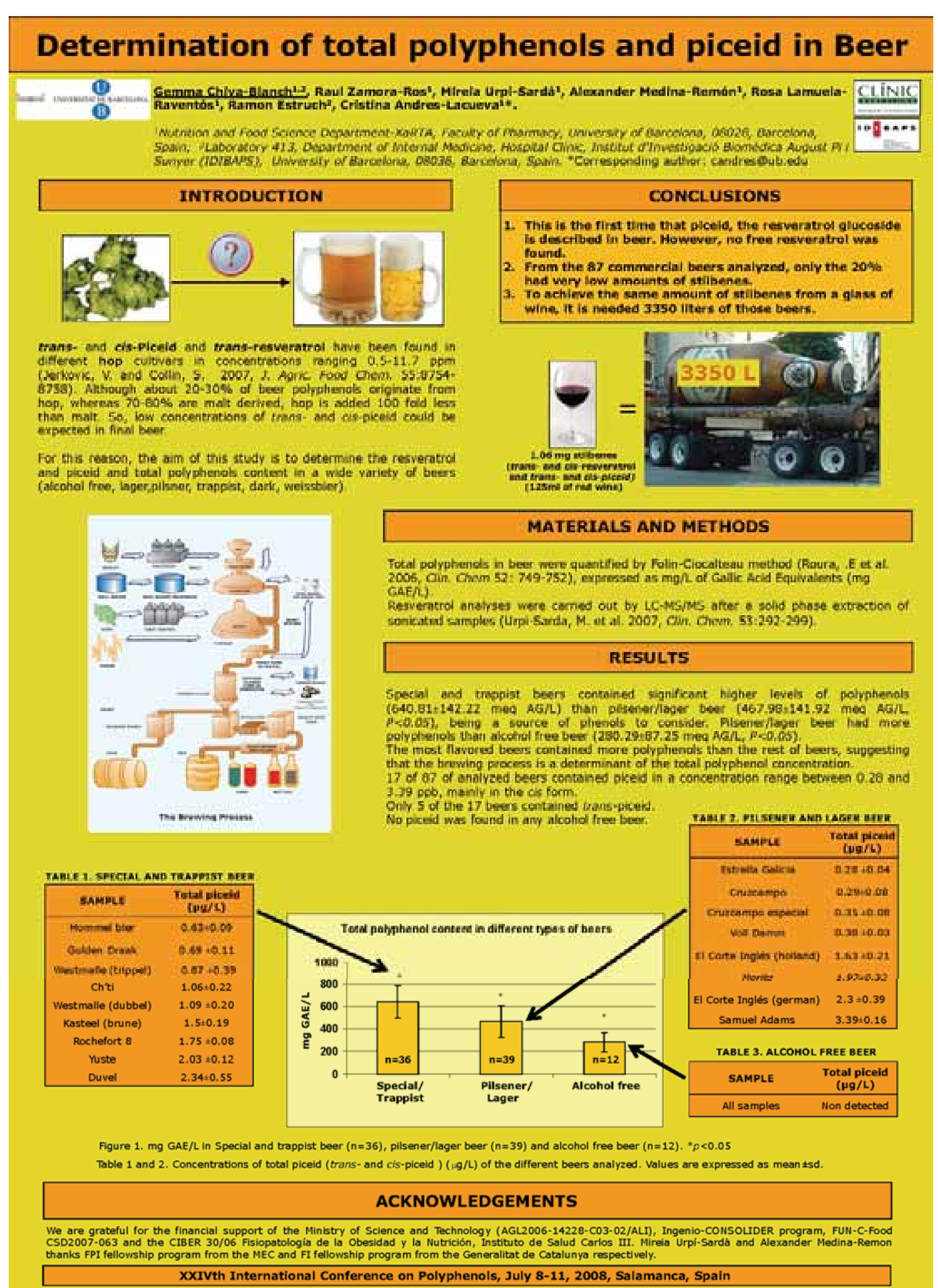


TABLE 1. SPECIAL AND TRAPPIST BEER

SAMPLE	Total piceid (µg/L)
Hommel bier	0.43±0.09
Golden Draak	0.69 ±0.11
Westmalle (trippel)	0.87 ±0.39
Ch'ti	1.06±0.22
Westmalle (dubbel)	1.09 ±0.20
Kasteel (brune)	1.5±0.19
Rochefort 8	1.75 ±0.08
Yuste	2.03 ±0.12
Duvel	2.34±0.55

TABLE 2. PILSENER AND LAGER BEER

SAMPLE	Total piceid (µg/L)
Estrella Galicia	0.28 ±0.04
Cruzcampo	0.29±0.08
Cruzcampo especial	0.35 ±0.08
Voll Damm	0.38 ±0.03
El Corte Inglés (holland)	1.53 ±0.21
Moritz	1.97±0.32
El Corte Inglés (german)	2.3 ±0.39
Samuel Adams	3.39±0.16

TABLE 3. ALCOHOL FREE BEER

SAMPLE	Total piceid (µg/L)
All samples	Non detected

Comunicació 15: Poster

Khan, N.; Monagas, M.; Andres-Lacueva, C.; <u>Urpi-Sarda, M.;</u> Estruch, R.

Anti-inflammatory properties of phenolic acids derived from microbial metabolism

XXIV th International Conference on Polyphenols, Salamanca, Espanya (2008)

Comunicació 16: Poster

Urpi-Sarda, M.; Monagas, M.; Khan, N.; Lamuela-Raventos, R.; Santos-Buelga, C.; Sacanella, E.; Castell, M.; Andres-Lacueva, C.

Epicatechin, procyanidin and phenolic microbial metabolites after cocoa intake in humans and rats by liquid chromatography tandem mass spectrometry

12as Jornadas de Análisis Instrumental, Barcelona, Espanya (2008)

Comunicació 17: Poster

Chiva-Blanch, G.; Zamora-Ros, R.; Urpi-Sarda, M.; Medina-Remon, A.; Estruch, R.; Andres-Lacueva, C.

High sensitive determination of resveratrol in beer by liquid chromatography tandem mass spectrometry

12as Jornadas de Análisis Instrumental, Barcelona, Espanya (2008)