



Universitat de Barcelona
Facultat de Farmàcia
Departament de Nutrició i Bromatologia

Títol: Els polifenols presents en els aliments com a
components funcionals en la prevenció i possible
tractament de la hipertensió arterial.

Alexander Medina Remón
2010

Universitat de Barcelona
Facultat de Farmàcia
Departament de Nutrició i Bromatologia
Programa de Doctorat
Nutrició i Metabolisme
Bienni 2005-2007

**Títol: Els polifenols presents en els aliments com a
components funcionals en la prevenció i possible
tractament de la hipertensió arterial.**

Memòria presentada per Alexander Medina Remón per a optar al títol de
doctorat per la Universitat de Barcelona, dirigida per:

Dra. Rosa Maria Lamuela Raventós

Dra. Cristina Andrés Lacueva

Alexander Medina Remón

Alexander Medina Remón

2010

Aquest treball ha estat finançat per:



Centro de Investigaciones Biomédicas En Red
Fisiopatología de la Obesidad y Nutrición.
*CIBERObn CB06/03 és una iniciativa de l'Institut
de Salut Carlos III.*



Unión Europea

Fondo Europeo de Desarrollo Regional
Una manera de hacer Europa



Ministeri de Ciència i Innovació "Red de Grupo" G03/140 AGL2005-05597;
AGL2006-14228-C03-02/01; AGL2007-66638-C02-01; RETICS RD06/0045/0003



Programa INGENIO CONSOLIDER 2010,
FUN-C-FOOD (CSD2007-063)



Fundació
Bosch i Gimpera
Universitat de Barcelona



Generalitat de Catalunya
www.gencat.cat



**Agència
de Gestió d'Ajuts
Universitaris
i de Recerca**

AGAUR

Beca pre-doctoral 2006 FI 01399
Projectes d'Investigació per a potenciar
grups d'investigació consolidats:
2005 SGR 00156; 2009 SGR 724

*A: Alejandro Domínguez Espadas,
per il·luminar-me el camí...
pel seu amor i dedicació tots aquests anys... moltes gràcies!!!*

Agraïments:

Llarg i no sempre fàcil o clar ha estat el camí fins aquí. A vegades un esbarzer inesperat, una pedra inamovible, un pantà aparentment infranquejable o una cruïlla difícil ens fan dubtar, vacil·lar i fins i tot detenir-nos. És llavors quan la mà amiga ens ajuda a treure l'esbarzer, quan l'experiència d'altres ens aconsella com saltar la pedra, com franquejar el pantà o quin camí triar. Sense això no es pot arribar enlloc, no es pot aconseguir cap fita, no es pot viure. A aquestes mans amigues, a aquestes experiències tan valuoses, a aquests desitjos tan sincers d'ajuda, vull adreçar els meus més profunds agraïments amb la certesa d'una gratitud inoblidable i indestructible.

I aquestes mans, experiències i desitjos tenen amos, cossos, noms, y ells són:

- Primer de tot les dues directores d'aquesta tesi, la Dra. Rosa Maria Lamuela Raventós i la Dra. Cristina Andrés Lacueva, per confiar en mi a finals de l'any 2005, últim dia per a sol·licitar les beques FI i en el que tot va passar tan de pressa. A partir de llavors la meua vida va començar a canviar sense jo saber-ho; de cop em vaig veure immers en un món universitari que ja gairebé tenia oblidat. Voldria agrair la meravellosa tasca exercida durant aquests quatre anys i mig, els coneixements transmesos, però sobretot, per fer-me molt més fàcil el camí fins aquí, aconseguint que em sentís un més d'aquest grup de recerca. Sense elles aquesta tesi no s'hagués pogut compondre amb la qualitat que espero haver aconseguit.
- Al Dr. Ramon Estruch pel seu especial interès en els polifenols i la salut, salvaguardant el punt de vista mèdic. Però sobretot pel seu treball ràpid i eficient sempre que l'hem necessitat, portant-lo tot amb aquest bon humor que el caracteritza.
- Als meus pares, per disposar en mi una gran confiança des de les meves primeres passes per la vida, deixant-me lliure el camí en la recerca de la veritat; recolzant-me en totes les meves decisions; per ensenyar-me a estimar en silenci... Per ser com són.

- A Alejandro Domínguez Espadas, per ser el meu company tots aquests anys, cuidant-me i aconsellant-me, compartint llargues nits davant l'ordinador, unes vegades distraient la meva atenció i altres intentant formar aquest trencaclosques.
- Al meu germanet estimat Adonis, per seguir les meves passes allà on jo estigués, per exigir cada vegada més i no descansar fins a estar al meu costat, per saber buscar el millor de mi. Al meu germà Rafe, perquè tot i ser tan callat sempre ha estat aquí. A la meva germana Eglexis, per somiar al meu costat des de petits i donar-nos compte que seríem inseparables.
- A les meves primeres però mai oblidades companyes de laboratori: la Dra. Maria Lourdes Mata-Bilbao (Lulú), la Dra. Elena Roura i la meva especial amiga Anna Barrionuevo, per no dubtar a l'hora de mostrar-me els secrets guardats en carpetes o arxius, però que van ser molt millor compresos amb paraules, fent-me recordar aquelles classes de la Universitat de La Habana en aquell moment oblidades.
- Al Dr. Raúl Zamora-Ros, per aquelles llargues xerrades sobre estadística, que creia interminables, però que em van servir per endinsar-me en el món de la bioestadística, a més de comprendre que havia de llegir-me aquells totxos interminables. A la Dra. Mireia Urpí-Sardá, al Dr. Rafael Llorach, a Maria Rotchés, María Boto, Anna Vallverdú, Anna Tresserra, Palmira Valderas, Nasiruddin Khan, Gemma Chiva, Paola Quifer, Rosa Vázquez, Montse Rabassa, Miriam, Sara, Giuseppe, Sonia, Marta, Nacho i Leandro Cotos companys de grup i laboratori. Els que, més que suportat, m'han patit al llarg d'aquests anys i els que s'han convertit en autèntics companys de treball; alguns des del principi, altres una mica més recentment.
- A la Dra. Elvira López, la Dra. Susana Buxaderas, el Dr. Joan Bosch, la Dra. Montse Riu, la Dra. Estefanía Vichy, Joan Gallardo, Arnau Serra, María Ángeles, Paula, la Dra. Carolina Moltó, Mariluz Latorre i la resta

de companys del departament; per fer més agradable i divertit el camí, per acollir-me en aquest departament com un més.

- A Daniel Tomás Hechavaría el meu més fidel amic des de fa moltíssims anys, per donar suport a totes les meves decisions sense qüestionar res, per seguir-me fins a aquesta ciutat tan meravellosa, encara quan jo estava a punt de marxar, per plorar al meu costat quan ho hem necessitat, per no titubejar davant les adversitats.
- A Yoel Monzón Monzón, pels seus innumbrables esforços des de la distància, per apostar per la meua carrera professional des del dia en que ens vam conèixer, però sobre totes les coses per la seva amistat incondicional, moltes gràcies amic.
- A Rodrigo Izquierdo, pel seu meravellós treball en disseny final, hi ha qui ha nascut per això... altres ens limitem a idolatrar l'art dels magnífics.
- A Ricard Trench, Alberto Amador, Anabel Amador, Angelito, Juan José Burgos-Bosch, Erick Douglas Elliot, Rap Ramírez, Víctor, Iván Cruella, Raúl García, José Diego, José Antonio, Pedrito, Stefan Marxer, Brian Riordan i Ezequiel Barzabal. Per cada granet de sorra, cada pedra o maó posat en els fonaments d'aquest castell, per brindar-me la seva ajuda il·limitada, per estar a prop en els bons moments i en els dolents, donant-me l'alè necessari per a seguir endavant.
- A Maria i Joan Rotchés pel seu especial interès en què aquest manuscrit sigui llegit en català, però sobretot pel magnífic treball realitzat en el poc temps que els he deixat, moltes gràcies!!
- A les institucions públiques, sense les quals la ciència en aquests temps no seria possible, al Ministerio de Ciencia e Innovación, al Centro de Investigaciones Biomédicas en Red, Fisiopatología de la Obesidad y Nutrición, del *Instituto de Salud Carlos III*, a la Generalitat de Catalunya, la Agencia de Gestión de ayudas Universitarias y de Investigación (AGAUR), a la Fundació "Bosch i Gimpera" i la Universitat de Barcelona, pel suport econòmic aportat, tan a nivell de projectes d'investigació com

a través de la beca pre-doctoral concedida i que ha fet possible la meva dedicació completa per a la realització d'aquesta tesi doctoral.

- No podria acabar sense fer menció a part a tot el Departament de Nutrició i Bromatologia, de la Facultat de Farmàcia de la Universitat de Barcelona els que em van obrir les portes de les seves instal·lacions i em van ajudar en la mesura de les seves possibilitats i els meus requeriments.

A tots els demás i als -que no he anomenat sense cap intenció manifesta- arribin els meus més profunds agraïments i els meus millors desitjos de que la molt bona sort els acompanyi sempre.

Abreviatures

AHA: American Heart Association

AHS: Adventist Health Study

AUC: Àrea sota la corba

Cmax: Concentració màxima en plasma

COMT: Catecol-O-metil transferasa

CURES: Chennai Urban Rural Epidemiological Study

DASH: Dietary Approaches-a-STOP-Hypertension

DM: Dieta mediterrània

DMT: Dieta mediterrània tradicional

ECV: Malaltia cardiovascular

F&V: Fruïtes, verdures i hortalisses

FFQ: Qüestionari de freqüència de consum

GAE: Equivalent d'àcid gàl·lic

HDL: Lipoproteïna d'alta densitat

HPLC: Cromatografia líquida d'alta eficàcia

HT: Hidroxitirosol.

IAM: Infart agut de miocardi

IC: Interval de confiança

IWHS: Iowa Women's Health Study

LC-MS/MS: Cromatografia líquida acoblada a un detector de masses en tàndem

LDL: Lipoproteïna de baixa densitat

MAX: Mode d'intercanvi aniònic

Med-frutos secs: Dieta mediterrània-fruits secs

Med-VOO: Dieta mediterrània-oli d'oliva verge

NHS: Nurses' Health Study

PA: Pressió arterial

PAF: Risc relatiu poblacional

PREDIMED: PREvenció amb Dieta MEDiterrània

PUFA: Àcids grassos poliinsaturats

RR: Risc relatiu

SPE: Extracció en fase sòlida

SULT: Sulfotransferasses

SUN: Seguiment Universitat de Navarra

TAC: Capacitat antioxidant total

TCA: Taules de composició d'aliments

TP: Polifenols totals

TPE: Polifenols totals excretats

UDP: Uridina difosfat

UNESCO: Organització de les Nacions Unides per a l'Educació, la Ciència i la Cultura

VOO: Oli d'oliva verge

Índex

1. INTERÈS.....	1
2. OBJECTIUS.....	5
3. ANTECEDENTS BIBLIOGRÀFICS	9
3.1 COMPOSTOS FENÒLICS	9
3.1.1 Química, classificació i distribució de polifenols en els aliments. Metodologia analítica per a la seva determinació en aliments i plantes	9
 Capítol de llibre “ <i>Phenolic Compounds: Chemistry and Occurrence in Fruits and Vegetables</i> ”; del llibre “ <i>Fruit and Vegetable Phytochemicals: Chemistry, Nutritional Value and Stability</i> ”. Pàgines 53-88; Cristina Andrés-Lacueva, <u>Alexander Medina-Remón</u> , Rafael Llorach, Mireia Urpi-Sarda, Nasiruddin Khan, Gemma Chiva-Blanch, Raul Zamora-Ros, Maria Rotchés-Ribalta, Rosa M. Lamuela-Raventós. Copyright © 2010 Blackwell Publishing.	
DOI:10.1002/9780813809397.ch2US:http://dx.doi.org/10.1002/9780813809397.ch2	
3.2 BIODISPONIBILITAT DELS COMPOSTOS FENÒLICS.....	52
3.2.1 Generalitats	52
3.2.2 Factors que afecten la biodisponibilitat y farmacocinètica dels polifenols	57
3.2.2.1 Els aliments com matriu	57
3.2.2.2 Altres factors que poden afectar la biodisponibilitat	59
3.2.2.3 Efecte del processat dels aliments	60
3.3 EFECTES BENEFICIOSOS DELS POLIFENOLS	63
3.3.1 Radicals lliures i envelliment.....	63
3.3.2 Aterosclerosi, lipoproteïnes i plaquetes	66
3.3.3 Estudis epidemiològics. Malalties cardio i cerebro vasculars....	70

3.4. BIOMARCADORS	74
3.4.1. Enquestes alimentàries.....	74
3.4.2. Característiques dels marcadors nutricionals o biològics	75
3.4.3. Avantatges dels marcadors biològics respecte a l'estimació dietètica o marcadors dietètics.....	77
3.5. DIETA MEDITERRÀNIA	79
3.5.1. L'estudi PREDIMED.....	87
3.5.2. Dieta mediterrània i malalties.....	88
 4. RESULTATS.....	 93
4.1 Publicació 1: Mètode ràpid de Folin-Ciocalteu per a la determinació de compostos fenòlics totals urinari, com a biomarcador del consum total de polifenols ingerits, utilitzant plaques de 96 cartutxos per a extracció en fase sòlida.....	94
<i>Alexander Medina-Remón, Ana Barrionuevo-González, Raúl Zamora-Ros, Cristina Andrés-Lacueva, Ramón Estruch, Miguel-Ángel Martínez-González, Javier Díez-Espino and Rosa M. Lamuela-Raventós. Analítica Chimica Acta. 2009, 634(1): 54-60</i>	
4.2 Publicació 2: Distribució normal de l'excreció urinària de polifenols en adolescents masculins i canvis associats amb la intervenció nutricional de suc de tomàquet (<i>Lycopersicon esculentum</i>).....	104
<i>Laila Hussein, Alexander Medina, Anna Barrionuevo, Rosa Maria Lamuela-Raventós, Cristina Andres-Lacueva. International Journal of Food Sciences and Nutrition. 2009, 60(4): 302-311</i>	
4.3 Publicació 3: Excreció de polifenols totals i pressió arterial en subjectes amb un alt risc cardiovascular	117
<i>Medina-Remón A., Zamora-Ros R., Rotchés-Ribalta M., Andres-Lacueva C., Martínez-González M.A., Covas M.I., Corella D., Salas-Salvadó J., Gómez-Gracia E., Ruiz-Gutiérrez V., García de la Corte F.J., Fiol M., Pena M.A., Saez G.T., Ros E., Serra-Majem L., Pinto X., Warnberg J., Estruch R. and Lamuela-Raventós R.M. Nutrition Metabolism and Cardiovascular Diseases. 2009, doi:10.1016/j.numecd.2009.10.019</i>	

4.4 Publicació 4: Consum de polifenols i pressió arterial..... **128**

Alexander Medina-Remón, Ramón Estruch, Cristina Andres-Lacueva, and Rosa-Maria Lamuela-Raventos. *Archives of Biochemistry and Biophysics* (En proceso de revisión)

4.5 Publicació 5: El seguiment d'un patró de dieta mediterrània suplementada amb fruits secs o oli d'oliva verge, incrementa l'excreció de polifenols totals, produint una disminució significativa de la pressió arterial. (El PREDIMED després d'un any d'intervenció)..... **146**

Medina-Remón A., Estruch R., Andres-Lacueva C., Martínez-González M.A., Covas M.I., Corella D., Salas-Salvadó J., Gómez-Gracia E., Ruiz-Gutiérrez V., García de la Corte F.J., Fiol M., Pena M.A., Saez G.T., Ros E., Serra-Majem L., Pinto X., Warnberg J. and Lamuela-Raventos R.M. (En proceso de revisión)

5. DISCUSSIÓ GLOBAL..... **177**

6. CONCLUSIONS..... **185**

7. BIBLIOGRAFIA **189**

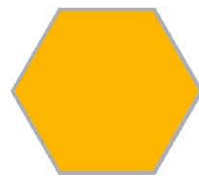
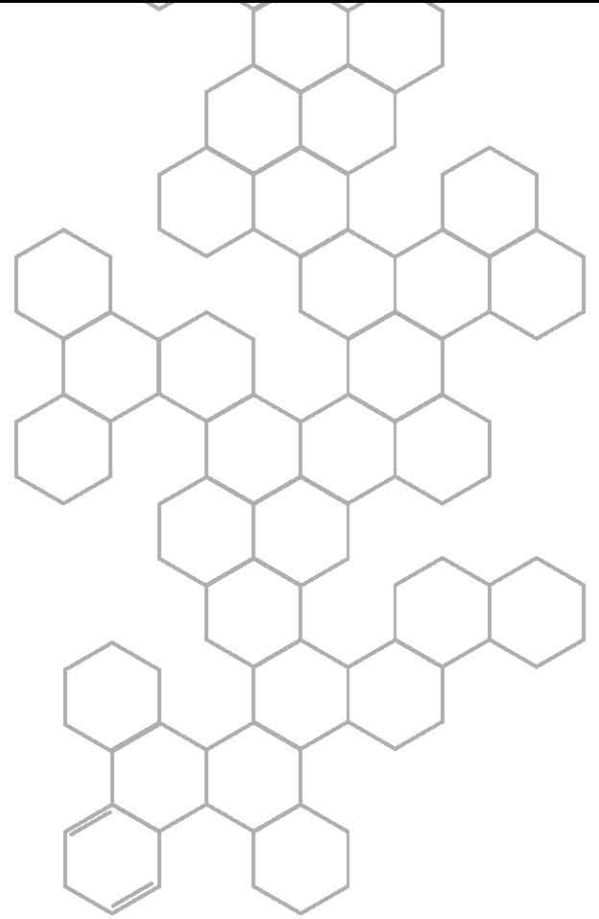
8. ANNEXES **205**

8.1 ALTRES PUBLICACIONS EN REVISTES **205**

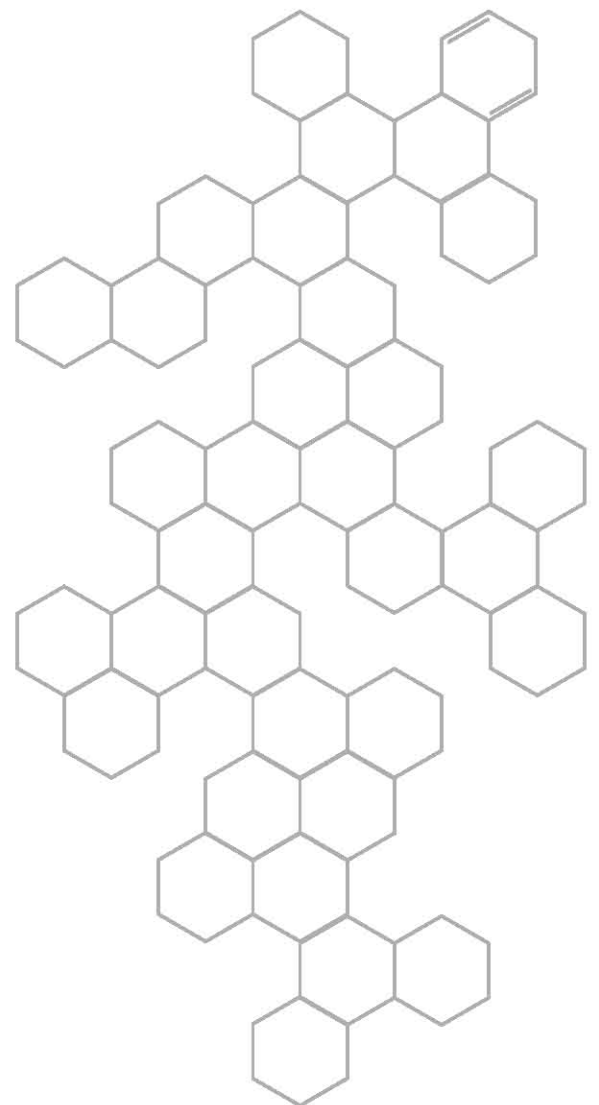
Publicació 6: Perfil de metabolits del plasma i orina en humans després del consum de polifenols de l'ametlla [*Prunus dulcis* (Mill.) D.A. Webb]..... **205**

Mireia Urpi-Sarda; Ignacio Garrido; María Monagas; Carmen Gómez-Cordovés; Alexander Medina-Remón; Cristina Andres-Lacueva; Begoña Bartolomé. J Agric.Food Chem. 2010, 57 (21): 10134-10142 Erratum in: J Agric Food Chem. 2009 Dec 23;57(24):11581-2.

8.2 COMUNICACIONS EN CONGRESSOS..... **215**



INTERÈS



1. Interès

La hipertensió és el problema més important de salut pública i la principal causa de mort i discapacitat en els països desenvolupats. Una quarta part de la població adulta del món pateix hipertensió; de manera que afecta aproximadament a mil milions de persones, causant 7.6 milions de morts prematures (Lawes et al. 2008).

La prevenció de la mateixa és un dels principals objectius en salut pública. La resposta a aquest repte ha d'impulsar una major promoció dels esforços de sensibilització, estudis de factors de risc de la hipertensió, i l'avaluació de l'impacte dels canvis d'estil de vida.

Una dieta saludable com la Mediterrània (Estruch et al. 2006) o la dieta DASH (Dietary Approaches-a-STOP-Hypertension) (Appel et al. 1997) i millorar l'estil de vida com per exemple, augmentant l'activitat física o reduint el pes corporal són factors que poden actuar en la disminució de la pressió arterial (Mancia et al. 2007). Les dietes mediterrània i DASH, riques en fruites, verdures i hortalisses (F&V), són riques en compostos fitoquímics antioxidants que confereixen propietats beneficioses en la prevenció de malalties associades a l'estrès oxidatiu (Alonso et al. 2004; Harnden et al. 2010).

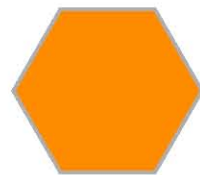
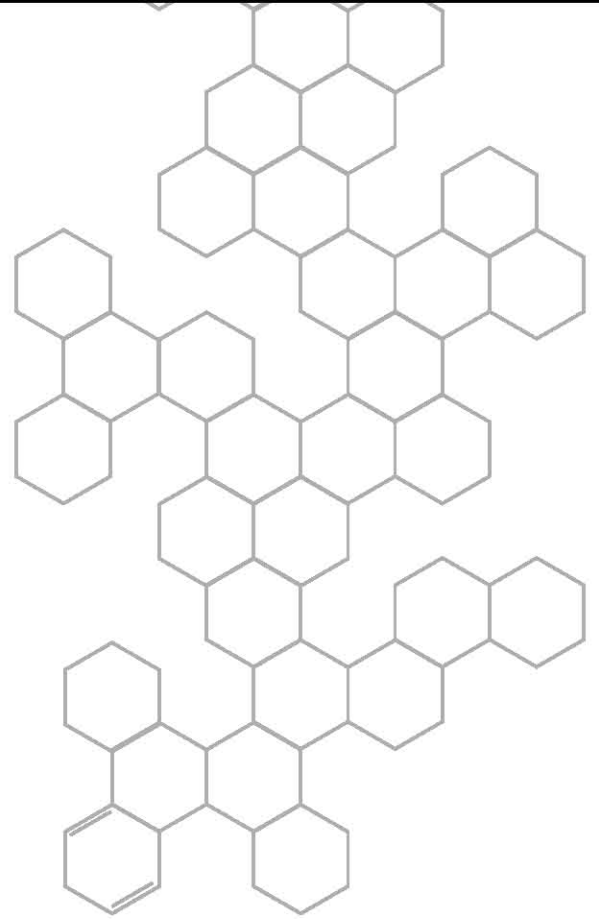
Diversos estudis epidemiològics relacionen de forma inversa el consum d'aliments rics en polifenols com el cacau, F&V, te, oli d'oliva verge, nous o vi i el risc d'hipertensió (Agudo et al. 2007; Alonso et al. 2004; Covas et al. 2001; Covas et al. 2006; Grassi et al. 2005; Manach et al. 2005a). L'elevat consum de peix i productes lactis baixos en greix, també pot reduir el risc d'hipertensió (Alonso et al. 2005; Bao et al. 1998; Engberink et al. 2009). Pel contrari, un elevat consum de cereals refinats, carn o productes càrnics s'associen amb un major risc cardiovascular (Appel et al. 1997; Mancia et al. 2007). No obstant això, a part del sodi i potasi ingerits en la dieta, fins ara hi ha pocs estudis sobre els efectes dels components de la dieta en la pressió arterial (PA), el consum d'àcid oleic s'ha correlacionat positivament amb una menor prevalència hipertensió (Alonso et al. 2006; Perona et al. 2004) a més de la vitamina C o altres compostos antioxidants (Guxens et al. 2009; Rodrigo et al. 2008).

Interès

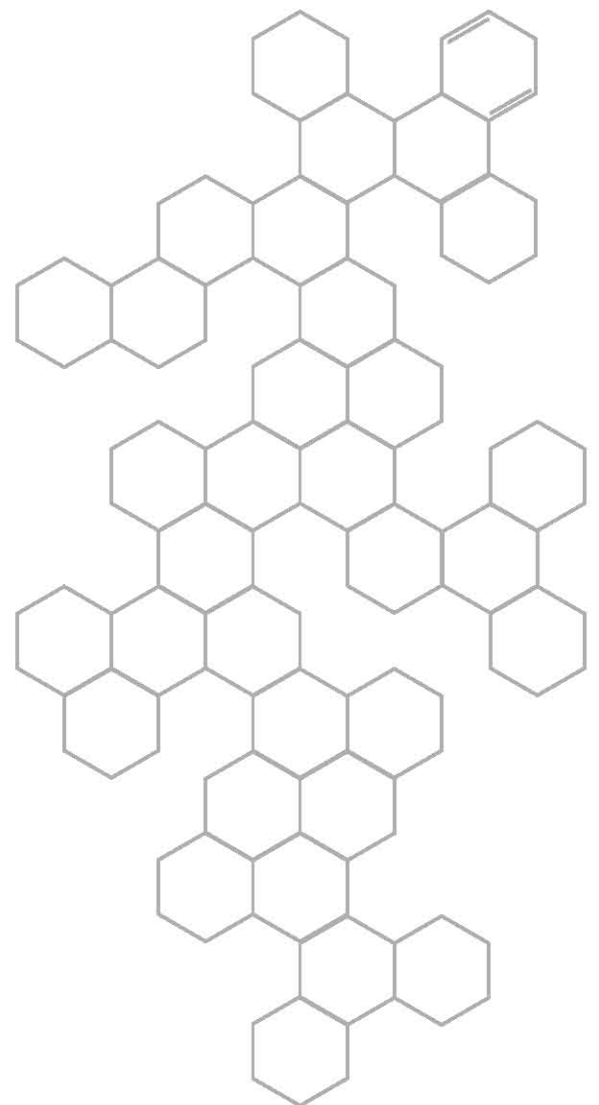
Els polifenols són quantitativament la principal font dietètica d'antioxidants, tanmateix, els efectes biològics d'aquests compostos depenen de la seva biodisponibilitat, la seva cinètica i el temps d'exposició (Roura et al. 2007a). Els polifenols més freqüents en la dieta humana no són necessàriament els més actius *in vivo*, ja sigui perquè tenen una menor activitat intrínseca, perquè són difícils d'absorbir a l'intestí o perquè són ràpidament metabolitzats i eliminats (Manach et al. 2004).

Un cop absorbits, els polifenols es metabolitzen en l'organisme, formant metabòlits que podran mantenir i/o modificar substancialment l'activitat biològica dels compostos de partida. La major part dels polifenols ingerits amb la dieta (75-99%) no s'excreten en orina, i les quantitats detectades varien d'un compost fenòlic a un altre (Scalbert and Williamson 2000). Aquest fet pot ser degut a la disminució de l'absorció a través del tracte gastrointestinal, la seva excreció amb la bilis o la seva metabolització per la microbiota del còlon o pels propis teixits. És per això que per a observar o detectar altes concentracions en plasma o orina es requereix la ingestió contínua d'aliments rics en polifenols (van het Hof et al. 1999).

Fins ara, cap estudi previ ha determinat l'excreció de polifenols totals en mostres d'orina preses de forma puntual, com biomarcador de la ingesta total de polifenols de la dieta, per a correlacionar aquest biomarcador amb la pressió arterial en pacients d'alt risc cardiovascular.



OBJECTIUS



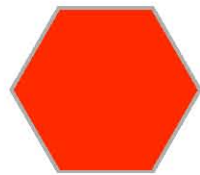
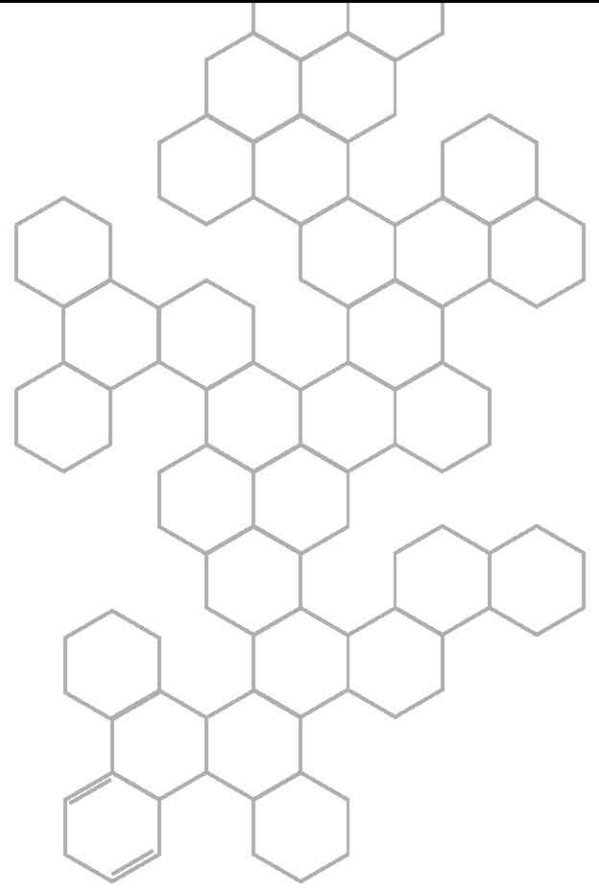
2. Objectius

Objectius principals.

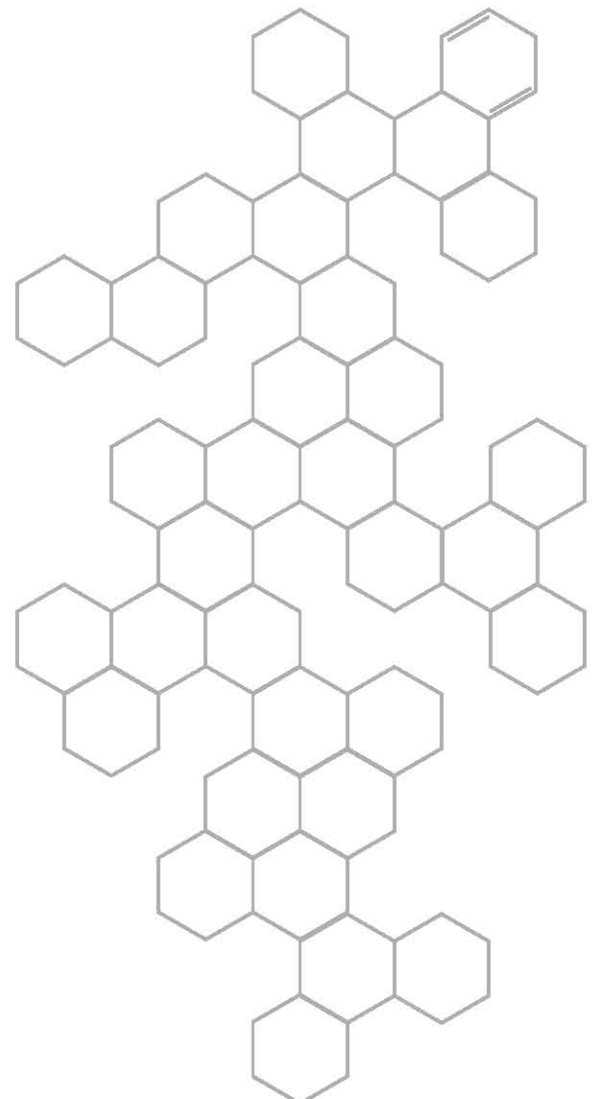
1. Posar a punt i validar la metodologia per a l'anàlisi dels polifenols totals en mostres d'orina.
2. Correlacionar el consum de polifenols totals mesurat mitjançant la seva excreció en orina amb el diagnòstic d'hipertensió arterial i les xifres de PA sistòlica i diastòlica

En concret, aquesta tesi té els següents objectius específics:

1. Desenvolupar i validar un mètode sensible, específic, ràpid i senzill per a la quantificació de polifenols totals en mostres d'orina.
2. Correlacionar la ingesta d'aliments rics en polifenols determinada mitjançant una enquesta de freqüència de consum amb l'excreció de polifenols totals en orina determinada mitjançant una metodologia validada en diferents estudis clínics transversals i d'intervenció, amb l'objectiu de poder utilitzar aquest paràmetre com a biomarcador del consum d'aquests compostos.
3. Correlacionar l'excreció urinària de polifenols totals amb la incidència de hipertensió en una població adulta d'alt risc cardiovascular.
4. Valorar si una ingesta d'aliments rics en polifenols, avaluada a través de l'excreció de polifenols totals, s'associa amb les xifres de pressió arterial sistòlica i diastòlica en una població adulta amb alt risc cardiovascular.
5. Avaluar si la dieta mediterrània suplementada amb fruits secs o oli d'oliva verge es correlacionen positivament amb els polifenols totals excretats en les mostres d'orina puntual, i si aquest increment s'associa amb les xifres de pressió arterial sistòlica i diastòlica, en una població mediterrània d'avançada edat i alt risc cardiovascular.



ANTECEDENTS BIBLIOGRÀFICS



3. Antecedents Bibliogràfics

3.1 COMPOSTOS FENÒLICS.

3.1.1 Química, classificació i distribució de polifenols en els 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, s'exposen en format de capítol de llibre *"Phenolic compounds. Chemistry and occurrences in fruit and vegetables"* Editat a Blackwell Publishing, 2010; del llibre *"Fruit and Vegetable Phytochemicals: Chemistry, Nutritional Value and Stability"*

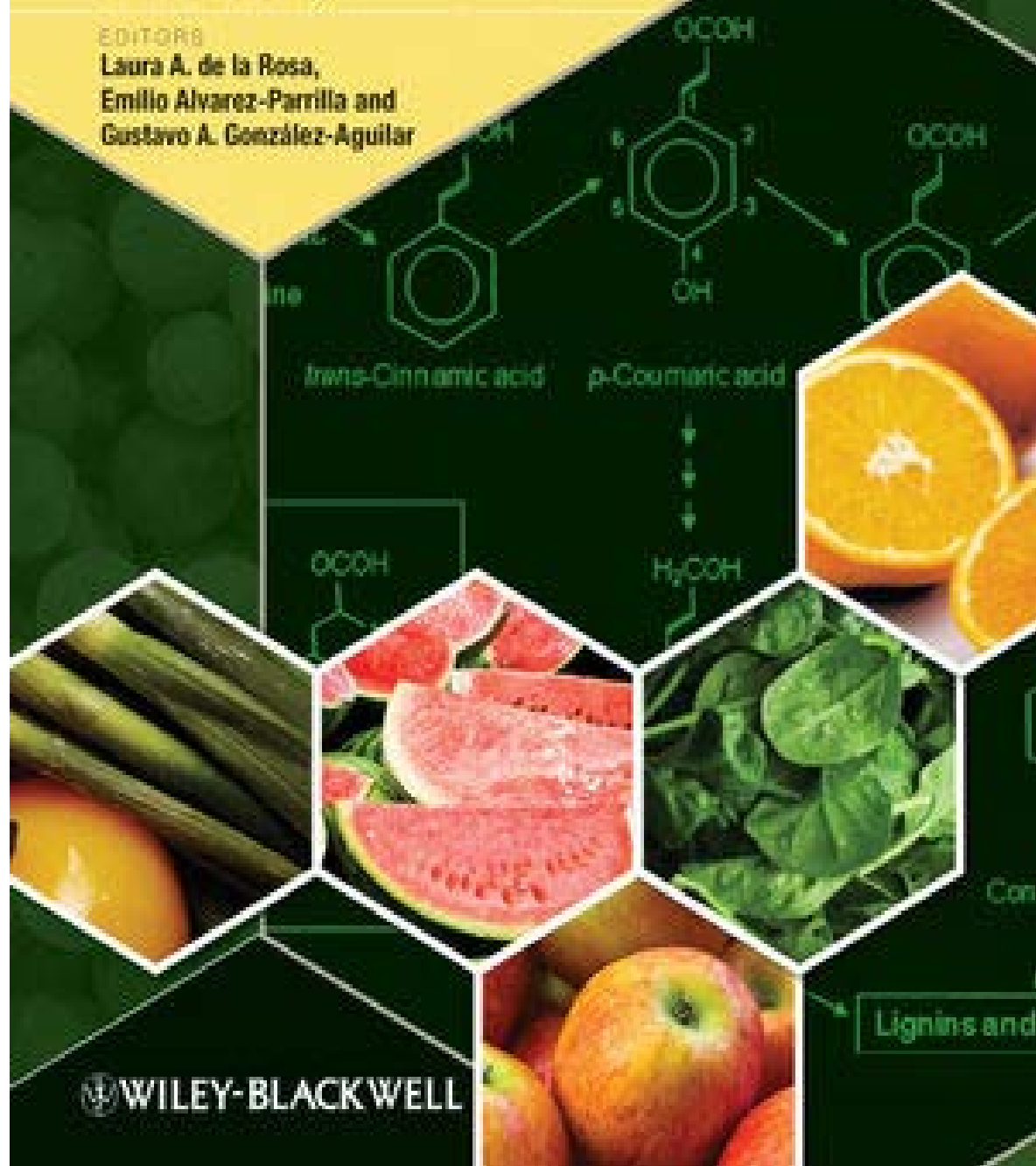
Cristina Andrés-Lacueva, Alexander Medina-Remón, Rafael Llorach, Mireia Urpi-Sarda, Nasiruddin Khan, Gemma Chiva-Blanch, Raul Zamora-Ros, Maria Rotchés-Ribalta, Rosa M. Lamuela-Raventós.

Fruit and Vegetable Phytochemicals

Chemistry, Nutritional Value and Stability

EDITORS

**Laura A. de la Rosa,
Emilio Alvarez-Parrilla and
Gustavo A. González-Aguilar**



Fruit and Vegetable Phytochemicals

**Chemistry, Nutritional Value,
and Stability**

Fruit and Vegetable Phytochemicals

**Chemistry, Nutritional Value,
and Stability**

Laura A. de la Rosa

Emilio Alvarez-Parrilla

Gustavo A. González-Aguilar

 **WILEY-BLACKWELL**

A John Wiley & Sons, Inc., Publication

Edition first published 2010
© 2010 Blackwell Publishing

Blackwell Publishing was acquired by John Wiley & Sons in February 2007. Blackwell's publishing program has been merged with Wiley's global Scientific, Technical, and Medical business to form Wiley-Blackwell.

Editorial Office

2121 State Avenue, Ames, Iowa 50014-8300, USA

For details of our global editorial offices, for customer services, and for information about how to apply for permission to reuse the copyright material in this book, please see our website at www.wiley.com/wiley-blackwell.

Authorization to photocopy items for internal or personal use, or the internal or personal use of specific clients, is granted by Blackwell Publishing, provided that the base fee is paid directly to the Copyright Clearance Center, 222 Rosewood Drive, Danvers, MA 01923. For those organizations that have been granted a photocopy license by CCC, a separate system of payments has been arranged. The fee codes for users of the Transactional Reporting Service are ISBN-13: 978-0-8138-0320-3/2010.

Designations used by companies to distinguish their products are often claimed as trademarks. All brand names and product names used in this book are trade names, service marks, trademarks or registered trademarks of their respective owners. The publisher is not associated with any product or vendor mentioned in this book. This publication is designed to provide accurate and authoritative information in regard to the subject matter covered. It is sold on the understanding that the publisher is not engaged in rendering professional services. If professional advice or other expert assistance is required, the services of a competent professional should be sought.

Library of Congress Cataloging-in-Publication Data

de la Rosa, Laura A.

Fruit and vegetable phytochemicals : chemistry, nutritional value and stability /
Laura A. de la Rosa, Emilio Alvarez-Parrilla, Gustavo A. González-Aguilar. – 1. ed.
p. cm.

Includes bibliographical references and index.

ISBN 978-0-8138-0320-3 (hardback : alk. paper)

1. Phytochemicals. 2. Polyphenols. 3. Carotenoids. 4. Fruit—Analysis. 5. Vegetables—
Analysis. I. Alvarez-Parrilla, Emilio. II. González-Aguilar, Gustavo A. III. Title.

QK898.P764R67 2009

615'.321—dc22

2009031846

A catalog record for this book is available from the U.S. Library of Congress.

Set in 10/12pt Times by Aptara® Inc., New Delhi, India
Printed in Singapore

1 2010

Contents

Contributors	vii
Preface	xi
Chapter 1. The Contribution of Fruit and Vegetable Consumption to Human Health <i>Elhadi M. Yahia</i>	3
Chapter 2. Phenolic Compounds: Chemistry and Occurrence in Fruits and Vegetables <i>Cristina Andrés-Lacueva, Alex Medina-Remon, Rafael Llorach, Mireia Urpi-Sarda, Nasiruddin Khan, Gemma Chiva-Blanch, Raul Zamora-Ros, Maria Rotches-Ribalta, and Rosa M. Lamuela-Raventós</i>	53
Chapter 3. Synthesis and Metabolism of Phenolic Compounds <i>Mikal E. Saltveit</i>	89
Chapter 4. Enzymatic and Nonenzymatic Degradation of Polyphenols <i>José Manuel López-Nicolás, and Francisco García-Carmona</i>	101
Chapter 5. Chemistry of Flavonoids <i>Rong Tsao and Jason McCallum</i>	131
Chapter 6. Flavonoids and Their Relation to Human Health <i>Alma E. Robles-Sardin, Adriana Verónica Bolaños-Villar, Gustavo A. González-Aguilar, and Laura A. de la Rosa</i>	155
Chapter 7. Chemistry, Stability, and Biological Actions of Carotenoids <i>Elhadi M. Yahia and José de Jesús Ornelas-Paz</i>	177
Chapter 8. Dietary Fiber and Associated Antioxidants in Fruit and Vegetables <i>Fulgencio Saura-Calixto, Jara Pérez-Jiménez, and Isabel Goñi</i>	223

vi Contents

Chapter 9.	Emerging Technologies Used for the Extraction of Phytochemicals from Fruits, Vegetables, and Other Natural Sources <i>Marleny D. A. Saldaña, Felix M. C. Gamarra, and Rodrigo M. P. Siloto</i>	235
Chapter 10.	Methods of Analysis of Antioxidant Capacity of Phytochemicals <i>Nuria Grigelmo-Miguel, M^a Alejandra Rojas-Graü, Robert Soliva-Fortuny, and Olga Martín-Belloso</i>	271
Chapter 11.	Phytochemical Changes in the Postharvest and Minimal Processing of Fresh Fruits and Vegetables <i>Gustavo A. González-Aguilar, J. Fernando Ayala-Zavala, Laura A. de la Rosa, and Emilio Alvarez-Parrilla</i>	309
Chapter 12.	Quality Loss of Fruits and Vegetables Induced by Microbial Growth <i>Saul Ruiz Cruz and Sofia Arvizu-Medrano</i>	341
Index		357

2 Phenolic Compounds: Chemistry and Occurrence in Fruits and Vegetables

Cristina Andrés-Lacueva*, Alex Medina-Remon, Rafael Llorach, Mireia Urpi-Sarda, Nasiruddin Khan, Gemma Chiva-Blanch, Raul Zamora-Ros, Maria Rotches and Rosa M. Lamuela-Raventós

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 2.1, the flavonoids group comprises the compounds with a C6-C3-C6 structure: flavanones, flavones, dihydroflavonols, flavonols, flavan-3-ols, anthocyanidins, isoflavones, and proanthocyanidins. The nonflavonoids group is classified according to the number of carbons that they have (Figure 2.2) and comprises the following subgroups: simple phenols, benzoic acids, hydrolyzable tannins, acetophenones and phenylacetic acids, cinnamic acids, coumarins, benzophenones, xanthenes, 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 (see Figure 2.1).

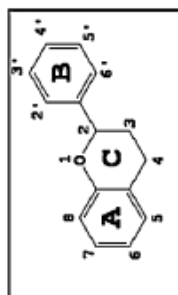
Flavonoids in plants usually occur glycosylated mainly with glucose or rhamnose, but they can also be linked with galactose, arabinose, xylose, glucuronic acid, or other sugars. The number of glycosyl moieties usually varies from one to three; nevertheless, flavonoids have been identified with four and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

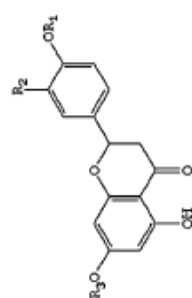
Flavanones are represented by the saturated three-carbon chain and an oxygen atom in the C4 position.

* Corresponding author: Cristina Andrés-Lacueva. Associate Professor, Nutrition and Food Science Department, Pharmacy School, University of Barcelona. Av Joan XXIII s/n, Barcelona 08028 (Spain).

FLAVONOIDS

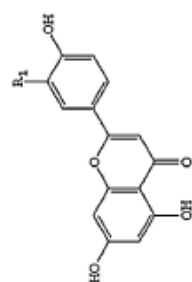


Flavanones



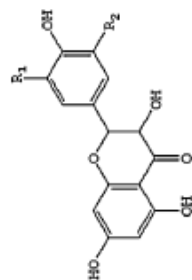
Naringenin: $R_1=H$, $R_2=H$, $R_3=H$, $R_4=H$
 Hesperetin: $R_1=CH_3$, $R_2=OH$, $R_3=H$

Flavones



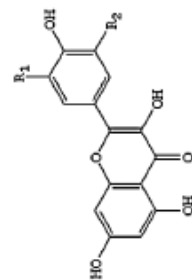
Apigenin: $R_1=H$, $R_2=OH$
 Luteolin: $R_1=OH$, $R_2=OH$

Dihydroflavonols



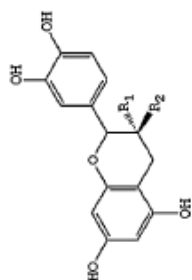
Dihydrokaempferol: $R_1=H$, $R_2=H$, $R_3=H$
 Dihydroquercetin: $R_1=OH$, $R_2=H$, $R_3=H$
 Dihydromyricetin: $R_1=OH$, $R_2=OH$, $R_3=OH$

Flavonols



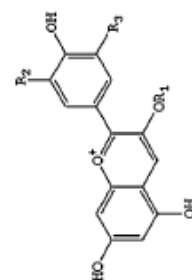
Kaempferol: $R_1=H$, $R_2=H$, $R_3=H$
 Quercetin: $R_1=OH$, $R_2=H$, $R_3=H$
 Myricetin: $R_1=OH$, $R_2=OH$, $R_3=OH$

Flavan-3-ols



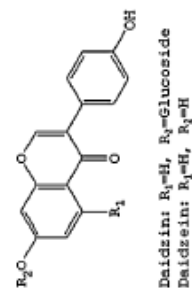
(-)-Epicatechin: $R_1=OH$, $R_2=H$, $R_3=H$
 (+)-Catechin: $R_1=H$, $R_2=OH$, $R_3=H$

Anthocyanidins



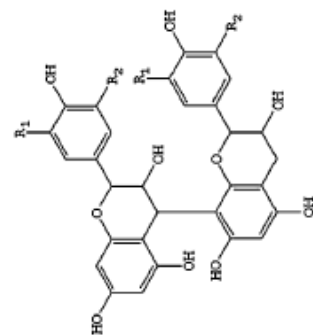
Cyanidin: $R_1=H$, $R_2=OH$, $R_3=H$
 Pelargonidin: $R_1=H$, $R_2=H$, $R_3=H$
 Peonidin: $R_1=H$, $R_2=H$, $R_3=OCH_3$

Isoflavones



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

Proanthocyanidins



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

Figure 2.1. Chemical structures of flavonoids.

NONFLAVONOIDS

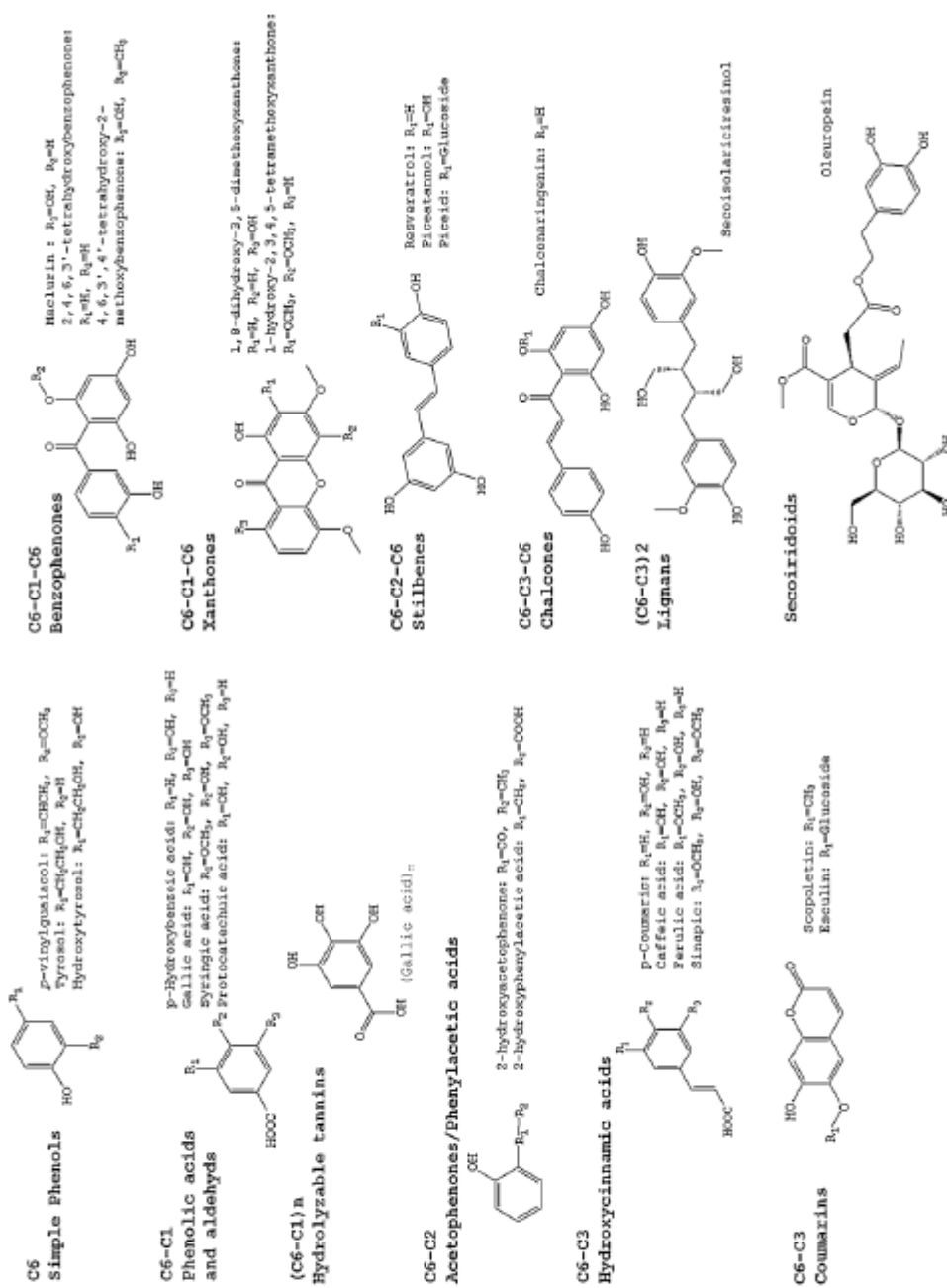


Figure 2.2. Chemical structures of nonflavonoids.

56 Fruit and Vegetable Phytochemicals

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

Anthocyanins are based on the flavylum salt structure and are water-soluble pigments in plants. They are 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, which 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 2000a).

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.

Nonflavonoids

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, such as alcoholic chains, in their structure. *Phenolic acids* (C6-C1) with the same structure as simple phenols have a carboxyl 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 *xanthenes* 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-oxanthracene. More than 500 xanthenes are currently known to exist in nature, and approximately 50 of them are found in the mangosteen with prenyl substituents.

Stilbenes have a 1,2-diphenylethylene as their basic structure (C6-C2-C6). Resveratrol, the most widely 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. *Trans* forms can be isomerized to *cis* forms by UV radiation (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 isomerase 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, aryl-naphthalene, 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 best-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).

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 oxidases (PPO) and peroxidases (POD), are considered important factors in the process of phenolic oxidation (Tomás-Barberán and Espín 2001). Processes such as 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 catechin 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 to the identification and quantification of the phenolic compound of fruit

58 *Fruit and Vegetable Phytochemicals*

and vegetables. Asami and others (2003) found that the total phenolic content of freeze-dried samples of marionberries and strawberries was higher than that of the air-dried samples. In this context, frozen samples should be thawed before phenolic extraction. This procedure could provoke a loss of phenolic compounds because some of them show 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 Forthcoming) 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 20 hr at 20°C 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, and 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, such as gallic acid, to highly polymerized phenolics, such as procyanidins and tannins. Also, from the quantitative point of view, the profile 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 genistein and daidzein, which are restricted to the Fabaceae (e.g., soya; 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 extensively 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 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 a 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 Hall (2008) studied the influence of the different solvents (water, ethanol, and acetone), and mixtures of them, on the extraction of phenolic compounds 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 (resveratrol 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 10 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 caffeoylquinic 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 that could facilitate the extraction from the matrix, and a negative effect, due to the higher temperature that 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-methylphenol (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 using reversed-phase C₁₈-bonded silica gel as stationary phase. Elution systems are

60 *Fruit and Vegetable Phytochemicals*

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 used, could drastically affect the separation of phenolic compounds and also further ionization.

Mass Spectrometry Methods for Identification and Quantification

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 CE, gas chromatography (GC), and liquid chromatography (LC), including HPLC and UPLC (ultra performance liquid chromatography).

An important part of the mass spectrometry methods are the ionization sources and the analyzers. Several ionization sources are available. Among these, electrospray ionization (ESI) is used the most because of the wide range of molecules that it covers. Other important sources are those related to atmospheric pressure ionization, including atmospheric-pressure photoionization (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 protons (negative ionization) or by a gain of protons (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).

Various 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, whereas the ion trap has been used for both 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).

Mass spectrometry applied to characterization of phenolic compounds has been widely 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 mobilities 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, a 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 nonvolatile. Samples used in GC are heavily processed before being ready for analysis. This process includes cleanup, solid-phase extraction, and often a derivatization process. Derivatization is carried out to generate a volatile phenolic compound. A variety of reagents are used for derivatization, 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)-trifluoroacetamide (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, GC-MS studies have been carried out using a flame ionization detector (FID); however, in recent years there have also been studies that used electron impact ionization. Additionally, an important variability has been shown concerning the chromatographic conditions, including different kinds of columns and temperature range. The author reported 12 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 vinifera*, 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 regard to the source ionization, it seems that ESI is used more frequently than other sources, such as APCI or APPI. Another important aspect of this technique is the ionization of phenolic compounds. Negative ionization seems to be more suitable

62 *Fruit and Vegetable Phytochemicals*

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 fragments 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 MSⁿ experiments using an ion trap (Kang and others 2007). In this context, de Rijke and others (2003) carried out a study with 15 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 the precursor ion and one of its daughter fragments, is used to characterize a particular compound. This behavior 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 2005, 2007). 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 past few years an important challenge in 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 glycosylation 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 2004, 2005; 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) interglycosidic linkages and to distinguish among the flavonoid isomers with two glucoses, three glucoses, and four glucoses. These authors have proposed the LC-MSⁿ as a powerful tool to characterize the *O*-glycosylated *C*-glycosyl flavones. 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-MSⁿ for the characterization of phenolic acids, predominantly chlorogenic acids (Clifford and others 2003, 2005, 2006a,b,c). For example, using LC-MS³ 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 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 cocrystallized 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 cocrystallization, the laser is fired and the matrix absorbs energy and allows a soft ionization of the samples. Afterward 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 soya products 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 Methods for Identification and Quantification

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 ^1H NMR, ^{13}C NMR, correlation spectroscopy (COSY), heteronuclear chemical shift correlation NMR (C-H HECTOR), nuclear Overhauser effect in the

64 *Fruit and Vegetable Phytochemicals*

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 and selective, and it 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 behavior. 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 280 nm, hydroxycinnamic acids at 320 nm, flavonols, flavones, and dihydroflavonols at 365 nm, and anthocyanins at 520 nm (Ibern-Gómez and others 2002; Merken Hand Beecher 2000). Hydrolyzable tannins show a characteristic shoulder at 300 nm, suitable for identifying them (Arapitsas and others 2007). For stilbenes, maximum absorbance of *trans*-forms are at 306 nm and at 285 nm for *cis*-forms (Lamuela-Raventós and others 1995).

Spectrophotometric Assays for the Determination of Specific Phenolic Groups

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 they may determine a specific phenolic substance such as sinapine (Ismail 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-colored 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 Proanthocyanidins**Vanillin Assay**

The vanillin method is based on the condensation of the vanillin reagent with proanthocyanidins 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 behavior in acidic media; anthocyanins exist in these media as an equilibrium between the colored oxonium ion and the colorless pseudobase form. Using an average extinction coefficient, the total content of anthocyanins may be estimated from the absorption of the total extracts at 520 nm (Moskowitz and Hrazdina 1981).

Determination of Flavan-3-ols**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 the enzyme-linked immunosorbent assay (ELISA), a method has been developed to quantify phenolic compounds such as isoflavones (Vergne and others 2007).

Occurrence

Polyphenols represent a wide variety of diverse structures from different subclasses, which 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 (>180 mg of gallic acid equivalent (GAE)/100 g fresh weight (FW)); the vegetables with the highest concentration are artichokes, parsley, and Brussels sprouts (>250 mg of GAE/100 g 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 490 mg/100 g FW) (US Department of Agriculture 2007a). Other abundant sources (ranging between 10 and 100 mg/100 g 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, at 30 (Lamuela-Raventós and others 2001), 4.5, and 3.1 mg/100 mL 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 2.1). In many other vegetables and fruits, quercetin is frequently present in low concentrations around 0.1 and 5 mg/100 g FW (US Department of Agriculture 2007a). Vegetables (0.1–26.7 mg/100 g FW) and some spices, such as chives, tarragon, and fennel (6.5–19 mg/100 g FW), are characteristic sources of kaempferol, whereas fruits are a poor source (down to 0.1 mg/100 g) (US Department of Agriculture 2007a). Myricetin, which is the third most abundant flavonol, is found in some spices, such as parsley, fennel, and oregano

Table 2.1. Flavonoids in fruits and vegetables

Subclass	Polyphenol	Food	Content mg/100 g FW	Reference
Flavonols	Quercetin	Onions	21.4	US Department of Agriculture 2007a
		Asparagus	12.4	
		Berries	10.7	
		Lettuce	7.1	
	Kaempferol	Kale	26.7	US Department of Agriculture 2007a
		Endive	10.1	
		Spinach	7.6	
		Berries	0.4	
	Myricetin	Berries	5.7	US Department of Agriculture 2007a
		Grape	0.4	
		Red cabbage	0.2	
	Isorhamnetin	Onions	5.0	US Department of Agriculture 2007a
		Pears	0.3	
Flavones	Apigenin	Artichokes	4.7	US Department of Agriculture 2007a
		Celery	2.3	
		Red onion	0.4	
		Lettuce	0.16	
	Luteolin	Pepper	5.0	US Department of Agriculture 2007a
		Artichokes	2.3	
		Red grape	1.3	
		Oranges	1.1	
Flavonones	Eriodictyol	Lemon, raw	21.4	US Department of Agriculture 2007a
		Lemon, juice	4.9	
		Orange, juice	0.2	
	Hesperetin	Lime, raw	43.0	US Department of Agriculture 2007a
		Lemon, raw	27.9	
		Orange, raw	27.3	
		Citric fruit, juice	10.5	
	Naringenin	Grapefruit, raw	21.3	US Department of Agriculture 2007a
		Orange, raw	15.3	
		Artichokes, raw	12.5	
		Ripe tomatoes, raw	0.7	
Isoflavones	Genistein	Soya bean	64.8	US Department of Agriculture 2007b
		Soya milk	6.1	
		Tofu	13.3	
	Daidzein	Soya bean	34.5	US Department of Agriculture 2007b
		Soya milk	4.5	
		Tofu	8.5	
	Glycitein	Soya bean	13.8	US Department of Agriculture 2007b
		Soya milk	0.6	
		Tofu	2.3	

68 Fruit and Vegetable Phytochemicals

Table 2.1. (Continued)

Subclass	Polyphenol	Food	Content mg/100 g FW	Reference
Flavan-3-ols	Catechin	Peaches	12.2	US Department of Agriculture 2007a
		Berries	11.2	
		Red grape	10.1	
		Bananas	6.1	
	Epicatechin	Red grape	8.7	US Department of Agriculture 2007a
		Apricot	5.5	
		Apples	5.5	
	Epigallocatechin	Peaches	1.1	US Department of Agriculture 2007a
		Apples	1.0	
		Berries	0.6	
	Epicatechin 3 gallate	Red grape	2.8	US Department of Agriculture 2007a
		Plums	0.8	
		Apples/Pears	0.01	
	Epigallocatechin 3-gallate	Apples	0.5	US Department of Agriculture 2007a
		Berries	0.6	
		Plums	0.4	
	Gallocatechin	Berries	0.5	US Department of Agriculture 2007a
		Plums	0.1	
		Pomegranate/ persimmons	0.2	
Proantho- cyanidins	Dimers	Berries	9.5	Gu and others 2004; US Department of Agriculture 2004
		Plums	30.1	
		Apples	11.3	
		Peaches/apricot/ nectarines	6.2	
		Kiwi	1.1	
	Trimers	Berries	7	Gu and others 2004; US Department of Agriculture 2004
		Plums	20.9	
		Apples	7.1	
		Peaches/apricot/ nectarines	2.3	
		Kiwi	0.9	
	4-6mers	Berries	23.5	Gu and others 2004; US Department of Agriculture 2004
		Plums	57.8	
		Apples	24.5	
		Peaches/apricot/ nectarines	9.5	
		Kiwi	3.2	

Table 2.1. (Continued)

Subclass	Polyphenol	Food	Content mg/100 g FW	Reference
Antho- cyanidins	7–10mers	Berries	21.9	Gu and others 2004; US Department of Agriculture 2004
		Plums	33.8	
		Apples	20.8	
		Peaches/apricot/ nectarines	5.6	
		Kiwi	2.6	
	Polymers	Berries	151.7	Gu and others 2004; US Department of Agriculture 2004
		Plums	57.3	
		Apples	29.7	
		Peaches/apricot/ nectarines	10.1	
		Kiwi	0	
	Cyanidin	Berries	189.9	US Department of Agriculture 2007a
		Plums	12.0	
		Red cabbage	72.9	
	Delphinidin	Berries	97.9	US Department of Agriculture 2007a
		Eggplant	13.8	
		Red grape	3.7	
	Malvidin	Blueberries	61.4	US Department of Agriculture 2007a
		Red grape	34.7	
	Pelargonidin	Strawberries	31.3	US Department of Agriculture 2007a
		Radish	25.7	
	Petunidin	Berries	33.9	US Department of Agriculture 2007a
		Red grapes	2.1	
	Peonidin	Berries	21.5	US Department of Agriculture 2007a
		Cherries	4.5	
		Red grape	2.9	

(2–19.8 mg/100 g FW), and it is also present in brewed tea (0.5–1.6 mg/100 mL FW) and red wine (0–9.7 mg/100 mL 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 0.2 mg/100 g FW (see Table 2.1). Isorhamnetin is the least abundant flavonol; it has been detected in a few foods, such as some spices: fennel 9.3 mg/100 g FW, chives 5.0–8.5 mg/100 g FW, tarragon 5 mg/100 g FW; in almonds it ranged between 1.2 and 10.3 mg/100 g FW (US Department of Agriculture 2007a). In vegetables and fruits it is only present in onions and pears (see Table 2.1).

70 *Fruit and Vegetable Phytochemicals***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 25 to 630 mg/100 g 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 (see Table 2.1). However, luteolin is present in both vegetables and fruits (see Table 2.1).

Flavanones

Citric fruits, both raw and as derived products, such as juices and jams, are the main sources of flavanones (Manach and others 2004). In lesser concentrations we also found eriodictyol in almonds (0.03–0.6 mg/100 g FW), hesperitin in mint (0–21.9 mg/100 g FW), and naringenin in artichokes and ripe tomatoes (0–22.9 and 0–1.5 mg/100 g 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. Eriodictyol is the characteristic flavonone of lemon and lemon juice (see Table 2.1). 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). Soya bean and its processed products, such as soya milk, tofu, tempeh, and miso, are the main source of genistein, daidzein, and glycerin (US Department of Agriculture 2007b). We can also find isoflavones in lower concentrations in beans and broadbeans (0.01 to 0.04 mg/100 g 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 colors of most fruits and vegetables. Berries are the main dietary source of anthocyanidins (66.8–947.5 mg/100 g FW) (US Department of Agriculture 2007a). Other fruits, such as red grapes, 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 and 150 mg/100 g FW (US Department of Agriculture 2007a). Anthocyanidins are poorly distributed (<10 mg/100 g 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 in berries, where they are present in both skin and flesh (Manach and others 2004).

Berries, such as blueberries, bilberries, and black currants, 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 (see Table 2.1).

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 1.5 mg/100 g 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 (0–70 mg/100 mL FW), cocoa powder (19.7–127.7 mg/100 g FW) (Andrés-Lacueva and others 2008), red wine (0.2–55.6 mg/100 mL FW), nuts (0–4 mg/100 g FW), beer (0–10 mg/100 mL FW), and fruits (US Department of Agriculture 2007a). Catechin and epicatechin are present in many fruits at concentrations of 0.5–3 and 0.5–6 mg/100 mL FW, respectively (see Table 2.1). Epigallocatechin, epicatechin 3-gallate, epigallocatechin 3-gallate, and galocatechin are found in several fruits, such as berries, red grapes, plums, apples, and peaches, normally at very low concentrations (less than 1 mg/100 g FW) (see Table 2.1); however, they are present in high amounts in tea (6.8–395 mg/100 g FW) (US Department of Agriculture 2007a). Chocolate is also a good source of epigallocatechin. Theaflavin (0–5.3 mg/100 mL FW), thearubigins (7.8–139 mg/100 mL FW), theaflavin 3-3'-digallate (0–4.9 mg/100 mL FW), theaflavin 3'-gallate (0–4.1 mg/100 mL FW), and theaflavin 3-gallate (0–3.2 mg/100 mL FW) are flavan-3-ols that are detected only 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, 8084 mg/100 g FW, and sorghum, 3937 mg/100 g FW. Other important sources of PAs are beans, red wine, nuts, and chocolate, their content ranging between 180 and 300 mg/100 g FW. In fruits, berries and plums are the major sources, with 213.6 and 199.9 mg/100 g FW, respectively. Apples and grapes are intermediate sources of PAs (60 to 90 mg/100 g FW), and the content of PAs in other fruits is less than 40 mg/100 g FW. In the majority of vegetables PAs are not detected, but they can be found in small concentrations in Indian squash (14.8 mg/100 g FW) (Gu and others, 2004; US Department of Agriculture, 2004).

Table 2.1 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.

Nonflavonoids

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-flavor 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 the 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 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, whereas those of tyrosol are not (Visioli and Galli 1998).

Phenolic acids

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

Benzoic Aldehydes

Benzoic aldehydes mainly cover syringaldehyde and vanillin. Natural vanilla is prepared from the seeds (beans) of *Vanilla planifolia*, which may contain some 21 mg/100 g FW total phenols, including the major components vanillin (19.4 mg/100 g FW), 4-hydroxybenzaldehyde (1 mg/100 g FW), and vanillic acid (0.4 mg/100 g FW) (Clifford 2000b). 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

19.75 µg/100 mL for syringaldehyde, coniferaldehyde, sinapaldehyde, and vanillin, respectively (Panossian and others 2001).

Hydrolyzable Tannins

The potent antioxidant properties of pomegranate juice have been attributed to its high content of punicalagin isomers, unique ellagitannins that can reach levels exceeding 200 mg/100 mL juice (Cerdà and others 2003a,b). Total hydrolyzable tannins in barley flour, oak wood, and green tea were determined spectrophotometrically as 870, 1120, and 590 mg/100 g, respectively (Taubert and others 2005). Grape seed extract has a high tannin content of 535.6 mg/100 g (Ahn 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 ± 0.66 mg/100 g (Ahn and others 2002). Although citrus fruits do not themselves contain tannins, orange-colored juices often contain food dyes with tannins. Apple juice, grape juice, and berry juices are all high in tannins. Most legumes contain tannins. Red-colored beans contain the most tannins, and white-colored 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 soya bean made with the strain *Bacillus licheniformis* as a starter, but has not been present in extracts of nonfermented soya bean. The phenylacetic acid produced by *Bacillus licheniformis* during the fermentation of soya bean is one of the main compounds of antimicrobial activity of Chungkook-Jang, a traditional Korean fermented-soya 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-dihydroxycinnamic), 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 2.2). 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 800–2000 mg/100 g DW. It is found chiefly in the outer part of the grain, in the *trans* form, which is transformed into arabinoxylans and

Table 2.2. Nonflavonoids in foods

Subclass	Polyphenol	Foods	Content (mg/100g FW)	Reference
Phenolic alcohols	Tyrosol	Olive oils	0.29–2.44 [£]	De la Torre-Carbot and others 2005
	Hydroxytyrosol	Olive oils	0.70–6.35 [£]	De la Torre-Carbot and others 2005
Volatile phenols	Eugenol	Passion fruit	92	Chassagne and others 1997
		Passion fruit juice	172	Chassagne and others 1997
		Passion fruit peel	0.0035	Chassagne and others 1997
		Wines	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
	Ellagic acid	Strawberries	0.4–2.9	Da Silva-Pinto and others 2007
Phenolic (benzoic) acids		Muscadine grapes	66.7	Pastrana-Bonilla and others 2003
		Muscadine grapes	8.6	Pastrana-Bonilla and others 2003
	Gallic acid	White currant	1.8	Maatta and others 2001
	4-Hydroxybenzoic acid	Redcurrant	0.3	Maatta and others 2001
	Protocatechuic acid	Raspberry	6–10	Macheix and Fleuriet 1990
	Syringaldehyde	Ripe walnut fruit	33.83	Colaric and others 2005
	Vanillin	Orange	0.02	Goodner and others 2000
		Tangerine	0.35	
		Lemon	0.041	
		Lime	0.035	
Benzoic aldehydes		Grapefruit juice	0.06	

Hydrolyzable tannins	Ellagitannins	Pomegranate juice	156.1 ^f	Seeram and others 2006
		Strawberry	19.8 ± 0.2	Mertens-Talcott and others 2003
		Blueberry	0.9 ± 0.1	
		Raspberry	17.9 ± 0.3	
		Blackberry	42.4 ± 0.4	
		Longan seed	23.3	Soong and Barlow 2006
		Longan seed	156	Soong and Barlow 2006
		Mango kernel	1550	Berardini and others 2004
Hydroxycinnamic acids	Gallotannins	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
	<i>p</i> -Coumaric acid	Orange juices	7.9–4.46	Rapisarda and others 1998
		Citrus ^g	1.8–19.3 ^h	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
		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
	Caffeic acid	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
		Blueberries	3.02–16.97	Sellappan and others 2002
		Blackberries	2.99–3.51	Sellappan and others 2002
		Cranberries	0.8–8.8	Zuo and others 2002

Table 2.2. (Continued)

Subclass	Polyphenol	Foods	Content (mg/100g FW)	Reference
Hydroxycinnamates (esters of hydroxy- cinnamic acids)	Sinapic acid	Orange juices	3.07–6.37 ^g	Rapisarda and others 1998
		Citrus ^g	3.6–158 ^h	Bocco and others 1998
		Carrot	1.57	Kang and others 2008
		Beet root	1.3–14.3	Ng and others 1998
		Coffee drink	9	Mattila and Kumpulainen 2002
		Broccoli	105.9	Yeh and Yen 2005
		Eggplant	93.6	Yeh and Yen 2005
		Asparagus	46.1	Yeh and Yen 2005
		Cranberries	0–21.18	Zuo and others 2002
		Orange juices	0.78–3.59 ^g	Rapisarda and others 1998
	Chlorogenic acids	Citrus ^g	3.0–95.4 ^h	Bocco and others 1998
		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
Coumarins	<i>trans</i> -Resveratrol	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 ^h	Guerrero and others 2001
		Tomato	8.5	Buta and Spaulding 1997
		Noni fruit juice	6100 ^g	Suroño and others 2008
Stilbenes		Red grapes	0.25	Zamora-Ros and others 2007

Chalcones	<i>trans</i> -Piceid	White grapes	0.07	Zamora-Ros and others 2007
		Red grapes	0.06	
		White grapes	0.025	
		Cherry tomatoes	15.3	
Dihydro-chalcones	Chalconaringenin	Apples	2.1	Slimestad and Verheul 2005
		Apples	1	Burda and others 1990
		<i>Brassica</i>	0.599	Burda and others 1990
		Kale	0.972	Milder and others 2005
Lignans	Phloretin xylogalactoside	Apricot	0.105	
		Strawberry	0.117	
		Peach	0.080	
		Pear	0.155	
	Pinoresinol	<i>Brassica</i>	0.1691	Milder and others 2005
		Kale	0.315	
		Apricot	0.314	
		Strawberry	0.212	
	Secoisolarici-resinol	Peach	0.186	
		Pear	0.034	
		<i>Brassica</i>	0.019	
		Pear	0.004	
	Matairesinol	Kale	0.038	Milder and others 2005
		Strawberry	0.005	
		Peach	0.027	
		Apricot	0.031	
Secoiridoids	Oleuropein aglycon	<i>Brassica</i>	0.012	Milder and others 2005
		Olive fruit	1.45	

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

78 Fruit and Vegetable Phytochemicals

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 major sources of ferulic acid. Low concentrations of ferulic acid have been reported in blueberries, blackberries, cranberries, orange juice, carrots, potatoes, beetroot, apples, coffee, and others (see Table 2.2). *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 in some cranberry varieties. However, the contents in these fruits are clearly higher than in orange juice (see Table 2.2).

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 2006a,b,c). Coffee drink, apples, prunes, pears, and sweet cherries are the highest sources of chlorogenic acids. Moderately good values are found in sour cherries, apricots, nectarines, peaches, quince, and potatoes (see Table 2.2). 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 16.3 mg/100 g FW, less than that found in the Occidental pear (30.9 mg/100 g 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 6 days in cold storage varies between 7 and 30 mg/100 g FW (Truong and others 2007). For dry lyophilized potato powder, chlorogenic acid levels range from 3.28 to 637 mg/100 g FW (Im and others 2008). Steam-cooked potato strips retain 42% of the initial chlorogenic acid, whereas frying preserves only 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 300 mg/100 g FW (Clifford 2000b). A new coumarin, isoschinallyl, was isolated from the fruits of *Poncirus trifoliata* Raf. (Xu 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 (700 mg/100 g), cassia leaf oil (up to 8730 mg/100 g), and lavender oil. Coumarin is also found in fruits (e.g., bilberries and cloudberries), green tea, and other foods, such as chicory (Lake 1999). Simple coumarins (esculetin and scopoletin) may also be present in carrots, but in some cases the roots seem to be free of the furanocoumarins (Mercier and others 1993). Parsnip root may contain some 4 mg/

100 g total furanocoumarins (Wawrzynowicz and Waksmundzka Hajnos 1990). Celery may contain a significant amount (up to 8.5 mg/100 g) but has a very variable quantity of furanocoumarins, with simple coumarins (esculetin and scopoletin) contributing a modest 0.1 mg/100 g (Diawara and others 1995). The total coumarin content (mainly meranzin derivatives) of the flavedo of bitter orange, grapefruit, and pomelo is 710, 250 and 510 mg/100 g, respectively (McHale and others 1987). Scopoletin content is particularly high in noni fruit juice (see Table 2.2).

Benzophenones

Benzophenones are distributed scarcely in foods. They are mainly present in the *Garcinia* genus, such as in fruits of *Garcinia 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 (0–15 mg/100 g FW) (Barreto and others 2008).

Xanthones

A total of 515 xanthones have been identified in 20 families of higher plants, mainly in the Bonnetiaceae and Clusiaceae families (Vieira and Kijjoa 2005). Mangosteen fruit, a typical southeast Asian fruit, is the characteristic dietary source of xanthones (less than 7–8 mg/100 g 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 (see Table 2.2) 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 (0.006 mg/100 g FW), pistachios (0.007 mg/100 g FW), and berries (0.008 mg/100 g FW), but red wines (0.558 mg/100 mL 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 analyses convert 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 (see Table 2.2) 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 (0.29–0.21 mg/100 g), but pinoresinol, lariciresinol, and matairesinol are also present in substantial amounts (0.55–3.32 mg/100 g). Lignan content in beverages (wine, beer, tea, coffee) has been found as follows: South African red wine (91.3 µg/100 mL), French red wine (78.9 µg/100 mL), Ceylonese black tea (77.1 µg/100 mL), English-blend black tea (71.2 µg/100 mL), coffee (18–31 µg/100 mL), soya milk (37.7 µg/100 mL), chocolate milk (2.2 µg/100 mL) (Milder and others 2005). Lignans are also found in *Brassica* vegetables, kale, and fruits such as apricot, strawberry, peach, and pear (see Table 2.2).

Secoiridoids

Oleuropein (the most abundant bitter principle) and its analog ligstroside, both secoiridoid biophenols, were extracted from Hojiblanca black olives (Piperno and others 2004). Secoiridoids are present exclusively in plants of the Oleraceae family (Tripoli and others 2005). Oleuropein is present in high amounts (6000 ± 90 mg/100 g 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).

References

- Aaby K, Hvattum E and Skrede G. 2004. Analysis of flavonoids and other phenolic compounds using high-performance liquid chromatography with coulometric array detection: relationship to antioxidant activity. *J Agric Food Chem* 52(15):4595–4603.
- Ahn HS, Jeon TI, Lee JY, Hwang SG, Lim Y and Park DK. 2002. Antioxidative activity of persimmon and grape seed extract: *in vitro* and *in vivo*. *Nutr Res* 22: 1265–1273.
- Andrés-Lacueva C, Lamuela-Raventós RM, Jauregui O, Casals I, Izquierdo-Pulido M and Permanyer J. 2000. An LC method for the analysis of cocoa phenolics. *LC GC Eur* 13: 902–904.
- Andrés-Lacueva C, Monagas M, Khan N, Izquierdo-Pulido M, Urpi-Sarda M, Permanyer J and Lamuela-Raventós RM. 2008. Flavanol and flavonol contents of cocoa powder products: influence of the manufacturing process. *J Agric Food Chem* 56(9):3111–3117.
- Arapitsas P, Menichetti S, Vincieri FF and Romani A. 2007. Hydrolyzable tannins with the hexahydroxydiphenoyl unit and the m-depsidic link: HPLC-DAD-MS identification and model synthesis. *J Agric Food Chem* 55(1):48–55.
- Arts ICW and Hollman PCH. 1998. Optimization of a quantitative method for the determination of catechins in fruits and legumes. *J Agric Food Chem* 46(12):5156–5162.
- Asami DK, Hong YJ, Barrett DM and Mitchell AE. 2003. Comparison of the total phenolic and ascorbic acid content of freeze-dried and air-dried marionberry, strawberry, and corn grown using conventional, organic, and sustainable agricultural practices. *J Agric Food Chem* 51(5):1237–1241.
- Baggenstoss J, Poisson L, Kaegi R, Perren R and Escher F. 2008. Coffee roasting and aroma formation: application of different time-temperature conditions. *J Agric Food Chem* 56(14):5836–5846.
- Barreto JC, Trevisan MT, Hull WE, Erben G, de Brito ES, Pfundstein B, Wurtele G, Spiegelhalter B and Owen RW. 2008. Characterization and quantitation of polyphenolic compounds in bark, kernel, leaves, and peel of mango (*Mangifera indica* L.). *J Agric Food Chem* 56(14):5599–5610.
- Berardini N, Carle R and Schieber A. 2004. Characterization of gallotannins and benzophenone derivatives from mango (*Mangifera indica* L. cv. 'Tommy Atkins') peels, pulp and kernels by high-performance liquid chromatography/electrospray ionization mass spectrometry. *Rapid Commun Mass Spectrom* 18(19):2208–2216.

- Betés-Saura C, Andrés-Lacueva C and Lamuela-Raventós RM. 1996. Phenolics in white free-run juice and wines from Penedès by high-performance liquid chromatography: changes during vinification. *J Agric Food Chem* 44(10):3040–3046.
- Bocco A, Cuvelier ME, Richard H and Berset C. 1998. Antioxidant activity and phenolic composition of citrus peel and seed extracts. *J Agric Food Chem* 46(6):2123–2129.
- Bradshaw MP, Prenzler PD and Scollary GR. 2001. Ascorbic acid-induced browning of (+)-catechin in a model wine system. *J Agric Food Chem* 49(2):934–939.
- Brat P, George S, Bellamy A, Du CL, Scalbert A, Mennen L, Arnault N and Amiot M J. 2006. Daily polyphenol intake in France from fruit and vegetables. *J Nutr* 136(9):2368–2373.
- Brune M, Rossander L and Hallberg L. 1989. Iron absorption and phenolic compounds: importance of different phenolic structures. *Eur J Clin Nutr* 43(8):547–557.
- Burda S, Oleszek W and Lee CY. 1990. Phenolic compounds and their changes in apples during maturation and cold storage. *J Agric Food Chem* 38(4):945–948.
- Buta JG and Spaulding DW. 1997. Endogenous levels of phenolics in tomato fruit during growth and maturation. *J Plant Growth Regul* 16(1):43–46.
- Carpinella MC, Ferrayoli CG and Palacios SM. 2005. Antifungal synergistic effect of scopoletin, a hydroxycoumarin isolated from *Melia azedarach* L. fruits. *J Agric Food Chem* 53(8):2922–2927.
- Cassidy A, Handley B and Lamuela-Raventós RM. 2000. Isoflavones, lignans and stilbenes – origins, metabolism and potential importance to human health. *J Sci Food Agric* 80(7):1044–1062.
- Cerdá B, Cerón JJ, Tomás-Barberán FA and Espín JC. 2003a. Repeated oral administration of high doses of pomegranate ellagitannin punicalagin to rats for 37 days is not toxic. *J Agric Food Chem* 51(11):3493–3501.
- Cerdá B, Llorach R, Cerón JJ, Espín JC and Tomás-Barberán FA. 2003b. Evaluation of the bioavailability and metabolism in the rat of punicalagin, an antioxidant polyphenol from pomegranate juice. *Eur J Nutr* 42(1):18–28.
- Chassagne D, Crouzet J, Bayonove CL and Baumes RL. 1997. Glycosidically bound eugenol and methyl salicylate in the fruit of edible *Passiflora* species. *J Agric Food Chem* 45(7):2685–2689.
- Chen H, Zuo Y and Deng Y. 2001. Separation and determination of flavonoids and other phenolic compounds in cranberry juice by high-performance liquid chromatography. *J Chromatogr A* 913(1–2):387–395.
- Clifford MN. 2000a. Anthocyanins—nature, occurrence and dietary burden. *J Sci Food Agric* 80(7):1063–1072.
- Clifford MN. 2000b. Miscellaneous phenols in foods and beverages—nature, occurrence and dietary burden. *J Sci Food Agric* 80(7):1126–1137.
- Clifford MN, Johnston KL, Knight S and Kuhnert N. 2003. Hierarchical scheme for LC-MSⁿ identification of chlorogenic acids. *J Agric Food Chem* 51(10):2900–2911.
- Clifford MN, Knight S, Kuhnert N. 2005. Discriminating between the six isomers of dicaffeoylquinic acid by LC-MSⁿ. *J Agric Food Chem* 53(10):3821–3832.
- Clifford MN, Knight S, Surucu B and Kuhnert N. 2006a. Characterization by LC-MSⁿ of four new classes of chlorogenic acids in green coffee beans: dimethoxycinnamoylquinic acids, diferuloylquinic acids, caffeoyl-dimethoxycinnamoylquinic acids, and feruloyl-dimethoxycinnamoylquinic acids. *J Agric Food Chem* 54(6):1957–1969.
- Clifford MN, Marks S, Knight S and Kuhnert N. 2006b. Characterization by LC-MSⁿ of four new classes of *p*-coumaric acid-containing diacyl chlorogenic acids in green coffee beans. *J Agric Food Chem* 54(12):4095–4101.
- Clifford MN, Zheng W and Kuhnert N. 2006c. Profiling the chlorogenic acids of aster by HPLC-MSⁿ. *Phytochem Anal* 17(6):384–393.
- Colaric M, Veberic R, Solar A, Hudina M and Stampar F. 2005. Phenolic acids, syringaldehyde, and juglone in fruits of different cultivars of *Juglans regia* L. *J Agric Food Chem* 53(16):6390–6396.
- Coward L, Smith M, Kirk M and Barnes S. 1998. Chemical modification of isoflavones in soyfoods during cooking and processing. *Am J Clin Nutr* 68(6):1486S–1491S.
- Cui T, Nakamura K, Ma L, Li JZ and Kayahara H. 2005. Analyses of arbutin and chlorogenic acid, the major phenolic constituents in oriental pear. *J Agric Food Chem* 53(10):3882–3887.
- Cuyckens F and Claeys M. 2002. Optimization of a liquid chromatography method based on simultaneous electrospray ionization mass spectrometric and ultraviolet photodiode array detection for analysis of flavonoid glycosides. *Rapid Commun Mass Spectrom* 16(24):2341–2348.

82 Fruit and Vegetable Phytochemicals

- Cuyckens F and Claeys M. 2004. Mass spectrometry in the structural analysis of flavonoids. *J Mass Spectrom* 39(1):1–15.
- Cuyckens F and Claeys M. 2005. Determination of the glycosylation site in flavonoid mono-*O*-glycosides by collision-induced dissociation of electrospray-generated deprotonated and sodiated molecules. *J Mass Spectrom* 40(3):364–372.
- Cuyckens F, Shahat AA, Van den Heuvel H, Abdel-Shafeek KA, El-Messiry MM, Seif-El Nasr MM, Pieters L, Vlietinck AJ and Claeys M. 2003. The application of liquid chromatography-electrospray ionization mass spectrometry and collision-induced dissociation in the structural characterization of acylated flavonol *O*-glycosides from the seeds of *Carrichtera annua*. *Eur J Mass Spectrom* 9(4):409–420.
- Czerny M and Grosch W. 2000. Potent odorants of raw arabica coffee. Their changes during roasting. *J Agric Food Chem* 48(3):868–872.
- Da Silva Pinto M, Lajolo FM and Genovese MI. 2007. Bioactive compounds and antioxidant capacity of strawberry jams. *Plant Foods Hum Nutr* 62(3):127–131.
- De la Torre-Carbot K, Jauregui O, Gimeno E, Castellote AI, Lamuela-Raventós RM and Lopez-Sabater M. 2005. Characterization and quantification of phenolic compounds in olive oils by solid-phase extraction, HPLC-DAD, and HPLC-MS/MS. *J Agric Food Chem* 53(11):4331–4340.
- de Rijke E, Out P, Niessen WM, Ariese F, Gooijer C and Brinkman UA. 2006. Analytical separation and detection methods for flavonoids. *J Chromatogr A* 1112(1–2):31–63.
- de Rijke E, Zappey H, Ariese F, Gooijer C and Brinkman UA. 2003. Liquid chromatography with atmospheric pressure chemical ionization and electrospray ionization mass spectrometry of flavonoids with triple-quadrupole and ion-trap instruments. *J Chromatogr A* 984(1):45–58.
- Devanand LL. 2006. Significance of sample preparation in developing analytical methodologies for accurate estimation of bioactive compounds in functional foods. *J Sci Food Agric* 86(14):2266–2272.
- Diawara MM, Trumble JT, Quirós CF and Hansen R. 1995. Implications of distribution of linear furanocoumarins within celery. *J Agric Food Chem* 43(3):723–727.
- Donovan JL, Meyer AS and Waterhouse AL. 1998. Phenolic composition and antioxidant activity of prunes and prune juice (*Prunus domestica*). *J Agric Food Chem* 46(4):1247–1252.
- Escribano-Bailón M and Santos-Buelga C. 2003. Polyphenol extraction from foods. In: Santos-Buelga C, Williamson G, editors. *Methods in Polyphenol Analysis*. Cambridge: The Royal Society of Chemistry, pp. 1–12.
- Es-Safi NE, Meudec E, Bouchut C, Fulcrand H, Ducrot PH, Herbet G and Cheynier V. 2008. New compounds obtained by evolution and oxidation of malvidin 3-*O*-glucoside in ethanolic medium. *J Agric Food Chem* 56(12):4584–4591.
- Esti M, Cinquanta L and La Notte E. 1998. Phenolic compounds in different olive varieties. *J Agric Food Chem* 46(1):32–35.
- Fernandez de Simon B, Cadahia E and Jalocho J. 2003. Volatile compounds in a Spanish red wine aged in barrels made of Spanish, French, and American oak wood. *J Agric Food Chem* 51(26):7671–7678.
- Ferreres F, Gil-Izquierdo A, Andrade PB, Valentão P and Tomás Barberán FA. 2007. Characterization of C-glycosyl flavones O-glycosylated by liquid chromatography–tandem mass spectrometry. *J Chromatogr A* 1161(1–2):214–223.
- Ferreres F, Llorach R and Gil-Izquierdo A. 2004. Characterization of the interglycosidic linkage in di-, tri-, tetra- and pentaglycosylated flavonoids and differentiation of positional isomers by liquid chromatography/electrospray ionization tandem mass spectrometry. *J Mass Spectrom* 39(3):312–321.
- Floridi S, Montanari L, Marconi O and Fantozzi P. 2003. Determination of free phenolic acids in wort and beer by coulometric array detection. *J Agric Food Chem* 51(6):1548–1554.
- Folin O, Denis W. 1912. On phosphotungstic-phosphomolybdic compounds as color reagents. *J Biol Chem* 12: 239–243.
- Fulcrand H, Mane C, Preys S, Mazerolles G, Bouchut C, Mazauric JP, Souquet JM, Meudec E, Li Y, Cole RB and Cheynier V. 2008. Direct mass spectrometry approaches to characterize polyphenol composition of complex samples. *Phytochemistry*. Forthcoming.
- Gollücke APB, Catharino RR, de Souza JC, Eberlin MN and de Queiroz Tavares D. 2008. Evolution of major phenolic components and radical scavenging activity of grape juices through concentration process and storage. *Food Chem* 112(4):868–873.

- Gómez-Caravaca AM, Carrasco-Pancorbo A, Cañabate-Díaz B, Segura-Carretero A and Fernández-Gutiérrez A. 2005. Electrophoretic identification and quantitation of compounds in the polyphenolic fraction of extra-virgin olive. *Electrophoresis* 26(18):3538–3551.
- González-Barrio R, Beltrán D, Cantos E, Gil MI, Espín JC and Tomás-Barberán F A. 2006. Comparison of ozone and UV-C treatments on the postharvest stilbenoid monomer, dimer, and trimer induction in var. 'Superior' white table grapes. *J Agric Food Chem* 54(12):4222–4228.
- Goodner KL, Jella P and Rouseff RL. 2000. Determination of vanillin in orange, grapefruit, tangerine, lemon, and lime juices using GC-olfactometry and GC-MS/MS. *J Agric Food Chem* 48(7):2882–2886.
- Gu L, Kelm MA, Hammerstone JF, Beecher G, Holden J, Haytowitz D, Gebhardt S and Prior RL. 2004. Concentrations of proanthocyanidins in common foods and estimations of normal consumption. *J Nutr* 134(3):613–617.
- Guerrero G, Suárez M and Moreno G. 2001. Chlorogenic acids as a potential criterion in coffee genotype selections. *J Agric Food Chem* 49(5):2454–2458.
- Harnly JM, Bhagwat S and Lin LZ. 2007. Profiling methods for the determination of phenolic compounds in foods and dietary supplements. *Anal Bioanal Chem* 389(1):47–61.
- Hartzfeld PW, Forkner R, Hunter MD and Hagerman AE. 2002. Determination of hydrolyzable tannins (gallo-tannins and ellagitannins) after reaction with potassium iodate. *J Agric Food Chem* 50(7):1785–1790.
- Heck CI, Schmalko M and Gonzalez de Mejia E. 2008. Effect of growing and drying conditions on the phenolic composition of mate teas (*Ilex paraguariensis*). *J Agric Food Chem* 56(18):8394–8403.
- Hernández-Montes E, Pollard SE, Vauzour D, Jofre-Montseny L, Rota C, Rimbach G, Weinberg PD and Spencer JPE. 2006. Activation of glutathione peroxidase via Nrf1 mediates genistein's protection against oxidative endothelial cell injury. *Biochem Biophys Res Commun* 346(3):851–859.
- Herrera MC and Luque de Castro MD. 2005. Ultrasound-assisted extraction of phenolic compounds from strawberries prior to liquid chromatographic separation and photodiode array ultraviolet detection. *J Chromatogr A* 1100(1):1–7.
- Hollman PC, van Trijp JM, Mengelers MJ, de Vries JH and Katan M B. 1997. Bioavailability of the dietary antioxidant flavonol quercetin in man. *Cancer Lett* 114(1–2):139–140.
- Huck WC, Stecher G, Scherz H and Bonn G. 2005. Analysis of drugs, natural and bioactive compounds containing phenolic groups by capillary electrophoresis coupled to mass spectrometry. *Electrophoresis* 26(7–8):1319–1333.
- Ibern-Gómez M, Andrés-Lacueva C, Lamuela-Raventós RM and Waterhouse AL. 2002. Rapid HPLC analysis of phenolic compounds in red wines. *Am J Enol Vitic* 53(3):218–221.
- Im HW, Suh BS, Lee SU, Kozukue N, Ohnisi-Kameyama M, Levin CE and Friedman M. 2008. Analysis of phenolic compounds by high-performance liquid chromatography and liquid chromatography/mass spectrometry in potato plant flowers, leaves, stems, and tubers and in home-processed potatoes. *J Agric Food Chem* 56(9):3341–3349.
- Ismail F and Eskin NAM. 1979. A new quantitative procedure for determination of Sinapine. *J Agric Food Chem* 27(4):917–918.
- Kang J, Hick LA and Price WE. 2007. A fragmentation study of isoflavones in negative electrospray ionization by MSⁿ ion trap mass spectrometry and triple quadrupole mass spectrometry. *Rapid Commun Mass Spectrom* 21(6):857–868.
- Kang YH, Parker CC, Smith AC and Waldron KW. 2008. Characterization and distribution of phenolics in carrot cell walls. *J Agric Food Chem* 56(18):8558–8564.
- Karas M, Bachmann D, Bahr U, Hillenkamp F. 1987. Matrix-assisted ultraviolet laser desorption of non-volatile compounds. *Int J Mass Spectrom Ion Process* 78: 53–68.
- Kim DO, Heo HJ, Kim YJ, Yang HS and Lee CY. 2005. Sweet and sour cherry phenolics and their protective effects on neuronal cells. *J Agric Food Chem* 53(26):9921–9927.
- Kim Y, Cho JY, Kuk JH, Moon JH, Cho JI, Kim YC and Park KH. 2004. Identification and antimicrobial activity of phenylacetic acid produced by *Bacillus licheniformis* isolated from fermented soybean, Chungkook-Jang. *Curr Microbiol* 48(4):312–317.
- Lafont F, Aramendia M, García I, Borau V, Jiménez C, Marinas JM and Urbano F. 1999. Analyses of phenolic compounds by capillary electrophoresis electrospray mass spectrometry. *Rapid Commun Mass Spectrom* 13(7):562–567.
- Lake B. 1999. Coumarin metabolism, toxicity and carcinogenicity. relevance for human risk assessment. *Food Chem Toxicol* 37(4):423–453.

84 Fruit and Vegetable Phytochemicals

- Lamuela-Raventós RM, Andrés-Lacueva C, Permanyer J and Izquierdo-Pulido M. 2001. More antioxidants in cocoa. *J Nutr* 130(8S Suppl):2109S–2114S.
- Lamuela-Raventós RM, Romero-Pérez A, Waterhouse A and de la Torre-Boronat M. 1995. Direct HPLC analysis of *cis*- and *trans*-resveratrol and piceic isomers in Spanish red *Vitis vinifera* wines. *J Agric Food Chem* 43(2):281–283.
- Le Tutour B and Guedon D. 1992. Antioxidative activities of *Olea europaea* leaves and related phenolic compounds. *Phytochemistry* 31: 1173–1178.
- Li P, Wang XQ, Wang HZ and Wu YN. 1993. High performance liquid chromatographic determination of phenolic acids in fruits and vegetables. *Biomed Environ Sci* 6(4):389–398.
- Llorach R, Espín JC, Tomás-Barberán FA and Ferreres F. 2003. Valorization of cauliflower (*Brassica oleracea* L. var. botrytis) by-products as a source of antioxidant phenolics. *J Agric Food Chem* 51(8):2181–2187.
- Llorach R, Tomás-Barberán FA and Ferreres F. 2004. Lettuce and chicory byproducts as a source of antioxidant phenolic extracts. *J Agric Food Chem* 52(16):5109–5116.
- Maatta K, Kamal-Eldin A and Törrönen R. 2001. Phenolic compounds in berries of black, red, green, and white currants (*Ribes* sp.). *Antioxid Redox Signal* 3(6):981–993.
- Macheix JJ, Fleuriet A and Billot J. 1990. *Fruit Phenolics*. Boca Raton, FL: CRC Press.
- Manach C, Scalbert A, Morand C, Rémésy C and Jiménez L. 2004. Polyphenols: food sources and bioavailability. *Am J Clin Nutr* 79(5):727–724.
- Mané C, Souquet JM, Olle D, Verries C, Veran F, Mazerolles G, Cheynier V and Fulcrand H. 2007. Optimization of simultaneous flavanol, phenolic acid, and anthocyanin extraction from grapes using an experimental design: application to the characterization of champagne grape varieties. *J Agric Food Chem* 55(18):7224–7233.
- Marshall A, Bryant D, Latypova G, Hauck B, Olyott P, Morris P and Robbins M. 2008. A high-throughput method for the quantification of proanthocyanidins in forage crops and its application in assessing variation in condensed tannin content in breeding programmes for *Lotus corniculatus* and *Lotus uliginosus*. *J Agric Food Chem* 56(3):974–981.
- Masullo M, Bassarello C, Suzuki H, Pizza C and Piacente S. 2008. Polyisoprenylated benzophenones and an unusual polyisoprenylated tetracyclic xanthone from the fruits of *Garcinia cambogia*. *J Agric Food Chem* 56(13):5205–5210.
- Mattila P, Hellström J and Törrönen R. 2006. Phenolic acids in berries, fruits, and beverages. *J Agric Food Chem* 54(19):7193–7199.
- Mattila P and Kumpulainen J. 2002. Determination of free and total phenolic acids in plant-derived foods by HPLC with diode-array detection. *J Agric Food Chem* 50(13):3660–3667.
- Mattivi F, Reniero F and Korhammer S. 1995. Isolation, characterization, and evolution in red wine vinification of resveratrol monomers. *J Agric Food Chem* 43(7):1820–1823.
- Matus-Cádiz MA, Daskalchuk TE, Verma B, Puttick D, Chibbar RN, Gray GR, Perron CE, Tyler RT and Hucl P. 2008. Phenolic compounds contribute to dark bran pigmentation in hard white wheat. *J Agric Food Chem* 56(5):1644–1653.
- McDougall G, Martinussen I and Stewart D. 2008. Towards fruitful metabolomics: high throughput analyses of polyphenol composition in berries using direct infusion mass spectrometry. *J Chromatogr B* 871(2):362–369.
- McHale D, Khopkar PP and Sheridan JB. 1987. Coumarin glycosides from citrus flavedo. *Phytochemistry* 26(9):2547–2549.
- Mercier J, Ponnampalam R, Berard LS and Arul J. 1993. Polyacetylene content and UV-induced 6-methoxymellein accumulation in carrot cultivars. *J Sci Food Agric* 63(3):313–316.
- Merken HM and Beecher GR. 2000. Measurement of food flavonoids by high-performance liquid chromatography: a review. *J Agric Food Chem* 48(3):577–599.
- Mertens-Talcott SU, Talcott S T and Percival S S. 2003. Low concentrations of quercetin and ellagic acid synergistically influence proliferation, cytotoxicity and apoptosis in MOLT-4 human leukemia cells. *J Nutr* 133(8):2669–2674.
- Mezadri T, Villano D, Fernández-Pachón MS, García-Parrilla MC and Troncoso AM. 2008. Antioxidant compounds and antioxidant activity in acerola (*Malpighia emarginata* DC.) fruits and derivatives. *J Food Comp Anal* 21(4):282–290.
- Michalkiewicz A, Biesaga M and Pyrzynska K. 2008. Solid-phase extraction procedure for determination of phenolic acids and some flavonols in honey. *J Chromatogr A* 1187(1–2): 18–24.

- Milder IE, Arts IC, van de Putte B, Venema DP and Hollman PC. 2005. Lignan contents of Dutch plant foods: a database including lariciresinol, pinoresinol, secoisolariciresinol and matairesinol. *Br J Nutr* 93(3):393–402.
- Moco S, Bino RJ, Vorst O, Verhoeven HA, de Groot J, van Beek TA, Vervoort J and de Vos CH. 2006. A liquid chromatography-mass spectrometry-based metabolome database for tomato. *Plant Physiol* 141(4):1205–1218.
- Monagas M, Garrido I, Lebrón-Aguilar R, Bartolome B and Gómez-Cordobés C. 2007. Almond (*Prunus dulcis* (Mill.) D.A. Webb) Skins as a potential source of bioactive polyphenols. *J Agric Food Chem* 55(21):8498–8507.
- Moreno NJ, Marco AG and Azpilicueta CA. 2007. Influence of wine turbidity on the accumulation of volatile compounds from the oak barrel. *J Agric Food Chem* 55(15):6244–6251.
- Moskowitz AH and Hrazdina G. 1981. Vacuolar contents of fruit subepidermal cells from vitis species. *Plant Physiol* 68(3):686–692.
- Murray R, Mendez J and Brown S. 1982. *The Natural Coumarins: Occurrence, Chemistry and Biochemistry*. Chichester, UK: John Wiley & Sons.
- Negi N, Ochi A, Kurosawa M, Ushijima K, Kitaguchi Y, Kusakabe E, Okasho F, Kimachi T, Teshima N, Ju-Ichi M, Abou-Douh AM, Ito C and Furukawa H. 2005. Two new dimeric coumarins isolated from *Murraya exotica*. *Chem Pharm Bull (Tokyo)* 53(9):1180–1182.
- Ng A, Harvey AJ, Parker ML, Smith AC and Waldron KW. 1998. Effect of oxidative coupling on the thermal stability of texture and cell wall chemistry of beet root (*Beta vulgaris*). *J Agric Food Chem* 46(8):3365–3370.
- Ong PKC and Acree TE. 1998. Gas chromatography/olfactory analysis of lychee (*Litchi chinensis* Sonn.). *J Agric Food Chem* 46(6):2282–2286.
- Oszmianski J, Wojdylo A and Kolniak J. 2008. Effect of l-ascorbic acid, sugar, pectin and freeze-thaw treatment on polyphenol content of frozen strawberries. *LWT-Food Sci Technol*. Forthcoming.
- Panosian A, Mamikonyan G, Torosyan M, Gabrielyan E and Mkhitarian S. 2001. Analysis of aromatic aldehydes in brandy and wine by high-performance capillary electrophoresis. *Anal Chem* 73(17):4379–4383.
- Pastrana-Bonilla E, Akoh CC, Sellappan S and Krewer G. 2003. Phenolic content and antioxidant capacity of muscadine grapes. *J Agric Food Chem* 51(18):5497–5503.
- Piperno A, Toscano M and Uccella NA. 2004. The Cannizzaro-like metabolites of secoiridoid glucosides in some olive cultivars. *J Sci Food Agric* 84(4):341–349.
- Polster J, Dithmar H and Walter F. 2003. Are histones the targets for flavan-3-ols (catechins) in nuclei? *Biol Chem* 384(7):997–1006.
- Prasain JK, Wang CC and Barnes S. 2004. Mass spectrometric methods for the determination of flavonoids in biological samples. *Free Radic Biol Med* 37(9):1324–1350.
- Rapisarda P, Bellomo SE and Intelisano S. 2001. Storage temperature effects on blood orange fruit quality. *J Agric Food Chem* 49(7):3230–3235.
- Rapisarda P, Carollo G, Fallico B, Tomaselli F and Maccarone E. 1998. Hydroxycinnamic acids as markers of Italian blood orange juices. *J Agric Food Chem* 46(2):464–470.
- Reed JD. 1995. Nutritional toxicology of tannins and related polyphenols in forage legumes. *J Anim Sci* 73(5):1516–1528.
- Reed JD, Krueger CG and Vestling MM. 2005. MALDI-TOF mass spectrometry of oligomeric food polyphenols. *Phytochemistry* 66(18):2248–2263.
- Robards K, Prenzler PD, Tucker G, Swatsitang P and Glover W. 1999. Phenolic compounds and their role in oxidative processes in fruits. *Food Chem* 66(4):401–436.
- Robbins RJ. 2003. Phenolic acids in foods: An overview of analytical methodology. *J Agric Food Chem* 51(10):2866–2887.
- Romero-Pérez AI, Ibern-Gómez M, Lamuela-Raventós RM and de la Torre-Boronat MC. 1999. Piceid, the major resveratrol derivative in grape juices. *J Agric Food Chem* 47(4):1533–1536.
- Romero-Pérez AI, Lamuela-Raventós RM, Andrés-Lacueva C and Torre-Boronat MC. 2001. Method for the quantitative extraction of resveratrol and piceid isomers in grape berry skins. Effect of powdery mildew on the stilbene content. *J Agric Food Chem* 49(1):210–215.
- Ruiz D, Egea J, Gil MI and Tomás-Barberán FA. 2005. Characterization and quantitation of phenolic compounds in new apricot (*Prunus armeniaca* L.) varieties. *J Agric Food Chem* 53(24):9544–9552.

86 Fruit and Vegetable Phytochemicals

- Sakho M, Chassagne D, Jaus A, Chiarazzo E and Crouzet J. 1997. Enzymatic maceration: effects on volatile components of mango pulp. *J Food Sci* 63(6):975–978.
- Sánchez-Rabaneda F, Jáuregui O, Casals I, Andrés-Lacueva C, Izquierdo-Pulido M and Lamuela-Raventós RM. 2003. Liquid chromatographic/electrospray ionization tandem mass spectrometric study of the phenolic composition of cocoa (*Theobroma cacao*). *J Mass Spectrom*. 38(1):35–42.
- Seeram NP, Henning SM, Zhang Y, Suchard M, Li Z and Heber D. 2006. Pomegranate juice ellagitannin metabolites are present in human plasma and some persist in urine for up to 48 hours. *J Nutr* 136(10):2481–2485.
- Sellappan S, Akoh CC and Krewer G. 2002. Phenolic compounds and antioxidant capacity of Georgia-grown blueberries and blackberries. *J Agric Food Chem* 50(8):2432–2438.
- Servili M, Baldioli M, Selvaggini R, Macchioni A and Montedoro GF. 1999. Phenolic compounds of olive fruit: one and two-dimensional nuclear magnetic resonance characterization of nüzhenide and its distribution in the constitutive parts of fruit. *J Agric Food Chem* 47(1):12–18.
- Shier WT, Shier AC, Xie W and Mirocha CJ. 2001. Structure-activity relationships for human estrogenic activity in zearalenone mycotoxins. *Toxicol* 39 (9):1435–1438.
- Silva BM, Andrade PB, Ferreres F, Domingues AL, Seabra RM and Ferreira MA. 2002. Phenolic profile of quince fruit (*Cydonia oblonga* Miller) (pulp and peel). *J Agric Food Chem* 50(16):4615–4618.
- Singleton VL, Orthofer R and Lamuela-Raventós RM. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. In: Abelson JN, Simon MI, editors. *Methods in Enzymology*, Volume 299. San Diego, CA: Academic Press, pp. 152–178.
- Slimestad R and Verheul MJ. 2005. Content of chalconaringenin and chlorogenic acid in cherry tomatoes is strongly reduced during postharvest ripening. *J Agric Food Chem* 53(18):7251–7256.
- Soler-Rivas C, Espín JC and Wichers HJ. 2000. Oleuropein and related compounds. *J Sci Food Agric* 80(7):1013–1023.
- Soong YY and Barlow PJ. 2006. Quantification of gallic acid and ellagic acid from longan (*Dimocarpus longan* Lour.) seed and mango (*Mangifera indica* L.) kernel and their effects on antioxidant activity. *Food Chem* 87(3):524–530.
- Stalikas C. 2007. Extraction, separation, and detection methods for phenolic acids and flavonoids. *J Sep Sci* 30(18):3268–3295.
- Stobiecki M. 2000. Application of mass spectrometry for identification and structural studies of flavonoid glycosides. *Phytochemistry* 54(3):237–256.
- Suárez B, Picinelli A and Mangas JJ. 1996. Solid-phase extraction and high-performance liquid chromatographic determination of polyphenols in apple musts and ciders. *J Chromatogr A* 727(2):203–209.
- Sun BS, Ricardo-da-Silva JM and Spranger MI. 1998. Critical factors of vanillin assay for catechins and proanthocyanidins. *J Agric Food Chem* 46: 4267–4274.
- Surono IS, Nishigaki T, Endaryanto A and Wasposito P. 2008. Indonesian biodiversities, from microbes to herbal plants as potential functional foods. *J Fac Agric, Shun Shu Univ.* 44(1–2):23–27.
- Tan XJ, Li Q, Chen XH, Wang ZW, Shi ZY, Bi KS and Jia Y. 2008. Simultaneous determination of 13 bioactive compounds in *Herba Artemisiae Scopariae* (Yin Chen) from different harvest seasons by HPLC-DAD. *J Pharm Biomed Anal* 47(4–5):847–853.
- Taubert D, Grimberg G and Schomig E. 2005. Tannic acid in plant dust causes airway obstruction. *Thorax* 60(9):789–791.
- Teow CC, Truong V-D, McFeeters RF, Thompson RL, Pecota KV and Yencho GC. 2007. Antioxidant activities, phenolic and [beta]-carotene contents of sweet potato genotypes with varying flesh colours. *Food Chem* 103(3):829–838.
- Tomás-Barberán FA and Clifford MN. 2000. Flavanones, chalcones and dihydrochalcones—nature, occurrence and dietary burden. *J Sci Food Agric* 80(7):1073–1080.
- Tomás-Barberán FA and Espín JC. 2001. Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. *J Sci Food Agric* 81(9):853–876.
- Tomás-Barberán FA, Gil MI, Cremin P, Waterhouse AL, Hess-Pierce B and Kader AA. 2001. HPLC-DAD-ESIMS analysis of phenolic compounds in nectarines, peaches, and plums. *J Agric Food Chem.* 49(10):4748–4760.
- Tovar MJ, Motilva MJ and Romero MP. 2001. Changes in the phenolic composition of virgin olive oil from young trees (*Olea europaea* L. cv. Arbequina) grown under linear irrigation strategies. *J Agric Food Chem* 49(11):5502–5508.

- Tripoli E, Giammanco M, Tabacchi G, Di Majo D, Giammanco S and La Guardia M. 2005. The phenolic compounds of olive oil: structure, biological activity and beneficial effects on human health. *Nutr Res Rev* 18(1):98–112.
- Truong VD, McFeeters RF, Thompson RT, Dean LL and Shofran B. 2007. Phenolic acid content and composition in leaves and roots of common commercial sweetpotato (*Ipomea batatas* L.) cultivars in the United States. *J Food Sci* 72(6):C343–C349.
- Tudela JA, Cantos E, Espín JC, Tomás-Barberán FA and Gil MI. 2002. Induction of antioxidant flavonol biosynthesis in fresh-cut potatoes. Effect of domestic cooking. *J Agric Food Chem* 50(21):5925–5931.
- Tura D and Robards K. 2002. Sample handling strategies for the determination of biophenols in food and plants. *J Chromatogr A* 975(1):71–93.
- Urpi-Sardà M, Jáuregui O, Lamuela-Raventós RM, Jaeger W, Miksits M, Covas MI and Andrés-Lacueva C. 2005. Uptake of diet resveratrol into the human low-density lipoprotein. Identification and quantification of resveratrol metabolites by liquid chromatography coupled with tandem mass spectrometry. *Anal Chem* 77(10):3149–3155.
- Urpi-Sardà M, Zamora-Ros R, Lamuela-Raventós R, Cherubini A, Jauregui O, de la Torre R, Covas MI, Estruch R, Jaeger W and Andrés-Lacueva C. 2007. HPLC–tandem mass spectrometric method to characterize resveratrol metabolism in humans. *Clin Chem* 53(2):292–299.
- US Department of Agriculture. 2004. USDA database for the proanthocyanidin content of selected foods. Beltsville, MD: USDA.
- US Department of Agriculture. 2007a. USDA database for the flavonoid content of selected foods. Beltsville, MD: USDA.
- US Department of Agriculture. 2007b. USDA–Iowa state university database on the isoflavone content of foods. Beltsville, MD: USDA.
- Vallejo F, Tomás-Barberán FA and Ferreres F. 2004. Characterisation of flavonols in broccoli (*Brassica oleracea* L. var. *italica*) by liquid chromatography–UV diode-array detection–electrospray ionisation mass spectrometry. *J Chromatogr A* 1054(1–2):181–193.
- Vergne S, Titier K, Bernard V, Asselineau J, Durand M, Lamothe V, Potier M, Perez P, Demotes-Mainard J, Chantre P, Moore N, Bennetau-Pelissero C and Sauvant P. 2007. Bioavailability and urinary excretion of isoflavones in humans: effects of soy-based supplements formulation and equol production. *J Pharm Biomed Anal* 43(4):1488–1494.
- Vieira LMM and Kijjoo A. 2005. Naturally-occurring xanthones: recent developments. *Curr Med Chem* 12(21):2413–2446.
- Visioli F and Galli C. 1998. Olive oil phenols and their potential effects on human health. *J Agric Food Chem* 46(10):4292–4296.
- Walker EB. 2007. HPLC analysis of selected xanthones in mangosteen fruit. *J Sep Sci* 30(9):1229–1234.
- Wang J and Sporns P. 2000a. MALDI-TOF MS analysis of food flavonol glycosides. *J Agric Food Chem* 48(5):1657–1662.
- Wang J and Sporns P. 2000b. MALDI-TOF MS analysis of isoflavones in soy products. *J Agric Food Chem* 48(12):5887–5892.
- Waridel P, Wolfender J-L, Ndjoko K, Hobby KR, Major HJ and Hostettmann K. 2001. Evaluation of quadrupole time-of-flight tandem mass spectrometry and ion-trap multiple-stage mass spectrometry for the differentiation of C-glycosidic flavonoid isomers. *J Chromatogr A* 926(1):29–41.
- Waterhouse AL and Lamuela-Raventós RM. 1994. The occurrence of piceid, a stilbene glucoside, in grape berries. *Phytochemistry* 37: 571–573.
- Wawrzynowicz T and Waksmundzka Hajnos M. 1990. The application of systems with different selectivity for the separation and isolation of some furocoumarins. *J Liq Chromatogr* 13(20):3925–3940.
- Werner E, Heilier JF, Ducruix C, Ezan E, Junot C and Tabet JC. 2008. Mass spectrometry for the identification of the discriminating signals from metabolomics: Current status and future trends. *J Chromatogr B Analyt Technol Biomed Life Sci* 871(2):143–163.
- Wilson TC and Hagerman AE. 1990. Quantitative determination of ellagic acid. *J Agric Food Chem* 38: 1678–1683.
- Wojdyło A, Oszmianski J and Laskowski P. 2008. Polyphenolic compounds and antioxidant activity of new and old apple varieties. *J Agric Food Chem* 56(15):6520–6530.

88 Fruit and Vegetable Phytochemicals

- Wolfender JL, Ndjoko K and Hostettmann K. 2003. Application of LC-NMR in the structure elucidation of polyphenols. In: Santos-Buelga C, Williamson G, editors. *Methods in Polyphenol Analysis*. Cambridge, UK: Royal Society of Chemistry, pp. 128–156.
- Xia YQ, Guo TY, Zhao HL, Song MD, Zhang BH and Zhang BL. 2007. A novel solid phase for selective separation of flavonoid compounds. *J Sep Sci* 30(9):1300–1306.
- Xu GH, Kim JA, Kim SY, Ryu JC, Kim YS, Jung SH, Kim MK and Lee SH. 2008. Terpenoids and coumarins isolated from the fruits of *Poncirus trifoliata*. *Chem Pharm Bull (Tokyo)* 56(6):839–842.
- Yamaguchi F, Ariga T, Yoshimura Y and Nakazawa H. 2000. Antioxidative and anti-glycation activity of garcinol from *Garcinia indica* fruit rind. *J Agric Food Chem* 48(2):180–185.
- Yeh CT and Yen GC. 2005. Effect of vegetables on human phenolsulfotransferases in relation to their antioxidant activity and total phenolics. *Free Radic Res* 39(8):893–904.
- Zadernowski R, Czaplicki S and Naczek M. 2009. Phenolic acid profiles of mangosteen fruits (*Garcinia mangostana*). *Food Chem* 112(3):685–689.
- Zamora-Ros R, Andrés-Lacueva C, Lamuela-Raventós RM, Berenguer T, Jakyszyn P, Martínez C, Sánchez MJ, Navarro C, Chirlaque MD, Tormo MJ, Quirós JR, Amiano P, Dorronsoro M, Larrañaga N, Barricarte A, Ardanaz E and González CA. 2007. Concentrations of resveratrol and derivatives in foods and estimation of dietary intake in a Spanish population: European Prospective Investigation into Cancer and Nutrition (EPIC)-Spain cohort. *Br J Nutr* 100(1):188–196.
- Zhao B and Hall CA III. 2008. Composition and antioxidant activity of raisin extracts obtained from various solvents. *Food Chem* 108(2):511–518.
- Zuo Y, Wang C and Zhan J. 2002. Separation, characterization, and quantitation of benzoic and phenolic antioxidants in American cranberry fruit by GC-MS. *J Agric Food Chem* 50(13):3789–3794.

3.2 BIODISPONIBILITAT DELS COMPOSTOS FENÒLICS.

3.2.1 Generalitats.

Existeixen varies definicions de biodisponibilitat. Unes la defineixen com “l'índex i grau en que un ingredient es absorbit i es torna disponible en el lloc d'acció”; una altra definició alternativa més habitualment usada en nutrició, és “la fracció del component consumit que apareix en la circulació sanguínia” (Erdman, Jr. et al. 2007).

Des del punt de vista de la nutrició, la biodisponibilitat s'expressa freqüentment com la proporció de la dosi ingerida que és excretada en la orina comparada amb l'excretada en femta (Rechner et al. 2002). En el cas dels compostos liposolubles, no seran directament excretats en l'orina sinó que apareixeran com metabòlits hidrosolubles. Part d'aquests compostos liposolubles poden emmagatzemar-se en els teixits adiposos, de forma tal que la ingesta total menys l'excreció no reflectirà la història real del destí biològic d'aquests metabòlits (Tomás-Barberán 2003).

Per tant, en el present treball sempre que es parli de biodisponibilitat dels polifenols es referirà a un conjunt de processos: a l'alliberament i la digestió en l'estómac i en el tracte intestinal, al transport a través de la membrana intestinal cap al corrent sanguini (absorció), a la seva distribució en els teixits, al seu metabolisme i finalment a la seva eliminació.

La biodisponibilitat dels polifenols difereix àmpliament d'un polifenol a un altre; aquells més abundants en la nostra dieta i que ingerim amb ella, no són necessàriament els que després trobarem en majores concentracions com a metabòlits actius en els nostres teixits (Manach et al. 2005b). L'estructura química del polifenol determina el seu ràtio d'absorció a través del tracte gastrointestinal, metabolisme i finalment la seva activitat biològica.

El 2005, Manach i col·laboradors, (Manach et al. 2005b) van recopilar dades de 97 estudis de biodisponibilitat, on s'havia investigat sobre la cinètica i capacitat d'absorció dels polifenols en humans després de que aquests ingerissin una única dosi de polifenols en forma de compost pur, extracte vegetal o amb el

mateix aliment o beguda que els contenien. Amb aquests 97 estudis van descriure els valors mitjans de la concentració màxima en plasma (C_{max}), el temps per a arribar a aquesta C_{max} , l'àrea sota la corba de concentració plasmàtica-temps (AUC), el temps mitjà d'eliminació i l'excreció urinària relativa de 18 polifenols.

En aquesta publicació van concloure que la majoria dels polifenols s'absorbeixen poc a través de l'intestí i es metabolitzen extensivament o s'eliminen ràpidament. Els metabòlits presents en la sang, resultats de la digestió i de l'activitat hepàtica, generalment difereixen del seu compost originari. Les concentracions plasmàtiques dels metabòlits totals varien des de 0 fins a 4 $\mu\text{mol/L}$ amb un consum de 50 mg d'equivalents d'aglicones, amb una excreció urinària relativa entre el 0.3% a 43% de la dosi ingerida, en funció dels polifenols. El temps de C_{max} també difereix força d'un polifenol a un altre, establint el rang entre 1.5h i 5.5h.

L'àcid gàl·lic i les isoflavones són els polifenols millor absorbits pels humans, seguits de les catequines, les flavonoles, i els glucòsids de quercetina, però amb una cinètica diferent. Les pitjor absorbides són, les proantocianidines, les galocatequines del te i les antocianines. Les dades d'absorció per a altres polifenols són encara limitades (Manach et al. 2005b).

El metabolisme dels polifenols presenta aspectes comuns en els diferents grups de compostos fenòlics (Scalbert and Williamson 2000). Les aglicones poden absorbir-se des de l'intestí prim, però molts polifenols estan presents en els aliments en forma d'ésters, glicòsids o polímers que no poden ser absorbits directament i han de ser prèviament hidrolitzats pels enzims intestinals o la microbiota colònica (Manach et al. 2005b).

Els polifenols que no són absorbits en l'intestí prim arriben al còlon, on els enzims de la microbiota colònica hidrolitzen els glicòsids en aglicones, així com altres enzims que també permeten la metabolització de les aglicones. S'ha demostrat que la microbiota colònica pot metabolitzar àmpliament els polifenols produint així varis àcids aromàtics (Urpi-Sarda et al. 2009b).

Antecedents Bibliogràfics

Durant el curs de l'absorció, els polifenols són conjugats en l'enteròcit de l'intestí prim i posteriorment en el fetge, donant-se principalment reaccions de metilació, sulfatació i/o glucuronidació. Aquest mecanisme de conjugació és molt eficaç i, per tant, les aglicones estan gairebé sempre absents o en molt baixes concentracions en l'organisme, després del seu consum a dosis nutricionals (Manach et al. 2004). També cabria destacar que cada vegada més augmenten els articles relacionats amb altres conjugacions com, per exemple, la glutathionització (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 en la seva estructura (Wu et al. 2002). La metilació generalment passa en la posició 3' del polifenol, però també es formen una menor proporció de conjugats metilats en la posició 4'. Aquest enzim es troba en tots els teixits, però la seva major activitat és en el fetge i el ronyó (Piskula and Terao 1998). D'altra banda, les sulfotransferases (SULT) catalitzen la transferència d'un grup sulfat des de la 3'-fosfoadenosin-5'-fosfosulfat a un grup hidroxil present en varis substrats com els esterols, àcids biliars i polifenols, entre altres.

Les UDP-glucuronosiltransferases són enzims de membranes localitzats en el reticle endoplasmàtic de molts teixits i que catalitzen la transferència de l'àcid glucurònic a esterols, àcids biliars, polifenols i altres constituents dietètics. La glucuronidació té lloc en els enteròcits i després al fetge; s'han detectat al voltant de 15 isoformes d'UDP-glucuronosiltransferases en humans que tenen amples especificitats i diversa distribució en teixits (Fisher et al. 2001). 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). La proporció secretada depèn del polifenol i varia entre 0 y 52% (Crespy et al. 2003).

Els tres tipus de conjugacions varien en funció de la naturalesa del substrat i de la dosis ingerida. La sulfatació té generalment una elevada afinitat i una baixa capacitat comparada con la glucuronidació. Per tant, quan la dosis ingerida s'incrementa, té lloc un desplaçament de la sulfatació cap a la glucuronidació (Koster et al. 1981). La relació entre la sulfatació i la glucuronidació sembla que

està afectada per l'espècie, el sexe i la matriu de l'aliment (Piskula 2000; Roura et al. 2008).

Els metabòlits dels polifenols, generalment no es troben lliures en el torrent sanguini; l'albumina és una de les proteïnes que actuen com a transportador dels polifenols, però la seva afinitat pels polifenols varia en funció de la seva estructura química. El grau d'unió a l'albumina pot tenir conseqüències per al rati d'eliminació dels metabòlits i per a la seva distribució en les cèl·lules i els teixits. La concentració cel·lular és proporcional a les concentracions de metabòlits lliures (Manach et al. 2004).

Els metabòlits de polifenols poden seguir dos camins d'excreció, la via biliar o la via urinària. Els d'alt pes molecular, que estan extensivament conjugats, presenten una major probabilitat de ser excretats mitjançant la bilis, mentre que els conjugats de menor pes s'excreten preferiblement per l'orina (Crespy et al. 2003). El total de metabòlits excretats en orina es correlaciona amb les concentracions plasmàtiques màximes. Aquesta excreció és bastant elevada per les flavanones dels fruits cítrics (4-30%) i fins i tot més elevada per a les isoflavones 16-66% (Manach et al. 2003).

Els percentatges d'excreció d'altres polifenols, per exemple els antocians solen ser molt baixos (0.005-0.1%) (Cui et al. 2006; Matsumoto et al. 2001); encara que en un estudi anterior a aquests dos es van detectar elevades concentracions d'antocians, de fins a un 5%, després del consum de vi negre (Lapidot et al. 1998). Aquests valors baixos d'excreció podrien indicar una elevada excreció biliar o un excessiu metabolisme d'aquests compostos.

En la següent figura 1 es pot observar un esquema de l'absorció i metabolisme dels polifenols, la formació dels metabòlits i conjugats en humans. La degradació d'algunes procianidines pot succeir a l'estomac per acció del medi àcid. Tots els polifenols experimenten un alt metabolisme al jejú i a l'ili de l'intestí prim. Els metabòlits resultants entren llavors a la vena porta per a ser transportats fins al fetge on patiran més transformacions i conjugacions. Els polifenols no absorbits a nivell de l'intestí prim arribaran llavors a l'intestí gros on podran ser degradats per la microbiota colònica donant lloc a àcids fenòlics de baix pes molecular que podran ser reabsorbits posteriorment. La majoria de metabòlits s'eliminaran via renal. La distribució d'aquests compostos a la

cèl·lula i teixits encara roman una mica incerta, donat a las baixes concentracions en les que es troben en plasma (Spencer 2003).

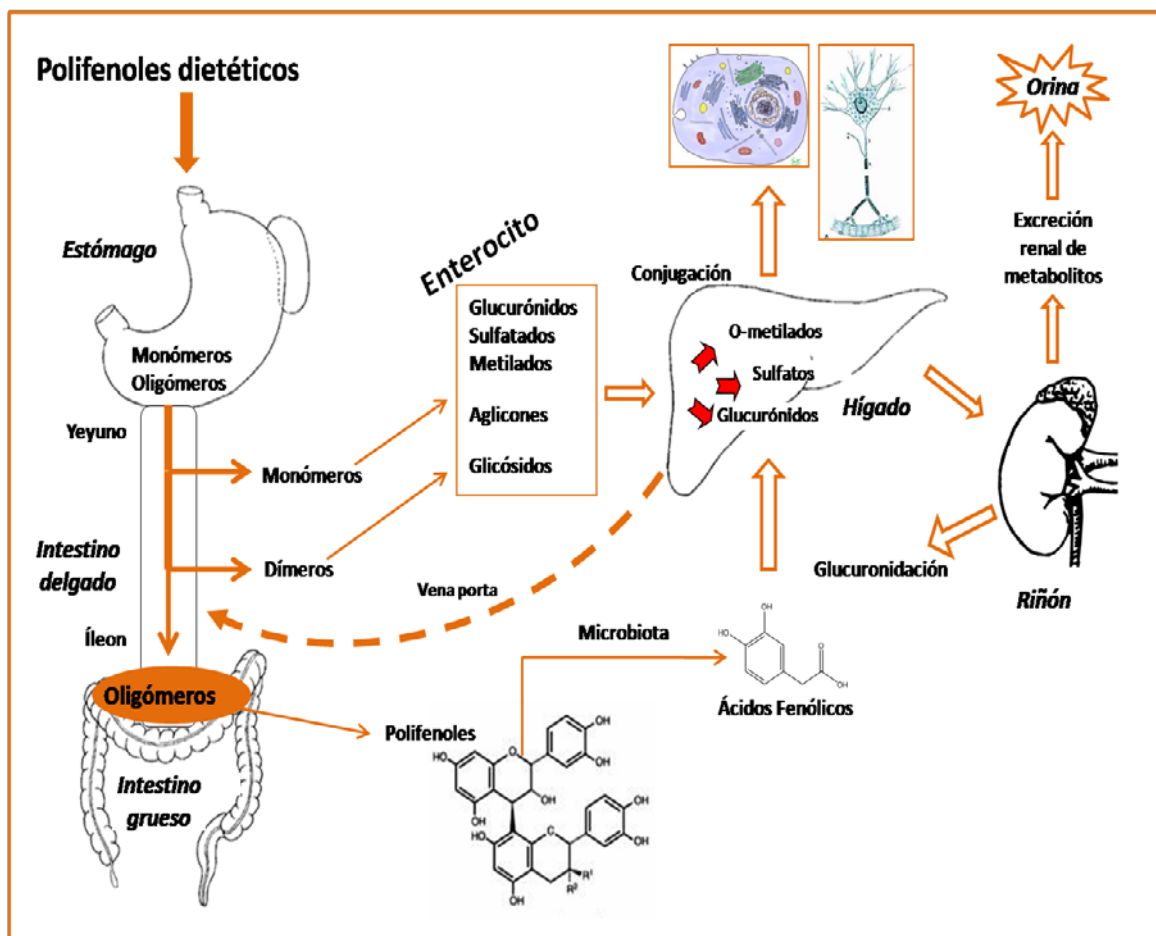


Figura 1: Esquema de l'absorció i biotransformació dels polifenols de la dieta *in vivo* (Spencer 2003).

3.2.2 Factors que afecten la biodisponibilitat i farmacocinètica dels polifenols.

3.2.2.1 Els aliments com a matriu.

Els polifenols són consumits generalment com a components dels aliments que els contenen, conjuntament amb altres macronutrients com les proteïnes, hidrats de carboni, lípids, micronutrients i també amb altres compostos com l'alcohol (Manach et al. 2004). La unió dels polifenols a certs components dels aliments com proteïnes, lípids o polisacàrids és possible, la qual cosa pot afectar la seva absorció. Els enzims i transportadors involucrats en l'absorció i metabolisme de polifenols poden veure's també induïts o inhibits per la presència de determinats micronutrients o xenobiòtics de la dieta. A més, altres efectes indirectes de la dieta sobre altres paràmetres de la fisiologia del tracte digestiu (pH, fermentació intestinal, excreció biliar, trànsit intestinal, buidat gàstric, etc.) poden tenir també conseqüències en l'absorció d'aquests compostos (Manach and Donovan 2004).

Existeixen relativament pocs estudis que hagin tractat de determinar la influència dels components dels aliments en la biodisponibilitat o farmacocinètica dels polifenols. Aquests estudis realitzats tant en humans com en animals, mostren aquests components de la dieta que poden millorar l'absorció dels polifenols. L'àcid tartàric per exemple pot millorar la biodisponibilitat dels polifenols del vi (Yamashita et al. 2002)

Els factors que donen lloc a una millor absorció dels flavonoides, en particular, la quercetina i els seus metabòlits, són principalment la naturalesa del sucre adherit, i en segon lloc, la solubilitat, modificat per l'etanol, greixos i emulsionants. Les flavanones com l'hesperidina i els àcids fenòlics es veuen fortament afectats pel tipus de sucre unit. Aquests últims es poden lligar de forma covalent a la matriu de segó de cereal (Scholz and Williamson 2007).

En rates, la co-administració de lípids tals com la lecitina i oli de soja o emulsionants como éster de sacarosa d'àcids grassos, ésters de poliglicerol d'àcids grassos i taurocolat de sodi no han tingut efectes estadísticament significatius en l'absorció de la quercetina, malgrat que aquests components augmenten l'acumulació de formes conjugades de la quercetina y de

l'isorhamnetina en plasma de rata. Tanmateix, la combinació de lípids i emulsionants millora l'absorció de la quercetina significativament (Azuma et al. 2002).

L'administració de 30 micromols de quercetina per kg de pes corporal en els menjars de porcs en creixement, incrementa la biodisponibilitat en un 38% ($P < 0.05$) y el 12% ($P > 0.05$) després de la ingesta d'una dieta estàndard de porc suplementada amb triglicèrids d'àcids grassos de cadena mitjana y triglicèrids d'àcids grassos de cadena llarga, respectivament, en comparació amb una dieta estàndard (Lesser et al. 2006).

Visioli i col·laboradors, van comparar l'excreció humana d'hidroxitiroso (HT) quan es consumeix com un component natural de l'oli d'oliva verge extra, quan s'afegeix a l'oli d'oliva refinat, o quan s'afegeix al iogurt (com una aproximació d'aliment funcional). L'alta excreció d'HT lliure (234% de HT administrat) va suggerir que la hidròlisi de la oleuropeïna (component fenòlic majoritari de la polpa de les olives verdes) administrada en humans es produeix *in vivo*. D'altra banda, l'excreció d'HT va ser molt més gran després de la seva administració com a component natural de l'oli d'oliva verge extra (44.2% del HT administrat) que després de la addició i administració d'oli d'oliva refinat (23% de la HT administrat) o iogurt (5.8% de la HT administrat) (Visioli et al. 2003).

Altres estudis reflecteixen com alguns components de la dieta poden disminuir la biodisponibilitat dels polifenols o tenir efectes nuls sobre ells. Goldberg col·laboradors, van provar l'eficiència d'absorció de tres dels components polifenòlics presents en el vi negre (trans-resveratrol, [+] -catequina i quercetina) quan s'administra per via oral a humans sans. Cada polifenol es va administrar de forma aleatòria a intervals de 4 setmanes en tres matrius diferents: vi blanc (11,5% d'etanol), suc de raïm y suc de verdures/homogeneizat. El trans-resveratrol es va absorbir de forma més eficient que la [+] -catequina i la quercetina. En aquest estudi es van observar alguns efectes significatius de la matriu per a les concentracions sèriques de polifenols; però en el cas de l'orina, les diferents matrius no van produir increments significatius. L'absorció d'aquests tres polifenols va ser aproximadament equivalent tant en la matriu aquosa com l'alcohòlica (Goldberg et al. 2003).

L'avaluació de l'activitat antioxidant de te verd i negre *in vitro*, amb i sense llet, ha sigut provada en dos grups de cinc adults sans. Ambdós tes van inhibir la peroxidació *in vitro* de forma dosis-depenent. El te verd va ser sis vegades més potent que el te negre y l'addició de llet a qualsevol dels tes no va modificar apreciablement el seu potencial antioxidant *in vitro*. *In vivo*, la ingestió de te va produir un augment significatiu de la capacitat antioxidant total del plasma ($P < 0.05$), similar en ambos tes. Quan el te es consumia amb llet, la seva activitat *in vivo* va ser totalment inhibida (Serafini et al. 1996). No obstant això Roura i col·laboradors, uns anys després, van avaluar la possible interacció de la llet en l'absorció de (-)-epicatequina de cacau en pols en 21 voluntaris sans. En aquest estudi la llet no va alterar la biodisponibilitat dels polifenols del cacau en pols (Roura et al. 2007b).

3.2.2.2 Altres factors que poden afectar la biodisponibilitat.

Existeixen altres factors que afecten l'absorció i/o biodisponibilitat dels polifenols dins dels quals es troben el sexe i l'edat. Cassidy *i col.*, 2006, van analitzar l'efecte de l'edat, el sexe i la matriu dels aliments en la biodisponibilitat de les aglicones y glucòsids d'isoflavones en les 3 diferents formes en que estan presents naturalment en els aliments de soja, llet de soja, proteïna vegetal texturitzada i el tempeh (producte alimentari procedent de la fermentació de la soja que es presenta en forma de pastel), observant que després d'administrar els diferents tipus d'aliments de soja, les concentracions sèriques d'isoflavones en tots els individus i grups es van incrementar ràpidament, observant-se concentracions sèriques de genisteïna superiors a les concentracions de daidzeïna. Les dones d'aquest estudi van arribar a un pic de concentració de daidzeïna superior al dels homes, demostrant que les dones tenen una major eficàcia d'absorció d'aquests compostos.

El consum de tempeh (que conté principalment aglicones d'isoflavones) dóna lloc a nivells més alts de daidzeïna i genisteïna en sèrum i una major àrea sota la corba associada, en comparació amb la proteïna vegetal texturitzada (principalment glucòsids d'isoflavones). No obstant, la llet de soja s'absorbeix més ràpidament i els nivells màxims d'isoflavones provinents d'aquest aliment, s'aconsegueixen abans que amb els altres aliments de soja. En aquest estudi

no es van observar diferències en la producció d'equol amb l'edat o el sexe (Cassidy et al. 2006).

Si bé el procés d'envelliment compromet la capacitat del cos per a obtenir els nutrients dels aliments, la nutrició, al mateix temps afecta la manera en que les persones envelleixen. Amb l'edat la capacitat d'absorció dels diferents components de la dieta va disminuint, ja sigui pel propi envelliment de les cèl·lules o per les diferents patologies que van apareixent amb els anys (Mirie 1997).

3.2.2.3 Efecte del processat dels aliments

El processat dels aliments normalment involucra tractament amb calor i/o homogeneïtzació, ambdós processos podrien trencar la matriu cel·lular de l'aliment. L'efecte sobre la matriu cel·lular determina la biodisponibilitat dels diferents components funcionals, però les dades publicades dels efectes del tractament amb calor no són consistents. Van het Hof i col·laboradors, (van het Hof et al. 2000a; van het Hof et al. 2000b) van trobar que el tractament amb calor podia tenir un efecte perjudicial en el contingut en micronutrients dels vegetals però a la vegada la biodisponibilitat de certs nutrients podia augmentar.

Així mateix, Sahlin i col·laboradors, van mostrar que la cocció té un efecte perjudicial en l'àcid ascòrbic, fenols totals, contingut en licopè i capacitat antioxidant. El fregit causa encara més pèrdues d'antioxidants amb la consegüent disminució de l'activitat antioxidant, però no tots els aliments processats contenen menys antioxidants. D'aquesta manera, varis estudis han observat que el processat i la cocció poden facilitar l'extracció del licopè del tomàquet (Sahlin et al. 2004). La matriu cel·lular determina la disponibilitat de diferents nutrients i l'increment de disponibilitat pot ser degut a que els nutrients es separen o s'extreuen de les seves estructures.

En alguns estudis que utilitzen aliments como la patata o el peix (Andrikopoulos et al. 2002; Kalogeropoulos et al. 2007) s'ha observat que hi ha un intercanvi entre els polifenols i l'aliment. En el peix fregit amb oli d'oliva verge, hi ha una pèrdua parcial d'antioxidants en l'oli mentre que les concentracions augmenten en el peix. Les diferències en la polaritat dels antioxidants semblen governar la

seva distribució entre l'oli de fregida i l'aigua de l'aliment (Kalogeropoulos et al. 2007).

En el cas de bullir o cuinar a pressió es produeix un fenomen de lixiviació que condueix a una pèrdua del 64% de carotenoides totals i una pèrdua del 49% dels fenols totals (Bunea et al. 2008). La concentració dels àcids fenòlics és més gran en las capes externes de la majoria de verdures i hortalisses (Turkmen et al. 2005) i aquests són extremadament exposats a l'aigua de cocció reduint el poder antioxidant d'alguns aliments com el pèsol, els espinacs, la coliflor i el cabdell de col (Andlauer et al. 2003).

No obstant això, malgrat que els fenols se solen emmagatzemar en les verdures i hortalisses en xarxes de pectina o cel·lulosa, aquests poden ser alliberats en el processament tèrmic; alguns fenols individuals a vegades pot augmentar ja que la calor pot trencar les estructures supramoleculares, alliberant els sucres glicòsids fenòlics lligats, que reaccionaran millor amb el reactiu de Folin-Ciocalteu (Bunea et al. 2008).

D'altra banda, l'ebullició pot disminuir l'activitat al disminuir l'àcid ascòrbic, mentre que la major inactivitat es pot produir com a conseqüència de la inactivació dels enzims oxidatius com l'ascorbato oxidasa (Yamaguchi T et al. 2001). També, l'escalfament mitjançant microones reté els components actius en el teixit dels aliments cuits (Yamaguchi T et al. 2001). En el procés de fregida amb oli d'oliva, els aliments perden aigua, però augmenta el seu contingut d' α -tocoferol, polifenols i fins i tot àcids terpenics de l'oli d'oliva (Kalogeropoulos et al. 2007).

Jimenez-Monreal i col·laboradors, van mesurar la capacitat antioxidant per part de 20 verdures i hortalisses sotmeses a ebullició, pressió de cocció, fornejat, cuinat en microones, a la planxa i fregit, expressada com la capacitat de captació de radicals hidròxil $\text{OH}^\bullet$, respecte a les mostres fresques (Jimenez-Monreal et al. 2009). La remolatxa, la mongetera i l'all generalment van mantenir la seva activitat antioxidant després dels diferents tractaments de cocció. El pebrot, les bledes, les cols de Brusel·les i la col van tenir les majores pèrdues de la capacitat antioxidant. Mentre que l'api va augmentar la seva capacitat antioxidant en tots els mètodes de cocció, excepte en l'ebullició que va perdre un 14% (Taula 1).

Taula 1: Percentatges de pèrdua de la capacitat d'atrapar radicals OH[•] en els vegetals i hortalisses sotmesos a diferents mètodes de cocció (Jimenez-Monreal et al. 2009).

% Pèrdua						
Mètode de cocció						
Vegetals	Bullit	Cuit a pressió	Fornejat	Microones	Planxa	Fregida
Carxofa	9.3 ± 2.02	10.0 ± 2.51	8.8 ± 1.13	8.3 ± 1.89	8.1 ± 1.53	5.4 ± 2.16
Espàrrecs	9.1 ± 1.46	9.6 ± 0.38	—	7.4 ± 1.8	9.2 ± 0.85	8.0 ± 1.37
Remolatxa	—	8.2 ± 1.72	8.0 ± 1.00	—	—	—
Fabes	14.5 ± 1.11	17.5 ± 1.25	13.9 ± 1.21	14.9 ± 1.54	14.7 ± 1.22	15.6 ± 1.46
Bròquil	15.2 ± 1.91	—	—	—	15.9 ± 1.33	12.2 ± 1.76
Col de Brusel·les	22.7 ± 1.90	20.6 ± 0.66	16.7 ± 1.31	18.3 ± 2.07	28.1 ± 1.63	20.7 ± 1.14
Coliflor	32.2 ± 1.53	28.4 ± 1.33	27.4 ± 1.45	10.4 ± 1.95	—	17.0 ± 1.67
Pastanaga	16.2 ± 1.56	19.0 ± 1.71	22.5 ± 1.66	—	12.9 ± 1.60	20.1 ± 0.93
Api	13.8 ± 1.03	-12.8 ± 2.40	-42.7 ± 2.80	-39.3 ± 1.53	-42.3 ± 3.45	-27.4 ± 2.0
Albergínia	20.4 ± 0.89	7.5 ± 1.56	—	—	—	14.3 ± 1.19
All	—	—	5.5 ± 1.31	—	6.3 ± 1.61	—
Fesols	—	—	—	—	11.5 ± 1.49	7.1 ± 0.90
Porro	8.7 ± 0.63	7.6 ± 1.24	—	—	12.5 ± 1.13	17.3 ± 0.92
Blat de moro	8.3 ± 1.43	8.6 ± 1.40	7.2 ± 1.07	—	7.7 ± 1.10	—
Ceba	9.1 ± 1.32	5.3 ± 1.89	—	7.0 ± 1.73	—	—
Pèsols	14.0 ± 1.93	12.2 ± 1.97	—	—	12.0 ± 1.32	17.4 ± 1.53
Pebrot	74.8 ± 0.88	72.4 ± 0.84	71.3 ± 1.32	70.4 ± 0.71	62.2 ± 0.69	24.4 ± 1.11
Espinacs	30.6 ± 1.05	—	9.7 ± 1.31	13.5 ± 1.19	17.8 ± 1.38	19.2 ± 1.20
Bledes	43.0 ± 0.89	48.8 ± 1.06	21.7 ± 1.75	31.3 ± 1.37	22.6 ± 0.99	48.1 ± 1.46
Carbassó	21.3 ± 0.64	23.3 ± 1.17	16.9 ± 1.31	6.2 ± 0.77	15.6 ± 1.15	20.7 ± 1.19

Les diferències estadístiques van ser analitzades per ANOVA ($P < 0.01$).

— indica que no hi ha pèrdues detectades.

Els valors negatius indiquen un increment de l'activitat antioxidant.

3.3 EFECTES BENEFICIOSOS DELS POLIFENOLS.

Els compostos fenòlics són substàncies essencials responsables en part de les propietats sensorials dels aliments d'origen vegetal. Alguns polifenols són responsables del gust amarg, com per exemple la naringina de les aranges, la neohesperidina de les taronges amargants o la oleuropeïna present en les olives. Les proantocianidinas (tanins condensats) i els tanins hidrolitzables confereixen astringència a les fruites i alguns fenols simples, essent els responsables directes de l'aroma de determinades fruites, com el eugenol present en els plàtans. Els derivats dels àcids hidroxicinàmics, com l'àcid cafeic, ferúlic o sinàpic, tenen un efecte directe sobre les característiques organolèptiques dels aliments que els contenen, afectant indirectament la seva qualitat si són oxidats pels enzims oxidants que es troben de forma natural en els teixits vegetals (Tomás-Barberán 2003).

Des del punt de vista de la seva activitat biològica els polifenols tenen la capacitat de captar radicals lliures i, per tant, se'ls confereix una activitat antioxidant que podria estar directament relacionada amb la prevenció de malalties lligades a l'estrés oxidatiu, com les malalties cardiovasculars, degeneratives i alguns tipus de càncer.

Alguns estudis clínics i epidemiològics han demostrat una clara associació entre el consum d'aliments rics en polifenols i determinades malalties; a continuació es descriu amb més detall, algunes activitats o accions beneficioses que s'han atribuït als polifenols com a resultat d'un gran nombre d'estudis científics.

3.3.1 Radicals lliures i envelliment

Els radicals lliures i altres espècies reactives d'oxigen i nitrogen es formen com a resultat del desenvolupament normal de les reaccions metabòliques oxidatives que es produeixen en la cèl·lula. Aquestes molècules també es formen com a conseqüència d'alguna malaltia, els contaminants ambientals, el fum del tabac, fàrmacs, drogues o alcohol. En cas de no ser desactivats pels antioxidants, aquestes molècules altament reactives, poden reaccionar, alterant l'estructura i funcionament de varis components de la cèl·lula (Beckman i Ames 1998; Gutteridge i Halliwell 2000; McCord 2000).

L'estrès oxidatiu pot passar quan l'equilibri entre la generació de radicals lliures i altres espècies reactives d'oxigen i nitrogen, i les defenses antioxidants és desfavorable. Per contrarestar el dany i l'estrès oxidatiu, l'organisme ha desenvolupat un complex mecanisme endògen de defensa antioxidant. Els mecanismes de defensa antioxidants comprenen components enzimàtics i no enzimàtics que prevenen la formació de radicals lliures, extreuen els radicals abans de que es produeixin els danys, reparen el dany oxidatiu, eliminen les molècules afectades i prevenen les mutacions (Gutteridge and Halliwell 2000; Halliwell 1996; Lindsay and Astley 2002).

Els polifenols naturals actuarien com substàncies antioxidants, exercint un paper citoprotector en situacions de dany cel·lular, com seria el cas de l'estrès oxidatiu. Aquesta elevada activitat antioxidant ve donada tant per la facilitat amb la que els compostos fenòlics cedeixen el protó dels seus grups hidroxils com per l'estabilitat del fenol oxidat producte de la cessió de l'electró.

L'activitat dels fenols es deu principalment als seus hidroxils. Pel que precedeix, com més grups hidroxils, més activitat antioxidant, però més important que la quantitat és la posició. Quan aquests hidroxils estan en posició O, la molècula resulta més activa. Això es degut a que la deslocalització electrònica resulta més efectiva i, a més, s'incrementa l'activitat quelant de la molècula. Aquesta circumstància es pot veure en l'anell B dels flavonoides, on les molècules més actives tenen els hidroxils en posició 3' y 4'. Els flavonoides tenen altres punts de quelació, la cetona en posició 4' del anell C pot interaccionar amb l'hidroxil en posició 3' o amb l'hidroxil en posició 5' de l'anell A. Aquest hidroxil situat en la posició 5', juntament amb el situat en la posició 7', són també dues propietats importants per incrementar l'activitat antioxidant dels flavonoides (Pietta 2000; Rice-Evans i Miller 1997; Silva et al. 2002) (Figura 2).

L'envelliment és un procés natural que avança de forma inalterable en tots els organismes vius. Els mecanismes bioquímics involucrats en l'envelliment són encara confusos, un dels factors més coneguts i rellevants és el genètic. Tanmateix, actualment, els factors mediambientals on s'inclou l'alimentació, estan augmentant el seu protagonisme donat que podrien estar involucrats en l'expressió i/o alteració gènica (Corella i Ordovas 2009).

Assaigs amb animals (Ingram et al. 2004; Sinclair 2005) i humans (Willcox et al. 2007a; Willcox et al. 2008; Willcox et al. 2007b) han mostrat que la reducció d'entre un 30 i un 40% de la ingesta calòrica habitual (*ad libitum*) és la forma més robusta i reproduïble de retardar l'envelliment i les malalties relacionades amb aquest procés i d'aquesta manera augmentar la esperança de vida. La restricció calòrica redueix, a més, la resistència insulínica en el teixit adipós (Bergamini et al. 2003).

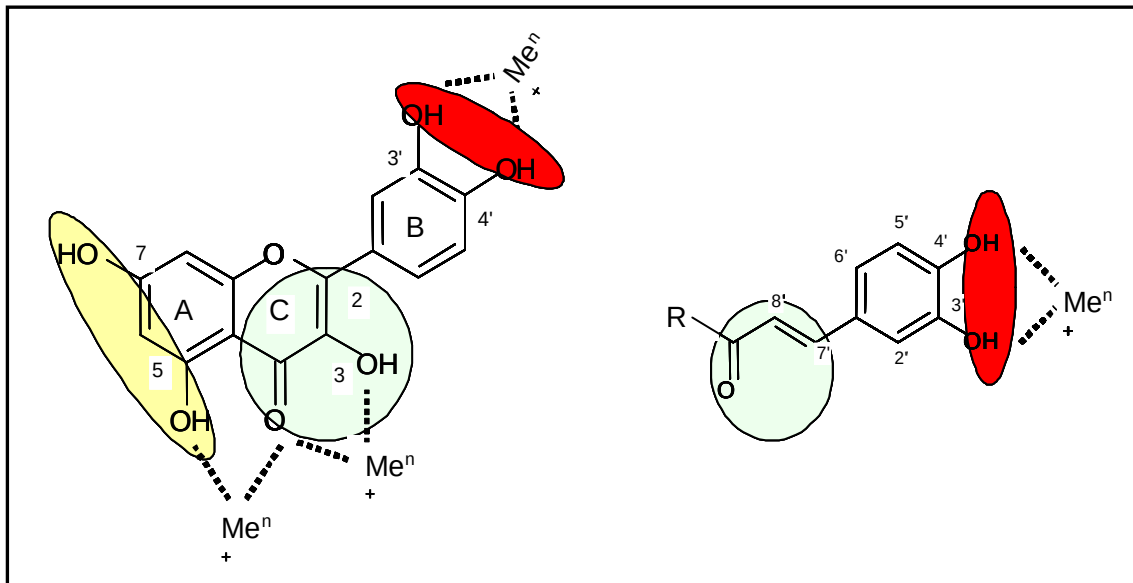


Figura 2: Relació estructura-activitat en els compostos fenòlics. En vermell, hidroxils en posició *orto*-; en els flavonoides, en groc hidroxils en *para*-. En verd la relació doble enllaç-cetona, punt que es veu reforçat per un hidroxil en els flavonoides. També podem observar els punts actius involucrats en la quelació de metalls.

3.3.2 Aterosclerosi, lipoproteïnes i plaquetes.

L'aterosclerosi i la trombosi associada amb la ruptura de les plaques vulnerables són les principals causes dels esdeveniments cardiovasculars, incloent la síndrome coronària aguda. La lipoproteïna de baixa densitat (LDL) té un paper clau en la patogènesi dels processos aterotrombòtics. Les LDLs modifiquen les propietats antitrombòtiques de l'endoteli vascular i canvien la contractilitat mitjançant la reducció de la disponibilitat d'òxid nítric endotelial i l'activació de les vies de senyalització proinflamatòria.

L'aterosclerosi és un procés inflamatori crònic a les parets de les grans artèries, això passa en resposta a una agressió sobre l'endoteli. La placa d'ateroma té el seu origen en la placa lipídica que s'observa en les grans artèries ja al naixement i es transforma amb el transcurs del temps en la placa d'ateroma. Les LDLs no només indueixen una alteració en les propietats antitrombòtiques derivades de l'endoteli vascular i en les propietats contràctils del vas com resultats d'una disminució en la disponibilitat d'òxid nítric endotelial i una activació de les vies de senyalització proinflamatòries, sinó que també afecten a la funció i interacció de les cèl·lules presents en la lesió ateroscleròtica (Badimon et al. 2009). Els símptomes se solen manifestar quan venen associats els factors de risc de l'aterosclerosi, tals com diabetis, hipertensió, consum de tabac, nivells de colesterol-LDL elevats ($>160\text{mg/dL}$) o de colesterol-HDL baixos ($<40\text{mg/dL}$).

Els factors de risc provoquen trencaments en la llum de les artèries de mitjà i gros calibre, en les que es dipositen substàncies greixoses, es produeix inflamació i finalment una reducció de la llum de les artèries amb la consegüent obstrucció del flux sanguini. El colesterol-LDL es diposita dins de les plaques d'ateroma quan la concentració de les LDL és elevada. Les cèl·lules de la paret arterial interpreten aquest dipòsit com una invasió activant al sistema immune que provoca una resposta inflamatòria.

En les zones de la paret vascular predisposades a les lesions ateroscleròtiques, amb l'augment de la permeabilitat, l'hipercolesterolèmia s'associa a un augment del trànsit de LDL a través de l'endoteli vascular. Això condueix a una acumulació de partícules de LDL en l'espai subendotelial, on interactuen amb proteoglicans i proteïnes que estimulen la seva modificació

(agregació, glicosilació, proteòlisi enzimàtica, oxidació, etc), la qual cosa augmenta la seva aterogenicitat i la retenció en la íntima vascular (Gleissner et al. 2007; Llorente-Cortes et al. 2000). La retenció de LDL en l'íntima arterial és un procés clau en la progressió de la iniciació de la lesió ateroscleròtica, perquè desencadena un procés inflamatori local. De fet, les lesions ateroscleròtiques són el resultat d'interaccions complexes entre cèl·lules inflamatòries, les plaquetes, els elements vasculars i les lipoproteïnes que regulen l'expressió de gens i proteïnes directament implicades en el procés de remodelat vascular (Badimon et al. 2009) (Figura 3).

Les cèl·lules immunitàries excitades són els monòcits circulants que penetren en la paret de l'artèria, es transformen en macròfags i comencen a fagocitar partícules de LDL, convertint-se en cèl·lules espumoses, el que fa créixer la vulnerabilitat de les plaques ateroscleròtiques. Així mateix, augmenta la trombogicitat de les plaques y de la sang; això últim associat a un augment en els nivells del factor tissular circulant i en la reactivitat de les plaquetes (Badimon et al. 2009). A mesura que avança la placa d'ateroma, es produeix un estretament o estenosi de l'artèria, inicialment parcial, fins a evolucionar a una completa obstrucció. A més, la placa d'ateroma és fràgil i pot trencar-se i sangrar o desprendre's de la paret de l'artèria i provocar, finalment, un trombe (Sesso et al. 2003; Tomás-Barberán 2003).

L'endoteli vascular és un òrgan capaç de percebre estímuls multifactorials (tant sistèmics com locals) i modificar el seu estat funcional per a contribuir a l'homeòstasi de les parets vasculars (Khazaei et al. 2008). Sota condicions fisiològiques, l'endoteli presenta una superfície amb propietats antitrombogèniques (Kato 2002) que permet l'intercanvi de nombroses substàncies entre la sang i els teixits, controla el to vascular i el trànsit de cèl·lules inflamatòries cap al llit vascular. L'augment de la permeabilitat de l'endoteli en presència de concentracions elevades de LDL ha sigut recentment associat amb l'activitat de la quinasa p21(Orr et al. 2007). Una gran part de les propietats antiaterogèniques i antitrombòtiques de l'endoteli vascular estan mediat per la seva capacitat de produir i alliberar substàncies com l'òxid nítric (NO), un inhibidor de l'agregació plaquetària amb una forta activitat vasodilatadora i una important funció anti-inflamatòria. El NO bloqueja

l'expressió de molècules pro inflamatòries com el factor de necrosis (NF) $\kappa\beta$ i molècules d'adhesió (ICAM-1, VCAM-1) (Bevilacqua et al. 1986); així com, l'adhesió i infiltració leucocitària (de Caterina 1999).

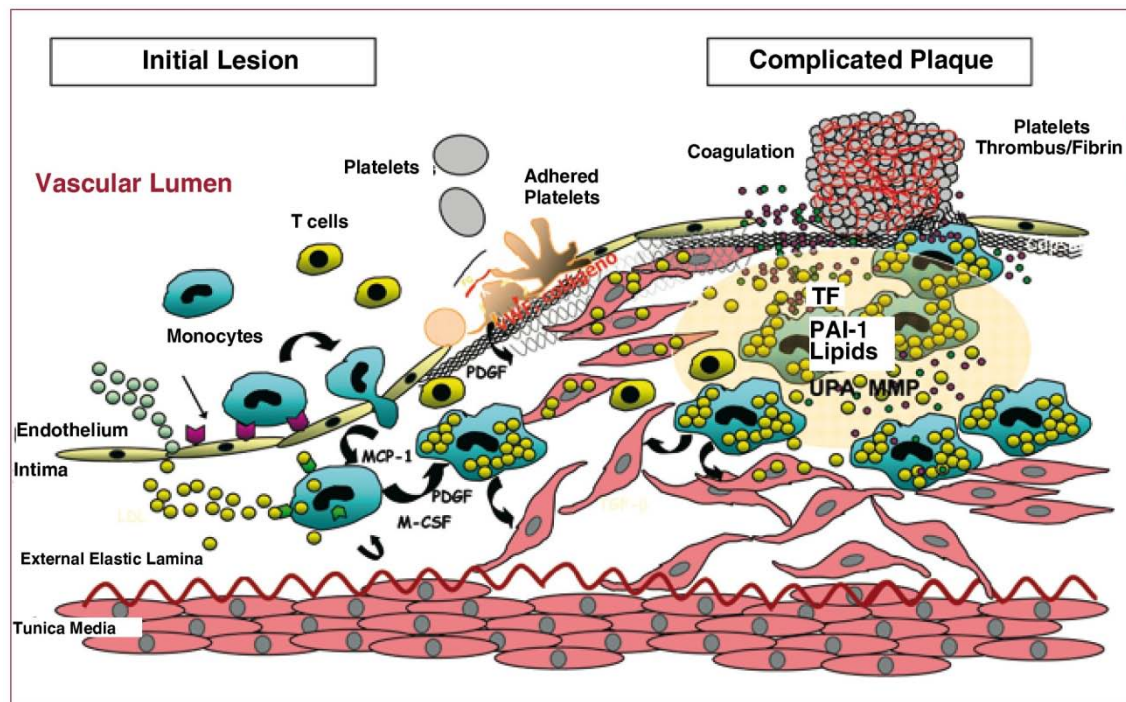


Figura 3: Representació esquemàtica de la progressió de la placa ateroscleròtica des de les etapes inicials de la disfunció endotelial a estadis més avançats amb la presència de plaques complicades. M-CSF: factor estimulant de colònies de macròfags; MCP-1: proteïna quimiotàctica de monòcits 1; MMP: metaloproteïnases; PAI-1: inhibidor de l'activador del plasminogen 1; PDGF: factor de creixement plaqueta-derivat; TF: factor tissular; UPA: activador del plasminogen urocinasa (Badimon et al. 2009).

Les connexions cèl·lula-cèl·lula, tals com unions tight o unions gap són estructures essencials en la regulació de la funció de la permeabilitat de l'endoteli. La formació d'unions gap (o unions en fenedura) estan regulades per la presència i funcionalitat de les conexines, proteïnes l'expressió de les quals es modifica durant la formació de les lesions ateroscleròtiques (Chadjichristos i Kwak 2007). Les unions gap, a més d'afavorir els processos de senyalització intercel·lular, regulen la vasodilatació depenent del NO (Figura 4).

Entre l'estria grassa i la placa vulnerable es troba l'estadi intermig de l'evolució de l'aterosclerosi, constituït per l'aparició de plaques d'ateroma. La fase 2 s'expressa amb la presència de plaques tipus IV i V, que histopatològicament corresponen a plaques vulnerables. Durant la seva evolució poden migrar cap a la fase 3, amb la progressió i consolidació de la placa d'ateroma, i s'expressen clínicament com una progressió del grau d'angina. També poden evolucionar cap a la fase 4, amb la ruptura o erosió, i produir un trombe parcial o total que desencadena una síndrome isquèmica aguda (Fuster et al. 1992). Aquestes plaques de tipus IV i V poden tenir diferent expressió fenotípica i manifestar-se amb diversa morfologia, amb ruptura, fisura-cicatrització i hemorràgia intraplaca. Amb menys freqüència es presenta com una erosió o amb calcificació de la placa (Badimon et al. 1999).

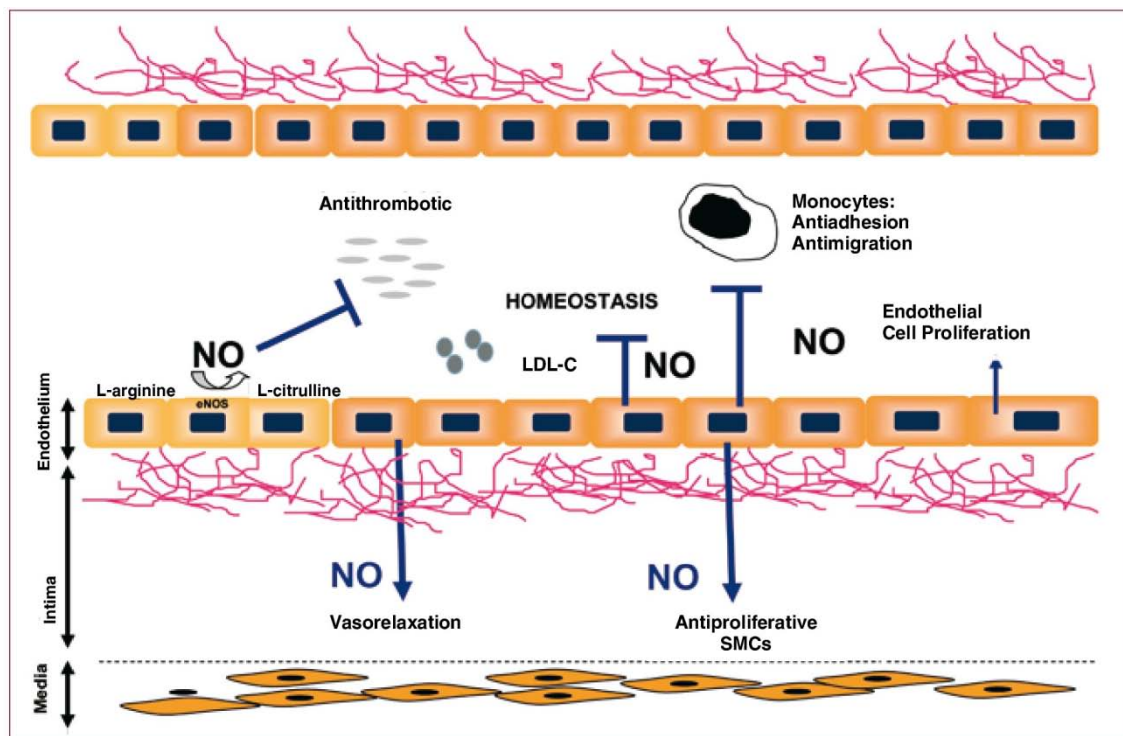


Figura 4: Esquema simplificat de l'efecte vasoprotector de l'òxid nítric. LDL-C: colesterol-LDL; NOS: òxid nítric sintetasa (Badimon et al. 2009).

3.3.3 Estudis epidemiològics. Malalties cardio i cerebro vasculars.

Durant els últims 30 – 35 anys, s'han portat a terme diferents estudis epidemiològics en diferents països amb l'objectiu d'avaluar l'efecte dels hàbits dietètics en el desenvolupament de malalties cardiovasculars.

En un d'aquests estudis, s'ha avaluat la mitjana d'ingesta de flavonoides i altres substàncies fenòliques en 16 grups que participaven en l'Estudi dels Set Països; on es va trobar que la ingesta de flavonoides estava inversament associada a la mortalitat per infart de miocardi (Hertog et al. 1995). En un estudi dissenyat amb 10.054 homes i dones, es va estudiar l'associació entre els flavonoides consumits dividit per quartils de consum i el risc de determinades malalties cròniques (Knekt et al. 2002). En aquest estudi les persones amb ingestes superiors de quercetina van tenir una menor mortalitat per cardiopatia isquèmica. El risc relatiu (RR) entre el quartil més alt i més baix va ser de 0.79. La incidència de malaltia cerebrovascular va ser menor a major ingesta de kaempferol, naringenina i hesperitina.

La presència de varis compostos antioxidants en les F&V que consumim a través de la dieta, pot explicar l'efecte decreixent de la PA en pacients hipertensos. Aquest efecte protector contra la malaltia cardiovascular (ECV) s'ha observat en nombrosos estudis epidemiològics (Agudo et al. 2007; Alonso et al. 2004). L'alt consum de F&V es va relacionar amb un menor risc de malalties cardiovasculars en un estudi amb 2.682 homes a Finlàndia (Rissanen et al. 2003). Radhika i col·laboradors (Radhika et al. 2008) també van examinar la relació entre la ingesta de F&V i els factors de risc d'ECV a la població xinesa al sud de l'Índia. La població d'aquest estudi va estar composta per 983 persones de 20 o més anys, seleccionades a partir de Chennai Urban Rural Epidemiological Study (CURES). L'anàlisi de regressió lineal va revelar que després d'ajustar per varis factors de confusió, el quartil més alt de consum de F&V va mostrar una relació inversa significativa amb la PA sistòlica ($\beta = -2.6$ mmHg; $P = 0.027$), en comparació amb el quartil més baix. Un elevat consum de F&V expliquen el 48% de l'efecte protector contra els factors de risc de ECV.

De la mateixa manera, la prevalença d'hipertensió no diagnosticada prèviament en l'estudi SUN (Seguiment Universitat de Navarra) (Alonso et al. 2004) es va associar inversament amb el consum de F&V en una població mediterrània

amb un consum molt elevat de greixos d'origen vegetal. El *Nurses' Health Study* (NHS) (Hu et al. 1998) és un estudi prospectiu, realitzat en 83.818 infermeres entre 30 y 55 anys d'edat i procedents de varis estats dels Estats Units, en aquest estudi el consum de F&V es va associar inversament amb la PA sistòlica y diastòlica, mentre que la ingesta de cereals i carn es van associar directament amb una PA sistòlica superior (Ascherio et al. 1996).

Al Chicago Western Electric Study el consum de proteïna vegetal, vitamines antioxidants basades en vitamina C i beta-carotè van estar inversa i significativament relacionats amb una variació anual mitjana de la PA, després de 8 anys del seguiment de 1714 empleats homes de mitjana edat (Stamler et al. 2002). D'altra banda, Hung i col·laboradors, (Hung et al. 2003) van avaluar l'associació del consum de F&V amb la malaltia arterial perifèrica, en una cohort de 44.059 homes amb absència de malaltia cardiovascular i diabetis, observant que el consum de F&V protegeix contra la malaltia arterial perifèrica. En el model ajustat per edat, els homes en el quintil més alt de consum de F&V en comparació amb els del quintil més baix, van tenir una disminució del RR de 0.55 (95% CI: 0.38-0.80) per la malaltia arterial perifèrica. No obstant, les associacions van ser molt dèbils després de l'ajust per tabaquisme i altres factors tradicionals de risc cardiovascular.

Reshef i col·laboradors, en un estudi clínic doble cec i creuat, van demostrar que amb aliments rics en flavonoides, suc i xocolata negra s'observa una reducció significativa de la PA i una millora de la vasodilatació del flux dilatat depenent de l'endoteli (Grassi et al. 2005; Reshef et al. 2005).

L'absorció intestinal i el metabolisme dels polifenols vegetals són un pas limitant dels efectes protectors d'aquesta classe de compostos, degut a que la rellevància clínica dels efectes de l'endoteli-depenents dels polifenols de la planta, depèn de la seva disponibilitat sistèmica.

El consum diari d'una dosi de 100 g de xocolata negra rica en polifenols (500 mg de polifenols) va disminuir tant la PA sistòlica com la diastòlica en 10 dies, en pacients amb hipertensió sistòlica aïllada (Taubert et al. 2003). La velocitat del flux coronari es va incrementar en voluntaris sans després de prendre una beguda rica en polifenols (1 g/kg d'etanol com a vi negre), però no després de beure la mateixa quantitat d'alcohol en forma de vodka o vi blanc, una beguda

alcohòlica sense polifenols o unes begudes amb contingut de polifenols mitjà, respectivament (Shimada et al. 1999). Després de la ingesta aguda de 500 ml de vi negre i de vi negre desalcoholitzat (Hashimoto et al. 2001), la vasodilatació depenent de l'endoteli va patir una millora. Una reducció en el consum d'àcids grassos totals i saturats i la intervenció amb oli d'oliva extra verge va afectar favorablement a la PA en pacients hipertensos (Ferrara et al. 2000).

En tots els fruits secs la majoria dels antioxidants es troben localitzats en la pel·lícula o closca tova exterior i més del 50% d'ells es perden quan es retira la pell (Blomhoff et al. 2006). Aquest fet, poques vegades es té en compte en els assaigs clínics amb fruits secs. Les nous són una excepció, perquè gairebé sempre es consumeixen com producte cru amb pells. Estudis recents han demostrat que les pells d'ametlla (Urpi-Sarda et al. 2009a) i cacahuet (Lou et al. 2004) són molt rics en antioxidants. Una anàlisi combinada de 4 estudis epidemiològics d'EE.UU. va mostrar que els subjectes en el grup de major consum de nous tenen aproximadament 35% menor risc d'incidència de ECV.

El *Adventist Health Study* (AHS) va ser el primer en despertar l'interès pels fruits secs i la reducció de risc de patir cardiopatia coronària. Aquest estudi prospectiu va incloure 31.208 Adventistes del Setè Dia de Califòrnia (Fraser et al. 1992). En la cohort de AHS, la freqüència de consum de fruits secs va mostrar una relació inversa amb el risc de patir un infart agut de miocardi (IAM) no mortal o mort per cardiopatia coronària. Els individus que consumien fruits secs d'1 a 4 vegades per setmana presentaven una reducció del 26% en el risc de patir un IAM no mortal i una reducció del 27% en el risc de morir per una cardiopatia coronària, en comparació amb els individus que ingerien fruits secs menys d'una vegada per setmana, mentre que les persones que consumien 5 vegades o més per setmana van mostrar una reducció del 48% en el risc de patir un IAM i una reducció del 38% en el risc de mort per cardiopatia coronària (Fraser et al. 1992).

El *Iowa Women's Health Study* (IWHS) es va realitzar en aproximadament 34.000 dones postmenopàusiques d'edats compreses entre 55 i 69 anys (Kushi et al. 1996). En aquest estudi, les dones pertanyents al quartil més alt de consum de fruits secs presentaven una reducció del 40% en el risc de patir una

cardiopatia coronària mortal, en comparació amb les del quartil inferior, però després de l'ajust per vitamina E consumida aquest risc es va reduir al 28%.

Els fruits secs a més protegeixen als PUFA *in vivo* contra l'oxidació, demostrant-se en varis assaigs clínics. Berry i col·laboradors, (Berry et al. 1992) van mostrar que l'oxidació dels lípids del plasma i de les LDL en voluntaris sans va ser menor després d'una dieta amb ametlles en comparació amb una dieta baixa en greixos. Jenkins i col·laboradors, en un estudi de dosi-resposta van comparar dues dosis d'ametlles amb una dieta baixa en greixos en subjectes hiperlipidèmics. La dosi més alta d'ametlles va produir una major reducció en els nivells de lípids del plasma sanguini. No obstant, las dues dosis van ser efectives reduint el colesterol LDL i la relació colesterol-LDL:colesterol-HDL (Jenkins et al. 2002).

Hyson i col·laboradors, (Hyson et al. 2002) van portar a terme un estudi per un període de 6 setmanes, per comparar els efectes del consum d'ametlles amb clofolla vs. oli d'ametlles, sobre els lípids del plasma i l'oxidació de LDL en homes i dones sans. L'efecte d'ambdós tractaments sobre els lípids plasmàtics no es va diferenciar respecte a les mides basals; però els triglicèrids plasmàtics, el colesterol total i el colesterol-LDL van disminuir significativament, 14, 4 i 6% respectivament, mentre que el colesterol-HDL va augmentar en un 6% (en ambdues intervencions). Els fruits secs, incloent el cacauet, s'han reconegut com a potencials en la millora del perfil de lípids en plasma. En estudis de cohort, el consum de nous ha estat associat amb un menor risc de ECV (Jenkins et al. 2008).

L'efecte protector dels compostos fenòlics sobre l'oxidació de les LDL ha sigut investigat en un estudi amb extracte d'oli d'oliva, olis d'oliva amb components fenòlics (extra verge) i sense components fenòlics (refinat) (Fito et al. 2000). En aquest estudi es va observar que l'oli amb fenols disminuïa l'oxidació de les LDL amb un retard en la formació de conjugats diens mediada per LDL.

En un estudi dins de la cort REGICOR (Registre Gironí del Cor), es va plantejar el colesterol-LDL com el major potencial per a prevenir les ECV entre els 35 i 74 anys [42% (95% Interval de confiança: 23,58)]. L'anàlisi estratificada per edat va mostrar que el risc atribuïble poblacional (PAF) per a pacients fumadors menors de 55 anys era de 64% (30,80); mentre que els fumadors de ≥ 55 anys,

la PAF per a la hipertensió va ser del 34% (1,61) (Grau et al. 2010). Tots aquests estudis també donen suport a l'opinió de que les dietes riques en polifenols, poden reduir la PA i millorar la funció endotelial en pacients hipertensos.

3.4. BIOMARCADORS

Un marcador biològic és una molècula biològica que es troba en la sang o en altres fluids o teixits corporals i és un signe d'un procés normal o anormal, o d'una afecció o malaltia. Un marcador biològic pot fer-se servir per a determinar la resposta del cos a un tractament per a la prevenció o cura d'una malaltia o afecció. En particular, el biomarcador nutricional és un compost extern, com poden ser els propis components dels aliments o els metabòlits originats a partir dels mateixos, uns metabòlits analitzats en mostres biològiques que serveixen per a determinar la seva exposició o ingesta a aquest aliment o component.

L'epidemiologia nutricional té com a principal objectiu avaluar els efectes de la dieta en el risc de patir determinades malalties. Per a comprendre de manera fiable com influeix la dieta és necessari conèixer de forma precisa la ingesta d'aliments de tots els participants. La valoració de la dieta es pot realitzar mitjançant biomarcadors nutricionals o a partir d'enquestes alimentàries.

3.4.1. Enquestes alimentàries

Per a conèixer el consum alimentari individual o poblacional es disposa de les enquestes alimentàries que estimen el consum durant un període de temps determinat. Existeixen diferents classificacions de les enquestes, però una de les més habituals és segons el període de temps que es vol avaluar:

- Mesures de la ingesta dietètica en dies concrets o actual.
 - Registre alimentari: mètode prospectiu consistent en sol·licitar a l'entrevistat que anoti en un formulari diàriament, durant varis dies, els aliments i begudes que va ingerint tant a casa com fora (els més freqüents són els de 3 dies).
 - Recordatori de 24 hores: mètode retrospectiu consistent en demanar al subjecte que recordi tots els aliments i begudes consumides 24 hores abans de la entrevista.

- Mesures de ingesta habitual.
 - Qüestionari de Freqüència de Consum (FFQ): mètode retrospectiu en el que s'administra al subjecte una llista tancada d'aliments i begudes sobre la que se li sol·licita la freqüència i la porció de consum durant un període de temps determinat (6 mesos, 1 any).
 - Història dietètica: mètode retrospectiu que inclou un o varis recordatoris de 24 hores, amb la finalitat de conèixer la ingesta actual, i un qüestionari de freqüència de consum per a conèixer la ingesta habitual.

Els avantatges i limitacions de les enquestes són en gran part influenciades pel mètode d'administració de les mateixes, aquestes poden realitzar-se mitjançant entrevista, ja sigui en persona, per via telefònica o autoadministrada. L'entrevista dóna millors resultats perquè l'entrevistador pot facilitar i amenitzar la resposta del qüestionari, però pot influenciar de forma diferent en la resposta dels participants introduint en l'estudi un biaix degut a l'entrevistador (Delgado M. et al. 2008).

3.4.2. Característiques dels marcadors nutricionals o biològics.

Com en qualsevol mesura analítica que es preï un biomarcador ha de complir els clàssics requisits d'exactitud, reproductibilitat, fiabilitat i validesa (Marshall 2003; Zamora-Ros et al. 2009). A més, qualsevol biomarcador nutricional per a ser considerat útil ha de satisfer els següents 4 criteris (Spencer et al. 2008):

1. Disposar d'un mètode analític qualitativa i quantitativament robust. És necessari treballar amb la metodologia adequada per a garantir la validesa dels resultats. En components minoritaris, com és el cas dels polifenols, la sensibilitat i especificitat de la tècnica adquireixen gran importància. Els avenços en tècniques d'anàlisi químic, com és el cas de LC-MS/MS, estan permetent l'estudi d'aquests components en mostres biològiques (Liu et al. 2002; Zamora-Ros et al. 2009)
2. Les concentracions del biomarcador en la mostra biològica (teixit o biofluid) han de ser sensibles als canvis en el consum del compost d'interès. Aquesta és la característica més important que ha de complir un biomarcador nutricional. En aquest apartat és important considerar la capacitat que té el biomarcador de discriminar als consumidors dels no consumidors, en el cas que sigui preceptiu, com en qualsevol prova diagnòstica, ha de presentar uns bons paràmetres de sensibilitat, especificitat i uns valors predictius alts, minimitzant els falsos

positius i negatius. També s'ha de destacar la capacitat de detectar petits increments en l'exposició, mostrant una bona correlació entre mesura i exposició.

3. El biomarcador ha de ser específic de la ingesta del component d'interès. Per aquest motiu, qualsevol variació en la seva concentració ha de ser el resultat en un canvi en el consum del component d'interès. El cas ideal seria que un únic aliment contingués el component d'interès, d'aquesta forma les variacions en el marcador serien degudes exclusivament a les variacions en el consum d'aquest aliment. Aquesta possibilitat és molt difícil, però alguns components són específics d'uns aliments molt concrets. Aquest és el cas d'alguns polifenols i el consum d'aliments rics en aquestes substàncies com el te, cafè, vi, soja, ceba, oli d'oliva (Spencer et al. 2008; Zamora-Ros et al. 2006).
4. La interpretació del biomarcador és més complexa que la enquesta alimentària degut a que el biomarcador té en compte la biodisponibilitat del component. En el cas dels polifenols, depenent de la dosi, del temps transcorregut des de la ingesta i de l'individu poden aparèixer diferents perfils metabòlics del mateix polifenol. Per aquest motiu, l'avançament en les tècniques analítiques és imprescindible per disposar del perfil total de metabòlits d'aquest polifenol.

Una limitació de l'ús dels biomarcadors és el temps de vida mitja dels components en la mostra biològica. Existeixen múltiples exemples, depenent del teixit o biofluid seleccionat, de biomarcadors d'ingesta a curt termini (2-6 hores), com podrien ser els polifenols en plasma, d'ingesta a mitjà termini (1-5 dies), com podria ser els polifenols en orina, i de llarg termini (setmanes o mesos), com per exemple seleni a les ungles (Marshall 2003). Depenent de la nostra investigació ens interessarà més seleccionar un teixit/biofluid o un altre, ja que la informació que ens reportarà el biomarcador serà totalment diversa: un biomarcador a curt termini ens informarà d'una ingesta aguda (consum puntual); i un biomarcador a mitjà/llarg termini ens informarà d'una ingesta regular o habitual (consum continuat en el temps).

Una altra limitació important és la gran variabilitat interindividual que existeix en la majoria de respostes metabòliques quan es dóna la mateixa dosi de component, aquesta és una situació molt freqüent en els estudis de biodisponibilitat de polifenols (Manach and Donovan 2004; Urpi-Sarda et al. 2005; Urpi-Sarda et al. 2007).

3.4.3. Avantatges dels marcadors biològics respecte a l'estimació dietètica o marcadors dietètics.

Sempre existeix un error en les estimacions dietètiques, el repte actual és entendre, estimar i tenir en compte aquest error durant l'anàlisi de les dades (Beaton et al. 1997); per conèixer aquest error és necessari disposar de biomarcadors que reflecteixin la ingesta real dels individus. Aquests biomarcadors poden arribar a substituir les estimacions dietètiques tradicionals (Kristal et al. 2005); encara que en algunes situacions, les estimacions dietètiques són encara imprescindibles degut a limitacions econòmiques i a la falta de biomarcadors útils (Kelemen 2006).

Existeixen varies raons per decantar-se per l'ús de biomarcadors nutricionals respecte a les convencionals enquestes dietètiques (Kelemen 2006; Potischman 2003). Els biomarcadors nutricionals són més precisos que les estimacions dietètiques. Les enquestes, sobre tot els FFQ, ofereixen uns inconvenients a l'hora d'identificar correctament l'aliment, degut a la gran gamma d'aliments presents al mercat, a l'àmplia varietat de tècniques culinàries disponibles i a les diferències en ingredients i preparació de les receptes i tecnologies culinàries. Un altre factor limitant és la quantificació d'aquests aliments, encara que es faciliti mitjançant mesures caseres i llibres de fotos sobre aquestes porcions. També s'ha de destacar que existeix la tendència dels participants a sobreestimar el consum d'aliments saludables (per exemple fruites i verdures) i infravalorar el consum d'aliments considerats menys saludables (aliments rics en greixos saturats i begudes alcohòliques, entre altres) (Spencer et al. 2008).

Les enquestes d'aliments són transformades a components mitjançant bases de dades o taules de composició d'aliments (TCA). Les TCA presenten moltes limitacions en quant al número d'aliments, variabilitat en composició dels aliments, mètodes analítics utilitzats i nombre de components disponibles, sobretot en el cas de components minoritaris com poden ser els polifenols. També s'ha de tenir en compte la complexitat de saber quines són les TCA més adequades per l'estudi i com utilitzar aquestes TCA per part de l'investigador.

Els biomarcadors proporcionen una mesura més propera de l'estat nutricional que les estimacions dietètiques; degut a que els biomarcadors integren en la mesura, la biodisponibilitat i el metabolisme del component. En canvi les TCA només informen de la quantitat present en l'aliment. La funció principal dels biomarcadors nutricionals, com s'ha comentat anteriorment, és la de conèixer de forma menys subjectiva que les enquestes alimentàries el consum d'un component o grup de components (ja es tracti d'un nutrient o no nutrient) o d'un aliment o grup d'aliments.

Una funció secundària dels biomarcadors nutricionals és la de valorar el compliment d'una intervenció nutricional. Comprovar objectivament que els participants realment han seguit el tractament o la suplementació, en aquest cas dietètica, de forma correcta; on les enquestes alimentàries no podrien substituir l'ús de biomarcadors. Per exemple, en l'estudi PREDIMED es suplementa la dieta habitual amb oli d'oliva verge o fruits secs i per verificar que els participants amb aquestes suplementacions estan seguint correctament les pautes s'analitza l'hidroxitirosol i el tirosol en orina (polifenols característics de l'oli d'oliva) i l'àcid oleic en plasma (àcid greixós característic de l'oli d'oliva) en el grup d'oli d'oliva i en el grup dels fruits secs l'àcid α -linolènic plasmàtic (característic de les nous) (Estruch et al. 2006).

Durant un estudi d'intervenció nutricional, els polifenols mai són consumits independentment sinó com una barreja complexa que inclou una ampla varietat de compostos biològicament actius que no han sigut totalment caracteritzats. La font i la possible interacció amb altres constituents de la dieta podrien afectar intensament la resposta metabòlica d'un individu, el que no sempre es té en compte quan s'estudien els efectes beneficiosos dels polifenols. Per tant, s'hauria de considerar una aproximació global que permeti avaluar les complexes relacions entre la salut i el consum de polifenols.

A part de la necessitat d'obtenir dades precises sobre el contingut polifenòlic en la dieta humana, també és crucial considerar la capacitat que tenen els polifenols d'entrar en la circulació sanguínia ja que la seva activitat biològica depèn altament de la seva absorció i metabolisme. L'absorció intestinal dels diferents polifenols està estretament lligada a la seva estructura molecular i a la font alimentària (Manach et al. 2004). Per exemple, les isoflavones són molt

més eficientment absorbides que les flavonoles i flavones, mentre les proantocianidines i antocianines són escassament absorbides (Manach et al. 2004).

Molts científics han triat l'ús de metabòlits específics presents en biofluids humans com marcadors potencials de l'exposició als polifenols. L'orina i el plasma han sigut freqüentment usats per predir el consum de polifenols a partir de dietes habituals d'individus no controlats (Noroozi et al. 2000; Radtke et al. 2002). Malgrat això, les mostres d'orina requereixen menys preparació pre-analítica i per aquesta raó són sovint preferentment analitzades en estudis de cohort on les mostres de plasma presenten un maneig més laboriós. A més, els compostos com els polifenols, dels quals les farmacocinètiques es caracteritzen per un curt temps de vida mitjana y una baixa concentració màxima en plasma, són més fàcils de detectar en orina.

3.5. DIETA MEDITERRÀNIA

La dieta mediterrània tradicional és l'herència de segles d'intercanvis de persones, cultures i aliments de tots els països de la conca mediterrània. Aquesta ha sigut la base dels hàbits alimentaris fins a mitjans del segle XX en tots els països de la regió, però ara està desapareixent progressivament degut a l'amplia difusió de l'economia de tipus occidental, la cultura urbana, la tecnologia, així com la globalització de la producció i consum d'aliments (Serra-Majem et al. 2009).

Des de la dècada dels 50, l'estudi dels set països, basat en les dades recollides a Grècia, Itàlia i Iugoslàvia, ens va donar a conèixer quins aliments eren més o menys consumits freqüentment en aquest àmbit. Conduint a la definició de la dieta mediterrània tradicional (DMT) com un patró de dieta rica en aliments vegetals (cereals, fruites, verdures, llegums, fruits secs, llavors i olives), amb oli d'oliva verge com principal font de greix afegit, junt amb una alta/moderada ingesta de peix i mariscs, un consum moderat d'ous, aus de corral i productes lactis (formatge y iogurt), un baix consum de carn vermella (principalment oví i cabrum), i un consum moderat d'alcohol, principalment com a vi i durant els

menjars. Aquesta va ser la dieta seguida principalment pels individus de les societats rurals.

L'estudi pioner dels "Set Països" i nombrosos estudis epidemiològics recents han establert el benefici de salut associats amb l'adherència al patró de DM, amb respecte a la millora de la síndrome metabòlica, diabetis tipus II, malalties cardiovasculars, algunes malalties neurodegeneratives i el càncer (Estruch et al. 2006; Meydani 2005; Salas-Salvado et al. 2008; Serra-Majem et al. 2006; Sofi et al. 2008; Trichopoulou et al. 2000; Trichopoulou et al. 2005). En un estudi més recent, amb 3.910 homes i 4.285 dones procedents de Girona, entre 35 i 74 anys d'edat, un increment de 5 unitats de l'escala de DM va mostrar una relació directa amb canvis de 0.74 i 1.15 unitats en la puntuació del component mental, en homes i dones, respectivament, després d'ajustar per possibles factors de confusió (Muñoz et al. 2009).

Aquest patró saludable de DMT s'ha popularitzat des de 1995 amb la famosa piràmide de la representació del món que posa de manifest gràficament els aliments a consumir diàriament, setmanalment o amb menor freqüència. Dins de la comunitat científica, existeix un consens sobre el que constitueix el patró de DMT, però s'han plantejat qüestions sobre la necessitat d'actualitzar les presents recomanacions tenint en compte el nou estil de vida, hàbits dietètics, factors ambientals i problemes de salut que enfronten els reptes de les noves generacions, diferents països i sectors de la població (especialment els de baixos ingressos).

La Declaració de Barcelona del 2007, amb la proposta dels científics presenta una nova eina de comunicació per a la població, professionals de la salut i les parts interessades; qui esperen el reconeixement, per part de la UNESCO, del patró de DMT com patrimoni cultural immaterial de la humanitat, considerant l'interès mundial d'aquest patró de DM. Amb aquesta representació s'espera contribuir a una millor adherència a aquest patró de dieta saludable y la seva forma de la vida en el Mediterrani i altres països en el futur pròxim.

El consens sobre una nova representació gràfica de la piràmide de DM té com a objectiu principal fomentar el diàleg entre els científics i experts en nutrició i salut pública, ciències de l'alimentació, antropologia, sociologia, agricultura i medi ambient a fi de tenir sobre la zona del Mediterrani una representació

comuna dels patrons de DM sense drets d'autor. Aquesta piràmide es planteja pel consens entre els experts científics mencionats i es basa en la investigació en el camp de la nutrició i la salut; l'evidència científica al voltant dels beneficis cap a la salut, que aporten els patrons dietètics de DM, justificats a través dels grans estudis epidemiològics publicats en cents d'articles en forma de revisions en les revistes científiques de les últimes dècades. Aquesta representació dels patrons dietètics de DM respon a la necessitat d'un marc comú entre els països mediterranis com pautes dietètiques basades en l'alimentació (World Health Organization. 2002).

El disseny final de la piràmide de la dieta mediterrània d'avui en dia i un breu text complementari per el públic en general ha sigut desenvolupat per l'opinió recollida pel Comitè Científic Internacional de la Fundació Dieta Mediterrània i les deliberacions "*in situ*" d'un grup representatiu dels membres reunits en el VIII Congrés Internacional sobre la Dieta Mediterrània en el Barcelona, 2010.

El fet d'expressar els objectius en termes dels aliments que les persones compren i mengen, com un patró de dieta basada en un model cultural, pot contribuir a un major respecte entre la població general. La DM és, però, no sols un patró dietètic sinó que també representa una forma de vida i un model cultural. L'objectiu de recomanacions va dirigit a la població adulta sana entre 18-65 anys i han d'adaptar-se a les necessitats especials dels nens, dones embarassades i altres condicions de salut.

La nova piràmide de DM (Figura 5) és una semi-quantitativa representació que incorpora les proporcions relatives de les porcions i la freqüència dels principals grups d'aliments que constitueixen el model de DM. Els aliments en la base de la piràmide han de proporcionar la major contribució de la ingesta d'energia. Així, diàriament, la consumició adequada dels tres elements principals dels menjars i dels aliments principals que complementen en una menor contribució a la ingesta energètica diària, ha de ser la base de la ingesta diària en el context de la DM.

Les fonts de proteïna figuren en una menor freqüència de consum i la contribució a la ingesta de energia, passen de consum diari a setmanal, i en el vèrtex de la piràmide dels aliments que es recomana en la dieta només ocasionalment. Cap aliment present en la piràmide ha de ser considerat dolent

Antecedents Bibliogràfics

per la salut i per tant no ha de ser evitat. És la varietat, la freqüència i la proporció dels aliments presentats en la dieta habitual i la seva contribució a la ingesta de nutrients el que fa que la dieta sigui saludable o no. Una ampla varietat d'aliments en la dieta redueix al mínim la possibilitat de deficiències d'un nutrient en particular. De fet, una major adhesió al patró de DM s'ha associat a un millor perfil de nutrients, amb una menor prevalença d'individus que mostren la ingesta inadequada de micronutrients en comparació amb altres models com l'occidental (Serra-Majem et al. 2009).

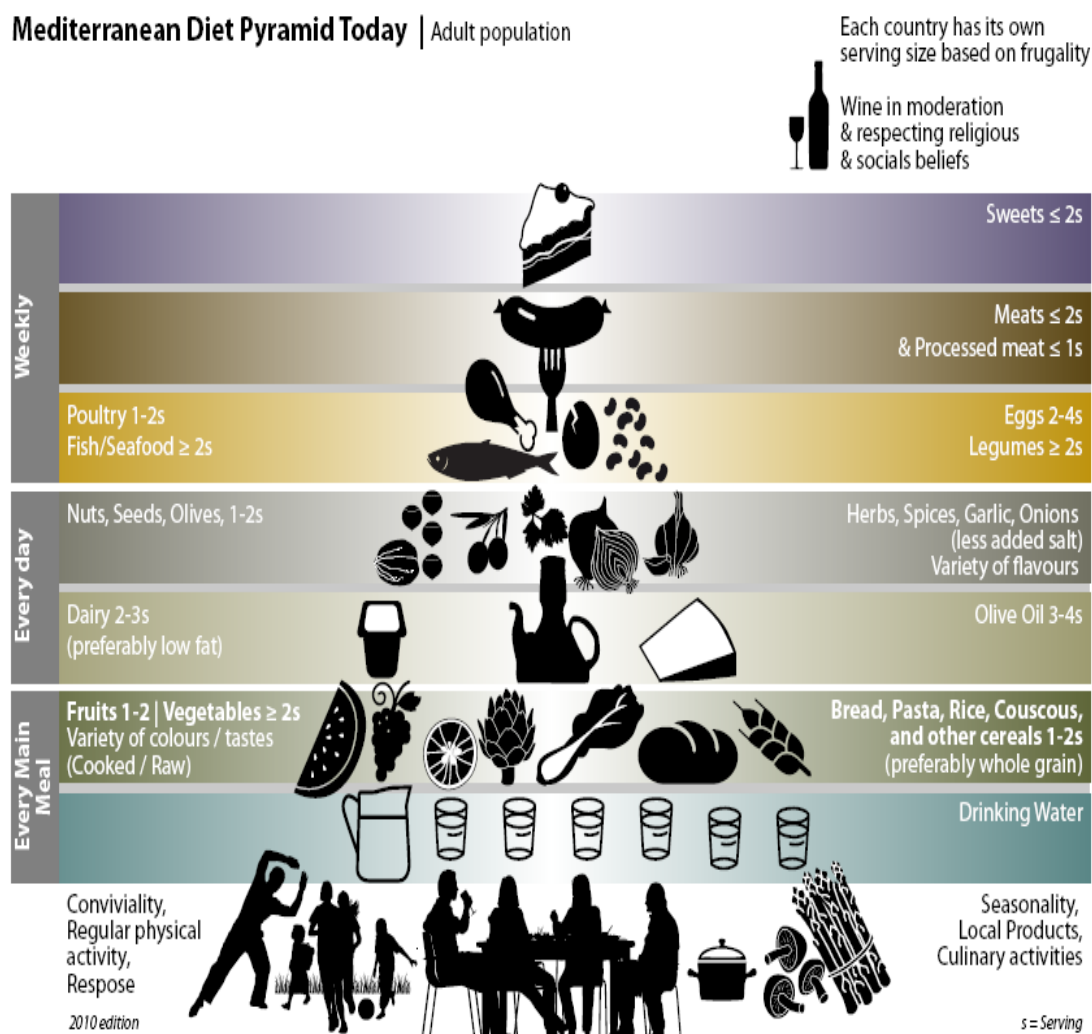


Figura 5: Consens sobre una nova representació gràfica de la piràmide de la Dieta Mediterrània.

En l'exterior, però en la base de la piràmide, s'introdueix el concepte de l'estacionalitat i la producció local. A més, la nova piràmide contribueix a definir un estil mediterrani de vida saludable; l'activitat física i la socialització durant els menjars estan representades en la base. Així es destaca la importància de la pràctica regular d'activitat física moderada (com a mínim 30 minuts al dia) com un complement bàsic de la dieta pel consum d'energia de compensació, pel manteniment del pes corporal saludable i per molts altres beneficis de salut. L'activitat física no només involucra als exercicis com el futbol, ballar, córrer, bicicleta, etc., sinó també caminar, pujar les escales en comptes d'utilitzar l'ascensor, les tasques domèstiques, entre altres. Destacant la importància de la pràctica d'activitat física a l'aire lliure i de preferència amb els demés. I per últim però no menys important, l'aspecte de la convivència és important pel valor social i cultural del menjar més enllà dels aspectes nutricionals. Al voltant de la taula i compartir el menjar en companyia de familiars i amics; les xifres de suport social i sentit de comunitat, proporciona una forma d'alleugeriment del frenètic ritme de la vida quotidiana i el gaudir recíprocament de la preparació d'aliments. La importància de les activitats culinàries, el temps i l'espai dedicat a ells també haurien d'estar presents. I, finalment, el repòs, per exemple, la migdiada, un descans després del menjar saludable no ha de ser oblidat.

Beure aigua (6-8 porcions, equivalent a 1.5-2L) és essencial per la vida i per preservar l'equilibri de l'aigua corporal. D'acord amb l'edat i altres condicions personals aquestes necessitats poden variar. També està present un consum moderat de vi (1 got/dia per les dones i 2 gots/dia per homes, com una referència genèrica) durant els menjars mentre es respecten les creences religioses i socials.

La mida de les porcions ha de basar-se en la frugalitat, per adaptar l'estil de vida urbà i modern, a les necessitats d'energia. Les porcions dels aliments representats en la part inferior han de ser més grans i consumits amb major freqüència, tanmateix els aliments en els nivells superiors s'han de consumir en quantitats més petites i amb menor freqüència.

La composició d'un menjar principal, doncs, ha de ser una combinació de cereals (de preferència integral), hortalisses, verdures i fruites. Les quantitats per menjar són: verdures i hortalisses (major o igual a 2 porcions de menjars,

Antecedents Bibliogràfics

en forma crua, en un dels dos menjars principals), cereals en forma de pa, pasta, arròs, cuscús y altres (1-2 porcions/menjar) i la fruita com els postres principals (1-2 porcions/menjar). El concepte de "varietat de colors i sabors" es sobremarca, en el cas de les F&V per la varietat de compostos antioxidants (figura 6). I en el cas dels cereals, els no refinats seran preferibles ja que el processament normalment elimina alguns valuosos nutrients como la fibra o els antioxidants.

Com els fonamentals elements esmentats de l'àpat principal, altres aliments vegetals han de constituir el nucli del model de DM perquè són importants fonts de vitamines, minerals, fibra, així com d'altres compostos amb potencial antioxidant que contribueixen al benestar general i la sacietat. Aquest nucli de DM, basat en aliments vegetals, és responsable de la prevenció de moltes malalties cròniques i del control de pes corporal (Buckland et al. 2008; Sofi et al. 2008). Tradicionalment, les verdures i altres aliments vegetals són cuinats amb oli d'oliva, el que multiplica el seu valor nutricional. Tanmateix, amb la finalitat d'assegurar la ingesta de vitamines i minerals al dia, algunes de les hortalisses han de consumir-se crues (un àpat al dia).



Figura 6: Exposició de fruites, verdures i hortalisses. Mercat de la Boqueria, Barcelona.

El patró de DM, en les quantitats suficients dels seus components, contribueix a la presència de la fibra, micronutrients, antioxidants i substàncies no nutritives que són necessàries per a la conservació de la salut. La varietat dels diferents components asseguren una varietat dels nutrients mencionats. A més, la preferència pels aliments de temporada, frescos i mínimament processats poden maximitzar el seu contingut en la dieta.

A nivell diari, els productes lactis estan presents en una quantitat moderada (2-3 porcions/dia) i la preferència pels productes lactis baixos en greixos s'accentua. Tradicionalment, els productes lactis de major consum encara que en quantitats baixes a moderades han sigut en forma de iogurt y formatge. La seva riquesa en calci per un costat és important per la salut òssia i el cor, però per l'altre, també poden ser una important font de greixos saturats.

L'oli d'oliva ha de ser la principal font de lípids de la dieta, en cas d'utilitzar oli d'oliva barrejat es recomana la seva utilització per cuinar i, en forma verge per a amanir les amanides. Les espècies, herbes, all i ceba són possibles solucions per reduir la ingesta de sal (contribuent al desenvolupament de la hipertensió entre els individus predisposats) i una forma d'introduir varietat d'aromes i sabor als plats, a més de ser una bona font de micronutrients i compostos antioxidants. A més, les herbes i espècies, també contribueixen a la identitat regional de plats mediterranis. Els fruits secs, llavors i olives han d'estar presents als plats, en el patró de tots els dies, ja que són bones fonts de greixos saludables, proteïnes, antioxidants naturals i fibra.

L'oli d'oliva verge (VOO) conté gran quantitat de polifenols (100-600 mg/kg) que tenen un gran efecte en l'estabilitat i les característiques nutricionals del producte. Alguns dels fenols simples més representatius són l'hidroxitirosol i el tirosol. Tanmateix, els compostos fenòlics s'eliminen quan l'oli és refinat (Tovar et al. 2001). El contingut fenòlic del VOO està influenciat per la varietat, ubicació, grau de maduresa i el tipus de procediment d'extracció d'oli usat (Esti et al. 1998; Gimeno et al. 2007).

Els fruits secs contenen compostos bioactius que expliquen els seus múltiples beneficis cardiovasculars. Fitoquímics tals com fitoesterols, compostos fenòlics

i arginina (Kris-Etherton et al. 2008). Els efectes biològics d'aquests compostos depenen de la seva biodisponibilitat, la seva cinètica i temps d'exposició (Roura et al. 2007a).

Es prefereixen fonts de proteïnes baixes en greixos saturats com el peix, els mariscs, les llegums, la carn d'aus de corral i els ous, i en menor quantitat i freqüència de consum de les carns vermelles i processades. En una escala setmanal; una varietat de fonts de proteïna podria figurar en (peix $\geq$ 2 porcions), llegums ($\geq$ 2 porcions), ous (2-4 porcions) i aus de corral (1-2 porcions). Els peixos i mariscs són una important font de proteïna saludable, i una gran varietat en el seu consum és recomanable (peix blau, peix magre i marisc). El peix (especialment aquells rics en lípids) i el consum de mariscs (figura 7) han estat reportats com a reductors del risc de malaltia coronària i amb propietats antiinflamatòries relacionades al seu contingut en àcids grassos poliinsaturats de cadena llarga ω -3 (Kris-Etherton et al. 2002).



Figura 7: Mariscs típics de la dieta mediterrània. Mercat de la Boqueria, Barcelona.

Els llegums comparteixen alguns dels atributs de la salut dels vegetals i també proporcionen proteïnes, encara que de qualitat moderada, que pot millorar-se si es combina amb cereals com l'arròs. Les aus de corral i els ous proporcionen proteïnes d'alta qualitat, les aus de corral són una bona font de proteïna magra, sense els alts nivells de greixos saturats que es troben en alguns talls de carn vermella. Els ous, inclosos els utilitzats per cuinar o fornejar, no haurien de superar els 4 per setmana. Les carns vermelles han de ser consumides en quantitats menors (≤ 2 porcions); preferiblement la de talls magres i les carns processades (≤ 1 porció) com a condiment per a afegir sabor als plats.

En el vèrtex superior, els aliments rics en sucre i els greixos poc saludables han sigut representats (dolços i pastels). Els dolços s'han de consumir en petites quantitats i només de tant en tant, guardar-los per a les celebracions. Alguns dels dolços tradicionals del Mediterrani són de mel. Tanmateix, aquests aliments són molt densos en energia i poden contribuir a l'augment de pes. Els sucres simples s'han associat amb major incidència de caries dental. Aquests es troben naturalment en moltes fruites, i també estan presents en els dolços i rebosteria; així com en begudes, suc de fruites, refrescs i begudes de cola.

3.5.1. L'estudi PREDIMED

L'estudi PREDIMED (*PRE*venició amb *Di*eta *MEDiterrània*) és un ampli estudi clínic de 5 anys, de grups paral·lels, multicèntric, aleatoritzat i controlat destinat a avaluar els efectes de la Dieta Mediterrània en la prevenció primària de malalties cardiovasculars (www.predimed.org; ISRCTN35739639). El mètode detallat de reclutament i el protocol de l'estudi s'han descrit anteriorment per Estruch i col·laboradors (Estruch et al. 2006).

A l'inici de l'estudi, els participants no devien presentar signes clínics de ECV havent de complir al menys un dels dos criteris següents: (1) diabetis mellitus tipus-2 i/o (2) tres o més factors de risc de ECV: hipertensió arterial (PA>140/90 mm Hg o tractament amb medicaments antihipertensius), dislipèmia, obesitat o sobrepès, tabaquisme o antecedents familiars de malaltia coronària d'aparició primerenca. A l'inici de l'estudi tots els participants van completar un validat semiquantitatiu FFQ amb 136 paràmetres (Martin-Moreno et al. 1993), la versió espanyola validada del qüestionari d'activitat física del temps d'oci "Minnesota Leisure Time Physical Activity Questionnaire" (Elosua et al. 1994), i un

Antecedents Bibliogràfics

qüestionari de 47 paràmetres sobre l'educació, estil de vida, història de les malalties i l'ús de medicaments. Infermeres capacitades mesuren els paràmetres antropomètrics com l'alçada, el pes i la cintura i la PA per triplicat amb un validador semi-automàtic OscilloMeter (Omron HEM-705CP; Hoofddorp, Països Baixos (Iglesias-Bonilla et al. 2002)).

Els participants d'ambdós grups d'intervenció amb DM van rebre assessorament personalitzat. Aquells pertanyents al grup de l'oli d'oliva (Med-VOO) van rebre oli d'oliva verge de forma gratuïta (1L per setmana) i aquells destinats al grup de fruits secs (Med-fruits secs) sobres de nous, avellanes i ametlles (30 g al dia).

Els participants assignats al grup control van rebre consells personals dirigits a un canvi de la dieta similar a la DMT, a més d'assessorament personal, junt amb un fulletó amb la recomanació per escrit a seguir: un baix consum de greixos, tal i com recomana la "American Heart Association" (Krauss et al. 2000). En els tres grups, la recomanació de la directriu general, inclou l'augment de la ingesta de fruites, verdures, hortalisses, llegums, peix o mariscs i carns blanques. Les recomanacions negatives inclouen l'eliminació o la reducció dels aliments perjudicials (carns vermella i processades, productes lactis rics en greixos, pastels comercials, aperitius i begudes endolcides amb sucre).

Una setmana després de la inclusió, la dietista delibera durant 1 hora en sessions de grups per separat (fins a 20 participants per grup). Cada sessió consisteix en parlar de la informació i subministrament del material escrit, amb la descripció dels aliments típics del Mediterrani i la llista de la compra de temporada, aliments i verdures comestibles i receptes de cuina (Zazpe et al. 2008).

3.5.2. Dieta mediterrània i malalties.

La relació entre els patrons d'alimentació en general rics en polifenols, com la dieta DASH (Dietary-Approaches-to-Stop-Hypertension) o la dieta mediterrània, y la PA també s'ha examinat en diferents estudis epidemiològics. La dieta DASH des de fa més de deu anys està sent àmpliament promoguda als EE.UU. per la prevenció i el tractament de la hipertensió (Appel et al. 1997). Aquest

patró de dieta ha sigut també avaluat en una població *free-living* del Regne Unit (Harnden et al. 2010); la PA sistòlica i diastòlica van disminuir significativament ($P<0.05$) en 4.6 i 3.9 mm Hg respectivament, en aquells individus que segueixen un estil de dieta-DASH. Després de 3 setmanes amb la dieta DASH, la PA sistòlica i diastòlica en pacients hipertensos obesos era inferior comparat amb els que segueixen una dieta habitual ($-7.6\pm 1.4/-5.3\pm 1.4$ mm Hg, $P<0.001/0.02$) i que els que segueixen una dieta habitual suplementada amb potassi, magnesi i fibra ($-6.2\pm 1.4/-3.7\pm 1.4$ mm Hg, $P<0.005/0.06$), mentre que la PA no va ser significativament diferent en la dieta habitual i suplementada (Al-Solaiman et al. 2010). En un altre estudi (Hodgson et al. 2004) amb 27 homes i dones que van seguir una dieta DASH o una dieta control, el grup de la dieta-DASH va mostrar una reducció significativa en PA sistòlica ($P<0.001$) i diastòlica ($P=0.005$).

Recentment, en l'estudi SUN (Núñez-Córdoba et al. 2009), l'adherència a la dieta mediterrània es va associar amb una reducció dels nivells mitjans de PA sistòlica i diastòlica després de 6 anys de seguiment, però no es va associar amb hipertensió arterial. Estruch i col·laboradors (Estruch et al. 2006) van comparar els efectes a curt termini sobre els marcadors intermedis de risc cardiovascular en l'estudi PREDIMED amb dues dietes mediterrànies suplementades amb VOO i fruits secs, davant una dieta baixa en greixos. Els participants inclosos en els grups de les dietes mediterrànies suplementades van mostrar una disminució significativa en la PA sistòlica i diastòlica després de 3 mesos de la intervenció en comparació amb el grup de dieta baixa en greixos. En comparació amb la dieta baixa en greixos, el canvi mitjà en la Med-VOO i Med-fruits secs van ser -0.39 mmol/L (95% CI, -0.70 a -0.07 mmol/L) i -0.30 mmol/L (CI, -0.58 a -0.01 mmol/L) respectivament, pels nivells de glucosa en plasma; -5.9 mm Hg (CI, -8.7 a -3.1 mm Hg) i -7.1 mm Hg (CI, -10.0 a -4.1 mm Hg) respectivament, per la PA sistòlica i -0.38 (IC -0.55 a -0.22) i -0.26 (IC -0.42 a -0.10) respectivament, per la relació colesterol-HDL/colesterol.

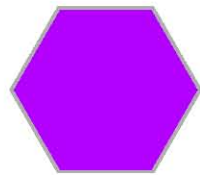
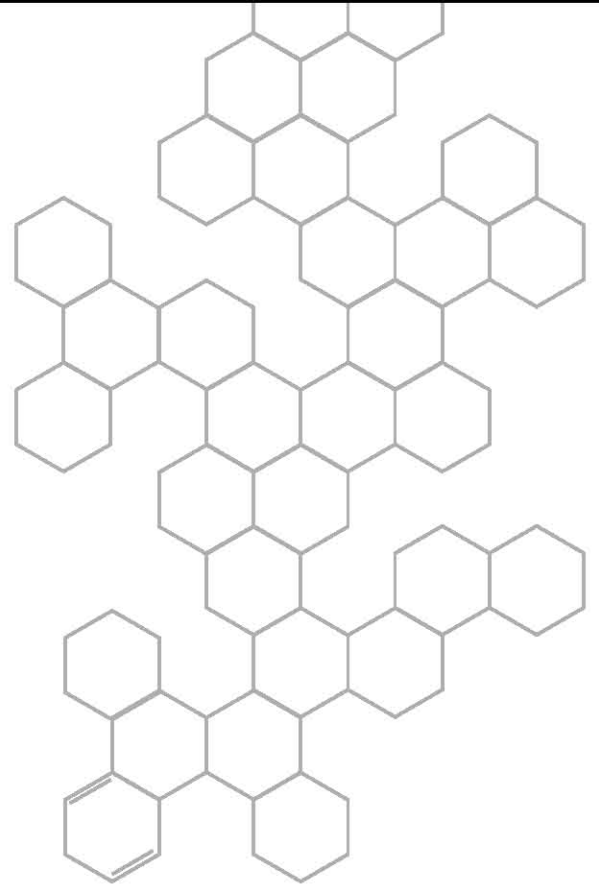
Altres sub-estudis (Fito et al. 2007) dins de l'estudi PREDIMED ha demostrat que la dieta mediterrània enriquida amb nous o VOO, després de 3 mesos d'intervenció, disminueix els nivells de LDL oxidada [-7.3 U/L (95% IC -11.2 a -3.3)] o [-10.6 U/L (IC 95%: -14.2 a -6.1)], respectivament, en comparació amb la

Antecedents Bibliogràfics

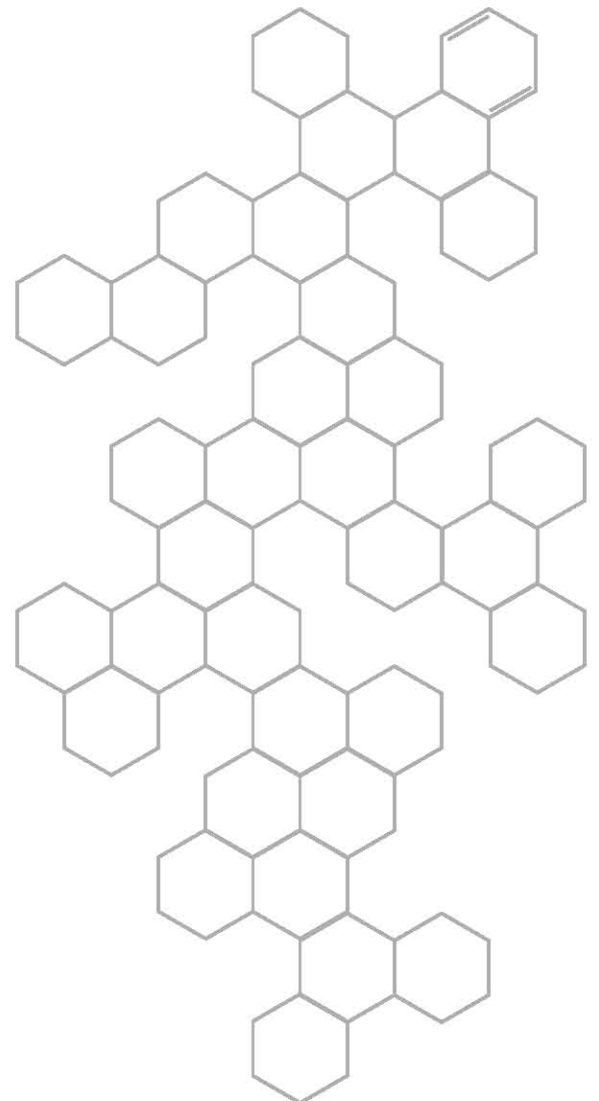
dieta de control, amb canvis no significatius. En altres sub-estudis també dins de l'estudi PREDIMED (Salas-Salvado et al. 2008) es va comparar l'efecte d'1-any d'intervenció sobre l'estat de síndrome metabòlic en 1.224 participants. La prevalència de la síndrome metabòlica després d'un any es va reduir en un 6.7%, 13.7% i 2.0% en la Med-VOO, Med-fruits secs, i els grups de la dieta control, respectivament. Després d'ajustar per factors diferents, el RR per la reversió de la síndrome metabòlica van ser de 1.3 (IC 95%: 0.8-2.1) pel grup Med-VOO i 1.7 (1.1-2.6) pel grup Med-fruits secs en comparació amb el grup control.

La dieta mediterrània, especialment rica en VOO, s'associa amb nivells més alts de la capacitat antioxidant del plasma. La capacitat antioxidant total (TAC) de plasma es va relacionar amb una reducció en el pes corporal després de 3 anys d'intervenció en una població d'alt risc cardiovascular amb una dieta mediterrània rica en VOO (Razquin et al. 2009). La TAC en els grups, control, VOO i fruits secs va ser de 2.01 ± 0.15 , 3.51 ± 0.14 i $3.02 \pm 0.14 \mu\text{M}$ Trolox, respectivament. En consonància amb els estudis anteriors, s'ha observat que l'adhesió a una DMT, pot ser capaç de reduir els factors de risc cardiovascular.

Totes aquestes evidències epidemiològiques recolzen la hipòtesi de partida de que una dieta rica en polifenols, com és el cas de la dieta mediterrània, pot prevenir l'augment de la PA, ajudant a disminuir els nivells elevats de PA.



RESULTATS



4. Resultats

En la següent secció s'exposen els resultats obtinguts dels treballs experimentals realitzats en aquesta tesi doctoral. Aquests resultats s'han concretat en 4 publicacions en revistes del *Science Citation Index*. A més d'una publicació en fase de preparació, però amb resultats previs que poden ser de gran interès social donat el possible paper protector dels polifenols en la hipertensió.

Previ a cada publicació hi ha un resum on es detallen els objectius, el disseny de l'estudi, la metodologia utilitzada i els resultats obtinguts, així com les principals conclusions.

4.1 Publicació 1: Mètode ràpid de Folin-Ciocalteu per a la determinació de compostos fenòlics totals urinaris, com a biomarcador del consum total de polifenols ingerits, utilitzant plaques de 96 cartutxos per a l'extracció en fase sòlida.

Alexander Medina-Remón, Ana Barrionuevo-González, Raúl Zamora-Ros, Cristina Andrés-Lacueva, Ramón Estruch, Miguel-Ángel Martínez-González, Javier Díez-Espino and Rosa M. Lamuela-Raventós. *Analítica Chimica Acta*. 2009, 634(1): 54-60

Resum:

L'epidemiologia nutricional té com a principal objectiu l'avaluació dels efectes de la dieta sobre el risc de patir alguna malaltia, i per comprendre com influeix la dieta és necessari conèixer de forma precisa i real la ingesta d'aliments dels participants de l'estudi. Aquesta ingesta es podria mesurar a través de biomarcadors nutricionals o mitjançant enquestes de consum dietètic. Els marcadors nutricionals tenen varis avantatges pels estudis clínics i epidemiològics al ser més precisos que les dades obtingudes a partir dels qüestionaris dietètics de consum. Pocs estudis han avaluat en quina mesura els polifenols totals excretats en orina són un biomarcador vàlid del consum total de polifenols. Fins ara són poques les cites bibliogràfiques que descriuen un mètode per determinar polifenols totals en una matriu tan complexa com l'orina, la qual té moltes substàncies que interfereixen amb el reactiu de Folin-Ciocalteu.

En aquest treball s'ha desenvolupat un mètode ràpid (Folin-Ciocalteu) per la determinació de polifenols totals en mostres d'orina, per mitjà de l'ús de plaques de 96 cartutxos Oasis® MAX, les quals ens permeten al mateix temps l'extracció en fase sòlida i l'eliminació de possibles interferències en un elevat nombre de mostres. El fet d'utilitzar plaques de 96 pouets ens ha permès realitzar tant la reacció colorimètrica com la lectura espectrofotomètrica a la mateixa placa en només uns pocs segons.

Es va realitzar un estudi prospectiu aleatoritzat creuat amb 12 voluntaris i un estudi transversal amb 60 voluntaris de l'estudi PREDIMED. En ambdós estudis es va avaluar com els polifenols totals excretats en mostres d'orina puntual preses al matí, estan correlacionats amb els polifenols totals ingerits, per ser considerats com un potencial biomarcador del consum de polifenols totals.

La metodologia va ser optimitzada; la sensibilitat i el rang de la polaritat dels polifenols mesurats en l'orina es van incrementar; els límits de detecció i quantificació van disminuir significativament. Els polifenols mesurats en solució estàndard i mostres d'orines van continuar estables sota les condicions d'emmagatzematge a -20 i -80°C, a curt i llarg termini, i després de varis cicles de congelació i descongelació en les condicions de manipulació.

Els polifenols totals excretats en l'orina es van correlacionar positivament amb els polifenols totals ingerits $r=0.48$ $P<0.01$ i $r= 0.257$ $P=0.04$ a l'assaig clínic i l'estudi transversal, respectivament.

En aquest treball, hem desenvolupat un mètode especialment adaptat per a l'anàlisi d'un gran nombre de mostres, que podria ser utilitzat per a la determinació de polifenols totals en mostres d'orina recollida de forma puntual, podent ser considerat com a biomarcador del consum de polifenols totals. Amb les plaques de lectura de 96 pouets es poden analitzar un major nombre de mostres utilitzant un menor volum i amb una major sensibilitat. Les plaques són també un format menys incòmode i millor per la manipulació automàtica. El mètode és ràpid, senzill i permet l'anàlisi d'un gran nombre de mostres al mateix temps, la qual cosa és útil en estudis epidemiològics on es és més factible la presa d'orines puntual al matí i hi ha un gran nombre d'individus.



This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

<http://www.elsevier.com/copyright>



Rapid Folin–Ciocalteu method using microtiter 96-well plate cartridges for solid phase extraction to assess urinary total phenolic compounds, as a biomarker of total polyphenols intake

Alexander Medina-Remón^{a,c}, Ana Barrionuevo-González^{a,c}, Raúl Zamora-Ros^{a,d}, Cristina Andres-Lacueva^{a,d}, Ramón Estruch^{b,c}, Miguel-Ángel Martínez-González^{c,e}, Javier Diez-Espino^f, Rosa M. Lamuela-Raventós^{a,c,*}

^a Nutrition and Food Science Department, XaRTA, INSA Pharmacy School, University of Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain

^b Department of Internal Medicine, Hospital Clinic, Institut d'Investigació Biomèdica August Pi i Sunyer (IDIBAPS), University of Barcelona, Barcelona, Spain

^c GIBER 06/003 Physiopathology of Obesity and Nutrition (CIBEROBN), and RETICS RD06/0045/0003, Instituto de Salud Carlos III, Spain

^d Ingenio-CONSOLIDER program, FUN-C-FOOD, Instituto de Salud Carlos III, Barcelona, Spain

^e Department of Preventive Medicine and Public Health, Clínica Universitaria, University of Navarra, Pamplona, Spain

^f Tafalla Health Center, Osasunbidea, Tafalla, Spain

ARTICLE INFO

Article history:

Received 6 June 2008

Received in revised form 28 October 2008

Accepted 8 December 2008

Available online 13 December 2008

Keywords:

Solid phase extraction

96-Well plate

Phenols

Folin–Ciocalteu

Biomarker

Urine

PREDIMED

Polyphenols stability

ABSTRACT

Nutritional markers have several advantages for epidemiologic and clinical assays, when compared to dietary data obtained by food frequency questionnaires. Few studies have assessed whether total polyphenol (TP) compounds provide a valid biomarker for TP intake. To date, there has been almost no literature describing methods to determine TP in complex matrices such as urine, which have many interfering substances.

We report a rapid Folin–Ciocalteu method to determine TP in urine samples using Oasis® MAX 96-well plate cartridges for solid phase extraction. These plates allow analysis of a high number of samples at the same time. We performed a prospective, randomized, crossover trial and one cross-sectional study with 60 volunteers from the PREDIMED trial, seeking to evaluate whether the TP in urine were correlated with polyphenol intake and could, therefore, be considered as a marker of intake of these compounds.

The assay was optimized; the sensitivity and the polarity range of urine polyphenols were increased and the detection and quantification limits were significantly reduced. The metabolites in standards solution and urine samples were stable under the storage and handling conditions. In the clinical trial and the cross-sectional study, TP excreted in spot urine samples were positively correlated with TP intake, $r = 0.48$, $P < 0.01$ and $r = 0.257$, $P = 0.04$, respectively.

The methodology described may be used to detect TP in urine samples, employing the high throughput of 96-well microtiter plates and reader. The method is fast and simple and it allows analysis of a large number of samples at the same time.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Total dietary polyphenol levels are approximately 1 g day^{-1} [1], higher than all other known dietary antioxidants, around 10 times higher than dietary vitamin C and 100 times higher than vitamin E and carotenoid levels. The health effects of polyphenols depend on their respective intakes, synergism and bioavailability, which can vary greatly [2]. Epidemiological studies have shown an inverse

association between the risk of myocardial infarction and the consumption of food rich in phenolic compounds: tea, wine, fruit and vegetables (F&V). Clear associations have been found between cancer risk and polyphenol consumption [3–7]. Nutritional markers have several advantages for epidemiologic and clinical assays, when compared to dietary data obtained by food frequency questionnaires (FFQ) [8]. Biomarkers of the intake of some nutrients are more precise than self-reported information based on recalled dietary assessment and thus provide a more objective measure of specific nutrient intake rather than the subjective information obtained by an FFQ. The development of biomarkers, measured in both blood and urine, is essential for making accurate estimates of polyphenol intake. However, the relationship between dietary intake and nutritional biomarkers is often highly complex [9].

* Corresponding author at: Nutrition and Food Science Department, XaRTA, INSA Pharmacy School, University of Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain. Tel.: +34 934034843; fax: +34 934035931.

E-mail address: lamuela@pub.edu (R.M. Lamuela-Raventós).

For most polyphenols, urinary excretion values have been reported to be consistent with plasma kinetics data. The Folin–Ciocalteu (F–C) assay for total phenols (TP) is affected by several interfering substances such as sulfur dioxide, ascorbic acid, sugar, aromatic amines, organic acid, Fe(II) and nonphenolic organic substances that react with the F–C reagent [10]. Roura et al. in their research on cocoa [11] studied these interferences after a single solid phase extraction (SPE) cleanup procedure and these substances were not in the eluate, so cannot react with F–C reagent. With the aim of increasing the number and recovery of a larger number of polyphenols from the diet, it was necessary to improve this methodology.

To our knowledge, there is no methodology available to analyze total phenols using microtiter 96-well plates, the use of which would allow a rapid determination for total phenols in urine samples at the same time. However, micro-well plates are used in new methods for the determination of some compounds in food [12,13] and biological sample [14–17]. The aim of the methodology was to have a fast method for total phenols in urine, which could then be used as a biomarker of phenol intake. Toward this end, we tested various solid phase extractions and selected the one with the best reproducible and recovery results in quantifying a high range of phenolic compounds from different foods. We applied Oasis® MAX 96-well plate cartridges for the first time, so that we could reduce analysis time from 11.5 to 2 h. The plate allows the analysis of a high number of samples at the same time for clinical and epidemiological studies. We conducted two trials, one prospective, randomized, crossover trial and one cross-sectional trial, which sought to evaluate whether the total polyphenols in urine are correlated with polyphenol intake and can, therefore, be considered as a marker of intake, bioavailability and accumulation of these compounds.

2. Material and methods

2.1. Standards and reagents

All samples and standards were handled with no exposure to light. Catechin, gallic acid, quercetin, caffeic acid, rutin and formic acid were purchased from Sigma®; epicatechin, F–C reagent and creatinine were obtained from Fluka. The 4-O-methylgallic acid, naringin and tyrosol from extrasyntheses; isoquercitrin was obtained from Chroma Dex; the picric acid, sodium hydroxide, hydrochloric acid 35% and acetic acid 99.8% from Panreac and anhydrous sodium acetate (2 M) from Merck. Methanol, acetonitrile and formic acid were obtained from Scharlau. Ultrapure water (Milli-Q) from Millipore System (Bedford, MA). Calibration synthetic urine was prepared as described by Miro-Casas et al. [18] to avoid any possible interference generated by the matrices with F–C reagent.

2.2. The stationary phase for the sample extraction procedure

Oasis® MCX, 30 mg (mixed-mode cation-exchange and reversed-phase solvent), Oasis® MAX, 30 mg (mixed-mode anion-exchange and reversed-phase solvent) and Oasis® HLB, 30 mg (hydrophilic-lipophilic-balanced cartridges, water-wettable and reversed-phase solvent), from Waters (Milford, U.S.A.) were tested using the 96-well plate SPE with its special characteristics. Ten representative polyphenols (4-O-methylgallic acid, catechin, gallic acid, tyrosol, naringin, epicatechin, caffeic acid, rutin, quercetin and isoquercitrin) with different polarity were prepared in synthetic urine (5, 10, 25 and 50 mg L⁻¹ for each standard) and 1 mL was loaded into each kind of stationary phase ($n = 6$). For Oasis® MAX cartridges, 1 mL of methanol 98% and 1 mL of sodium acetate 50 mM pH 7 were loaded to equilibrate the cartridges. The synthetic urine samples with standard were loaded into the

cartridges separately. These were then rinsed with sodium acetate 50 mM pH 7/5% methanol. The polyphenols were eluted with 1800 µL of 2% formic acid in methanol. Oasis® MCX was previously equilibrated with 1 mL of methanol and washed with 1 mL of 2% formic acid in methanol. The standards were eluted with 1 mL sodium acetate 50 mM pH 7/5% methanol and 1 mL of methanol 98%. The results were compared those obtained with Oasis® HLB.

To quantify the standards obtained with the SPE, each of eluates was analyzed using high-performance liquid chromatography (HPLC) according to the method of Andrés-Lacueva et al. [19], with minor modifications. The linear gradient profile with the following acetonitrile properties (v/v) was applied (t (min), % acetonitrile): (0, 0.2), (2, 4), (10, 12), (14, 16), (16, 16), (18, 17.6), (22, 19.2), (30, 24), (37, 48), (40, 80). Using a Hewlett-Packard (HP) 1050 (Palo Alto, CA) liquid chromatograph with diode array detector HP 1050 at 280 nm (4-O-methylgallic acid, catechin, gallic acid, tyrosol, naringin, and epicatechin), at 306 nm (caffeic acid), at 385 nm (rutin, quercetin and isoquercitrin).

2.3. Analysis of total polyphenols in urines

The F–C method described by Roura et al. [11], used for cocoa epicatechin metabolites, was improved in order to detect a high range of phenolic compounds from different foods in studies with large numbers of samples. Oasis® MAX 96-well plate SPE cartridges were selected because they provided the best recovery. Urine samples were thawed on a ice bed for 3 h; they were centrifuged for 10 min at 4 °C and 1 mL of supernatants, catechin and gallic acid standards for calibrated line (1, 2, 4, 6, and 8 mg L⁻¹) were diluted with 1 mL of water Milli-Q and acidified with 34 µL of hydrochloric acid at 35%; they were used to load the Oasis® MAX 96-well plate SPE cartridges separately. The extraction procedure described above for Oasis® MAX cartridges was applied and 15 µL of the eluted fractions were mixed with 170 µL of Milli-Q water in the thermo microtiter 96-well plate (nunc™, Roskilde, Denmark), adding 12 µL of F–C reagent and 30 µL of sodium carbonate (200 g L⁻¹). The multichannel pipette minimized differences in the times (3 s) of the F–C reaction between the eight lines of the 96-well plate, ensuring a similar reaction time for all samples analyzed on the same plate. The 96-well plate permitted less reagent to be used in a more environmentally friendly test. The mixtures were incubated for 1 h at room temperature in the dark. After the reaction period, 73 µL of Milli-Q water were added with the multichannel pipette. Absorbance was measured at 765 nm in UV/VIS Thermo Multiskan Spectrum spectrophotometers (Vantaa, Finland). This spectrophotometer allowed the absorbance of a 96-well plate to be read in only 10 s.

For creatinine in urine samples, Jaffé alkaline picrate method [20] was adapted to thermo microtiter 96-well plates, 3 µL of urine were mixed with 60 µL of aqueous picric acid solution (1%) and 5 µL of sodium hydroxide (10%). After shaking, the mixture was left 15 min in the dark at room temperature; 232 µL of Milli-Q water was added and the absorbance was measured at 500 nm in the UV/VIS spectrophotometers. In the absence of disease, creatinine concentrations in urine are usually very stable and can be used to estimate the urinary excretion of substances with only spot urine samples [21–23]. Total polyphenols were expressed as mg gallic acid equivalent (GAE) per g creatinine and mg catechin per g creatinine.

2.4. Folin–Ciocalteu assay validation

The method was validated with gallic acid and catechin standard to compare the results with previous results obtained by Roura et al. [11] expressed in catechin. To evaluate the linearity of the F–C assay a series of calibrators with 1, 2, 4, 6 and 8 mg L⁻¹ were prepared in synthetic urine. The absorbances were plotted against the

corresponding standard concentrations. The sensitivity of the method was evaluated determining the limits of detection (LoD) and limits of quantification (LoQ). These were calculated by measuring the analytical background response, reading 10 blanks at the maximum sensitivity allowed (765 nm) by the system. LoD was considered to be three times the standard deviation (S.D.) of 10 blank samples analyzed while LoQ was considered to be 10 times the S.D. [24].

Accuracy was evaluated by repeatedly spiking the matrix with known levels of analyte standards at five different concentrations. The percentage deviations of added analyte concentrations recovered from a blank matrix were used to estimate accuracy. Precision was calculated by dividing the S.D. by the concentration to obtain the coefficient of variation (CV), which when expressed on a percentage basis gives the relative standard deviation (R.S.D.). It was validated according to the recommendations of the AOAC INTERNATIONAL [24] for the validation of methods.

Short-term temperature stability was evaluated using three aliquots of each concentration (1, 2, 4, 6 and 8 mg L⁻¹) of gallic acid and catechin in Milli-Q water, previously frozen at -20 and -80 °C. The aliquots were thawed at room temperature, kept at that temperature for 2 h (equivalent to the mean sample preparation time for a 96-well plate) and then analyzed. We also evaluated stability after successive freeze and thaw cycles. Three aliquots of each concentration were stored at -20 and -80 °C for 24 h, and thawed at room temperature. After being completely thawed, the samples were refrozen for 24 h, and the freeze–thaw cycle was repeated two more times. Then the aliquots were analyzed on the third cycle. Lastly, long-term stability was determined by storing three aliquots of each concentration under the same conditions as the study samples (-80 °C) for a period of 2 years. The concentrations of all the stability samples were compared with the mean back-calculated values for the standards at appropriate concentrations from the first day of long-term stability testing. For sample stability the same processes were applied [25].

2.5. Study design and samples

We obtained the first urine samples from the volunteers in morning, at the same time. The morning urine samples were collected in sterilized bottles and the aliquots were immediately prepared and frozen at -80 °C until analysis. For the assay, thawing is necessary over an ice bed for 3 h, to avoid degradation of the polyphenols. The samples must be transported in a polystyrene box with dry ice to maintain their temperature. Low temperature and direct sublimation to a gas makes dry ice an effective coolant, since it is colder than water or ice and leaves no moisture as it changes state. The samples must be kept frozen at -80 °C in aliquots if they are required for a long period, and the same thawing procedures described above must be repeated for subsequent assays.

2.5.1. Clinical trial

This prospective, randomized, crossover trial included 12 healthy adult volunteers (8 women and 4 men; age range 24–54 years) with no previous relevant illnesses. The study was carried out in accordance with the Helsinki Declaration of 1975, as revised in 1996. The Ethical Committee for Human Experimentation of the Barcelona University, Spain approved the study, and written informed consent was obtained from each participant.

The volunteers were randomly separated into two groups; the first group began with a high polyphenol diet (HPD) for 3 days and then changed to a low polyphenol diet (LPD) for 3 days, after a 3-day intermediate period of taking the usual diet (UD). They were instructed by a dietician to abstain from food and beverages the required polyphenols for each type of diet. The volunteers' daily intakes were recorded using an FFQ. They received a list of rec-

ommended or restricted foods and beverages. They abstained from taking medication and vitamin supplements for 1 week before and during the study.

2.5.2. Cross-sectional study

The PREDIMED (PREvention with MEDiterranean Diet) 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>). We selected a subsample of 60 volunteers from the center at the University of Navarra (School of Medicine) to analyze their total urinary polyphenols; 29 men and 31 women between 56 and 80 years old having a body mass index between 20.4 and 36.6. At baseline, participants completed a 137-item validated FFQ [26] and the validated Spanish version of the Minnesota Leisure Time Physical questionnaire [27]. Exclusion and inclusion criteria have been described previously by Estruch et al. [28]. The volunteers were divided into tertiles depending on their daily intake of total F&V: the first tertile had a consumption less than 508.03 g day⁻¹ (low F&V consumers, LFVC), the second tertile (medium F&V consumers, MFVC) between 508.04 and 640.23 g day⁻¹, and the third tertile from 640.24 g day⁻¹ upwards (high F&V consumers, HFVC). The Institutional Review Board of the Hospital Clinic of Barcelona approved the study protocol, and written informed consent was obtained from each participant. Urine samples were collected before the FFQ, and were coded and stored at -80 °C until analysis.

TP intake from plant food and beverages (mg g⁻¹ fresh matter) in both studies was quantified according to Saura-Calixto and Goni [29] taking into account the FFQ. The edible portion of the daily amount consumed, per capita, for each food as eaten was divided into eight groups: cereals, vegetables, legumes, nuts, chocolate, fruit, oils and phenolic beverages (coffee, tea, wine, beer and fruit juices).

2.6. Statistical analysis

The statistical analyses were performed using the SPSS package program version 14.0. Descriptive statistical tests with the mean (S.D.) were used for the baseline characteristics of the participants. Variables were examined for normality and skewness (Kolmogorov and Levene tests) and were tested for carry-over and treatment–period interactions. The TP content in morning urine and phenol intake were not normally distributed in both studies. Because this was not normalised by the logarithmic transformation, non-parametric tests were used in the statistical analyses of the data. The Wilcoxon test was performed for related samples to compare changes in outcome variables in response to each intervention period in the clinical trial and the Mann–Whitney test for independent samples in the cross-sectional study. Spearman correlations (r_s) were performed to evaluate the association between TP content in morning urine and TP intake from selected food groups in both studies. *P*-values <0.05 (two-tailed) were considered to be significant. To compare groups in the cross-sectional study, we used the two-tailed *t*-test.

3. Results

3.1. SPE cartridge selection

Table 1 shows the recoveries for 10 polyphenol standards in the urine matrix, for Oasis® MCX, MAX and HLB. They were calculated using the mean recoveries at four different levels: 5, 10, 25 and 50 mg L⁻¹ for all standards. The best recoveries were obtained with Oasis® MAX cartridges. The AOAC INTERNATIONAL [24] establishes a good recovery for biological samples of between 80% and 110%. MCX

Table 1
Absolute recovery of 10 polyphenol standards in different cartridges quantified by HPLC.

Cartridges	MCX	MAX	HLB
Gallic acid, mean (S.D.)	71.23 (3.56)	100.25 (3.66)	88.42 (6.25)
Isoquercetin, mean (S.D.)	107.52 (8.98)	105.13 (8.07)	96.23 (9.79)
Quercetin, mean (S.D.)	80.92 (3.74)	95.74 (7.12)	79.23 (6.52)
Catechin, mean (S.D.)	70.43 (3.97)	92.52 (4.43)	93.7 (6.6)
Epicatechin, mean (S.D.)	109.78 (4.51)	108.05 (1.90)	105.62 (2.10)
4-O-Methylgallic acid, mean (S.D.)	104.42 (0.89)	97.87 (1.52)	94.11 (8.42)
Tyrosol, mean (S.D.)	94.32 (3.07)	98.13 (2.23)	96.38 (5.23)
Naringin, mean (S.D.)	103.23 (0.42)	101.16 (9.45)	95.44 (6.48)
Caffeic acid, mean (S.D.)	109.50 (0.57)	101.07 (2.88)	93.85 (7.3)
Rutin, mean (S.D.)	104.54 (2.17)	97.66 (0.72)	93.77 (9.56)

was ruled out because some polyphenol recoveries were lower than 80%: for gallic acid and for catechin, they were 71.23% and 70.43%, respectively, and 80.92% for quercetin. For HLB, the recovery values were similar to MAX in some standards, although of the two resins MAX was selected because of the higher results obtained, being approximately 100%. The gallic acid recovery was 88.44% in HLB compared to 100.25% in MAX cartridges; the recovery for quercetin in the HLB was slightly lower than 80%.

The assay was optimized and Oasis® MAX 96-well extraction plates were selected. This significant change allowed acidic, basic and neutral compounds to be detected, confirmed and quantified in different fractions in biological fluids. The sensitivity and the polarity range of the urine polyphenols were increased and the detection and quantification limits were significantly reduced. The 96-well plate makes the method particularly well adapted to the analysis of large batches of samples for clinical and epidemiological studies.

3.2. Validation of the method for the analysis of total polyphenols in urine

The F–C method was linear over the working range between 1 and 8 mg L⁻¹ for gallic acid and catechin. These two standards were selected as they are the ones most frequently used for F–C assays. Least-squares regression analysis gave the following result for the gallic acid calibration curve: mean (S.D.); slope, 0.082 (0.005); y-intercept, 0.030 (0.009); $r^2 = 0.997$ (0.001) and standard deviation of residuals, 0.077. For the catechin calibration curve: mean (S.D.); slope, 0.0778 (0.002); y-intercept, 0.065 (0.009); $r^2 = 0.995$ (0.003) and standard deviation of residuals, 0.072. The limit of detection (0.07 mg L⁻¹) and limit of quantification (0.11 mg L⁻¹) were determined for gallic acid and catechin standards.

Assay precision and accuracy were evaluated at five different concentrations (Table 2). The values obtained for precision “R.S.D. (%)” were lower than those established by AOAC INTERNATIONAL [24], which would be 7.3% at this concentration. Accuracy was measured according to the percentage of gallic acid and catechin added. The AOAC INTERNATIONAL [24] accepts values of 80–110% recovery for the values analyzed, and values were found between 82.5% and 105.7%.

Table 2
Evaluation of precision and accuracy for gallic acid and catechin calibrator in synthetic urine.

Calibrator concentration (mg L ⁻¹)	Means measured concentration (mg L ⁻¹) (n = 5)		Precision, R.S.D. (%)		Recovery, error (%)	
	Gallic acid	Catechin	Gallic acid	Catechin	Gallic acid	Catechin
1	0.9	0.9	0.6	1.1	82.5	85.2
2	2.1	2.0	0.8	0.8	100.7	104.6
4	4.1	4.2	3.5	3.6	105.7	103.0
6	6.0	5.9	5.1	5.3	101.8	99.8
8	7.9	8.0	7.2	6.7	97.7	99.2

R.S.D., relative standard deviation.

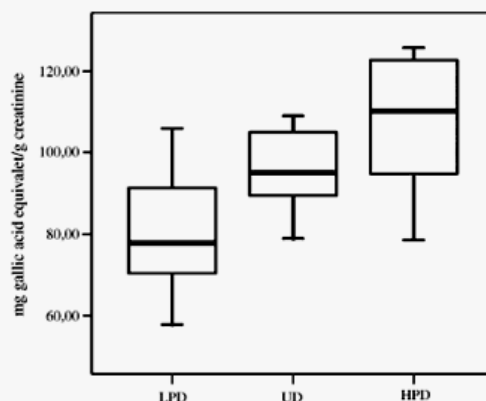


Fig. 1. Concentration of total phenolic “mg gallic acid equivalent per g creatinine” excreted in morning urine after the ingestion of the low (LPD), usual (UD) and high (HPD) polyphenol diets in the clinical trial.

The standards in Milli-Q water after freeze and thaw cycles remained stable for both temperature tested and at the different concentrations. At higher concentrations (6 and 8 mg L⁻¹) the gallic acid remained near 100%, while at smaller concentrations (1 and 2 mg L⁻¹) it was ~90%. Catechin standards frozen at –20 °C remained between 86% and 89% at all concentrations, while at –80 °C the standards remained at 89–95%. At room temperature (short-term stability) for 2 h, decreases in both standards (gallic acid and catechin) were observed; the amounts were reduced to ~86% when working with the lowest concentration at –20 °C, which was very similar to –80 °C (~89%). The standards remained higher (>96%) in the highest concentrations, and they were similar for both standards and both deep-freeze temperatures (–20 and –80 °C). For the rest of the concentrations at the two deep-freeze temperatures, the standards remained higher than 90%. For the long-term stability of standards, the level decreased to 93% and 95% for catechin and gallic acid, respectively, after 2 years, in both deep-freeze temperatures (–20 and –80 °C). We observed no statistically significant differences ($P > 0.05$) in total polyphenols in urine samples ($n = 24$), after testing freeze and thaw cycles and short-term and long-term stability. In conclusion, the phenolics in standards solution and urine samples were stable under the storage and sample handling conditions used for these assays; standards solution and urine samples should be stored at ≤ -20 °C over a long period of time, i.e., approximately 2 years in length or more.

3.3. Total polyphenols measured in urine samples

3.3.1. Clinical trial

The concentration of TP compounds excreted in morning urine after ingestion of different diets is shown in Fig. 1. TP excreted in the urine, expressed as mg GAE per g creatinine was 107.07 (17.01)

Table 3

Relative differences between the groups' total polyphenol intake, expressed as mg gallic acid equivalent, and between the groups' urinary excretion of total polyphenols, expressed as mg gallic acid equivalent/g creatinine, for different diets in the clinical trial.

Differences between	95% Confidence interval for the difference			
	Mean	Lower	Higher	P
TPI in HPD and TPI in UD	495.12	265.90	724.34	0.002
TPI in UD and TPI in LPD	389.39	271.95	506.84	0.002
TPI in HPD and TPI in LPD	884.51	429.74	124.06	0.002
UETP in HPD and UETP in UD	11.63	-0.91	24.18	0.06
UETP in UD and UETP in LPD	12.70	-0.59	25.99	0.06
UETP in HPD and UETP in LPD	24.34	11.81	36.87	0.002

TPI, total polyphenol intake; UETP, urinary excretion of total polyphenols; UD, normal polyphenol diet; HPD, high polyphenol diet; LPD, low polyphenol diet.

after the HPD. The UD consumed maintained the excretion of TP at the intermediate value of 95.44 (10.50), higher than 82.73 (13.43) in LPD. The intake of TP compounds in the HPD was 1255.02 (491.89) mg GAE per person per day; in UD and LPD, it was 759.90 (239.81) and 370.51 (121.98), respectively.

The Wilcoxon test for related samples for each intervention period showed a significant difference ($P=0.002$) between the urine excreted after the HPD and LPD; and a tendency to significant difference was found ($P=0.06$) when comparing UD with HPD and LPD. This statistical analysis was performed to compare the TP intake. The results showed a significant difference ($P<0.01$) between the TP intake for each intervention period. The differences between groups are shown in Table 3.

The Spearman correlation analyses (see Table 4) revealed a positive significant correlation between the TP excretion in morning urine and the consumption of TP from fruit ($P<0.05$), vegetables ($P<0.01$), F&V ($P<0.01$) and TP intake ($P<0.01$); there was also a significant negative correlation with cereals ($P<0.01$). No significant correlations were observed with the rest of the food groups.

3.3.2. Cross-sectional study

TP excreted in the urine for LFVC was 111.86 (34.74) mg GAE per g creatinine after the TP intake of 1293.38 (357.29) mg GAE per person per day. The excretion for NFVC was 141.87 (48.81) mg GAE per g creatinine and 160.73 (82.42) mg GAE per g creatinine for HFVC after the intake of 1364.01 (402.23) and 1768.87 (527.32) mg GAE per person per day, respectively. The daily F&V ingestion was 397.96 (91.44) g, 574.73 (39.57) g and 844 (202.74) g for low, normal and high intake, respectively. The Mann-Whitney test for independent samples showed a statistically significant difference in the mg GAE per g creatinine when LFVC were compared with NFVC ($P=0.033$) and HFVC ($P=0.023$); but there were no significant differences ($P=0.79$) when NFVC were compared with HFVC.

The Spearman correlation analyses (Fig. 2) revealed a positive significant correlation between the TP excretion in morning urine and total dietary polyphenols ($r=0.257$, $P=0.04$) and the total F&V

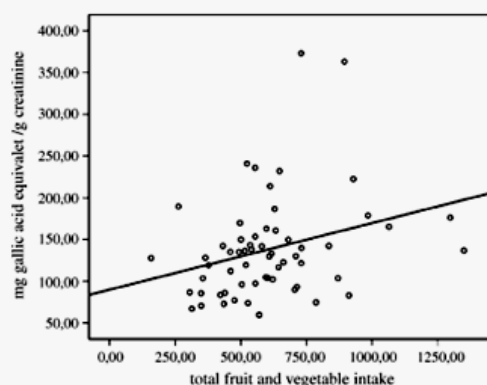


Fig. 2. Correlation between total fruit and vegetable intake (g day^{-1}) and concentration of total polyphenols (mg gallic acid equivalent per g creatinine) excreted in morning urine in the cross-sectional study.

intake ($r=0.339$, $P=0.008$). No correlation between the total urinary polyphenols and the other groups were found.

4. Discussion

In this work, we developed a method especially adapted to analyse large batches of samples, which could be used for TP and creatinine assay, the 96-well plates accommodate much larger sample volumes, with the potential for proportionally greater sensitivity. The well plates are also a less cumbersome format and better suited to automated manipulation, which proved faster than previous methods [10,11,29]. It was possible to analyze 96 urine samples in only 3 h (2 h for the sample preparation and 1 h for the reaction) by using the Oasis® MAX 96-well plate for the SPE and the thermo microtiter 96-well plate for the Folin-Ciocalteu reaction. This eliminated laborious sample preparation after the thawing period over a bed of ice (approximately 3 h) in contrast to other methods, which required ~12.5 h for 96 samples (11 h for sample preparation, 1 h for the reaction and 0.5 h to read the cuvettes), and it is a methodology that is much more environmentally friendly. This permitted the analysis of a large number of samples per day, which is ideal for clinical and epidemiological studies in which the subjects ingest a large variety of polyphenols with their usual diet. It was possible to increase the recovery of a high number of polyphenols using the SPE with MAX cartridges, due to the mixed mode anion exchange and reversed phase solvent with a high selectivity and sensitivity for all polyphenols compounds tested. The SPE diminishes the interference for TP analysis in urine samples.

Mennen et al. [30] and Krogholm et al. [31] studied the correlation between the consumption of polyphenol-rich foods and beverages and the concentration of polyphenols in urine samples determined by LC-MS/MS. Their results suggested that several polyphenols measured in urine samples can be used as biomarkers of polyphenol-rich food intake. In our investigation the total polyphenol food intake was positively correlated to the total polyphenol excreted in urine samples. Most correlations observed between urinary excretion of polyphenols and food intake were expected from their contents in food and established recoveries in urine in intervention studies of specific food items [32].

The intake of TP in the clinical trial on HPD, 1255.02 (491.89) mg per person per day was slightly higher than the daily intake of TP in the Spanish diet, estimated by Saura-Calixto and Goni [29] as 1171 mg per person per day. However, in our study the volunteers were intentionally consuming a HPD while Saura-Calixto estimated

Table 4

Correlation between consumption of different food groups and morning urinary polyphenols (mg gallic acid per g creatinine).

Food group	r	P
Fruits	0.332	0.048
Vegetables	0.504	0.002
Fruits and vegetables	0.501	0.002
Legumes	-0.106	0.539
Cereals	-0.644	0.001
Phenolic beverages	0.293	0.082
Oils	-0.094	0.586
Nuts	0.221	0.195
Chocolate	0.103	0.551
Total phenols	0.482	0.003

this from the general population. In the cross-sectional study the LFVC consumed 1293.38 (357.29) mg GAE per person per day similar to HPD in the clinical trial, and HFVC consumed 1768.87 (527.32) mg GAE per person per day higher than other studies. The cereals ingested could cause decreases in the TP excretion since when more cereals are consumed the intake of fruit ($r = -0.533$, $P = 0.001$) and vegetables is reduced ($r = 0.680$, $P < 0.001$).

In the cross-sectional study, TP in urine were a good indicator of polyphenol consumption. The present study has shown a significant correlation between urinary excretion of TP determined by the F–C method and the intake of F&V and TP intake. Very few authors [30,31,33] have examined the use of urinary concentrations of polyphenols as non-specific biomarkers of F&V consumption in free-living populations, and they required complicated equipment to do so. Since flavonoids are widely distributed in F&V, some investigators studied their value as biomarkers of F&V intake. In a controlled dietary intervention study [33], flavonoids from 24 h urine samples were measured by LC–MS after 6 weeks of a diet either low or high in fruit, vegetables and berries. Urinary quercetin, flavanone, and total flavonoid output were clearly higher after the high vegetable diet compared with the low vegetable diet. As well as individual level, a significant positive correlation between changes in F&V intake and changes in urinary flavonoid excretion was observed. A positive correlation was found between total polyphenol metabolites in 24 h urine samples and intake of F&V following 1 day consumption of a basic diet supplemented with 300 or 600 g of F&V [31]. However, to investigate the potential health effect of F&V in epidemiological studies, only spot urine and/or blood samples are collected and, rarely, 24 h urine samples. Furthermore, a recent study [30] has evaluated the correlation between the urinary excretion of several polyphenols from spot urine samples and the intake of polyphenol-rich food. This study suggests that several polyphenols measured by LC–MS in spot urine samples can be used as biomarkers of polyphenol-rich food intake.

Even though the sample for our cross-sectional study was relatively homogenous, there was a high between-subjects variation in the amount of TP excreted in urine, two high-excretors with TP excretion more than three times the mean of the other 58 volunteers (see Fig. 2). Exclusion of the 2 high-excretors from the analysis affects the correlation between TP urine excretion and total F&V intake ($r = 0.292$, $P = 0.02$), but the positive correlation is maintained. This high interindividual variation in phenol excretion with a few high-excretors had already been observed in previous studies, although this interindividual variation was lower [33–35].

Here we have developed a method for TP that is a marker of intake of polyphenols rich foods and is cheaper, more environmentally friendly, and simpler than the method described by Krogholm et al. [31] and Nielsen et al. [33], and it does not require LC–MS instrumentation. However, the quantification of a number of metabolites in 24 h urine samples may be a suitable approach in small-scale human intervention trials but may not be a realistic possibility in large-scale epidemiological studies, due to the problems of organizing the collection of 24 h urine samples from a large study population.

In summary, we have developed a new method for the analysis of total phenols in urine samples using Oasis® MAX 96-well plate cartridges for SPE. The method is fast, simple and allows the analysis of a large number of samples, as many as 96 samples, using the 96-well microtiter plates and reader. It is cheaper, more environmentally friendly, and simpler than other methods described. Validation is based on the results of a prospective, randomized, crossover trial study with different intervention periods, and it was corroborated with a free-living population in a cross-sectional study with 60 volunteers. Associations were found between TP excreted in morning urine and the TP and F&V intake in the clinical trial, and similar associations were found in the cross-sectional study. TP in urine

was correlated with its intake and can, therefore, be considered as a nutritional biomarker.

Acknowledgments

We would like to thank all of the volunteers involved in this study for their valuable cooperation. The authors express their gratitude for financial support from CICYT's (AGL2005-002823; 2006-14228) and "Red de Grupo" G03/140, the Spanish Ministries of Education and Science and of Health (PI 052443, PI 070240), RETICS RD06/0045/0003 from the "Instituto de Salud Carlos III" and the INGENIO-CONSOLIDER FUN-C-FOOD program (CSD2007-063), also from the Spanish Ministry of Education and Science. A.M.-R. and R.Z.-R. received support from the Generalitat de Catalonia for the training of researchers. We are also grateful to the University of Barcelona for a research and teaching grant.

References

- [1] A. Scalbert, G. Williamson, Dietary intake and bioavailability of polyphenols, *J. Nutr.* 130 (2000) 2073S–2085S.
- [2] J. Feher, G. Lengyel, Nutrition and cardiovascular mortality, *Orv. Hetil.* 147 (2006) 1491–1496.
- [3] M.I. Covas, K. Nyyssönen, H.E. Poulsen, J. Kaikkonen, H.J. Zunft, H. Kieseewetter, A. Gaddi, T.R. de la, J. Mursu, H. Baumler, S. Nascetti, J.T. Salonen, M. Fito, J. Virtanen, J. Marrugat, EUROLIVE Study Group, The effect of polyphenols in olive oil on heart disease risk factors: a randomized trial, *Ann. Intern. Med.* 145 (2006) 333–341.
- [4] C. Fortes, F. Forastiere, S. Farchi, S. Mallone, T. Trequattrini, F. Anatra, G. Schmid, C.A. Perucci, The protective effect of the Mediterranean diet on lung cancer, *Nutr. Cancer* 46 (2003) 30–37.
- [5] L.R. He, Han Dong Hao, Y.Y.H.S.U.O.T.C.M.S. Cardiovascular Sect., and -mail shqjh@online.sh.cn, Mechanisms of cardiovascular protection by resveratrol, *J. Med. Food* 7 (3) (2004) 290–298.
- [6] C. Manach, A. Mazur, A. Scalbert, Polyphenols and prevention of cardiovascular diseases, *Curr. Opin. Lipidol.* 16 (2005) 77–84.
- [7] A. Scalbert, C. Manach, C. Morand, C. Remesy, L. Jimenez, Dietary polyphenols and the prevention of diseases, *Crit. Rev. Food Sci. Nutr.* 45 (2005) 287–306.
- [8] J.R. Marshall, Methodologic and statistical considerations regarding use of biomarkers of nutritional exposure in epidemiology, *J. Nutr.* 133 (Suppl. 3) (2003) 881S–887S.
- [9] J.P. Spencer, M.M. bd El Mohsen, A.M. Minihane, J.C. Mathers, Biomarkers of the intake of dietary polyphenols: strengths, limitations and application in nutrition research, *Br. J. Nutr.* 99 (2008) 12–22.
- [10] V.L. Singleton, R. Orthofer, R.M. Lamuela-Raventos, Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent, *Oxid. Antioxid. Pt A299* (1999) 152–178.
- [11] E. Roura, C. Andrés-Lacueva, R. Estruch, R.M. Lamuela-Raventos, Total polyphenol intake estimated by a modified Folin–Ciocalteu assay of urine, *Clin. Chem.* 52 (2006) 749–752.
- [12] A.G. Gehring, D.M. Albin, S.A. Reed, S.I. Tu, J.D. Brewster, An antibody microarray, in multiwell plate format, for multiplex screening of foodborne pathogenic bacteria and biomolecules, *Anal. Bioanal. Chem.* (2008).
- [13] H.S.M. Abd-Rabboh, M.E. Meyerhoff, Determination of glucose using a coupled-enzymatic reaction with new fluoride selective optical sensing polymeric film coated in microtiter plate wells, *Talanta* 72 (2007) 1129–1133.
- [14] M.L. Christensen, T.H. Braunstein, M. Treiman, Fluorescence assay for mitochondrial permeability transition in cardiomyocytes cultured in a microtiter plate, *Anal. Biochem.* 378 (2008) 25–31.
- [15] P.S. Schrader, E.H. Burrows, R.L. Ely, High-throughput screening assay for biological hydrogen production, *Anal. Chem.* 80 (2008) 4014–4019.
- [16] T.H. Cho, J.W. Lee, M.H. Lee, Evaluating the cytotoxic doses of narrowband and broadband UVB in human keratinocytes, melanocytes, and fibroblasts, *Photodermatol. Photoimmunol. Photomed.* 24 (2008) 110–114.
- [17] H.J. Kim, H.M. Yun, T. Kim, G. Nam, E.J. Roh, E. Kostenis, H.Y. Choo, A.N. Pae, H. Rhim, Functional human 5-HT(6) receptor assay for high throughput screening of chemical ligands and binding proteins, *Comb. Chem. High Throughput. Screen.* 11 (2008) 316–324.
- [18] E. Miro-Casas, M.F. Albaladejo, M.I. Covas, J.O. Rodríguez, E.M. Colomer, R.M.L. Raventos, R. de la Torre, Capillary gas chromatography–mass spectrometry quantitative determination of hydroxytyrosol and tyrosol in human urine after olive oil intake, *Anal. Biochem.* 294 (2001) 63–72.
- [19] C. Andrés-Lacueva, R.M. Lamuela-Raventos, O. Jáuregui, An LC method for the analysis of cocoa phenolics, *LC–GC Eur.* (2000) 902–905.
- [20] M. Jaffé, Über den niederschlag welchen pikrinsäure in normalen harn erzeugt und über eine neue Reaction des Kreatinins, *Z. Physiol. Chem.* 10 (1886) 391–400.
- [21] J.M. Hodgson, S.Y. Chan, I.B. Puddey, A. Devine, N. Wattanapenpaiboon, M.L. Wahlqvist, W. Lukito, V. Burke, N.C. Ward, R.L. Prince, K.D. Croft, Phenolic acid

- metabolites as biomarkers for tea- and coffee-derived polyphenol exposure in human subjects, *Br. J. Nutr.* 91 (2004) 301–305.
- [22] S. Mir, E. Sendaroglu, Quantification of hypercalciuria with the urine calcium osmolality ratio in children, *Pediatr. Nephrol.* 20 (2005) 1562–1565.
- [23] F. Valianpour, N.G.G.M. Abeling, M. Duran, J.G.M. Huijmans, W. Kulik, Quantification of free sialic acid in urine by HPLC-electrospray tandem mass spectrometry: a tool for the diagnosis of sialic acid storage disease, *Clin. Chem.* 50 (2004) 402–409.
- [24] W. Horwitz, Official Method of Analysis of AOAC INTERNATIONAL (Appendix D and E), 2003.
- [25] Guidance for Industry, Bioanalytical Method Validation, U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Veterinary Medicine (CVM), 2001.
- [26] J.M. Martín-Moreno, P. Boyle, L. Gorgojo, P. Maisonneuve, J.C. Fernandez-Rodriguez, S. Salvini, W.C. Willett, Development and validation of a food frequency questionnaire in Spain, *Int. J. Epidemiol.* 22 (1993) 512–519.
- [27] R. Elosua, J. Marrugat, L. Molina, S. Pons, E. Pujol, Validation of the Minnesota Leisure Time Physical Activity Questionnaire in Spanish men: the MARATHOM Investigators, *Am. J. Epidemiol.* 139 (1994) 1197–1209.
- [28] R. Estruch, M.A. Martínez-González, D. Corella, J. Salas-Salvado, V. Ruiz-Gutiérrez, M.I. Covas, M. Fiol, E. Gómez-Gracia, M.C. López-Sabater, E. Vinyoles, F. Aros, M. Conde, C. Lahoz, J. Lapetra, G. Saez, E. Ros, Effects of a Mediterranean-style diet on cardiovascular risk factors: a randomized trial, *Ann. Intern. Med.* 145 (2006) 1–11.
- [29] F. Saura-Calixto, I. Goni, Antioxidant capacity of the Spanish Mediterranean diet, *Food Chem.* 94 (2006) 442–447.
- [30] L.I. Mennen, D. Sapinho, H. Ito, S. Bertrais, P. Galan, S. Hercberg, A. Scalbert, Urinary flavonoids and phenolic acids as biomarkers of intake for polyphenol-rich foods, *Br. J. Nutr.* 96 (2006) 191–198.
- [31] K.S. Krogholm, J. Haraldsdottir, P. Knuthsen, S.E. Rasmussen, Urinary total flavonoid excretion but not 4-pyridoxic acid or potassium can be used as a biomarker for the intake of fruits and vegetables, *J. Nutr.* 134 (2004) 445–451.
- [32] C. Manach, A. Scalbert, C. Morand, C. Remesy, L. Jimenez, Polyphenols: food sources and bioavailability, *Am. J. Clin. Nutr.* 79 (2004) 727–747.
- [33] S.E. Nielsen, R. Freese, P. Kleemola, M. Mutanen, Flavonoids in human urine as biomarkers for intake of fruits and vegetables, *Cancer Epidemiol. Biomarkers Prev.* 11 (2002) 459–466.
- [34] J.H. de Vries, P.C. Hollman, S. Meyboom, M.N. Buysman, P.L. Zock, W.A. van Staveren, M.B. Katan, Plasma concentrations and urinary excretion of the antioxidant flavonols quercetin and kaempferol as biomarkers for dietary intake, *Am. J. Clin. Nutr.* 68 (1998) 60–65.
- [35] I. Erlund, E. Meririnne, G. Alfthan, A. Aro, Plasma kinetics and urinary excretion of the flavanones naringenin and hesperetin in humans after ingestion of orange juice and grapefruit juice, *J. Nutr.* 131 (2001) 235–241.

4.2 Publicació 2: Distribució normal d'excreció urinària de polifenols en adolescents masculins i canvis associats amb la intervenció nutricional de suc de tomàquet (*Lycopersicon esculentum*)

Laila Hussein, Alexander Medina, Anna Barrionuevo, Rosa Maria Lamuela-Raventos, Cristina Andres-Lacueva. *International Journal of Food Sciences and Nutrition*. 2009, 60(4): 302-311

Resum:

Els flavonoides són metabòlits secundaris de les plantes àmpliament distribuïts en les fruites, verdures, llavors i flors, aportant una elevada capacitat antioxidant. Múltiples efectes biològics són atribuïts als flavonoides, els quals promouen l'estat de salut en general. El teixit de la pell dels tomàquets (*Lycopersicon esculentum*) contenen al voltant d'un 98% de flavonoides, però la resta també conté un alt contingut en polifenols. L'objectiu del present estudi va ser mesurar els patrons de distribució normal i els límits de referència clínica d'excreció de polifenols totals urinaris, entre els varons joves egipcis que consumeixen una típica dieta egípcia. A més, es van estudiar els canvis obtinguts en l'excreció de polifenols totals després d'una intervenció amb suc de tomàquet.

Un total de 49 homes joves egipcis d'entre 7 i 14 anys d'edat, de Giza Welfare, van ser reclutats per a l'estudi. Les excrecions urinàries de 24 hores van ser recollides durant dos i tres dies consecutius, a més d'una enquesta dietètica d'un període de 7 dies, abans i durant l'estudi. Els tomàquets escollits per l'estudi van ser batuts sencers i el suc va ser distribuït en porcions de 240 grams, els quals van ser subministrats durant 3 setmanes. Els polifenols totals van ser analitzats a través del mètode de Folin-Ciocalteu després de l'extracció en fase sòlida per l'eliminació de possibles interferències presents en les orines.

La mitjana de polifenols totals excretats 48.6 ± 5.5 mg equivalent d'àcid gàlic (GAE) en 24 hores equivalent a 89.5 ± 8.4 mg GAE/g creatinina en aquesta població. La mitjana de polifenols totals urinaris excretats al matí de forma

puntual, incrementa significativament ($P<0.05$) després de la intervenció amb suc de tomàquet (287.4 ± 64.3 mg GAE /g creatinina) comparat amb la respectiva basal. Mesurant l'excreció urinària de polifenols totals, es va demostrar una vegada més l'ús d'aquesta mesura com un bon biomarcador del consum de polifenols totals, a més de demostrar que els polifenols presents en el tomàquet són altament biodisponibles.

This article was downloaded by: [Consorti de Biblioteques Universitaries de Catalunya]

On: 22 July 2009

Access details: Access Details: [subscription number 789296668]

Publisher Informa Healthcare

Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



International Journal of Food Sciences and Nutrition

Publication details, including instructions for authors and subscription information:

<http://www.informaworld.com/smpp/title-content=t713425816>

Normal distribution of urinary polyphenol excretion among Egyptian males 7-14 years old and changes following nutritional intervention with tomato juice (*Lycopersicon esculentum*)

Laila Hussein ^{*}; Alexander Medina [†]; Ana Barrionuevo [‡]; Rosa M. Lammuela-Raventos [‡]; Cristina Andres-Lacueva ^{*}

^{*} Departments of Nutrition and Food Science, National Research Center, Egypt [†] Nutrition and Food Science Department, Pharmacy School, University of Barcelona, Barcelona, Spain

First Published on: 26 June 2008

To cite this Article Hussein, Laila, Medina, Alexander, Barrionuevo, Ana, Lammuela-Raventos, Rosa M. and Andres-Lacueva, Cristina(2008)Normal distribution of urinary polyphenol excretion among Egyptian males 7-14 years old and changes following nutritional intervention with tomato juice (*Lycopersicon esculentum*),International Journal of Food Sciences and Nutrition,60:4,302 — 311

To link to this Article: DOI: 10.1080/09637480701780047

URL: <http://dx.doi.org/10.1080/09637480701780047>

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: <http://www.informaworld.com/terms-and-conditions-of-access.pdf>

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

Normal distribution of urinary polyphenol excretion among Egyptian males 7–14 years old and changes following nutritional intervention with tomato juice (*Lycopersicon esculentum*)

LAILA HUSSEIN¹, ALEXANDER MEDINA²,
ANA BARRIONNEVO², ROSA M. LAMMUELA-RAVENTOS² &
CRISTINA ANDRES-LACUEVA²

¹Departments of Nutrition and Food Science, National Research Center, Egypt, and ²Nutrition and Food Science Department, Pharmacy School, University of Barcelona, Barcelona, Spain

Abstract

Background The urinary flavonoids are considered a reliable biomarker for the intake of polyphenol-rich foods.

Objectives To assess the normal distribution of urinary polyphenol [PP] excretion among healthy male children and adolescents on a typical Egyptian diet. To follow up the impact of nutritional intervention with tomato juice on the urinary excretion of [PP].

Subjects Forty-nine male subjects 7–14 years old collected a 24-h urine sample and filled a dietary record during a 7-day period. A daily serving of 230 g fresh tomato juice was followed for 18 days in a subgroup. Total urinary [PP] excretions were measured before and after termination of the intervention program. The total urinary [PP] was analyzed after a clean-up solid-phase extraction step by the Folin–Ciocalteu reagent in the 96 micro plates. The results were expressed as gallic acid equivalents (GAE).

Results The urinary [PP] excretion averaged 48.6 ± 5.5 mg GAE/24 h, equivalent to 89.5 ± 8.4 mg GAE/g creatinine. The mean urinary [PP] excretion increased significantly ($P < 0.05$) following the intervention with tomato juice (287.4 ± 64.3 mg GAE/g creatinine) compared with the respective mean baseline level (94.5 ± 8.92 mg GAE/g creatinine).

Conclusion Clinical laboratory reference limits for urinary polyphenols are presented for Egyptian male children and adolescents. Measuring the urinary polyphenol excretion proved a good biomarker for the dietary polyphenol intake and the results demonstrated that tomato [PP] was highly bioavailable in the human body.

Keywords: Male children and adolescents, nutritional intervention, tomato juice, urinary polyphenol excretion, biomarker

Introduction

Flavonoids are secondary plant metabolites widely distributed in fruits, vegetables, nuts, seeds, and flowers, and exert high antioxidant capacity (Duthie and Crozier 2000; Pietta 2000). Multiple biological effects were reported for the flavonoids, which

Correspondence: Professor Laila Hussein, Departments of Nutrition and Food Science, National Research Center, 12311 Giza-Dokki, Elbohous Street, Egypt. Tel: 202 24155744. Fax: 202 3370931. E-mail: egcairouw@yahoo.com

ISSN 0963-7486 print/ISSN 1465-3478 online © 2009 Informa UK Ltd
DOI: 10.1080/09637480701780047

promote the health status in general (Scalbert and Williamson 2000; Manach et al. 2004) and affect gut health in particular (Roura et al. 2006).

The peel tissue of tomato (*Lycopersicon esculentum*) contains roughly 98% of the flavonols (quercetin-glycosides and kaempferol-glycosides) and between 95% and 98% of the naringenin chalcone (Stewart et al. 2000; Verhoeven et al. 2002). The majority of tomatoes' [PP] are in the form of quercetin 3-rhamnosylglucoside or rutin (US Department of Agriculture 2003), with levels ranging between 1.3 and 22.2 mg flavonol/kg fresh weight in one study (de Pascual-Teresa et al. 2000) and between 5 and 30 mg/kg fresh weight in a second study (Stewart et al. 2000).

Naringenin (4',5,7-trihydroxyflavanone; molecular weight, 272.3) flavanone is present in levels ranging between 8 and 42 mg/kg whole red tomato (Bugianesi et al. 2002). Tomato flesh tissues with up to 78-fold excess flavonoid content were obtained by introducing a new biosynthetic branch through genetic engineering technology (Verhoeven et al. 2002; Schijlen et al. 2006). Tomatoes, however, are quite poor in their flavone content (Hertog and Hollman 1996).

Estimation of the dietary intake of [PP] is difficult due to limited availability of food composition databases and bias inherent to dietary assessment methods. Dietary biomarkers represent an objective alternative to the traditional food registration methods. The 24-h urinary total flavonoids excretion has been validated in intervention studies as a potential biomarker for the dietary intake of fruits and vegetables; its excretion changed in a linear manner with varying exposures (Krogholm et al. 2004; Roura et al. 2006).

The objective of the present study is to assess the normal distribution patterns and the clinical laboratory reference limits of urinary total polyphenol excretion among Egyptian young males consuming a typical Egyptian diet. The changes in the pattern following nutrition intervention with tomato juice were studied as well.

Materials and methods

Subjects

A total of 49 boys from Giza Wellfare Institute were enrolled in the study. Exclusion criteria included: regular intake of dietary supplementation or medicine <14 days before the study start; smoking; disliking the taste of tomato juice; >10 h/week physical activity; or participation in other experiments during the study period.

Table I. Characteristics of the study subjects.

Parameter	Mean $\pm$ standard error	Median	Range
Age (years)	10.7 $\pm$ 0.35	10	7–14
Body weight (kg)	32.2 $\pm$ 0.65	31	24.5–47
Body mass index (kg/m ²)	17.8 $\pm$ 0.51	17.2	15–27.4
Blood hemoglobin (g/dl)	11.0 $\pm$ 0.27	11.03	9–13.4
Urinary creatinine (g/24 h)	770.5 $\pm$ 87.0	677.2	249.6–1674.5
Urinary creatinine (mg/kg body weight)	25.6 $\pm$ 3.27	25.1	7.1–53.2
Urinary [PP] (GAE mg/24 h)	48.6 $\pm$ 5.5	46.4	7.3–113.8
Urinary [PP] (GAE mg/g creatinine)	89.5 $\pm$ 8.4	79.2	48.1–198.4

Detailed characteristics of the volunteers are presented in Table I. The study was approved by the Ethics Committee of the National Research Center. Informed written consent was obtained from the director of the Welfare Institute. The participants consumed three meals daily based on typical Egyptian dishes.

Timely 24-h urine excretions were collected on two or three consecutive days. The volume of the 24-h urine excretion was recorded and aliquots were saved frozen at -20°C for subsequent chemical analysis of urinary total polyphenols and creatinine.

Nutrition intervention with tomato juice and serving schedule

One batch of 200 kg local juicy tomato variety was purchased from the experimental station of the Department of Horticulture at the National Research Center. The fruits were harvested in June 2006, and upon delivery the fruits were freed from the green neck and were washed thoroughly by soaking in a sink containing running tap water. The tomatoes were blended in the Waring blender without any additives. The juice was distributed in 230 g portions in bottles (330-ml capacity) with blue caps. On the day of consumption, maize oil (4–5 g) was added with a dropper to each bottle. The bottles were turned upside down in the carton box to allow homogeneous distribution of the oil.

The design of the nutrition intervention was based on the rationale that subjects serve as their own control. A subgroup of 27 boys was selected randomly from the 49 boys who completed the 24-h urine collection.

The ice-cold tomato juice was served daily between 5:30 and 7:00 pm for 18 days.

Dietary assessment

A 7-day dietary food record was completed before and during the last week of the intervention period. The estimated [PP] intakes were calculated using our own analytical data and the available published data (US Department of Agriculture 2003).

Collection of urine samples

All urine samples were collected in 1,500-ml labeled plastic bottles. The volunteers received careful instruction about the importance of complete 24-h urine collection. Compliance to instructions was checked by analysis of the urinary creatinine. Successive 24-h urine collections continued until two urine collections with highest and close creatinine values were obtained. The volume of the 24-h urine samples was recorded and 10-ml; aliquots were stored at -20°C for total [PP] analysis.

Spot urine samples were collected prior to and 12 h after the last serving day of the tomato juice. On delivery of the urine samples, two to four 10-ml samples were distributed in vials, which were stored at -20°C for subsequent analysis.

Analysis of [PP] in the urine samples (Roura et al. 2006)

All chemicals were analytical grade. The solid-phase extraction (SPE) Cartridge (60 mg) from Waters Oasis reverse phase HLB (Mildford, MA, USA); with Folin-Ciocalteu reagent (Catalog No47641; Sigma, USA). MilliQ ultrapure

water (Millipore Iberica, Madrid, Spain) was used throughout the course of the study. All of the analytical steps were carried out in dim light.

For clean up with SPE, aliquots of the centrifuged urine samples (1 ml) were acidified with normal hydrochloric acid (17 μ l) and were loaded onto an Activated Waters Cartridge, preconditioned with 1 ml methanol and equilibrated with 1 ml of 1.5 mol/l formic acid in water. The SPE cartridge was washed twice with 2 ml of the same formic acid and 2 ml of water-methanol (95:5 by volume) to elute the impurities. Elution of the [PP] was completed with 1 ml of methanol containing 1 ml/l of formic acid (2%)-methanol-H₂O (2/60/38 v/v/v).

Aliquots of the elute (15 μ l) were pipetted into a 96-well plate for the colorimetric determination of the [PP]. After the successive addition of Folin-Ciocalteu reagent (10 μ l) and 20% sodium carbonate (32 μ l), the final volume was brought to 300 μ l with H₂O. The plates were left in the dark for 60 min and then the absorbance of the color was measured at 765 nm against a blank using a multiscan spectrum equipped with SkanIt Software version 2 (Thermo Electron Corporation, Spain).

A blank and standard working solutions of gallic acid (1–14 mg/l) were run in parallel. The detection limit of the method was 0.9 mg gallic acid/l. The results were expressed as gallic acid equivalents (GAE) per liter of urine.

Urinary creatinine

Aliquots of the urine samples (3 μ l) were pipetted into a 96-well plate for the determination of creatinine by the Jaffe (1886) alkaline picrate method. Aliquots (60 μ l) of aqueous picric acid solution (10 g/l) and a 60 μ l aliquot of sodium hydroxide (100 g/l) were added in this sequence, and after 15 min water (232 μ l) was added and the intensity of the color was read at 500 nm in the multiscan spectrum. The coefficient of variation of the repeatability of the assay was below 12%. Urinary [PP] excretion was expressed as mg gallic acid equivalent per 24 hours or as GAE per gram of creatinine.

Analysis of tomato juice for total [PP]

Accurately weighed portions (4 ± 0.5 g) of the tomato juice were extracted successively and vigorously in dim light with 5 ml distilled water and then with 20 ml absolute methanol (Andrés Lacueva et al. 2000). After centrifugation in the cold, the upper layer was aspirated and concentrated *in vacuo* to 4 ml. Clean up step on Waters 3 ml HLB (60 mg); cartridges were 3 ml well-plate equilibrated with the successive addition of absolute methanol (1 ml) and H₂O (1 ml). Aliquots (2 ml) were loaded on the SPE, washing with the successive addition of H₂O (10 ml) and 5% aqueous methanol (1 ml). The [PP]-containing fraction was collected after the addition of 5 ml absolute methanol. The elute was concentrated under a stream of nitrogen to 2 ml. Aliquots (20 μ l) were taken for the analysis of [PP] using the spectrophotometric Folin-Ciocalteu method as described above and the results were expressed as GAE per gram.

Statistical analysis

The results are displayed as the mean (standard error). Means between study groups were compared by Student's *t*-test. (Excel statistics version 5.0; Excel Software, NM, USA). *P* < 0.05 (two-tailed) was considered significant.

306 *L. Hussein et al.*

Results

Characteristics of the subjects are presented in Table I. The urinary total [PP] excretion averaged 48.6 ± 5.5 mg GAE/24 h, with median value of 46.4 mg GAE/24 h. Respective values of 89.5 ± 8.4 and 79.2 were obtained when the results were expressed as mg GAE/g creatinine (Table I).

Figure 1 illustrates the normal distribution of the urinary [PP] excretion among the 49 studied boys with a mean age of 10 years.

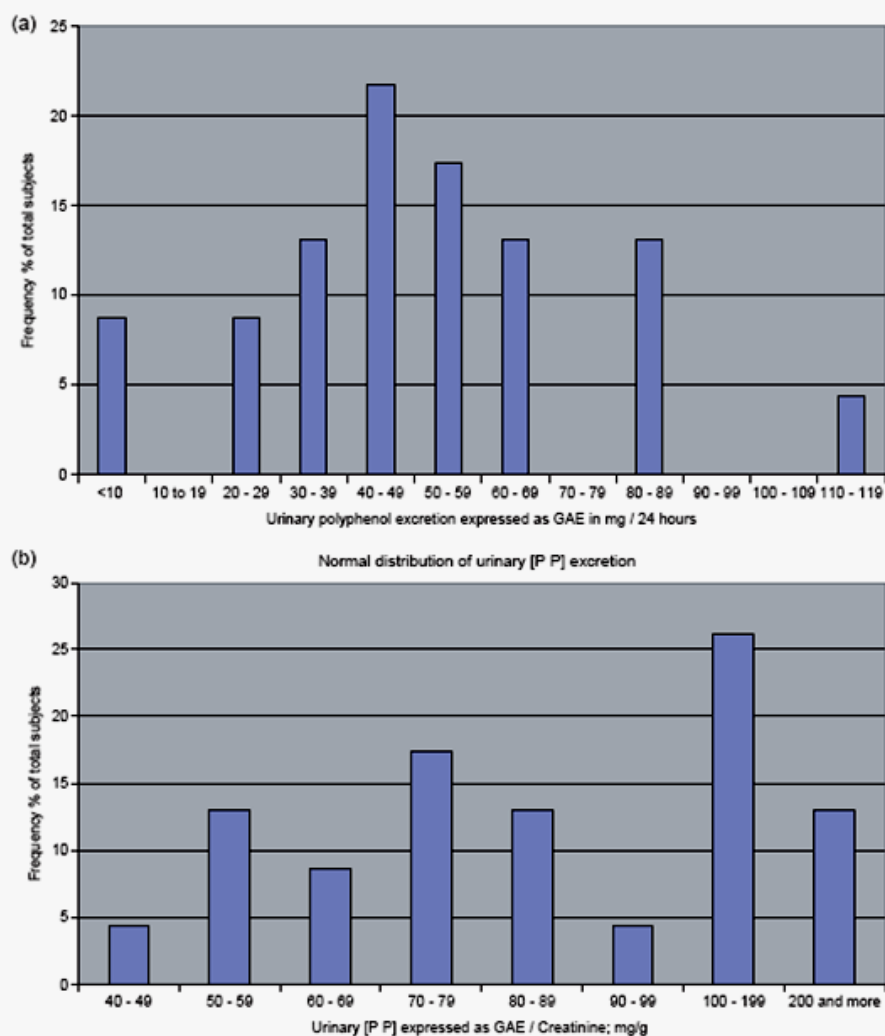


Figure 1. Normal distribution of urinary excretion of total polyphenols among healthy males. (a) 24-h urine [PP] expressed as GAE. (b) Spot urine sample [PP] expressed as mg GAE/g creatinine.

Tomato intervention study

Tomato juice polyphenols [PP] averaged 106.8 mg/kg; providing 25.6 ± 1.27 mg total [PP] per serving (230 g tomato juice).

The studied individuals who participated in the tomato intervention did not differ significantly with regard to age or physical characteristics. The 27 recruited boys completed the study with no adverse effects and enjoyed the consumption of the tomato juices. At the baseline, the mean urinary [PP] excretion was 105.1 ± 13.0 mg GAE/g creatinine.

The 18 day nutrition intervention was associated with a significant increase ($P < 0.05$) in the mean urinary [PP] excretion amounting to 269.8 ± 65.4 mg GAE/g creatinine (Figure 2). When the initial urinary concentrations of total polyphenols/creatinine were set equal to 100%, the percentage average increase following the nutrition intervention amounted to $185.9 \pm 59.5\%$.

Major dietary sources of polyphenols in the diet

The stewed faba beans (16 mg polyphenols/100 g) served on breakfast were among the major [PP] contributors in the diet. Onions (19.6 mg polyphenols/100 g) were incorporated in the preparation of all vegetables and meats. Oranges (55 mg polyphenols/100 g) contributed also to the [PP] supply. Berries were not consumed by the study group during the course of the study. Based on the dietary intake, the boys consumed on average 80 ± 15 mg total [PP] in their diet.

Discussion

Dietary biomarkers represent an objective alternative to the traditional food registration methods since the estimation of dietary intake of [PP] is difficult, owing to limited availability of food composition databases and bias inherent to dietary assessment methods (William 1990).

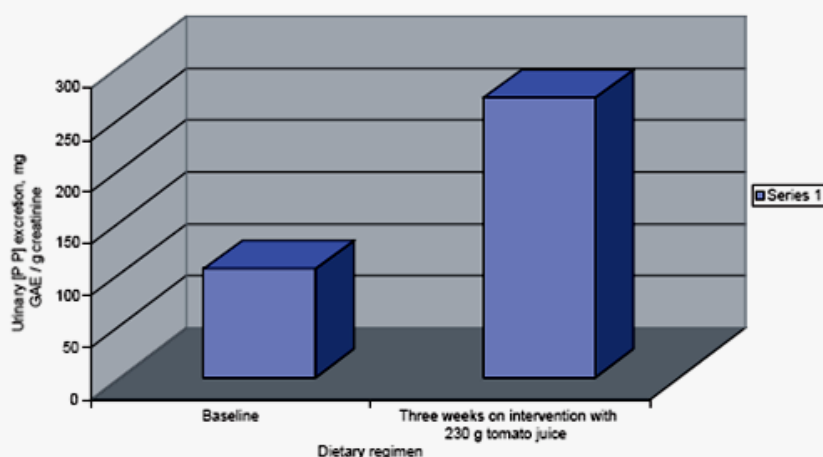


Figure 2. Impact of nutritional intervention with tomato juice on the urinary [PP] excretion among male adolescents.

An essential property of a biomarker is that it changes in a linear manner with varying exposures (William 1990). For most polyphenols, the urinary excretion values were consistent with the plasma kinetic data, and relative urinary excretion is currently used to estimate the minimal absorption rate (Manach et al. 2005).

Urine sampling is non-invasive and is easily accessible in nutritional surveys. The urinary flavonoids have also been validated as a reliable biomarker for fruits and vegetables intake (Manach et al. 2005; Roura et al. 2006).

In one study, the urinary flavonoid excretion in 24-h urine ($r_s = 0.86$) and in morning urine ($r_s = 0.59$) increased linearly with increasing fruit and vegetable intakes (Krogholm et al. 2004). Another study separated 15 flavonoids from the 24-h urine excreted by individuals following the consumption of six different polyphenol-rich beverages (Ito et al. 2005). Based on the pattern of flavonoid in the urine, the authors suggested that chlorogenic acid, gallic acid, epicatechin, naringenin or hesperetin could be used as specific biomarkers to evaluate the consumption of coffee, wine, tea, cocoa or citrus juices, respectively (Ito et al. 2005).

The collection of 24-h urine is possible only in the controlled metabolic studies, but is impractical and too costly for a large-scale cohort study. In an epidemiological setting, phenolic compounds in spot urine samples were considered a urinary biomarker in polyphenol exposure studies (Mennen et al. 2006; Roura et al. 2006).

The liquid chromatography instrumentations reported for the isolation and quantitation of the urinary flavonoids (Krogholm et al. 2004; Ito et al. 2005) are quite expensive and not practical in epidemiological studies. The method adopted in the present work for the analysis of urinary [PP] had been described (Roura et al. 2006), which depends on a solid phase extraction [SPE] step combined with the Folin–Ciocalteu assay (Singleton and Rossi, 1956 and Singleton et al., 1999). The Folin–Ciocalteu method also provides a measure of the reducing capacity of total phenolic content of botanicals in foods and dietary supplements (Prior et al. 2005).

The mean 24-h urinary [PP] excreted by the studied boys in the present study (48.6 ± 5.5 mg GAE/24 h) are higher than the respective figures reported earlier (1.829 ± 1.408 mg total flavonoids/24 h) among Danish (Krogholm et al. 2004) and French ($22.6 \mu\text{mol/day}$, equivalent to 4.25 mg GAE/day) (Ito et al. 2005; Mennen et al. 2006) samples. This great discrepancy may be attributed either to technical differences in the analytical methodologies or to variation in the sources and level of dietary flavonoids consumed with the diets.

The mean level of urinary [PP] excreted by our studied subjects (89.5 mg GAE/g creatinine) is coincides with the respective figures reported earlier (79.3 – 90.4 mg catechin/g creatinine) among Spanish adult male and female volunteers (Roura et al. 2006).

The initial urinary [PP] excreted by our studied subjects before (105.1 ± 13.0 mg GAE/g creatinine) and after the 18 days nutrition intervention (269.8 ± 65.4 mg GAE/g creatinine) with tomato juice (25.6 mg PP per serving $\times 18$ days = 461 mg PP) were superior to the respective mean total phenolic acids excreted in the urine of French subjects before (60.6 mg phenolic acids/g creatinine) and following the consumption of 80 g chocolate containing 439 mg proanthocyanidins (106.2 mg phenolic acids/g creatinine) (Rios et al. 2003).

In the present study, the 18 days nutrition intervention was associated with a percentage average increase of $185.9 \pm 59.5\%$ in the urinary [PP] excretion relative to the initial baseline level. An earlier report showed a four-fold increase in 24-h urinary

phenolic acids among adult non-smokers following the ingestion of 500 mg GAE oregano packed in gelatin capsules (Nurmi et al. 2006).

There is a growing interest in the development of food crops with enhanced levels of flavonoids designed for specific benefits to human health. The tomato is an excellent candidate given the large daily consumption of tomato and tomato-based products, being the major horticultural crop grown in the two Mediterranean countries; Egypt and Spain. The average [PP] content (25.6 ± 1.27 mg) of tomatoes growing in the warm sunny climate of Egypt ranked within the upper level reported earlier (5–30 mg PP/kg tomato) (Stewart et al. 2000).

The bioavailability of tomato ferulic acid in humans was investigated through the monitoring of the pharmacokinetics of urinary excretion in relation to intake, and the results showed that the peak time for maximal urinary excretion was approximately 7 h and the recovery of ferulic acid in the urine was 11–25% of that ingested (Bourne and Rice-Evans 1998). Another report showed that urinary recovery of naringenin in the 6–12 h urine samples accounted for 12.9% of the corresponding glycosides ingested (Ito et al. 2005). Furthermore, the plasma naringenin peaked (0.12 ± 0.03 $\mu\text{mol/l}$; equivalent to 32.7 $\mu\text{g/l}$) within 2 h among male adults consuming cooked tomato paste containing 3.8 mg naringenin (Bugianesi et al. 2002). Naringenin isomerizes to naringenin chalcone in simulated gut conditions (Gil-Izquierdo et al. 2003), suggesting its dehydrogenation to phloretin by the gut microflora.

In the present study, the amount of the tomato juice was kept such that it would be reasonable to consume as part of a normal diet. The 240 g fresh tomato juice served during the intervention period reflected a human intake of three medium-size tomato fruits, equivalent to 2.3 g tomato peel and providing 25.6 ± 1.27 mg total [PP] expressed as GAE.

The interindividual variability in the 24-h urinary excretion of [PP] had been reported by other investigators (Krogholm et al. 2004; Ito et al. 2005). Differences were attributed to variations in intestinal absorption; polyphenol interactions with other food ingredients and/or genetic polymorphisms of selected intestinal enzymes (rapid and slow excretors) are among the confounding factors.

Although this study was based on a male cohort, there is no reason to doubt that the result would not be applicable to women.

A recent study showed that tomato benefits could be due to a synergistic action between lycopene with some polyphenols, thus enhancing its antioxidant properties (Fuhrman et al. 2000). The total antioxidant capacity of tomatoes correlates with the color intensity of intact tomatoes (Wold et al. 2004).

In conclusion, the present study demonstrates that urinary [PP] is a useful biomarker of polyphenol intake in an epidemiological setting.

Acknowledgements

The authors would like to thank the volunteers involved in this study as well as their supervisors.

Conflict of interest

This study was partially supported by grants from the Spanish Ministry of Education and Science (MEC), AGL2005-002823, AGL2007-66638-C02-01/ALI and CONSOLIDER program (CB 06/02/0079). AM is the recipient of a fellowship from the Generalitat de Catalunya (2006FI 01399).

References

- Andrés-Lacueva C, Lamuela-Raventós RM, Jáuregui O. 2000. An LC method for the analysis of cocoa phenolics. *LC-GC Eur J* 12:902–905.
- Bourne LC, Rice-Evans C. 1998. Bioavailability of ferulic acid. *Biochem Biophys Res Commun* 253:222–227.
- Bugianesi R, Catasta G, Spigno P, DUva A, Maiani G. 2002. Naringenin from cooked tomato paste is bioavailable in men. *J Nutr* 132:3349–3352.
- de Pascual-Teresa S, Santos-Buelga C, Rivas-Gonzalo JC. 2000. Quantitative analysis of flavan-3-ols in Spanish foodstuffs and beverages. *J Agric Food Chem* 48:5331–5337.
- Duthie G, Crozier A. 2000. Plant-derived phenolic antioxidants. *Curr Opin Lipidol* 11:43–47.
- Fuhrman B, Volkova N, Rosenblat M, Aviram M. 2000. Lycopene synergistically inhibits LDL oxidation in combination with vitamin E, glabridin, rosmarinic acid, carnosic acid of garlic. *Antioxid Red Sign* 2:491–506.
- Gil-Izquierdo A, Gil MI, Tomas-Barberan FA, Ferreres F. 2003. Influence of industrial processing on orange juice flavanone solubility and transformation to chalcones under gastrointestinal conditions. *J Agric Food Chem* 51:3024–3028.
- Hertog MG, Hollman PC. 1996. Potential health effects of the dietary flavonol quercetin. *Eur J Clin Nutr* 50:63–71.
- Ito H, Gonthier MP, Manach C, Morand C, Mennen L, Rémésy C, Scalbert A. 2005. Polyphenol levels in human urine after intake of six different polyphenol-rich beverages. *Br J Nutr* 94:500–509.
- Jaffe M. 1886. Über den Niederschlag welchen Pikrinsäure in normalen Harn erzeugt und über eine neue Reaction des Kreatinins. *Z Physiol Chem* 10:391–400.
- Krogholm KS, Haraldsdottir J, Knuthsen P, Rasmussen SE. 2004. Urinary total flavonoid excretion, but not 4-pyridoxic acid or potassium, can be used as a biomarker for the intake of fruits and vegetables. *J Nutr* 134:445–451.
- Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L. 2004. Polyphenols: food sources and bioavailability. *Am J Clin Nutr* 79:727–747.
- Manach C, Williamson G, Morand C, Scalbert A, Rémésy C. 2005. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am J Clin Nutr (Suppl)*:81:230S–242S.
- Mennen LJ, Sapinho D, Ito H, Bertrais S, Galan P, Hercberg S, Scalbert A. 2006. Urinary flavonoids and phenolic acids as biomarkers of intake for polyphenol-rich foods. *Br J Nutr* 96:191–198.
- Nurmi A, Mursu J, Nurmi T, Nyyssö-Nen K, Alftan G, Hiltunen R, Kaikonen J, Salonen JT, Voutilainen S. 2006. Consumption of juice fortified with oregano extract markedly increases excretion of phenolic acids but lacks short- and long-term effects on lipid peroxidation in healthy nonsmoking men. *J Agric Food Chem* 54:5790–5796.
- Pietta PG. 2000. Flavonoids as antioxidants. *J Nat Prod* 63:1035–1042.
- Prior R, Wu X, Schaich K. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J Agric Food Chem* 53:3101–3113.
- Rios LY, Gonthier MP, Rémésy C, Milla I, Lapierre C, Lazarus SP, Williamson G, Scalbert A. 2003. Chocolate intake increases urinary excretion of polyphenol-derived phenolic acids in healthy human subjects. *Am J Clin Nutr* 77:712–718.
- Roura E, Lacueva CA, Estruch R, Lamuela-Raventós RM. 2006. Total polyphenol intake estimated by a modified Folin-Ciocalteu assay of urine. *Clin Chem* 52:749–752.
- Scalbert A, Williamson G. 2000. Dietary intake and bioavailability of polyphenols. *J Nutr* 130:2073S–2085S.
- Schijlen EG, de Vos R, Jonker H, van den Broeck H, Molthoff J, vanTunen A, Martens S, Bovy A. 2006. Pathway engineering for healthy phytochemicals leading to the production of novel flavonoids in tomato fruit. *Plant Biotechnol J* 4:433–444.
- Sánchez-Rabaneda F, Jáuregui O, Casals J, Andre-Lacueva C, Izquierdo-Pulido M, Lamuela-Raventós RM. 2003. Liquid chromatography/electrospray ionization tandem mass spectrometric study of the phenolic composition of cacao (*Theobroma cacao*). *J Mass Spectrom* 38:35–42.
- Singleton VL, Rossi JA. 1965. Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. *Am J Enol Vitic* 16:44–58.
- Singleton VL, Orthofer R, Lamuela-Raventós RM. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol* 299:152–178.
- Stewart AJ, Bozonnet S, Mullen W, Jenkins GI, Lean ME, Crozier A. 2000. Occurrence of flavonols in tomatoes and tomato-based products. *J Agric Food Chem* 48:2663–2669.

Intervention with tomato juice and urinary polyphenol excretion 311

- US Department of Agriculture. 2003. Database for the flavonoid content of selected foods. Beltsville, MD: Agricultural Research Service, Beltsville Human Nutrition Research Center, US Department of Agriculture. Available online at: <http://www.nal.usda.gov/fnic/foodcomp>. (Accessed October 2007).
- Verhoeven ME, Bovy A, Collins G, Muir S, Robinson S, de Vos CHR, Colliver S. 2002. Increasing antioxidant levels in tomatoes through modification of the flavonoid biosynthetic pathway. *J Exp Bot* 53:2099–2106.
- William W. 1990. Nutritional epidemiology. In: Monographs in epidemiology and biostatistics. Oxford: Oxford University Press.
- Wold AB, Rosenfeld HJ, Holte K, Baugerød H, Blomhoff R, Haffner K. 2004. Colour of post-harvest ripened and vine ripened tomatoes (*Lycopersicon esculentum* Mill.) as related to total antioxidant capacity and chemical composition. *Int J Food Sci Technol* 39:295–302.

This paper was first published online on iFirst on 26 June 2008.

4.3 Publicació 3: Excreció de polifenols totals i pressió arterial en subjectes amb un alt risc cardiovascular.

Medina-Remón A., Zamora-Ros R., Rotchés-Ribalta M., Andres-Lacueva C., Martínez-González M.A., Covas M.I., Corella D., Salas-Salvadó J., Gómez-Gracia E., Ruiz-Gutiérrez V., García de la Corte F.J., Fiol M., Pena M.A., Saez G.T., Ros E., Serra-Majem L., Pinto X., Warnberg J., Estruch R. and Lamuela-Raventos R.M. *Nutrition Metabolism and Cardiovascular Diseases*. 2009, doi:10.1016/j.numecd.2009.10.019

Resum:

Una dieta saludable i modificacions en l'estil de vida són els primers passos en la prevenció de la hipertensió, un important problema mundial a nivell de salut pública. L'objectiu del present treball ha sigut avaluar l'efecte del consum dels polifenols en la hipertensió arterial en una població adulta amb alt risc cardiovascular. Com a paràmetre per determinar la ingesta de polifenols totals s'han utilitzat els polifenols totals excretats (TPE), biomarcador de la ingesta de polifenols.

S'ha portat a terme un sub-estudi transversal amb dades de referència de l'estudi PREDIMED (www.predimed.org). Als participants, 263 homes i 326 dones amb edats entre 55-80, se'ls van prendre les dades antropomètriques, la pressió arterial, i un qüestionari de freqüència de consum. Els polifenols totals excretats es van determinar utilitzant el mètode de Folin-Ciocalteu, adaptat i validat pel nostre grup d'investigació a mostres biològiques i els resultats van ser dividits en quartils de TPE.

Es va observar una correlació positiva i estadísticament significativa entre els TPE i la ingesta total de cafè, vi i fruites-verdures-hortalisses (F&V). La ingesta d'una porció de 100 g de F&V (Beta = 0.150; $P < 0.001$) va contribuir més als TPE que una de 100 mL de cafè (Beta = 0.141; $P = 0.001$) i que una porció de 100 mL de vi (Beta = 0.120; $P = 0.019$). L'anàlisi de regressió logística va mostrar una associació inversa entre els TPE (per quartils) i la prevalència

Resultats

d'hipertensió; els participants del quartil més alt de TPE urinària van tenir una reducció de la prevalència d'hipertensió en comparació amb el quartil més baix (OR = 0.64; $P = 0.016$). En l'anàlisi de regressió lineal múltiple la PA sistòlica ($\beta = -1.731$; $P = 0.024$) i diastòlica ($\beta = -1.264$; $P = 0.003$) es van associar inversament amb la TPE urinària després d'ajustar per possibles factors de confusió.

La ingesta de polifenols, avaluats a través dels TPE en orina, es va associar negativament amb els nivells de PA i la prevalència de la hipertensió en una població mediterrània d'edat avançada amb alt risc cardiovascular. Una intervenció dietètica dirigida a incrementar la ingesta d'aliments rics en polifenols, podria ser eficient en la prevenció i el tractament de la hipertensió.

available at www.sciencedirect.comjournal homepage: www.elsevier.com/locate/nmcdNutrition,
Metabolism &
Cardiovascular Diseases

Total polyphenol excretion and blood pressure in subjects at high cardiovascular risk

A. Medina-Remón^{a,b,c}, R. Zamora-Ros^{a,d}, M. Rotchés-Ribalta^{a,d},
C. Andres-Lacueva^{a,d}, M.A. Martínez-González^{c,e}, M.I. Covas^{b,f},
D. Corella^{b,g}, J. Salas-Salvadó^{b,h}, E. Gómez-Gracia^{c,i}, V. Ruiz-Gutiérrez^{c,j},
F.J. García de la Corte^{b,k}, M. Fiol^{b,l}, M.A. Peña^m, G.T. Saez^{c,n}, E. Ros^{b,o},
L. Serra-Majem^{c,p}, X. Pinto^{c,q}, J. Warnberg^{c,r,s}, R. Estruch^{b,c,t},
R.M. Lamuela-Raventós^{a,b,c,*}, on behalf of the PREDIMED Study Investigators

^a Nutrition and Food Science Department, XaRTA, INSA, Pharmacy School, University of Barcelona, Barcelona, Spain

^b CIBER CB06/03 Fisiopatología de la Obesidad y la Nutrición, (CIBEROBN), Spain

^c RETICS RD06/0045, Instituto de Salud Carlos III, Spain

^d Ingenio-CONSOLIDER Program, FUN-C-FOOD, Barcelona, Spain

^e Department of Preventive Medicine and Public Health, School of Medicine, University of Navarra, Pamplona, Spain

^f Cardiovascular Epidemiology Unit, Municipal Institute for Medical Research (IMIM), Barcelona, Spain

^g Department of Epidemiology, Preventive Medicine and Public Health, School of Medicine, University of Valencia, Valencia, Spain

^h Human Nutrition Unit, School of Medicine, IISPV, University Rovira i Virgili, Reus, Spain

ⁱ Department of Epidemiology, School of Medicine, University of Malaga, Malaga, Spain

^j Instituto de la Grasa, Consejo Superior de Investigaciones Científicas, Sevilla, Spain

^k Department of Family Medicine, Primary Care Division of Sevilla, San Pablo Health Center, Sevilla, Spain

^l Institut Universitari d'Investigació en Ciències de la Salut (IUNICS), Palma de Mallorca, Spain

^m Clinical Trials Unit, Hospital Txangorritxu, Vitoria, Spain

ⁿ Department of Biochemistry and Molecular Biology Service of Clinical Analysis, CDB Hospital General Universitario, Universitat de Valencia, Spain

^o Lipid Clinic, Endocrinology and Nutrition Service, Institut d'Investigacions Biomèdiques August Pi Sunyer (IDIBAPS), Hospital Clínic, Barcelona, Spain

^p Department of Clinical Sciences, University of Las Palmas de Gran Canaria, Palmas de Gran Canaria, Spain

^q Lipid Unit, Department of Internal Medicine, IDIBELL-Hospital Universitari de Bellvitge, L'Hospitalet de Llobregat, Fipec, Barcelona, Spain

^r Immunonutrition Research Group, Instituto del Frío-ICTAN (CSIC), Madrid, Spain

^s Department of Biosciences and Nutrition, Karolinska Institutet, Stockholm, Sweden

^t Department of Internal Medicine, Hospital Clínic, IDIBAPS, University of Barcelona, Spain

Received 20 July 2009; received in revised form 22 October 2009; accepted 23 October 2009

Acronyms: GFR, glomerular filtration rate; BP, blood pressure; TPs, total polyphenols; TPE, total polyphenol excretion; F&V, fruit and vegetables; SPE, solid phase extraction; F=C, Folin–Ciocalteu; GAE, gallic acid equivalent; CHD, coronary heart disease; ORs, odds ratios.

* Corresponding author. Nutrition and Food Science Department, XaRTA, INSA Pharmacy School, University of Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain. Tel.: +34 934034843; fax: +34 934035931.

E-mail address: lamuela@ub.edu (R.M. Lamuela-Raventós).

0939-4753/\$ - see front matter © 2009 Elsevier B.V. All rights reserved.

doi:10.1016/j.numcd.2009.10.019

Please cite this article in press as: Medina-Remón A, et al., Total polyphenol excretion and blood pressure in subjects at high cardiovascular risk, Nutr Metab Cardiovasc Dis (2009), doi:10.1016/j.numcd.2009.10.019

KEYWORDS

Polyphenol;
Urinary polyphenol;
Folin–Ciocalteu;
Fruits;
Vegetables;
Coffee;
Wine;
Hypertension;
Blood pressure;
Mediterranean diet;
Biomarker

Abstract *Background and aims:* Dietary factors are critical for the prevention and treatment of hypertension, but data on the effects of specific nutrients on blood pressure (BP) are scarce. The aim of this study was to assess the relationship between total polyphenol excretion (TPE) in urine, as an objective measurement of total polyphenol intake and BP in an elderly population at high cardiovascular risk.

Methods and results: Cross-sectional substudy of 589 high-risk participants entering in the PREDIMED trial. BP was measured and TPE was determined in urine by Folin–Ciocalteu assay. A significant positive association was observed between TPE in urine and daily intake of fruit and vegetables (F&V), coffee or wine after adjusting for potential confounders. The intake of 100 g of F&V ($\text{Beta} = 0.150; P < 0.001$) had a greater contribution to TPE than 100 mL of coffee ($\text{Beta} = 0.141; P = 0.001$), and the latter two foods contributed more than the consumption of 100 mL of wine ($\text{Beta} = 0.120; P = 0.019$). An inverse association was observed between urinary TPE and the prevalence of hypertension. Participants in the highest quartile of urinary TPE had a reduced prevalence of hypertension compared to those in the lowest quartile (Odds Ratio = 0.64; 95% confidence interval 0.45 to 0.92; $P = 0.015$). Systolic and diastolic BP were inversely associated with urinary TPE after adjustment for potential confounders ($P = 0.024$ and $P = 0.003$, respectively).

Conclusions: Polyphenol intake, assessed via TPE in urine, was negatively associated with BP levels and prevalence of hypertension in an elderly Mediterranean population at high cardiovascular risk. Participants with the highest intake of polyphenol-rich foods showed the lowest BP measurements.

© 2009 Elsevier B.V. All rights reserved.

Introduction

Hypertension is a major global public health problem [1]. A healthy diet and lifestyle modification are the first steps for the management of hypertension [2]. Adoption of a healthier food pattern such as the Mediterranean diet [3] or the DASH (Dietary-Approaches-to-Stop-Hypertension) diet has also been advocated. In addition, there is considerable evidence that the consumption of fruit and vegetables (F&V) is inversely associated with high BP, whereas a high consumption of refined cereals or of meat/meat products is associated with a higher risk [2,4,5].

However, besides sodium and potassium dietary intake, few studies have analyzed the effects of dietary nutrients, such as oleic acid [6,7] vitamin C or other antioxidant compounds [8,9] on BP. Some studies have also evaluated the effects of polyphenol-rich foods such as cocoa [10], however up to now no studies have evaluated the effects of total dietary polyphenol intake on this issue.

The total dietary amount of polyphenol is approximately 1 g/d [11], being an amount that is higher than any other known dietary antioxidants; in fact, it is around 10 times higher than dietary vitamin C and 100 times higher than vitamin E and carotenoid intake. On the other hand, epidemiologic data have shown an inverse association between the risk of overall mortality or cardiovascular disease and the consumption of polyphenol-rich foods such as fruit and vegetables, tea, olive oil and wine [12–14].

We undertook, therefore, a substudy within a larger clinical trial, the PREDIMED study [3,15], in order to evaluate whether polyphenol-rich food intake, assessed as the excretion of total polyphenols (TPs) in spot urine samples, was associated with average BP and/or with the prevalence of hypertension. To our knowledge this is the first study to assess this relationship using biochemical analysis of TPs in

spot urine samples, and directly measuring BP in a large sample of elderly individuals at high cardiovascular risk.

Methods

Subjects and design

The PREDIMED (*PREvención con Dieta MEDiterránea*) study is a large, parallel-group, multicenter, randomized, controlled 5-year clinical trial aimed to assess the effects of the Med-Diet on the primary prevention of cardiovascular disease (www.predimed.org; ISRCTN35739639). The detailed recruitment method and study protocol have been described previously [3].

From October-2003 to July-2004, we selected 612 potential participants in primary health centers affiliated with the Hospital Clinic of Barcelona and the University of Navarra (Pamplona) in Spain. Eligible participants were free of cardiovascular disease at baseline and fulfilled at least one of the following two criteria: (1) type-2 diabetes mellitus and/or (2) three or more coronary heart disease (CHD) risk factors [16]. The participants provided written informed consent and the study protocol was approved by the Institutional Review Boards of the two participating centres.

Measurements

At baseline, all participants completed a validated semi-quantitative food frequency questionnaire (FFQ) with 136-items [17], the validated Spanish version [18] of the Minnesota Leisure Time Physical Activity Questionnaire, and a 47-item questionnaire about education, lifestyle, history of illnesses and medication use. Trained nurses measured BP thrice with a validated semi-automatic oscillometer (Omron HEM-705CP [19]; Hoofdorp, The

Please cite this article in press as: Medina-Remón A, et al., Total polyphenol excretion and blood pressure in subjects at high cardiovascular risk, *Nutr Metab Cardiovasc Dis* (2009), doi:10.1016/j.numecd.2009.10.019

Netherlands). Creatinine-estimated glomerular filtration rate (GFR) was calculated using the Modification of Diet in Renal Disease (MDRD) formula in all participants [20]. Energy and nutrient intake was derived from Spanish food composition tables [21]. The morning urine samples were collected; the aliquots were immediately prepared, coded, shipped to a central laboratory and frozen at -80°C until analysis. TP consumption from plant food and beverages (mg/g fresh matter) was quantified according to Saura-Calixto F et al. [22] and Brat P et al. [23], from the FFQ.

Analysis of total polyphenols in urine

Solid Phase Extraction (SPE) with 96-well plate cartridges (Oasis[®] MAX) was performed in the urine samples to avoid any interference with F-C reagent. For all the spot urine samples TPs and creatinine were analyzed as described by Medina-Remón A. et al. [24]. TPE was expressed as mg gallic acid equivalent (GAE)/g of creatinine.

Statistical analysis

Analyses were performed using SPSS software v14.0 (Chicago, USA). The baseline characteristics of the participants were expressed as means or percentages and standard deviations (SD). Variables were examined for normality and skewness (Kolmogorov and Levene tests). The variable urinary TPE, which was not normally distributed and was not normalized by the logarithmic transformation, were Box-Cox transformed with $\alpha = 0.00001$ and $\lambda = 0.25$. Participants were divided into quartiles according to their urinary TPE (not normalized).

Pearson correlation coefficients were used to examine the unadjusted correlation between the urinary TPE and TP intake according to FFQ, as well as in the different food groups using units of 100 g/day. Multivariate linear regression models were used to assess the relationship between the TPE normalized in urine as the dependent variable, and TP intake (100 mg), total F&V (100 g), wine (100 mL) and coffee (100 mL), as the exposure variable with adjustment for potential confounders (see below). The relationship between the urinary TPE quartile and systolic or diastolic BP was assessed by linear regression models adjusted for potential confounders. Four models were fitted with each independent variable. Logistic regression models were fitted to assess the relationship between urinary TPE (categorized in quartiles) and hypertension status. Odds ratios (ORs) and 95% confidence intervals (CI) were calculated using the lowest quartile as the reference category. P -values < 0.05 (two-tailed) were considered to be significant. Diagnostics for the detection of outliers, multicollinearity, homoscedasticity, and the normality and independence of errors were assessed when appropriate.

Results

Participant characteristics

Twenty-three out of 612 presumably eligible participants were excluded from entry in the study; 11 declined to participate and 12 did not meet inclusion criteria. Thus, 589

participants entered the study, 263 men[mean age 66.3 (6.1) y] and 326 women[mean age 69.2 (5.6) y]. By study design, participants were older than 55 years, mostly overweight[mean body mass index 29.36 (3.37) kg/m²], and with a sizeable burden of cardiovascular risk factors (32.6% diabetics, 80.8% hypertensive, 62.6% with dyslipidemia, 16.1% active smokers and 34.8 % had a family history of early-onset CHD).

Food and nutrient intake and urinary total polyphenol excretion

Almost all of participants adhered to a Mediterranean-style diet at baseline with a relatively high consumption of vegetables, fruits, legumes, olive oil and nuts, and a moderate intake of fish and wine, and lower, but also a relatively high intake of meat, meat products, pastries and dairy products. However, when nutrient intake was estimated, large between-subject variability in daily TPs intake was observed (from 122.96 to 3298.17 mg/day). This variability was consistent with the variation observed when the TPE in urine was analyzed. In fact, a weak but significant Pearson correlation was obtained between dietary intake of TPs from FFQ and urinary TPE measured ($r = 0.179$; $P < 0.001$).

Table 1 shows the average food consumption of study participants divided according to quartiles of urinary TPE expressed as mg GAE/g creatinine. Significant increasing trends across quartiles of TPE were observed for the intake of fruits, vegetables, total F&V, dairy products, fish and TP intake; whereas decreasing trends across TPE quartiles were observed for total alcohol intake, cereals, olive oil, pastries, cakes or sweets, as well as total energy intake.

The linear regression analyses of TPE in spot urine samples and TP intake (100 mg), total F&V intake (100 g), wine intake (100 mL) and coffee intake (100 mL) are presented in Table 2 with various models. The remaining variables are not shown because they did not exhibit any significant association with urinary TPE adjusted for potential confounding factors. We observed a significant positive association between urine TPE and daily intake of F&V, as well as with daily intake of coffee in the unadjusted model ($\beta = 0.131$; $P < 0.001$ and $\beta = 0.261$; $P < 0.05$, respectively). After adjusting for potential confounding factors in models 2, 3 and 4, the association remained statistically significant in the multivariate regression analysis. TPE concentration in spot urine samples showed a significant inverse association with the intake of wine, $\beta = -0.090$, $P < 0.05$ in the unadjusted model; but after adjusting for potential confounding factors this association reversed and became positive and statistically significant ($\beta = 0.121$, $P = 0.019$).

The standardized coefficients (Beta) are the regression coefficients obtained with the regression model using the standardized values (measured in standard deviation units), and are therefore independent of measurement units. The standardized coefficients (Table 2) from this model showed that total phenol intake (Beta = 0.283), F&V intake (Beta = 0.150) contributed more to urinary TPE than coffee (Beta = 0.141), and both contributed to a greater extent than wine (Beta = 0.120).

Table 1 Daily intake of selected foods according to quartiles of excreted total urinary polyphenols, expressed as mg GAE / g creatinine.

	Urine mg GAE/ g creatinine concentration quartile				P for trend ^a
	Q1 (<89.0)	Q2 (89.1–119.5)	Q3 (119.6–160.2)	Q4 (>160.3)	
Urine total polyphenol (mg GAE/ g creatinine) ^b	72.8 (11.6)	103.1 (8.2)	138.2 (11.1)	226.1 (69.8)	<0.001
No. of subjects	147	148	147	147	0.742
Olive oil (g)	48.7 (14.3)	47.3 (13.7)	45.1 (14.6)	45.0 (15.7)	0.014
Total nuts (g)	8.6 (10.7)	9.2 (11.3)	9.6 (12.2)	9.1 (10.9)	0.680
Vegetables (g)	253.1 (80.2)	261.1 (81.1)	262.6 (84.0)	272.7 (92.1)	0.053
Legumes (g)	17.9 (7.0)	17.9 (7.5)	17.7 (7.0)	16.5 (7.6)	0.107
Fruits (g)	304.6 (143.6)	319.3 (141.3)	315.1 (132.9)	360.8 (151.2)	0.002
Total fruits and vegetables (g)	557.7 (176.8)	580.4 (173.3)	577.7 (162.1)	633.5 (190.1)	0.001
Fish or seafood (g)	86.5 (34.0)	82.2 (35.8)	88.7 (36.9)	94.1 (39.0)	0.030
Meat or meat products (g)	132.0 (49.6)	129.7 (43.7)	134.3 (48.5)	134.2 (47.5)	0.531
Pastries, cakes or sweets (g)	34.8 (31.6)	27.9 (24.8)	25.3 (25.9)	23.7 (28.7)	<0.001
Cereals (g)	243.7 (106.7)	235.8 (91.6)	228.7 (98.3)	211.3 (83.3)	0.003
Milk and dairy products (mL)	352.1 (214.8)	378.8 (216.5)	396.5 (222.1)	403.1 (215.1)	0.033
Wine (mL)	121.1 (147.0)	81.5 (137.9)	101.1 (161.5)	81.3 (149.3)	0.071
Coffee (mL)	63.3 (47.7)	69.6 (52.9)	67.5 (48.3)	74.5 (56.0)	0.097
Tea (mL)	3.92 (16.4)	3.25 (11.4)	4.16 (12.6)	5.47 (20.4)	0.332
Chocolate (g)	3.1 (6.2)	2.4 (5.2)	2.3 (5.5)	1.9 (4.8)	0.063
Natural orange juice (mL)	23.3 (54.3)	25.19 (57.8)	13.4 (41.2)	19.1 (50.8)	0.199
Total polyphenol intake (mg GAE)	1075.6 (354.9)	1057.5 (320.2)	1086.2 (322.3)	1222.5 (439.8)	0.001
Alcohol (g)	15.7 (17.9)	10.2 (16.0)	12.2 (18.5)	9.9 (17.8)	0.018
Fibre (g)	22.1 (6.2)	22.0 (5.5)	21.9 (5.2)	22.5 (6.2)	0.606
Cholesterol (g)	353.6 (119.7)	328.6 (93.7)	340.5 (113.4)	342.1 (89.4)	0.561
Sodium (mg/d)	3347.7 (959.2)	3088.0 (905.4)	3123.2 (1006.5)	3145.4 (877.2)	0.100
Potassium (mg/d)	3926.7 (722.3)	3929.4 (700.9)	3994.8 (805.5)	4029.7 (659.7)	0.161
Total energy, Kcal/d	2380.1 (586.8)	2238.0 (472.0)	2205.1 (547.4)	2138.5 (476.8)	<0.001

^a One-factor ANOVA was used for continuous variables and χ^2 -test for categorical variables.^b Mean (standard deviation). GAE: gallic acid equivalent.

Urinary polyphenol analysis and cardiovascular risk factors

Table 3 shows the profile of cardiovascular risk factors of participants according to the quartile of urinary TPE. A higher polyphenol excretion in urine was associated with older age (P for trend < 0.001), lower systolic (P for trend = 0.015) and diastolic BP (P for trend < 0.001), and a lower, but not significant, use of anti-hypertensive medication. However, participants in the highest quartile of TPE reported a lower educational level than those in the lower quartile categories, had a higher prevalence of diabetes, but were less likely to be smokers, and to have a family history of CHD.

On multivariate linear regression analyses, systolic and diastolic BP exhibited a monotonic inverse association with TPE in spot urine samples (quartiles) after adjustment for potential confounders (Table 4). The non-standardized coefficients, $\beta = -1.731$ ($P = 0.024$) and $\beta = -1.264$ ($P = 0.003$) represent the expected change of systolic and diastolic BP,

respectively, corresponding to an increase from a TPE to the upper quartile.

Logistic regression analysis showed an inverse association between urinary TPE (by quartile) and the prevalence of hypertension (Table 5). Compared to the participants in the lowest quartile of TPE (<88.99 mg GAE/g creatinine), participants in the highest quartile (>160.23 mg GAE/g creatinine) had a significantly reduced prevalence of hypertension (OR = 0.71, CI 0.53 to 0.95; $P = 0.022$) in the unadjusted model. In all three models, a significant difference was observed between the top and the bottom quartiles for the prevalence of hypertension, after adjustment for potential confounders. When the analysis was adjusted for all possible confounding factors (model 4), participants in the highest quartile had a 36% reduced odds of hypertension (OR = 0.64, CI 0.45 to 0.92; $P = 0.015$), compared to those in the lowest quartile. Finally, BP correlated better with urinary TPE than with TP intake assessed by FFQ. In fact, a highly significant association between polyphenol intake assessed via TPE in urine and

Please cite this article in press as: Medina-Remón A, et al., Total polyphenol excretion and blood pressure in subjects at high cardiovascular risk, *Nutr Metab Cardiovasc Dis* (2009), doi:10.1016/j.numecd.2009.10.019

Table 2 Multivariate linear regression analysis with total polyphenol excreted normalized, in spot urine samples (mg GAE/g creatinine) as the dependent variable, and total polyphenol (100 mg/d), total fruit and vegetable (100 g/d), wine (100 mL/d) and coffee (100 mL/d) intakes, as exposure variable, adjusted for potential confounders.

	Model	β	SE	Beta	P	95 % CI
Total polyphenol intake (100 mg/d)	Model 1	0.073	0.017	0.179	<0.001	0.041 to 0.106
	Model 2	0.110	0.016	0.268	<0.001	0.078 to 0.141
	Model 3	0.117	0.017	0.286	<0.001	0.085 to 0.150
	Model 4	0.116	0.020	0.283	<0.001	0.077 to 0.154
Fruit and Vegetables (100 g)	Model 1	0.131	0.035	0.155	<0.001	0.064 to 0.199
	Model 2	0.112	0.033	0.132	0.001	0.047 to 0.177
	Model 3	0.107	0.034	0.126	0.002	0.040 to 0.174
	Model 4	0.127	0.036	0.150	<0.001	0.056 to 0.198
Wine (100 mL)	Model 1	-0.090	0.041	-0.090	0.029	-0.171 to -0.009
	Model 2	0.063	0.045	0.063	0.164	-0.026 to 0.152
	Model 3	0.075	0.047	0.075	0.107	-0.016 to 0.166
	Model 4	0.121	0.051	0.120	0.019	0.020 to 0.222
Coffee (100 mL)	Model 1	0.261	0.120	0.089	0.030	0.025 to 0.497
	Model 2	0.321	0.115	0.110	0.006	0.095 to 0.548
	Model 3	0.373	0.116	0.128	0.001	0.144 to 0.602
	Model 4	0.414	0.119	0.141	0.001	0.181 to 0.647

β : Non-standardized coefficient (regression line coefficient); SE: Standard error; Beta: Standardized coefficient; CI: Confidence interval; P: two-sided test of significance; Model 1: unadjusted; Model 2 was adjusted by sex, age and weight; Model 3 as in Model 2 plus smoking status, physical activity, educational level and energy expenditure in physical activity; Model 4 was adjusted as in Model 3 plus total fish/seafood, olive oil, beer, chocolate, natural orange juice, tea, cereals, milk and dairy products, meat or meat products, nuts, pastries/cakes/sweets, fruits and vegetables, wine and coffee intake; GAE: Gallic acid equivalent.

systolic (P for trend = 0.024) and diastolic BP (P for trend = 0.003) was observed (Table 4), whereas polyphenol intake assessed via FFQ tended to be associated with BP values, but here the association did not reach the statistical significance.

Discussion

In the current study, we observed an inverse association between urinary TPE, an objective measurement of polyphenol intake, and the risk of hypertension in a large Spanish cohort of elderly participants at high cardiovascular risk. In addition, systolic and diastolic BP measurements correlated negatively with urinary TPE after adjustment for potential confounders. The results from the present study also provide evidence that total phenol, F&V, coffee and wine intake in the Mediterranean diet are positively correlated with the excretion of TPs in spot urine samples. The intake of F&V contributed more to urinary TPE than coffee, and wine consumption. In addition, the absolute daily consumption of F&V in grams was 781% and 852% higher than the daily intake of coffee and wine, respectively.

F&V, and beverages such as coffee, tea and red wine, constitute the main sources of polyphenols [25]. Polyphenols represent a wide variety of structures from different subclasses, that explain the difficulties to estimate the TP content in these foods, mainly F&V [23]. Polyphenols are quantitatively the main dietary antioxidant source; however, the biological effects of these compounds depends on their bioavailability, their kinetics and exposure time [26]. The most common polyphenols in the human diet are not necessarily the most active *in vivo*, either because

they have a lower intrinsic activity than others or because they are poorly absorbed from the intestine, highly metabolized, or rapidly eliminated [25].

Most dietary polyphenols (75–99%) are not found in urine, and the quantities detected intact vary from one phenolic compound to another [11]. This fact may be due to their reduced absorption through the gut barrier, their excretion to the bile or their metabolism by the colonic microflora or our own tissues. To acquire high plasma concentrations requires repeated ingestion of polyphenol-rich foods [27].

The BP lowering effects of fruit- and vegetable-rich diets in hypertensive patients have mainly been attributed to the presence of multiple antioxidants. Two different double-blind cross-over trials [10,28] with polyphenol rich food (dark chocolate versus white and a rich versus low flavonoid sweet juice) showed that the polyphenols rich food reduced BP.

The clinical relevance of the endothelium-dependent effects of plant polyphenols is dependent upon their systemic availability. For this reason, intestinal absorption and metabolism of the plant polyphenols are a rate-limiting step for the protective effects of this class of compounds. In healthy volunteers, the coronary flow-velocity reserve increased 30 min after drinking red wine (1 g/kg ethanol), a polyphenol-rich beverage, but not after drinking the same quantity of alcohol as vodka or white wine, a polyphenol-free beverage or an alcoholic beverage with medium polyphenolic content, respectively [29]. Endothelium-dependent vasodilatation also improved in men, after the acute intake of 500 mL of red wine and de-alcoholized red wine [30]. In patients with mild isolated systolic

Please cite this article in press as: Medina-Remón A, et al., Total polyphenol excretion and blood pressure in subjects at high cardiovascular risk, *Nutr Metab Cardiovasc Dis* (2009), doi:10.1016/j.numecd.2009.10.019

Table 3 Baseline characteristics of the study participants by quartile of total polyphenol excretion expressed as mg GAE/g creatinine.

	Urine mg GAE/ g creatinine concentration quartile				P for trend ^a
	Q1 (<88.99)	Q2 (89–119.46)	Q3 (119.47–160.22)	Q4 (>160.23)	
No. of subjects	147	148	147	147	
Age, (y) mean (SD)	66.0 (5.7)	68.3 (6.1)	68.0 (5.8)	69.3 (6.0)	< 0.001
Women, n (%)	51 (34.7)	83 (56.1)	85 (57.8)	107 (72.8)	< 0.001
Weight (kg), mean (SD)	80.0 (11.1)	75.3 (11.2)	74.1 (10.3)	71.7 (11.2)	< 0.001
BMI, (kg/m ²), mean (SD)	29.6 (2.9)	29.6 (3.7)	29.2 (3.7)	29.1 (3.4)	0.171
Overweight or obese (BMI ≥25 Kg/m ²), n (%)	138 (93.9)	137 (92.6)	128 (87.1)	135 (91.8)	0.263
Systolic BP (mm Hg), mean (SD)	156.0 (15.6)	152.2 (18.3)	155.0 (21.6)	149.1 (16.0)	0.015
Diastolic BP (mmHg), mean (SD)	88.9 (10.2)	86.2 (9.8)	85.5 (11.8)	83.4 (8.5)	< 0.001
Hypertension, n (%)	123 (83.7)	126 (85.1)	113 (76.9)	114 (77.6)	0.067
Diabetes, n (%)	31 (21.1)	47 (31.8)	52 (35.4)	62 (42.2)	< 0.001
Dyslipidemia, n (%)	78 (53.1)	103 (69.6)	93 (63.3)	96 (65.3)	0.097
Current smoker n (%)	39 (26.5)	20 (13.5)	22 (15.0)	14 (9.5)	< 0.001
Family history of CHD, n (%)	45 (30.6)	46 (31.1)	47 (32.0)	67 (45.6)	0.008
Medication, n (%)					
ACE inhibitors	119 (81.0)	114 (77.0)	106 (72.1)	107 (72.8)	0.063
Diuretics	11 (7.5)	7 (4.7)	7 (4.8)	9 (5.8)	0.639
Statins or other hypolipidemic drugs	42 (28.6)	70 (47.3)	69 (46.9)	65 (44.2)	0.01
Insulin	3 (2.0)	4 (2.7)	12 (8.2)	9 (6.1)	0.024
Oral hypoglycemic drugs	17 (11.6)	26 (17.6)	26 (17.6)	36 (24.5)	0.006
Aspirin or other antiplatelet drugs	29 (19.7)	28 (18.9)	37 (25.2)	30 (20.4)	0.582
Educational level, n (%)					
Primary school	104 (70.7)	112 (75.7)	108 (73.5)	121 (82.3)	0.041
High school	24 (16.3)	18 (12.2)	28 (19.0)	18 (12.2)	0.685
University	19 (12.9)	17 (11.5)	10 (6.8)	7 (4.8)	0.006
Energy expenditure in physical activity (kcal/d), mean (SD)	256.0 (215.5)	245.9 (220.7)	293.1 (231.0)	255.3 (205.3)	0.576
Glomerular filtration rate, mL/min	77.2 (16.4)	78.1 (14.6)	78.1 (18.1)	79.5 (18.9)	0.264

^a ANOVA-one factor was used for continuous variables and χ^2 -test for categorical variables. BMI: body mass index (calculated as weight in kilograms divided by height in square meters); BP: blood pressure; ACE: angiotensin-converting enzyme; CHD: coronary heart disease.

hypertension, intake of consecutive daily doses of 100 g of polyphenol-rich dark chocolate (500 mg of polyphenols), but not polyphenol-free chocolate, decreased both systolic and diastolic BPs within 10 days [31]. Dietary interventions

with extra-virgin olive oil and a reduction in total and saturated fatty acid intake favorably affect BP control in pharmacologically treated hypertensive patients [32]. All these studies support the view that polyphenol-rich diets

Table 4 Multivariate linear regression analyses with systolic blood pressure and diastolic blood pressure as the dependent variables, and quartile of total polyphenol excreted in spot urine samples (mg GAE/g creatinine) as exposure variable, adjusted for potential confounders.

	Model	β	SE	Beta	P	95 % CI
Systolic blood pressure	Model 1	-1.743	0.712	-0.104	0.015	-3.141 to -0.345
	Model 2	-1.895	0.741	-0.113	0.011	-3.350 to -0.440
	Model 3	-1.895	0.743	-0.113	0.011	-3.354 to -0.436
	Model 4	-1.731	0.765	-0.103	0.024	-3.233 to -0.228
Diastolic blood pressure	Model 1	-1.705	0.397	-0.180	<0.001	-2.485 to -0.925
	Model 2	-1.438	0.408	-0.152	<0.001	-2.238 to -0.637
	Model 3	-1.405	0.409	-0.148	0.001	-2.208 to -0.602
	Model 4	-1.264	0.422	-0.133	0.003	-2.092 to -0.435

β : Non-standardized coefficient (regression line coefficient); SE: Standard error; Beta: Standardized coefficient; CI: Confidence interval; P: two-sided test of significance; Model 1: unadjusted; Model 2: adjusted by sex, age and weight; Model 3 adjusted as in Model 2 plus smoking status, physical activity, and educational level; Model 4 adjusted as in Model 3 plus additionally adjusted for medication use (angiotensin-converting enzyme inhibitors, diuretics, statins or other hypolipidemic drugs, insulin, oral hypoglycemic drugs and aspirin or other antiplatelet drugs) consumed in the last month, sodium and potassium intake and glomerular filtration rate (GFR); GAE: Gallic acid equivalent.

Please cite this article in press as: Medina-Remón A, et al., Total polyphenol excretion and blood pressure in subjects at high cardiovascular risk, *Nutr Metab Cardiovasc Dis* (2009), doi:10.1016/j.numecd.2009.10.019

Table 5 Multivariate adjusted odds ratios (95% confidence intervals) for cardiovascular risk factors (hypertension) according to quartiles of total polyphenol excretion expressed as mg GAE/g creatinine using the lowest quartile group as reference category.

	Urine mg GAE/ g creatinine concentration quartile				P for trend
	Q1 (<88.99)	Q2 (89–119.46)	Q3 (119.47–160.22)	Q4 (>160.23)	
Hypertension, n (%)	123 (83.7)	126 (85.1)	113 (76.9)	114 (77.6)	0.067
Model 1	1.00	1.82 (0.33–10.13)	0.67 (0.29–1.55)	0.71 (0.53–0.95)	0.021
Model 2	1.00	1.40 (0.21–9.24)	0.61 (0.25–1.47)	0.64 (0.46–0.89)	0.006
Model 3	1.00	1.29 (0.19–8.49)	0.55 (0.22–1.37)	0.65 (0.47–0.91)	0.007
Model 4	1.00	1.39 (0.19–10.34)	0.55 (0.20–1.48)	0.64 (0.45–0.92)	0.047

Model 1, unadjusted; Model 2 was adjusted for sex, age and weight; Model 3 adjusted as in Model 2 plus smoking status, physical activity, educational level and energy expenditure in physical activity; Model 4 was adjusted as in Model 3 plus medication intake: ACE inhibitor, diuretics, statins (hypolipidemic drugs), insulin, oral hypoglycemic drugs, aspirin or other antiplatelet drug supplements taken in the last month, sodium and potassium intake and glomerular filtration rate (GFR); GAE: Gallic acid equivalent.

may improve endothelium function and reduce BP in hypertensive subjects.

F&V seem to be essential items in the prevention of dietary diseases. Some epidemiological evidence supports that a diet rich in F&V may help prevent BP from increasing and help decrease elevated BP levels for those with high normal BP or hypertension [33]. In the SUN study [4], the prevalence of non-previously diagnosed hypertension was inversely associated with F&V consumption in a Mediterranean population with a very high intake of both fat- and plant-derived foods. In the Nurses' Health Study, intake of F&V was also inversely associated with systolic and diastolic BP, whereas the intake of cereals and meat was directly associated with systolic BP [34]. After the 8-year follow-up in 1714 employed middle-aged men in the Chicago Western Electric Study, vegetable protein, beta-carotene, and an antioxidant vitamin score based on vitamin C and beta-carotene were inversely and significantly related to an average annual change in BP [35]. Thus, in all mentioned studies a high consumption of F&V was associated with a decrease in BP levels. In the current study performed in a free-living Spanish high-risk population, we observed a similar effect when consumption of F&V was high. However, until now, no study has tried to correlate the biomarker of TP intake, determined in spot urine samples, with BP measurements or with the prevalence of hypertension. Taking into account that greater excretion of polyphenols in urine is determined by high TP consumption, we suggest that the observed independent inverse association of objectively measured TPE in urine samples with BP is very probably related to a favourable effect of TP intake in preventing raised BP levels.

Recently, in the SUN study [36] adherence to the Mediterranean diet was not associated with hypertension. However, it was associated with reduced changes in mean levels of systolic and diastolic BP after a 6-year follow-up, suggesting that adherence to a Mediterranean-type diet could contribute to the prevention of age-related changes in BP. Estruch et al. [3] compared the short-term effects of 2 Mediterranean diets versus those of a low-fat diet on intermediate markers of cardiovascular risk in an intervention feeding trial, participants included in the Mediterranean diet groups supplemented with either olive oil or nuts showed a significant decrease in systolic and diastolic

BP measurements after 3-months of intervention compared to the low-fat diet group. Our results also add to the increasing evidence that polyphenol-rich diets decrease BP.

Our study has some limitations. First, the participants were elderly subjects at high risk for cardiovascular disease, thus the results can not be extrapolated to the general population. However, since most were hypertensive subjects, our results confirm the usefulness of polyphenol-rich diets in the management of hypertension. A second limitation of our study is its cross-sectional nature which does not allow definitive establishment of causal relationships. However, the current study has the strength of the objective measurement of polyphenol intake by determination of urinary TPE which allows a more precise correlation to be obtained with biological variables than data obtained by FFQ [37,38]. Another limitation is that the colonic microflora catalyze the breakdown of the polyphenol itself to more simple compounds, such as valerolactones or other chemical structures that are not able to further reduce the chemicals present in the F–C reagent and the behavior of these compounds in the SPE has not been studied.

In summary, we report that a high TPE in spot urine is negatively associated with BP levels in an elderly Mediterranean population. Thus, a high intake of polyphenol-rich diet may help to decrease BP in elderly hypertensive populations in order to lower their cardiovascular risk. Compared with the lowest quartile of TPE, in the participants in the highest quartile, the odds of hypertension were reduced by 36% after adjustment for potential confounding factors. However, the observation that systolic and diastolic BP decrease when TPE increases needs to be confirmed in further intervention studies. A longer follow-up of the whole PREDIMED trial will eventually provide the evidence to confirm our results.

Acknowledgements

We would like to thank all of the volunteers involved in the PREDIMED study. This work has received financial support from AGL2005-05597; AGL2006-14228-C03-02, AGL2007-66638-C02-01, RETICS RD06/0045/0003, and in part by the CONSOLIDER INGENIO 2010 Programme, FUN-C-FOOD

Please cite this article in press as: Medina-Remón A, et al., Total polyphenol excretion and blood pressure in subjects at high cardiovascular risk, *Nutr Metab Cardiovasc Dis* (2009), doi:10.1016/j.numecd.2009.10.019

CSD2007-063 all from the Spanish Ministry of Science and Innovation (MICINN) and grant PI070240 from Instituto de Salud Carlos III, Spain. The CIBERobn CB06/03 is an initiative from the Instituto de Salud Carlos III, Spain. None of the funding sources played a role in the design, collection, analysis or interpretation of data, in the writing of the report or in the decision to submit the paper for publication. None of the authors have any conflict of interest. A. M-R received support from the Catalonia Generalitat for training of researchers.

Appendix

Other PREDIMED Investigators

Nutrition and Food Science Department, XaRTA, INSA. Pharmacy School, University of Barcelona, Barcelona, Spain: Mireia Urpi-Sarda, PhD; Rafael Llorach, PhD; Carmen López-Sabater, PhD.

Hospital Clinic, Institut d'Investigacions Biomèdiques August Pi Sunyer, Barcelona, Spain: Mercè Serra, RD; Ana Pérez-Heras, RD; Concha Viñas, RD; Emilio Sacanella, MD, PhD; Saul Romero, MD; Rosa Casas, BS.

University of Navarra, Primary Care Division, Pamplona, Spain: Estefania Toledo, MD, PhD; Pilar Buil-Cosiales, MD, and Javier Díez-Espino, MD.

University of Valencia, Valencia, Spain: Olga Portolés, PhD; José Vicente Sorlí, MD.

University Rovira i Virgili, Reus (Tarragona), Spain: Mónica Bulló, PhD; Josep Basora, MD; Nancy Babio, PhD.

Instituto de la Grasa, Consejo Superior de Investigaciones Científicas, Sevilla, Spain: Javier S. Perona, PhD.

Municipal Institute for Medical Research, Barcelona, Spain: Montserrat Fitó, PhD; Jaime Marrugat, MD, PhD; Roberto Elosúa, MD, PhD.

University of Málaga, Málaga, Spain: Juan José Sánchez-Luque, MD, PhD; Virginia Velasco-García, MD.

University Institute for Health Sciences Investigation, Palma de Mallorca, Spain: Fernando Rigo, MD; Guillem Frontera, MD.

Primary Care Division, Catalan Institute of Health, Barcelona, Spain: Carmen Cabezas, MD.

Hospital Txagorritxu, Vitoria, Spain: E. Sanz MD.

Hospital Universitario Virgen del Rocío, Sevilla, Spain: Adoración Nieto, MD, PhD.

Department of Family Medicine, Primary Care Division of Sevilla, San Pablo Health Center, Sevilla: José Lapetra, MD, PhD; José Manuel Santos, MD, PhD; Pablo Iglesias, MD, PhD; Manuel Ortega, MD, PhD.

References

- [1] Lawes CM, Vander HS, Rodgers A. Global burden of blood-pressure-related disease, 2001. *Lancet* 2008 May 3;371(9623):1513–8.
- [2] Mancia G, De BG, Dominiczak A, Cifkova R, Fagard R, Germano G, et al. 2007 Guidelines for the Management of Arterial Hypertension: The Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). *J Hypertens* 2007 Jun;25(6):1105–87.
- [3] Estruch R, Martínez-González MA, Corella D, Salas-Salvado J, Ruiz-Gutiérrez V, Covas MI, et al. Effects of a Mediterranean-style diet on cardiovascular risk factors: a randomized trial. *Ann Intern Med* 2006 Jul 4;145(1):1–11.
- [4] Alonso A, de la FC, Martín-Annau AM, de IJ, Martínez JA, Martínez-González MA. Fruit and vegetable consumption is inversely associated with blood pressure in a Mediterranean population with a high vegetable-fat intake: the Seguimiento Universidad de Navarra (SUN) Study. *Br J Nutr* 2004 Aug;92(2):311–9.
- [5] Appel LJ, Moore TJ, Obarzanek E, Vollmer WM, Svetkey LP, Sacks FM, et al. A Clinical trial of the effects of dietary patterns on blood pressure. *N Engl J Med* 1997 Apr 17;336(16):1117–24.
- [6] Perona JS, Canizares J, Montero E, Sánchez-Domínguez JM, Catalá A, Ruiz-Gutiérrez V. Virgin olive oil reduces blood pressure in hypertensive elderly subjects. *Clin Nutr* 2004 Oct;23(5):1113–21.
- [7] Alonso A, Ruiz-Gutiérrez V, Martínez-González MA. Mono-unsaturated fatty acids, olive oil and blood pressure: epidemiological, clinical and experimental evidence. *Pub Health Nutr* 2006 Apr;9(2):251–7.
- [8] Rodrigo R, Prat H, Passalacqua W, Araya J, Bachler JP. Decrease in oxidative stress through supplementation of vitamins C and E is associated with a reduction in blood pressure in patients with essential hypertension. *Clin Sci (Lond)* 2008 May;114(10):625–34.
- [9] Guxens M, Fito M, Martínez-González MA, Salas-Salvado J, Estruch R, Vinyoles E, et al. Hypertensive status and lipoprotein oxidation in an elderly population at high cardiovascular risk. *Am J Hypertens* 2009 Jan;22(1):68–73.
- [10] Grassi D, Necozione S, Lippi C, Croce G, Valeri L, Pasqualetti P, et al. Cocoa reduces blood pressure and insulin resistance and improves endothelium-dependent vasodilation in hypertensives. *Hypertension* 2005 Aug;46(2):398–405.
- [11] Scalbert A, Williamson G. Dietary intake and bioavailability of polyphenols. *J Nutr* 2000 Aug;130(8S Suppl):2073S–85S.
- [12] Covas MI, Fito M, Marrugat J, Miro E, Farre M, de la TR, et al. Coronary disease protective factors: antioxidant effect of olive oil. *Therapie* 2001 Sep;56(5):607–11.
- [13] Covas MI, Nyyssonen K, Poulsen HE, Kaikkonen J, Zunft HJ, Kiesewetter H, et al. The effect of polyphenols in olive oil on heart disease risk factors: a randomized trial. *Ann Intern Med* 2006 Sep 5;145(5):333–41.
- [14] Manach C, Mazur A, Scalbert A. Polyphenols and prevention of cardiovascular diseases. *Curr Opin Lipidol* 2005 Feb;16(1):77–84.
- [15] Zazpe I, Sánchez-Tainta A, Estruch R, Lamuela-Raventós RM, Schröder H, Salas-Salvado J, et al. A large randomized individual and group intervention conducted by registered dietitians increased adherence to Mediterranean-type diets: the PREDIMED study. *J Am Diet Assoc* 2008 Jul;108(7):1134–44.
- [16] Nigg CR, Burbank PM, Padula C, Dufresne R, Rossi JS, Velicer WF, et al. Stages of change across ten health risk behaviors for older adults. *Gerontologist* 1999 Aug;39(4):473–82.
- [17] Martín-Moreno JM, Boyle P, Gorgojo L, Maisonneuve P, Fernandez-Rodríguez JC, Salvini S, et al. Development and validation of a food frequency questionnaire in Spain. *Int J Epidemiol* 1993 Jun;22(3):512–9.
- [18] Elosua R, Marrugat J, Molina L, Pons S, Pujol E. Validation of the Minnesota Leisure Time Physical Activity Questionnaire in Spanish men. The MARATHON Investigators. *Am J Epidemiol* 1994 Jun 15;139(12):1197–209.
- [19] Iglesias-Bonilla P, Mayoral-Sánchez E, Lapetra-Peralta J, Iborra-Oquendo M, Villalba-Alcalá F, Cayuela-Domínguez A. Validación de dos sistemas de automedida de presión arterial,

Please cite this article in press as: Medina-Remón A, et al., Total polyphenol excretion and blood pressure in subjects at high cardiovascular risk, *Nutr Metab Cardiovasc Dis* (2009), doi:10.1016/j.numecd.2009.10.019

- modelos OMRON HEM 705 CP y OMRON MI (HEM 422C2-E). Atención Primaria 2002;30(1):22–8.
- [20] Levey AS, Coresh J, Greene T, Stevens LA, Zhang YL, Hendriksen S, et al. Using standardized serum creatinine values in the modification of diet in renal disease study equation for estimating glomerular filtration rate. *Ann Intern Med* 2006 Aug 15;145(4):247–54.
- [21] Mataix J. Tabla de composición de alimentos (Food composition tables). Granada, Spain: Universidad de Granada; 2003 [Ref Type: Generic].
- [22] Saura-Calixto F, Goni I. Antioxidant capacity of the Spanish Mediterranean diet. *Food Chem* 2006 Feb;94(3):442–7.
- [23] Brat P, George S, Bellamy A, Du CL, Scalbert A, Mennen L, et al. Daily polyphenol intake in France from fruit and vegetables. *J Nutr* 2006 Sep;136(9):2368–73.
- [24] Medina-Remón A, Barrionuevo-González A, Zamora-Ros R, Andres-Lacueva C, Estruch R, Martínez-González MA, et al. Rapid Folín–Ciocalteu method using microtiter 96-well plate cartridges for solid phase extraction to assess urinary total phenolic compounds, as a biomarker of total polyphenols intake. *Anal Chim Acta* 2009 Feb 16;634(1):54–60.
- [25] Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Polyphenols: food sources and bioavailability. *Am J Clin Nutr* 2004 May;79(5):727–47.
- [26] Roura E, Almajano MP, Bilbao ML, Andres-Lacueva C, Estruch R, Lamuela-Raventós RM. Human urine: epicatechin metabolites and antioxidant activity after cocoa beverage intake. *Free Radic Res* 2007 Aug;41(8):943–9.
- [27] van het Hof KH, Wiseman SA, Yang CS, Tijburg LB. Plasma and lipoprotein levels of tea catechins following repeated tea consumption. *Proc Soc Exp Biol Med* 1999 Apr;220(4):203–9.
- [28] Reshef N, Hayari Y, Goren C, Boaz M, Madar Z, Knobler H. Antihypertensive effect of sweetie fruit in patients with stage I hypertension. *Am J Hypertens* 2005 Oct;18(10):1360–3.
- [29] Shimada K, Watanabe H, Hosoda K, Takeuchi K, Yoshikawa J. Effect of red wine on coronary flow-velocity reserve. *Lancet* 1999 Sep 18;354(9183):1002.
- [30] Hashimoto M, Kim S, Eto M, Iijima K, Ako J, Yoshizumi M, et al. Effect of acute intake of red wine on flow-mediated vasodilatation of the brachial artery. *Am J Cardiol* 2001 Dec 15;88(12):1457–60. A9.
- [31] Taubert D, Berkels R, Roesen R, Klaus W. Chocolate and blood pressure in elderly individuals with isolated systolic hypertension. *JAMA* 2003 Aug 27;290(8):1029–30.
- [32] Ferrara LA, Raimondi AS, d'Episcopo L, Guida L, lo Russo A, Marotta T. Olive oil and reduced need for antihypertensive medications. *Arch Intern Med* 2000 Mar 27;160(6):837–42.
- [33] Whelton PK, He J, Appel LJ, Cutler JA, Havas S, Kotchen TA, et al. Primary prevention of hypertension: clinical and public health advisory from The National High Blood Pressure Education Program. *JAMA* 2002 Oct 16;288(15):1882–8.
- [34] Ascherio A, Hennekens C, Willett WC, Sacks F, Rosner B, Manson J, et al. Prospective study of nutritional factors, blood pressure, and hypertension among US women. *Hypertension* 1996 May;27(5):1065–72.
- [35] Stamler J, Liu K, Ruth KJ, Pryer J, Greenland P. Eight-year blood pressure change in middle-aged men: relationship to multiple nutrients. *Hypertension* 2002 May;39(5):1000–6.
- [36] Nunez-Cordoba JM, Valencia-Serrano F, Toledo E, Alonso A, Martinez-Gonzalez MA. The Mediterranean diet and incidence of hypertension: The Seguimiento Universidad de Navarra (SUN) Study. *Am J Epidemiol* 2009 Feb 1;169(3):339–46.
- [37] Spencer JP, bd El Mohsen MM, Minihane AM, Mathers JC. Biomarkers of the intake of dietary polyphenols: strengths, limitations and application in nutrition research. *Br J Nutr* 2008 Jan;99(1):12–22.
- [38] Marshall JR. Methodologic and statistical considerations regarding use of biomarkers of nutritional exposure in epidemiology. *J Nutr* 2003 Mar;133(Suppl. 3):881S–7S.

4.4 Publicació 4: Consum de polifenols i pressió arterial.

Alexander Medina-Remón, Ramón Estruch, Cristina Andres-Lacueva, and Rosa-Maria Lamuela-Raventos.

Archives of Biochemistry and Biophysics (En procés de revisió)

Resum:

La hipertensió és un dels principals factors de risc cardiovascular, que es podria modificar mitjançant la dieta. D'altra banda, en diversos estudis epidemiològics s'observa una associació inversa entre el risc de malalties cardiovasculars i el consum d'aliments rics en polifenols. Degut a les dificultats en mesurar el consum de polifenols totals (TP) mitjançant els qüestionaris de freqüència de consum (FFQ), un nou mètode per a la determinació dels polifenols totals excretats (TPE) en orina, ha sigut descrit i provat, com a biomarcador del consum total de polifenols.

En aquest treball han sigut revisats els principals estudis epidemiològics que relacionen el consum de TP i la pressió arterial (PA). A més, es va descriure un mètode ràpid per a la determinació dels TP en mostres d'orina utilitzant el mètode de Folin-Ciocalteu, posterior a una extracció en fase sòlida amb plaques de 96 cartutxos Oasis® MAX. Aquesta determinació es va correlacionar amb la pressió arterial sistòlica i diastòlica. Aquest mètode és ràpid, senzill i permet l'anàlisi simultània d'un gran nombre de mostres.

En un sub-estudi transversal amb dades inicials de l'estudi PREDIMED es va observar com els TPE en mostres d'orina puntuals del matí es correlacionen millor que els TP ingerits mesurats a través dels FFQ.

S'observa una associació inversa entre els TPE en l'orina i la prevalència d'hipertensió; els participants en el quartil més alt de TPE en orina, presentaven una menor prevalència d'hipertensió, comparat amb els del primer quartil de TPE (ORs=0.64; 95% CI 0.45 a 0.92; $P=0.016$). La PA sistòlica i diastòlica van

ser inversament associades amb els TPE ($P=0.025$ and $P=0.002$, respectivament), després d'ajustar per factors confusors potencials.

Es va concloure que la metodologia utilitzada per a la determinació de polifenols totals en mostres d'orina pot ser molt útil per a determinar els polifenols totals ingerits, tant en estudis epidemiològics com en estudis clínics. A més, els polifenols ingerits, mesurats via TPE en orina, mostren una millor correlació amb els nivells de pressió arterial i la prevalència d'hipertensió que els polifenols mesurats mitjançant les FFQ.

Polyphenol Consumption on Blood Pressure

Alexander Medina-Remón^{a,b}, Ramón Estruch^{b,c}, Cristina Andres-Lacueva^{a,d}, and Rosa-Maria Lamuela-Raventós^{a,b*}.

^aNutrition and Food Science Department, XaRTA, INSA. Pharmacy School, University of Barcelona, Barcelona, Spain; ^bCIBER CB06/03 Fisiopatología de la Obesidad y la Nutrición, (CIBEROBN) and RETICS RD06/0045/0003, Instituto de Salud Carlos III, Spain; ^cDepartment of Internal Medicine, Hospital Clinic, IDIBAPS, University of Barcelona, Spain. ^dIngenio-CONSOLIDER program, FUN-C-FOOD, Barcelona, Spain.

*Corresponding author: Nutrition and Food Science Department, XaRTA, INSA Pharmacy School, University of Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain. Telephone +34-934034843. Fax +34-934035931; e-mail lamuela@ub.edu

ABSTRACT

Hypertension is one of the main cardiovascular risk factors, modifiable by diet. On the other hand, an inverse association between risk for cardiovascular disease and consumption of polyphenol-rich foods has been found in several epidemiologic studies. Due to difficulties in achieving reliable assessment of total polyphenol (TP) intake using data obtained by food frequency questionnaires (FFQ), a new method for measurement of total polyphenols excreted (TPE) in urine has been described and tested as a marker of TP intake.

We review the epidemiological studies on the relationship between polyphenol intake and blood pressure (BP) and we describe a rapid Folin-Ciocalteu method to determine TP in urine samples using Oasis® MAX 96-well plate cartridges for solid phase extraction to correlate with BP. This method is fast, simple and allows simultaneous analysis of a large number of samples. We then performed a cross-sectional substudy with baseline data from the PREDIMED (Prevención con Dieta Mediterránea) study in order to evaluate whether TPE in spot urine correlated better with clinical data (BP and hypertension status) than TP intake assessed by FFQ. The participants studied included 263 men and 326 women, aged 55–80, at high risk for coronary heart disease. FFQ was recorded, anthropometric data and BP, measured and TPE in urine determined. The volunteers were divided into quartiles according to their TPE in urine. An inverse association between urinary TPE and the prevalence of hypertension was observed; participants in the highest quartile of urinary TPE had a reduced prevalence of hypertension compared to those in the lowest quartile (ORs=0.64; 95% CI 0.45 to 0.92; $P=0.016$). Systolic and diastolic BP were also inversely associated with urinary TPE after adjustment for potential confounders ($P=0.025$ and $P=0.002$, respectively).

We conclude that the methodology used to measure TP in urine samples may be very useful to assess TP intake in epidemiological and clinical studies. In addition, polyphenol intake, assessed via TPE in urine, shows good correlations

with BP levels and the prevalence of hypertension in an elderly Mediterranean population than polyphenol intake assessed by FFQ.

Keywords: Polyphenol intake; biomarkers; urinary polyphenol; hypertension; blood pressure.

Abbreviations: blood pressure (BP); coronary heart disease (CHD); Folin-Ciocalteu (F-C); fruit and vegetables (F&V); gallic acid equivalent (GAE); odds ratios (ORs); solid phase extraction (SPE); total polyphenols (TP) and total polyphenol excretion (TPE)

Introduction

Hypertension is a major global public health problem, affecting approximately 1 billion individuals, causing 7.6 million premature deaths and 6% of all cases of disability-adjusted life years worldwide [1]. This disease is also one of the main modifiable cardiovascular risk factors in the elderly and the first step in its management is to follow a healthy diet such as the Mediterranean [2] or the DASH (Dietary-Approaches-to-Stop-Hypertension) diets [3] and/or to improve lifestyle, for instance, by reducing body weight and increasing physical activity [4]. The Mediterranean and DASH diets are both rich in fruits and vegetables (F&V), which have been considered rich sources of phytochemicals, and are inversely associated with high blood pressure (BP) [5;6].

Numerous epidemiological studies have demonstrated an inverse association between polyphenol-rich foods such as cocoa, F&V, tea, olive oil and wine [6-11] and the risk of overall mortality or cardiovascular disease, whereas a high consumption of refined cereals or meat/meat products is associated with a higher cardiovascular risk [4;6;12]. Fish consumption, another food characteristic of the Mediterranean diet, and low-fat dairy products, may also reduce the risk of hypertension [4; 13-17]. In addition, some studies have analysed the effects of sodium and potassium dietary intake, vitamin C or other antioxidant compounds on BP [18;19], but only a previous study by our group has evaluated the effects of total dietary polyphenol intake on this issue [20].

Total dietary polyphenol levels are roughly 1 g/d [21], which is around 10 times higher than dietary vitamin C and 100 times higher than vitamin E and carotenoid intake. The health effect of polyphenols depends on their intake, synergism and bioavailability [22]. Biomarkers of the intake of some nutrients are more precise and provide better objective measures than data obtained from food frequency questionnaires (FFQ). The development of biomarkers, measured in blood and urine, is essential for making accurate estimates of polyphenol intake. However, the relationship between dietary intake and nutritional biomarkers is often highly complex [23].

The aim of this work was to review the epidemiological studies on the relationship between polyphenol intake and BP, a fast method [24] for evaluation total polyphenols excreted (TPE) in spot urine samples that could be used as a marker of intake, bioavailability and accumulation of total polyphenols (TPs) and a cross-sectional trial [20] performed within a larger clinical trial, the PREDIMED study [2; 25]. This latter study was conducted to evaluate whether TPs excreted in spot urine samples as a biomarker of TP intake was associated with BP and/or with the prevalence of hypertension in elderly individuals at high cardiovascular risk.

Epidemiological studies on polyphenol intake and blood pressure

Polyphenols are the main dietary antioxidant source and represent a wide variety of structures from different subclasses. The biological effects of these compounds depend on their bioavailability, their kinetics and exposure time [26]. The main sources of these compounds are fruit, vegetables and beverages such as wine, coffee and tea which contain complex mixtures of often poorly characterized antioxidants [27], possibly explaining the difficulties in determining total phenol content in food [28].

The presence of multiple antioxidants in fruit- and vegetable-rich diets may explain the lowering effect on BP observed in hypertensive patients. A protect effect of F&V against cardiovascular disease (CVD) has been observed in numerous epidemiological studies [6; 7]. Thus, high intake of F&V correlated with a reduced risk of CVD in a study on 2682 men in Finland [29]. Radhika G. *et al.* [30] also examined the relationship between F&V intake and CVD risk factors in urban south Indians. The study population was comprised of 983 individuals aged 20 years or more, selected from the Chennai Urban Rural Epidemiological Study (CURES). Linear regression analysis revealed that after adjusting for potential confounder factors, the highest quartile of F&V intake showed a significant inverse association with systolic BP ($\beta = -2.6$ mmHg; $P = 0.027$), when compared with the lowest quartile. A high intake of F&V explained 48 % of the protective effect against CVD risk factors.

Similarly, the prevalence of non-previously diagnosed hypertension in the SUN study [6] was inversely associated with F&V consumption in a Mediterranean population with a very high intake of both fat- and plant-derived foods. In the Nurses' Health Study, intake of F&V was also inversely associated with systolic and diastolic BP, whereas the intake of cereals and meat was directly associated with systolic BP [31]. In the Chicago Western Electric Study vegetable protein, beta-carotene, and an antioxidant vitamin score based on vitamin C and beta-carotene were inversely and significantly related to an average annual change in BP, after the 8-year follow-up in 1714 employed middle-aged men [32]. On the other hand, Hung HC *et al.* [33] evaluated the association of F&V consumption with peripheral arterial disease in a cohort of 44,059 men initially free of cardiovascular disease and diabetes, reporting no evidence that F&V consumption protects against peripheral arterial disease. In the age-adjusted model, men in the highest quintile of F&V had a relative risk of 0.55 (95% CI = 0.38-0.80) for peripheral arterial disease, compared with those in the lowest quintile. However, the associations were greatly weakened after adjustment for smoking and other traditional cardiovascular risk factors.

In relation to other types of foods, flavonoid-rich juice intake [34] and flavonoid-rich dark chocolate significantly reduced BP and improved flow-dilated endothelium-dependent vasodilatation in a well-designed double-blind cross-over trial [10]. Intestinal absorption and metabolism of the plant polyphenols are a rate-limiting step for the protective effects of this class of compounds because the clinical relevance of the endothelium-dependent effects of plant polyphenols is dependent upon their systemic availability. The intake of consecutive daily doses of 100 g of polyphenol-rich dark chocolate (500 mg of polyphenols) decreased both systolic and diastolic BP in patients with mild isolated systolic hypertension within 10 days [35]. The coronary flow-velocity reserve was increased in healthy volunteers after drinking a polyphenol-rich beverage (1g/kg ethanol as red wine) but not after drinking the same quantity of alcohol as vodka or white wine, a polyphenol-free beverage or an alcoholic beverage with medium polyphenol content, respectively [36]. After acute intake of 500 mL of red wine and de-alcoholized red wine [37] endothelium-dependent vasodilatation was also improved. A reduction in total and saturated fatty acid intake and interventions with extra-virgin olive oil favourably affected BP in hypertensive patients [38]. All these studies also support the view that

Resultats

polyphenol-rich diets may reduce BP and improve endothelium function in hypertensive subjects.

Finally, the relationship between overall food patterns rich in polyphenols, such as DASH and Mediterranean diets, and BP has been also examined. The DASH diet is widely promoted in the USA for the prevention and treatment of hypertension [3]. This dietary pattern has been assessed in a free-living UK population [5] and systolic and diastolic BP decreased significantly ($P < 0.05$) by 4.6 and 3.9 mmHg, respectively, in those who followed a DASH-style diet. After 3 weeks on the DASH diet, systolic and diastolic BP in obese hypertensive was lower than on the usual diet ($-7.6 \pm 1.4 / -5.3 \pm 1.4$ mmHg, $P < 0.001/0.02$) and the usual diet supplemented with potassium, magnesium and fibre ($-6.2 \pm 1.4 / -3.7 \pm 1.4$ mmHg, $P < 0.005/0.06$), whereas BP was not significantly different in the usual and supplemented diets [39]. BP values were not different among these three diets in lean normotensives. In another study [40] with 27 men and women who followed a DASH or a control diet, the DASH-diet group showed a significant reduction in systolic ($P < 0.001$) and diastolic ($P = 0.005$) BP.

Recently, in the SUN study [41] adherence to the Mediterranean diet was associated with reduced mean levels of systolic and diastolic BP after a 6-year follow-up, but it was not associated with hypertension. Estruch *et al.* [2] compared the short-term effects of 2 Mediterranean diets versus those of a low-fat diet on intermediate markers of cardiovascular risk in an intervention feeding trial (the PREDIMED study). In this last study, participants included in the Mediterranean diet groups supplemented with either olive oil or nuts showed a significant decrease in systolic and diastolic BP measurements after 3 months of intervention compared to the low-fat diet group.

All these epidemiological evidence support the contention that a diet rich in F&V may prevent BP from increasing and may help to decrease elevated BP levels. However, only one study [20] has attempted to correlate the biomarker of TP intake, determined in spot urine samples, with BP measurements or with the prevalence of hypertension. Taking into account that greater excretion of polyphenols in urine is determined by high TP consumption, we suggested that the inverse association observed between the objectively measured TPE in urine samples with BP may be related to a favourable effect of TP intake on preventing raised BP levels (see later).

Measurements of total polyphenol intake

Nutritional markers have several advantages for epidemiologic and clinical assays when compared to dietary data obtained by FFQ. Few studies have assessed whether TP compounds provide a valid biomarker of TP intake. Mennen *et al* [42] and Krogholm *et al* [43] studied the correlation between the consumption of polyphenol-rich foods and beverages and the concentration of polyphenols in urine samples determined by LC-MS/MS. Their results suggested that several polyphenols measured in urine samples can be used as biomarkers of polyphenol-rich food intake. Only few authors [42-44] have examined the use of urinary concentrations of polyphenols as non-specific biomarkers of F&V consumption. Since flavonoids are widely distributed in F&V, some investigators studied their value as biomarkers of F&V intake.

In a controlled dietary intervention study, urinary quercetin, flavanone, and total flavonoid from 24-h urine samples were measured by LC-MS after six weeks

on a diet either low or high in fruit, vegetables and berries. A significant positive correlation between changes in F&V intake and those in urinary flavonoid excretion was observed [44]. A positive correlation was found between TP metabolites in 24-h urine samples and intake of F&V following 1 d consumption of a basic diet supplemented with 300 or 600 g of F&V [43]. However, in epidemiological studies, only spot urine samples and, rarely, 24-h urine samples are collected to investigate the potential health effect of F&V. Furthermore, a recent study [42] has evaluated the correlation between the urinary excretion of several polyphenols from spot urine samples and the intake of polyphenol-rich food suggesting that several polyphenols measured by LC-MS in spot urine samples can be used as biomarkers of polyphenol-rich food intake.

Our group recently described a new method to determine TP in complex matrices such as urine samples, which have many interfering substances. We reported a rapid Folin-Ciocalteu (F-C) method to determine TP in urine using Oasis® MAX 96-well plate cartridges for solid phase extraction (SPE) to avoid any interference with F-C reagent in the urine samples [24]. Briefly, 1 mL of methanol 98% and then 1 mL of sodium acetate 50mM pH =7 were loaded to equilibrate the cartridges; 1.2 mL of the urine samples, previously thawed on an ice bed for 3 h, were centrifuged for 10 min at 4° C, and 1 mL of supernatants was diluted with 1 mL of Milli-Q water and acidified with 34 µL of hydrochloric acid at 35%; they were used to load the cartridges. These were then rinsed with 1 mL of sodium acetate 50mM pH=7 / 5% methanol. The polyphenols were eluted with 1.8 mL of 2% formic acid in methanol. 15 µL of eluted fractions was mixed with 170 µL of Milli-Q water in the thermo microtiter 96-well plate (Nunc™, Roskilde, Denmark), adding 12 µL of F-C reagent and 30 µL of sodium carbonate (200 g/L). The mixtures were incubated at room temperature in the dark during 1 h; 73 µL of Milli-Q water was added after the reaction period. The absorbance was measured at 765 nm in UV/VIS Thermo Multiskan Spectrum spectrophotometers (Vantaa, Finland).

For creatinine determination in the thermo microtiter 96-well plate, 60 µL of aqueous picric acid solution (1%) was mixed with 3 µL of urine and 5 µL of sodium hydroxide (10%). The mixtures were incubated at room temperature in the dark during 15 min. After the reaction time, 232 µL of Milli-Q water was added and the absorbance was measured in the UV/VIS spectrophotometers at 500 nm. TPE was expressed as mg gallic acid equivalent (GAE)/ g of creatinine.

Correlations between estimations of total polyphenol intake and blood pressure in the PREDIMED trial

The PREDIMED (*PREvención con Dieta MEDiterránea*) study is a large, 5-year, parallel-group, multicentre, randomised, controlled clinical trial aimed to assess the effects of the Mediterranean Diet on the primary prevention of cardiovascular disease (www.predimed.org; ISRCTN35739639). The detailed recruitment method and study protocol have been described previously [2].

We selected 589 participants in primary health centres affiliated in Spain between October 2003 and July 2004. They were 263 men aged 53 to 82 years and 326 women aged 58 to 82 years, free of cardiovascular disease at baseline and who fulfilled at least one of the following two criteria: (1) type-2 diabetes

Resultats

mellitus and/or (2) three or more coronary heart disease (CHD) risk factors: hypertension (BP>140/90 mmHg or treatment with antihypertensive medication), dyslipidemia, obesity or overweight, smoking or family history of early-onset CHD. At baseline all participants completed a validated semiquantitative FFQ with 136 items [45], the validated Spanish version [46] of the Minnesota Leisure Time Physical Activity Questionnaire, and a 47-item questionnaire about education, lifestyle, history of illnesses and medication use. Trained nurses measured height and weight with a wall-mounted stadiometer and calibrated scales, respectively, as well as BP in triplicate with a validated semi-automatic oscillometer (Omron HEM-705CP; Hoofddorp, The Netherlands [47]). Energy and nutrients intake were derived from Spanish food composition tables [48]. The morning urine samples were frozen at -80°C until analysis. For the urine assay, thawing is necessary over an ice bed for 3 h, to avoid degradation of the polyphenols [24]. Total polyphenol consumption from plant food and beverages (mg/ g fresh matter) was quantified according to Saura-Calixto F *et al.* [49] and Brat P *et al.* [28], from the data of FFQ.

Participant characteristics from the PREDIMED substudy.

All 589 participants who entered the study were older than 55 years, mostly overweight [mean body mass index 29.36 (3.37) kg/m²], and with a sizeable burden of cardiovascular risk factors (32.6% diabetics, 80.8% hypertensive, 62.6% with dyslipidemia, 16.1% active smokers and 34.8 % had a family history of early-onset CHD). Table 1 shows the profile of cardiovascular risk factors of the participants according to the quartile of urinary TPE [20]. A higher polyphenol excretion in urine was associated with older age (*P* for trend<0.001) and lower systolic and diastolic BP (see figure 1). Participants in the highest quartile of TPE had a higher prevalence of diabetes and were less likely to be smokers.

Food and nutrient intake and urinary total polyphenols.

Table 2 shows the average food consumption of study participants divided according to quartiles of urinary TPE; Q1 (<89.0 mg GAE/g creatinine), Q2 (89.1-119.5 mg GAE/g creatinine), Q3 (119.6-160.2 mg GAE/g creatinine) and Q4 (>160.3 mg GAE/g creatinine) [20]. Significant increasing trends across quartiles of TPE were observed for the intake total F&V, fish or fish-food, milk and dairy products and TP intake. Whereas decreasing trends across TPE quartiles were observed for cereals, alcohol intake, and total energy intake. The daily variability of TP intake measured from FFQ was between 122.96 to 3298.17 mg/day. This variability correlated with the TPE variation observed in the urine as it was measured with a Pearson correlation between the two variables with a weak but significant correlation being obtained (*r*=0.179; *P*<0.001).

In the linear regression analyses [20] we observed a significant positive association between normalized urine TPE expressed as mg GAE/ g creatinine and TP intake (100mg), daily F&V intake (100 g) and daily coffee intake (100 mL) in the unadjusted model (β =0.073, *P*<0.001; β =0.131, *P*<0.001 and β =0.261, *P*<0.05, respectively), with β being the non-standardized regression coefficient. After adjusting for potential confounding factors (models 4), the association remained statistically significant: β =0.116, *P*<0.001; β =0.127, *P*<0.001 and β =0.414; *P*<0.05 for TP, F&V and coffee intake, respectively. TPE

concentration in spot urine samples showed a significant inverse association with the intake of wine (100 mL), $\beta=-0.090$, $P<0.05$ in the unadjusted model. However, after adjusting for potential confounding factors (model 2) this association reversed and became positive and statistically significant ($\beta=0.121$, $P=0.019$). The remaining group did not exhibit any significant association with urinary TPE adjusted for potential confounding factors.

The standardized coefficients (Beta) are the regression coefficients obtained with the regression model using the standardized values. The standardized coefficients from this model showed that total phenol intake (Beta=0.283) and F&V intake (Beta=0.150) contributed more to urinary TPE than coffee (Beta=0.141), and both contributed to a greater extent than wine (Beta=0.120).

Total polyphenols excreted and cardiovascular risk factors.

After adjustment for different potential confounding factors (sex, age, weight, smoking status, physical activity, educational level, energy expenditure in physical activity, medication intake: ACE inhibitor, diuretics, statins (hypolipidemic drugs), insulin, oral hypoglycaemic drugs, aspirin or other antiplatelet drug supplements taken in the last month, sodium and potassium intake and glomerular filtration rate), multivariate linear regression analyses with systolic and diastolic BPs as the dependent variables, and quartile of TPE in spot urine samples (mg GAE/g creatinine) as exposure variable, systolic and diastolic BP exhibited a monotonic inverse association with TPE in spot urine samples. The non-standardized coefficients, $\beta = -1.73$, $P=0.024$ (95 % CI -3.23 to -0.23) and $\beta= -1.26$, $P=0.003$ (95 % CI -2.09 to -0.44) represent the expected change in systolic and diastolic BP, respectively, corresponding to an increase in TPE to the upper quartile.

In a multivariate logistic regression analysis for cardiovascular risk factors (hypertension) according to quartiles of TPE expressed as mg GAE/ g creatinine using the lowest quartile group as the reference category, the participants in the lowest quartile of TPE (<88.99 mg GAE/g creatinine) had a significantly reduced prevalence of hypertension (OR=0.71, CI 0.53 to 0.95; $P=0.021$), compared with those in the highest quartile (>160.23 mg GAE/ g creatinine). After adjustment for all possible confounding factors, a significant difference was observed between the highest and the lowest quartiles for the prevalence of hypertension. Participants in the highest quartile had a 36% reduced odds ratio of hypertension (OR=0.64, CI 0.45 to 0.92; $P=0.015$), compared to those in the lowest quartile [20]. In fact, BP correlated better with urinary TPE than with TP intake assessed by FFQ.

Thus, the results of this study confirm that total phenols, F&V, and coffee and wine intake are positively correlated with the excretion of TPs in spot urine samples. F&V consumption is the main contributor to urinary TPE.

Conclusions

In summary, the Folin-Ciocalteu method for determination TP in urine is rapid and simple and allows the analysis of total phenols in urine samples [24]. This method is cheaper, more environmentally friendly and simpler than other methods described. In the cross-sectional trial [20] within the framework of the PREDIMED study, a high TPE in spot urine samples was negatively associated with BP levels in an elderly Mediterranean population [20]. Thus, a high intake

of polyphenol-rich diet may help to decrease BP in elderly hypertensive populations in order to lower their cardiovascular risk. On comparing the participants in the highest with those in the lowest quartile of TPE a 36% reduction was observed in the odds ratio of hypertension. However, the observation that systolic and diastolic BP decreases when TPE increases should be confirmed in further intervention studies. A longer follow-up of the whole PREDIMED trial, which includes two dietary interventions enriched in polyphenol-rich foods such as nuts and virgin olive oil will eventually provide evidence to confirm the results reported.

Acknowledgements

We would like to thank all of the volunteers involved in the PREDIMED study for their valuable cooperation. The authors express their gratitude for financial support from CICYT's (AGL2005-05597; AGL2006-14228-C03-02 and AGL2007-66638-C02-01), RETICS RD06/0045/0003 from the Spanish Ministry of Science and Innovation (MICINN) and grant PI070240 from Instituto de Salud Carlos III, Spain. The CIBERobn CB06/03 is an initiative from the Instituto de Salud Carlos III, Spain. This work has also been funded in part by the CONSOLIDER INGENIO 2010 Programme, FUN-C-FOOD CSD2007-063 and from MICINN. None of the funding sources played a role in the design, collection, analysis or interpretation of data, in the writing of the report or in the decision to submit the paper for publication. None of the authors have any conflict of interest. A. M-R received support from the Generalitat of Catalonia for training of researcher.

References

- [1] C.M.Lawes, H.S.Vander, and A.Rodgers, Global burden of blood-pressure-related disease, 2001, *Lancet* 371 (2008) 1513-1518.
- [2] R.Estruch, M.A.Martinez-Gonzalez, D.Corella, J.Salas-Salvado, V.Ruiz-Gutierrez, M.I.Covas, M.Fiol, E.Gomez-Gracia, M.C.Lopez-Sabater, E.Vinyoles, F.Aros, M.Conde, C.Lahoz, J.Lapetra, G.Saez, and E.Ros, Effects of a Mediterranean-style diet on cardiovascular risk factors: a randomized trial, *Ann.Intern.Med.* 145 (2006) 1-11.
- [3] L.J.Appel, T.J.Moore, E.Obarzanek, W.M.Vollmer, L.P.Svetkey, F.M.Sacks, G.A.Bray, T.M.Vogt, J.A.Cutler, M.M.Windhauser, P.H.Lin, N.Karanja, D.Simons-Morton, M.McCullough, J.Swain, P.Steele, M.A.Evans, E.R.Miller, D.W.Harsha, and The DASH Collaborative Research Group, A Clinical Trial of the Effects of Dietary Patterns on Blood Pressure, *N Engl J Med* 336 (1997) 1117-1124.
- [4] G.Mancia, B.G.De, A.Dominiczak, R.Cifkova, R.Fagard, G.Germano, G.Grassi, A.M.Heagerty, S.E.Kjeldsen, S.Laurent, K.Narkiewicz, L.Ruilope, A.Rynkiewicz, R.E.Schmieder, H.A.Boudier, A.Zanchetti, A.Vahanian, J.Camm, C.R.De, V.Dean, K.Dickstein, G.Filippatos, C.Funck-Brentano, I.Hellemans, S.D.Kristensen, K.McGregor, U.Sechtem, S.Silber, M.Tendera, P.Widimsky, J.L.Zamorano, S.Erdine, W.Kiowski, E.gabiti-Rosei, E.Ambrosioni, L.H.Lindholm, M.Viigimaa, S.Adamopoulos, E.gabiti-Rosei, E.Ambrosioni, V.Bertomeu, D.Clement, S.Erdine, C.Farsang, D.Gaita, G.Lip, J.M.Mallion, A.J.Manolis, P.M.Nilsson, E.O'Brien, P.Ponikowski, J.Redon, F.Ruschitzka, J.Tamargo, Z.P.van, B.Waeber, and B.Williams, 2007 Guidelines for the Management of Arterial Hypertension: The Task Force for the Management of

- Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC), *J.Hypertens.* 25 (2007) 1105-1187.
- [5] K.E.Harnden, K.N.Frayn, and L.Hodson, Dietary Approaches to Stop Hypertension (DASH) diet: applicability and acceptability to a UK population, *J.Hum.Nutr.Diet.* 23 (2010) 3-10.
 - [6] A.Alonso, F.C.de la, A.M.Martin-Arnau, I.J.de, J.A.Martinez, and M.A.Martinez-Gonzalez, Fruit and vegetable consumption is inversely associated with blood pressure in a Mediterranean population with a high vegetable-fat intake: the Seguimiento Universidad de Navarra (SUN) Study, *Br.J.Nutr.* 92 (2004) 311-319.
 - [7] A.Agudo, L.Cabrera, P.Amiano, E.Ardanaz, A.Barricarte, T.Berenguer, M.D.Chirlaque, M.Dorronsoro, P.Jakszyn, N.Larranaga, C.Martinez, C.Navarro, J.R.Quiros, M.J.Sanchez, M.J.Tormo, and C.A.Gonzalez, Fruit and vegetable intakes, dietary antioxidant nutrients, and total mortality in Spanish adults: findings from the Spanish cohort of the European Prospective Investigation into Cancer and Nutrition (EPIC-Spain), *Am.J.Clin.Nutr.* 85 (2007) 1634-1642.
 - [8] M.I.Covas, M.Fito, J.Marrugat, E.Miro, M.Farre, T.R.de la, E.Gimeno, M.C.Lopez-Sabater, R.Lamuella-Raventos, and de la Torre-Boronat MC, [Coronary disease protective factors: antioxidant effect of olive oil], *Therapie* 56 (2001) 607-611.
 - [9] M.I.Covas, K.Nyyssonen, H.E.Poulsen, J.Kaikkonen, H.J.Zunft, H.Kiesewetter, A.Gaddi, T.R.de la, J.Mursu, H.Baumler, S.Nascetti, J.T.Salonen, M.Fito, J.Virtanen, J.Marrugat, and EUROLIVE Study Group, The effect of polyphenols in olive oil on heart disease risk factors: a randomized trial, *Ann.Intern.Med.* 145 (2006) 333-341.
 - [10] D.Grassi, S.Necozone, C.Lippi, G.Croce, L.Valeri, P.Pasqualetti, G.Desideri, J.B.Blumberg, and C.Ferri, Cocoa reduces blood pressure and insulin resistance and improves endothelium-dependent vasodilation in hypertensives, *Hypertension* 46 (2005) 398-405.
 - [11] C.Manach, A.Mazur, and A.Scalbert, Polyphenols and prevention of cardiovascular diseases, *Curr.Opin.Lipidol.* 16 (2005) 77-84.
 - [12] L.J.Appel, T.J.Moore, E.Obarzanek, W.M.Vollmer, L.P.Svetkey, F.M.Sacks, G.A.Bray, T.M.Vogt, J.A.Cutler, M.M.Windhauser, P.H.Lin, N.Karanja, D.Simons-Morton, M.McCullough, J.Swain, P.Steele, M.A.Evans, E.R.Miller, D.W.Harsha, and The DASH Collaborative Research Group, A Clinical Trial of the Effects of Dietary Patterns on Blood Pressure, *N Engl J Med* 336 (1997) 1117-1124.
 - [13] L.Wang, J.E.Manson, J.E.Buring, I.M.Lee, and H.D.Sesso, Dietary intake of dairy products, calcium, and vitamin D and the risk of hypertension in middle-aged and older women, *Hypertension* 51 (2008) 1073-1079.
 - [14] A.Alonso, J.J.Beunza, M.Delgado-Rodriguez, J.A.Martinez, and M.A.Martinez-Gonzalez, Low-fat dairy consumption and reduced risk of hypertension: the Seguimiento Universidad de Navarra (SUN) cohort, *Am.J Clin.Nutr.* 82 (2005) 972-979.
 - [15] D.Q.Bao, T.A.Mori, V.Burke, I.B.Puddey, and L.J.Beilin, Effects of dietary fish and weight reduction on ambulatory blood pressure in overweight hypertensives, *Hypertension* 32 (1998) 710-717.
 - [16] T.A.Mori, D.Q.Bao, V.Burke, I.B.Puddey, G.F.Watts, and L.J.Beilin, Dietary fish as a major component of a weight-loss diet: effect on serum lipids, glucose, and

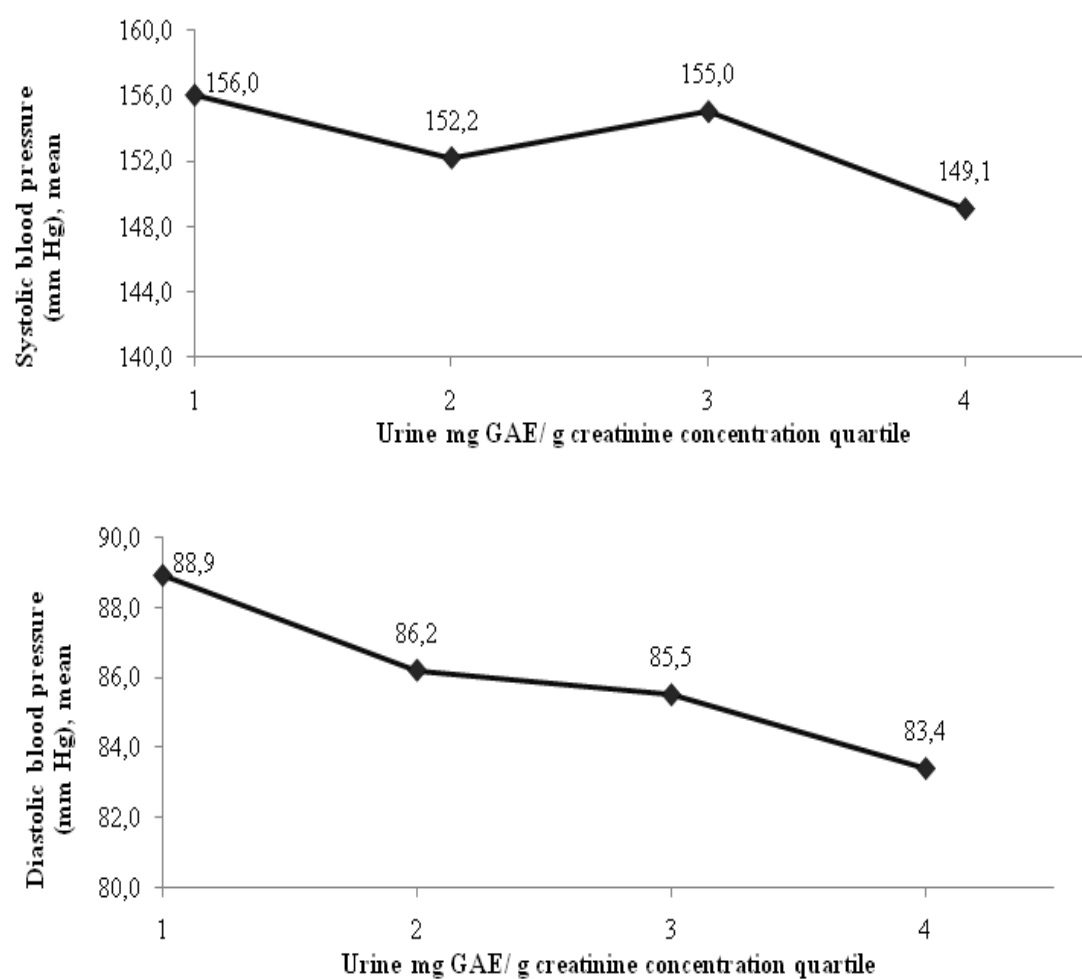
- insulin metabolism in overweight hypertensive subjects, *Am.J Clin.Nutr.* 70 (1999) 817-825.
- [17] M.F.Engberink, M.A.Hendriksen, E.G.Schouten, F.J.van Rooij, A.Hofman, J.C.Witteaman, and J.M.Geleijnse, Inverse association between dairy intake and hypertension: the Rotterdam Study, *Am.J Clin.Nutr.* 89 (2009) 1877-1883.
 - [18] R.Rodrigo, H.Prat, W.Passalacqua, J.Araya, and J.P.Bachler, Decrease in oxidative stress through supplementation of vitamins C and E is associated with a reduction in blood pressure in patients with essential hypertension, *Clin.Sci.(Lond)* 114 (2008) 625-634.
 - [19] M.Guxens, M.Fito, M.A.Martinez-Gonzalez, J.Salas-Salvado, R.Estruch, E.Vinyoles, M.Fiol, D.Corella, F.Aros, E.Gomez-Gracia, V.Ruiz-Gutierrez, J.Lapetra, E.Ros, J.Vila, and M.I.Covas, Hypertensive status and lipoprotein oxidation in an elderly population at high cardiovascular risk, *Am.J.Hypertens.* 22 (2009) 68-73.
 - [20] A.Medina-Remón, R.Zamora-Ros, M.Rotchés-Ribalta, C.Andres-Lacueva, MA Martínez-Gonzalez, MI Covas, D.Corella, J.Salas-Salvadó, E.Gómez-Gracia, V.Ruiz-Gutiérrez, FJ García de la Corte, M.Fiol, MA Pena, GT Saez, E.Ros, L.Serra-Majem, X.Pinto, J.Warnberg, R.Estruch, and RM Lamuela Raventos, Total polyphenol excretion and blood pressure in subjects at high cardiovascular risk., *Nutrition, Metabolism and Cardiovascular Diseases* (2009) doi:10.1016/j.numecd.2009.10.019.
 - [21] S.Arranz, F.Saura-Calixto, S.Shaha, and P.A.Kroon, High Contents of Nonextractable Polyphenols in Fruits Suggest That Polyphenol Contents of Plant Foods Have Been Underestimated, *J.Agric.Food Chem.* (2009).
 - [22] J.Feher and G.Lengyel, [Nutrition and cardiovascular mortality], *Orv.Hetil.* 147 (2006) 1491-1496.
 - [23] J.P.Spencer, M.M.bd El Mohsen, A.M.Minihane, and J.C.Mathers, Biomarkers of the intake of dietary polyphenols: strengths, limitations and application in nutrition research, *Br.J.Nutr.* 99 (2008) 12-22.
 - [24] A.Medina-Remón, A.Barrionuevo-González, R.Zamora-Ros, C.Andres-Lacueva, R.Estruch, MA Martínez-González, J.Diez-Espino, and RM Lamuela-Raventos, Rapid Folin-Ciocalteu method using microtiter 96-well plate cartridges for solid phase extraction to assess urinary total phenolic compounds, as a biomarker of total polyphenols intake, *Analytica Chimica Acta* 634 (2009) 54-60.
 - [25] I.Zazpe, A.Sanchez-Tainta, R.Estruch, R.M.Lamuela-Raventos, H.Schroder, J.Salas-Salvado, D.Corella, M.Fiol, E.Gomez-Gracia, F.Aros, E.Ros, V.Ruiz-Gutierrez, P.Iglesias, M.Conde-Herrera, and M.A.Martinez-Gonzalez, A large randomized individual and group intervention conducted by registered dietitians increased adherence to Mediterranean-type diets: the PREDIMED study, *J Am.Diet.Assoc.* 108 (2008) 1134-1144.
 - [26] E.Roura, M.P.Almajano, M.L.Bilbao, C.Andres-Lacueva, R.Estruch, and R.M.Lamuela-Raventos, Human urine: epicatechin metabolites and antioxidant activity after cocoa beverage intake, *Free Radic.Res* 41 (2007) 943-949.
 - [27] C.Manach, A.Scalbert, C.Morand, C.Remesy, and L.Jimenez, Polyphenols: food sources and bioavailability, *Am.J Clin.Nutr.* 79 (2004) 727-747.
 - [28] P.Brat, S.George, A.Bellamy, C.L.Du, A.Scalbert, L.Mennen, N.Arnault, and M.J.Amiot, Daily polyphenol intake in France from fruit and vegetables, *J.Nutr.* 136 (2006) 2368-2373.

- [29] T.H.Rissanen, S.Voutilainen, J.K.Virtanen, B.Venho, M.Vanharanta, J.Mursu, and J.T.Salonen, Low intake of fruits, berries and vegetables is associated with excess mortality in men: the Kuopio Ischaemic Heart Disease Risk Factor (KIHD) Study, *J Nutr.* 133 (2003) 199-204.
- [30] G.Radhika, V.Sudha, R.Mohan Sathya, A.Ganesan, and V.Mohan, Association of fruit and vegetable intake with cardiovascular risk factors in urban south Indians, *British Journal of Nutrition* 99 (2008) 398-405.
- [31] A.Ascherio, C.Hennekens, W.C.Willett, F.Sacks, B.Rosner, J.Manson, J.Witteman, and M.J.Stampfer, Prospective study of nutritional factors, blood pressure, and hypertension among US women, *Hypertension* 27 (1996) 1065-1072.
- [32] J.Stamler, K.Liu, K.J.Ruth, J.Pryer, and P.Greenland, Eight-year blood pressure change in middle-aged men: relationship to multiple nutrients, *Hypertension* 39 (2002) 1000-1006.
- [33] H.C.Hung, A.Merchant, W.Willett, A.Ascherio, B.A.Rosner, E.Rimm, and K.J.Joshipura, The association between fruit and vegetable consumption and peripheral arterial disease, *Epidemiology* 14 (2003) 659-665.
- [34] N.Reshef, Y.Hayari, C.Goren, M.Boaz, Z.Madar, and H.Knobler, Antihypertensive effect of sweetie fruit in patients with stage I hypertension, *Am.J.Hypertens.* 18 (2005) 1360-1363.
- [35] D.Taubert, R.Berkels, R.Roesen, and W.Klaus, Chocolate and blood pressure in elderly individuals with isolated systolic hypertension, *JAMA* 290 (2003) 1029-1030.
- [36] K.Shimada, H.Watanabe, K.Hosoda, K.Takeuchi, and J.Yoshikawa, Effect of red wine on coronary flow-velocity reserve, *Lancet* 354 (1999) 1002.
- [37] M.Hashimoto, S.Kim, M.Eto, K.Iijima, J.Ako, M.Yoshizumi, M.Akishita, K.Kondo, H.Itakura, K.Hosoda, K.Toba, and Y.Ouchi, Effect of acute intake of red wine on flow-mediated vasodilatation of the brachial artery, *Am.J.Cardiol.* 88 (2001) 1457-60, A9.
- [38] L.A.Ferrara, A.S.Raimondi, L.d'Episcopo, L.Guida, A.Io Russo, and T.Marotta, Olive Oil and Reduced Need for Antihypertensive Medications, *Arch Intern Med* 160 (2000) 837-842.
- [39] Y.Al-Solaiman, A.Jesri, W.K.Mountford, D.T.Lackland, Y.Zhao, and B.M.Egan, DASH lowers blood pressure in obese hypertensives beyond potassium, magnesium and fibre, *J Hum Hypertens* (2009).
- [40] L.Hodson, K.E.Harnden, R.Roberts, A.L.Dennis, and K.N.Frayn, Does the DASH diet lower blood pressure by altering peripheral vascular function[quest], *J Hum Hypertens* (2009).
- [41] J.M.Nunez-Cordoba, F.Valencia-Serrano, E.Toledo, A.Alonso, and M.A.Martinez-Gonzalez, The Mediterranean Diet and Incidence of Hypertension: The Seguimiento Universidad de Navarra (SUN) Study, *Am.J.Epidemiol.* 169 (2009) 339-346.
- [42] L.I.Mennen, D.Sapinho, H.Ito, S.Bertrais, P.Galan, S.Hercberg, and A.Scalbert, Urinary flavonoids and phenolic acids as biomarkers of intake for polyphenol-rich foods, *Br.J Nutr.* 96 (2006) 191-198.
- [43] K.S.Krogholm, J.Haraldsdottir, P.Knuthsen, and S.E.Rasmussen, Urinary total flavonoid excretion but not 4-pyridoxic acid or potassium can be used as a biomarker for the intake of fruits and vegetables, *J.Nutr.* 134 (2004) 445-451.

Resultats

- [44] S.E.Nielsen, R.Freese, P.Kleemola, and M.Mutanen, Flavonoids in human urine as biomarkers for intake of fruits and vegetables, *Cancer Epidemiol Biomarkers Prev* 11 (2002) 459-466.
- [45] J.M.Martin-Moreno, P.Boyle, L.Gorgojo, P.Maisonneuve, J.C.Fernandez-Rodriguez, S.Salvini, and W.C.Willett, Development and validation of a food frequency questionnaire in Spain, *Int.J.Epidemiol.* 22 (1993) 512-519.
- [46] R.Elosua, J.Marrugat, L.Molina, S.Pons, and E.Pujol, Validation of the Minnesota Leisure Time Physical Activity Questionnaire in Spanish men. The MARATHOM Investigators, *Am.J.Epidemiol.* 139 (1994) 1197-1209.
- [47] P.Iglesias-Bonilla, E.Mayoral-Sánchez, J.Lapetra-Peralta, M.Iborra-Oquendo, F.Villalba-Alcalá, and A.Cayuela-Domínguez, Validación de dos sistemas de automedida de presión arterial, modelos OMRON HEM 705 CP y OMRON MI (HEM 422C2-E), *Atención Primaria* 30 (2002) 22-28.
- [48] J.Mataix. Tabla de composición de alimentos (Food composition tables). 2003. Universidad de Granada: Granada, Spain. Ref Type: Generic
- [49] F.Saura-Calixto and I.Goni, Antioxidant capacity of the Spanish Mediterranean diet, *Food Chemistry* 94 (2006) 442-447.

Figure 1: Change in systolic and diastolic blood pressure according to quartiles of total polyphenol excretion expressed as mg gallic acid equivalent / g creatinine.



Resultats

Table 1: Characteristics of the study subjects classified by quartile of total polyphenol excreted in urine.

	Quartile of urinary total polyphenol concentration, mg GAE/ g creatinine				<i>P</i> for trend ¹
	Q1 (<89.0)	Q2 (89.1-119.5)	Q3 (119.6-160.2)	Q4 (>160.3)	
No. of subjects	147	148	147	147	
Age, (y) mean (SD)	66.0 (5.7)	68.3 (6.1)	68.0 (5.8)	69.3(6.0)	< 0.001
Women, n (%)	51 (34.7)	83 (56.1)	85 (57.8)	107 (72.8)	< 0.001
BMI, (kg/m ²), mean (SD)	29.6 (2.9)	29.6 (3.7)	29.2 (3.7)	29.1 (3.4)	0.171
Systolic BP (mm Hg), mean (SD)	156.0 (15.6)	152.2 (18.3)	155.0 (21.6)	149.1 (16.0)	0.015
Diastolic BP (mmHg), mean (SD)	88.9 (10.2)	86.2 (9.8)	85.5 (11.8)	83.4 (8.5)	< 0.001
Hypertension, n (%)	123 (83.7)	126 (85.1)	113 (76.9)	114 (77.6)	0.067
Diabetes, n (%)	31 (21.1)	47 (31.8)	52 (35.4)	62 (42.2)	< 0.001
Current smoker n (%)	39 (26.5)	20 (13.5)	22 (15.0)	14 (9.5)	< 0.001
Medication, n (%)					
ACE inhibitors	119 (81.0)	114 (77.0)	106 (72.1)	107 (72.8)	0.063
Diuretics	11 (7.5)	7 (4.7)	7 (4.8)	9 (5.8)	0.639
Statins or other hypolipidemic drugs	42 (28.6)	70 (47.3)	69 (46.9)	65 (44.2)	0.01
Insulin	3 (2.0)	4 (2.7)	12 (8.2)	9 (6.1)	0.024
Oral hypoglycemic drugs	17 (11.6)	26 (17.6)	26 (17.6)	36 (24.5)	0.006
Aspirin or other antiplatelet drugs	29 (19.7)	28 (18.9)	37 (25.2)	30 (20.4)	0.582
Educational level, n (%)					
Primary school	104 (70.7)	112 (75.7)	108 (73.5)	121 (82.3)	0.041
High school	24 (16.3)	18 (12.2)	28 (19.0)	18 (12.2)	0.685
University	19 (12.9)	17 (11.5)	10 (6.8)	7 (4.8)	0.006
Energy expenditure in physical activity (kcal/d), mean (SD)	256.0 (215.5)	245.9 (220.7)	293.1 (231.0)	255.3 (205.3)	0.576
Glomerular filtration rate, mL/min	77.2 (16.4)	78.1 (14.6)	78.1 (18.1)	79.5 (18.9)	0.264

¹ANOVA-one factor was used for continuous variables and χ^2 -test for categorical variables. BMI: body mass index (calculated as weight in kilograms divided by height in square meters); BP: blood pressure; ACE: angiotensin-converting enzyme. (Medina-Remón *et al.*, 2009)

Table 2: Baseline energy, daily intake of selected foods and nutrients classified according to the quartiles of total urinary polyphenols excreted.

	Quartile of urinary total polyphenol concentration (mg GAE/ g creatinine)				P for trend ^a
	Q1 (<89.0)	Q2 (89.1-119.5)	Q3 (119.6-160.2)	Q4 (>160.3)	
No. of subjects	147	148	147	147	
Urine total polyphenol (mg GAE/ g creatinine)	72.8 (11.6)	103.1 (8.2)	138.2 (11.1)	226.1 (69.8)	< 0.001
Fruits and vegetables (g)	557.7 (176.8)	580.4 (173.3)	577.7 (162.1)	633.5 (190.1)	0.001
Legumes (g)	17.9 (7.0)	17.9 (7.5)	17.7 (7.0)	16.5 (7.6)	0.107
Fish or seafood (g)	86.5 (34.0)	82.2 (35.8)	88.7 (36.9)	94.1 (39.0)	0.030
Meat or meat products (g)	132.0 (49.6)	129.7 (43.7)	134.3 (48.5)	134.2 (47.5)	0.531
Cereals (g)	243.7 (106.7)	235.8 (91.6)	228.7 (98.3)	211.3 (83.3)	0.003
Milk and dairy products (mL)	352.1 (214.8)	378.8 (216.5)	396.5 (222.1)	403.1 (215.1)	0.033
Wine (mL)	121.1 (147.0)	81.5 (137.9)	101.1 (161.5)	81.3 (149.3)	0.071
Coffee (mL)	63.3 (47.7)	69.6 (52.9)	67.5 (48.3)	74.5 (56.0)	0.097
Total nuts (g)	8.6 (10.7)	9.2 (11.3)	9.6 (12.2)	9.1 (10.9)	0.680
Tea (mL)	3.92 (16.4)	3.25 (11.4)	4.16 (12.6)	5.47 (20.4)	0.332
Chocolate (g)	3.1 (6.2)	2.4 (5.2)	2.3 (5.5)	1.9 (4.8)	0.063
Total polyphenol intake (mg GAE)	1075.6 (354.9)	1057.5 (320.2)	1086.2 (322.3)	1222.5 (439.8)	0.001
Alcohol (g)	15.7 (17.9)	10.2 (16.0)	12.2 (18.5)	9.9 (17.8)	0.018
Fibre (g)	22.1 (6.2)	22.0 (5.5)	21.9 (5.2)	22.5 (6.2)	0.606
Cholesterol (g)	353.6 (119.7)	328.6 (93.7)	340.5 (113.4)	342.1 (89.4)	0.561
Sodium (mg/d)	3347.7 (959.2)	3088.0 (905.4)	3123.2 (1006.5)	3145.4 (877.2)	0.100
Potassium (mg/d)	3926.7 (722.3)	3929.4 (700.9)	3994.8 (805.5)	4029.7 (659.7)	0.161
Total energy, Kcal/d	2380.1 (586.8)	2238.0 (472.0)	2205.1 (547.4)	2138.5 (476.8)	< 0.001

^aOne-factor ANOVA was used for continuous variables; mean (standard deviation). GAE: gallic acid equivalent. (Medina-Remón *et al.*, 2009)

4.5 Publicació 5: El seguiment d'un patró de dieta mediterrània suplementada amb fruits secs o oli d'oliva verge, incrementa l'excreció de polifenols totals, produint una disminució significativa de la pressió arterial. (El PREDIMED després d'un any d'intervenció).

Medina-Remón A., Estruch R., Andres-Lacueva C., Martínez-González M.A., Covas M.I., Corella D., Salas-Salvadó J., Gómez-Gracia E., Ruiz-Gutiérrez V., García de la Corte F.J., Fiol M., Pena M.A., Saez G.T., Ros E., Serra-Majem L., Pinto X., Warnberg J. and Lamuela-Raventos R.M.

En procés de revisió

Resum:

La hipertensió és un problema important de salut pública i la principal causa de mort i discapacitat en els països en desenvolupament. L'objectiu d'aquest estudi és avaluar si la intervenció durant un any amb una dieta tradicional mediterrània suplementada amb oli d'oliva verge (VOO) o nous, incrementa l'excreció de polifenols totals (TP) en mostres d'orina preses de forma puntual. Aquesta anàlisi bioquímica es va associar amb la mitjana de PA sistòlica i diastòlica en persones grans amb alt risc cardiovascular.

Es presenta un sub-estudi d'intervenció amb 1139 participants d'alt risc cardiovascular dins de l'estudi PREDIMED. Els participants van ser assignats a una dieta baixa en greixos (dieta control), n=379 o a dues dietes estil mediterrànies (Med), una suplementada amb VOO, n=394 i l'altra amb fruits secs, n=366. Les mesures antropomètriques van ser considerades al principi i al final de l'estudi, així com els polifenols totals excretats (TPE) determinats en les orines, mitjançant el mètode de Folin-Ciocalteu. Els resultats es van expressar en mg d'equivalent d'àcid gàl·lic (GAE) / g de creatinina.

Després de la intervenció amb Med-VOO i Med-fruits secs i ajustar per diferents covariables, els participants van mostrar un increment significatiu dels TPE urinaris (24.67 mg GAE/g de creatinina 95% IC: 11.62 a 37.72; $P < 0.001$ i

22.05 mg GAE/g de creatinina, 95% IC: 8.89 a 35.22; $P=0.001$, respectivament), en comparació amb la dieta de control. Després d'ajustar per totes les possibles covariables, els participants amb la mateixa PA sistòlica a l'inici de l'estudi, després de la intervenció amb Med-VOO i Med-fruits secs van tenir una reducció estadísticament significativa de -3.43 mm Hg (IC -5.60 a -1.26, $P=0.002$) i -2.27 mm Hg (IC -4.45 a -0.09, $P=0.042$), respectivament, en comparació amb la dieta control. En aquest mateix model ajustat, els participants amb la mateixa PA diastòlica a l'inici, després de la intervenció amb Med-VOO i Med-fruits secs van tenir una reducció de -1.38 mm Hg (IC: -2.48 a -0.28, $P=0.01$) i -0.70 mm Hg (IC: 1.80 a 0.41, $P=0.22$) respectivament, en comparació amb la dieta control, essent estadísticament significativa només en la primera comparació.

Una intervenció durant un any amb una dieta tradicional mediterrània suplementada amb fruits secs o bé VOO incrementa els polifenols totals excretats en l'orina, i disminueixen la PA sistòlica i diastòlica. Ambdues dietes mediterrànies van mostrar efectes beneficiosos sobre factors de risc cardiovascular.

Following a Mediterranean diet pattern supplemented with nuts or virgin olive oil increases total polyphenol excretion and produces a significant decrease in blood pressure. The PREDIMED randomized trial after one year.

Medina-Remón A^{a,b,c}, Estruch R^{b,c,d}, Andres-Lacueva C^{a,e}, Martínez-González MA^{c,f}, Covas MI^{b,g}, Corella D^{b,h}, Salas-Salvadó J^{b,i}, Gómez-Gracia E^{c,j}, Ruiz-Gutiérrez V^{c,k}, García de la Corte FJ^{b,l}, Fiol M^{b,m}, Pena MAⁿ, Saez GT^{c,o}, Ros E^{b,p}, Serra-Majem L^{c,q}, Pinto X^{c,r}, Warnberg J^{c,s,t} and Lamuela-Raventos RM^{a,b,c*}, on behalf of the PREDIMED Study Investigators.

^aNutrition and Food Science Department, XaRTA, INSA. Pharmacy School, University of Barcelona, Barcelona, Spain; ^bCIBER CB06/03 Fisiopatología de la Obesidad y la Nutrición, (CIBEROBN), and ^cRETICS RD06/0045/0003. Instituto de Salud Carlos III, Spain; ^dDepartment of Internal Medicine, Hospital Clinic, IDIBAPS, University of Barcelona; ^eIngenio-CONSOLIDER program, FUN-C-FOOD, Barcelona; ^fDepartment of Preventive Medicine and Public Health, School of Medicine, University of Navarra, Pamplona; ^gCardiovascular Epidemiology Unit, Municipal Institute for Medical Research (IMIM), Barcelona; ^hDepartment of Epidemiology, Preventive Medicine and Public Health, School of Medicine, University of Valencia, Valencia; ⁱHuman Nutrition Unit, School of Medicine, IISPV, University Rovira i Virgili, Reus; ^jDepartment of Epidemiology, School of Medicine, University of Malaga, Málaga; ^kInstituto de la Grasa, Consejo Superior de Investigaciones Científicas, Sevilla; ^lDepartment of Family Medicine, Primary Care Division of Sevilla, San Pablo Health Center, Sevilla; ^mInstitut Universitari d'Investigació en Ciències de la Salut (IUNICS), Palma de Mallorca; ⁿClinical Trials Unit, Hospital Txangorritxu, Vitoria; ^oDepartment of Biochemistry and Molecular Biology Service of Clinical Analysis, CDB Hospital General Universitario, Universitat de Valencia, Spain; ^pLipid Clinic, Endocrinology and Nutrition Service, Institut d'Investigacions Biomèdiques August Pi Sunyer (IDIBAPS), Hospital Clinic, Barcelona; ^qDepartment of Clinical Sciences, University of Las Palmas de Gran Canaria, Palmas de Gran Canaria; ^rLipid Unit, Department of Internal Medicine, IDIBELL-Hospital Universitari de Bellvitge, L'Hospitalet de Llobregat, Fipec, Barcelona, Spain; ^sImmunonutrition Research Group, Instituto del Frío-ICTAN (CSIC), Madrid, Spain; ^tDepartment of Biosciences and Nutrition, Karolinska Institutet, Stockholm, Sweden;

*Corresponding author: Nutrition and Food Science Department, XaRTA, INSA Pharmacy School, University of Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain. Telephone +34-934034843. Fax +34-934035931; e-mail lamuela@ub.edu

Key words: Hypertension, blood pressure, polyphenol biomarker, urinary polyphenol, Folin-Ciocalteu.

ABSTRACT

Background and Aims: Hypertension is a major public health problem and a leading cause of death and disability in developing countries. The aim of this study was to evaluate whether a one-year intervention with two traditional Mediterranean diets supplemented with virgin olive oil (VOO) or nuts increased the excretion of total polyphenols (TPs) in spot urine samples. These biochemical analyses were associated with average systolic and diastolic BP in elderly individuals at high cardiovascular risk.

Materials and methods: An intervention substudy of 1139 high-risk participants entering in the PREDIMED trial. They were assigned to a low-fat control diet, n=379 or to a Mediterranean-style (Med) diet groups, one with high intake of VOO, n=394 and the other with high intake of nuts, n=366. Anthropometrics measures were measured and total phenols excreted (TPE) was determined in urine by Folin-Ciocalteu assay, expressed as mg gallic acid equivalent (GAE)/ g of creatinine.

Results: After the intervention with Med-VOO and Med-nuts and adjusting by different covariates the participants showed a significant increases of urinary TPE (24.67 mg GAE/g creatinine, 95% CI 11.62 to 37.72; $P<0.001$ and 22.05 mg GAE/g creatinine, CI 8.89 to 35.22; $P=0.001$, respectively), compared with control diet. After adjusted by all possible covariates, participants with the same systolic BP at baseline, after the intervention with Med-VOO and Med-nuts had a reduction statistically significant of -3.43 mm Hg (CI -5.60 to -1.26; $P=0.002$) and -2.27 mm Hg (CI -4.45 to -0.09; $P=0.042$) respectively, compared with control diet. In this model, participants with the same diastolic BP at baseline, after the intervention with Med-VOO and Med-nuts have a reduction of -1.38 (CI -2.48 to -0.28; $P = 0.01$) and -0.70 mm Hg (CI -1.80 to 0.41; $P = 0.22$) respectively, compared with control diet, being statistically significant only in the first comparison.

Conclusions: A one-year intervention with a traditional Med-diet supplemented with either VOO or nuts increases total polyphenols excreted in urine samples from elderly participants, and decreases systolic and diastolic BP. Both Med-diets exhibited beneficial effects on cardiovascular risk factors.

Introduction

Hypertension is a major public health problem and a leading cause of death and disability in developing countries; one-quarter of the world's adult population has hypertension, affecting approximately 1 billion individuals, causing 7.6 million premature deaths and 6% of all cases of disability-adjusted life years worldwide [1]. The prevention is at the top of the public health agenda, the response to this challenge should drive greater promotion of awareness efforts, studies of risk factors for hypertension, and understanding of the impact of lifestyle changes. These efforts have the potential to reduce the emergence or lessen the toll of hypertension and its complication in many parts of the developing world.

The first step in its management of hypertension is to follow a healthy diet such as the traditional Mediterranean diet (TMD) [2] or the DASH (Dietary-Approaches-to-Stop-Hypertension) diets [3] and/or to improve lifestyle, for instance, by reducing body weight and increasing physical activity [4]. The Med and DASH diets are both rich in fruits and vegetables (F&V), which have been considered rich sources of phytochemicals, and are inversely associated with high blood pressure (BP) [5;6].

Adherences to the TMD in numerous epidemiological studies have demonstrated an inverse association with a reduction in coronary heart diseases; this protective effect has been attributed in part, to the richness of this diet in antioxidants [7]. Polyphenol-rich foods such as cocoa, F&V, tea, olive oil and wine and the risk of overall mortality or cardiovascular disease have been inverse association in numerous epidemiological studies [5;8-12]; high fish consumption, and low-fat dairy products, may also reduce the risk of hypertension [4;13-17]. Whereas a high consumption of refined cereals or meat/meat products is associated with a higher cardiovascular risk [4;5;18].

Olive oil is the main natural fat in the Mediterranean diet [7], the virgin olive oil (VOO) has the highest antioxidant phenolic content compared with other olive oils [19]; nuts are also typical from TMD, they are rich sources of nutrients and antioxidant phytochemicals [20].

In a previous study by our group polyphenol intake, assessed via total polyphenols excreted (TPE) in urine, was negatively associated with BP levels in an elderly Mediterranean population at high cardiovascular risk [21]. We undertook, therefore, a substudy within a larger clinical trial, the PREDIMED study, in order to evaluate whether an intervention during one year with two TMD supplemented with VOO or nuts and compared with a control diet, increased the excretion of total polyphenols (TPs) in spot urine samples. These measures were associated with average systolic and diastolic BP. To our knowledge this is the first intervention study to assess this relationship using biochemical analysis of TPs in spot urine samples, and directly measuring BP in a large sample of elderly individuals at high cardiovascular risk.

Materials and methods

Subjects

The PREDIMED (*PRE*vencción con *Di*eta *MEDiterránea*) study is a large, parallel-group, multicenter, randomized, controlled clinical trial of 5-year duration aimed to assess the effects of the Med-Diet on the primary prevention of cardiovascular disease (www.predimed.org; ISRCTN35739639). The detailed

recruitment method and study protocol have been described previously [2]. Up to December 2009, this trial includes 7234 high-risk participant allocated to three interventions: Mediterranean diet plus virgin olive oil (Med-VOO), Mediterranean diet plus nuts (Med-nuts) and control diet (low-fat diet).

From November-2007 to July-2009, we selected 1170 potential participants in primary health centres affiliated with seven different places in Spain. Eligible participants were community-dwelling men aged 55 to 80 years and women aged 60 to 80 years, who were free of cardiovascular disease at baseline and fulfilled at least one of the following two criteria: (1) type-2 diabetes mellitus and/or (2) three or more coronary heart disease (CHD) risk factors: hypertension (BP>140/90 mmHg or treatment with antihypertensive medication), dyslipidemia, obesity or overweight, smoking or family history of early-onset CHD. For the exclusion criteria was taken into account a history of CHD, any severe chronic illness, alcohol or drug addiction, history of food allergy or intolerance to olive oil or nuts, or low disposition to change their eating habits [22]. The participants provided written informed consent and the study protocol was approved by the Institutional Review Boards of the participating centres. This trial has been registered with the International Standard Randomised Controlled Trial Number (ISRCTN) 35739639.

Assessment and intervention

All participants completed at baseline a validated semiquantitative food frequency questionnaire (FFQ) with 136 items [23], the validated Spanish version [24] of the Minnesota Leisure Time Physical Activity Questionnaire, and a 47-item questionnaire about education, lifestyle, history of illnesses and medication use. Trained dietitians were responsible for all aspects of the intervention. Participants in both Med-diet intervention groups were given personalized advice for dietary changes directed to achieve a diet close to the traditional Med-diet. The participants from the Med-VOO received free virgin olive oil and the Med-nuts group free sachets of walnuts, hazelnuts and almond. Participants assigned to the control diet received personal advice together with a leaflet with written recommendation to follow a low-fat diet [25]. In the tree group, the general guideline recommendation included increasing the intake of fruit, vegetable, legumes, fish or seafood, and white meat; negatively recommendation included eliminating and/or reduction of detrimental foods (red and processed meats, fat-rich dairy products, commercial pastries, snacks and sugar-sweetened beverages). One week after the inclusion, the dietitian delivered in separate group session (up to 20 participants by group) during 1 hour; each group session consisted of information talk and provision of written material with description of typical Mediterranean foods and seasonal shopping list, meal plan and cooking recipes [26].

Clinical measurements

Trained nurses measured the height and weight with a wall-mounted stadiometer and calibrated scales, respectively, as well as the blood pressure in triplicate with a validated semi-automatic oscillometer (Omron HEM-705CP [27]; Hoofddorp, The Netherlands). Waist circumference was measured midway between the lowest rib and the iliac crest with an anthropometric tape. Urine and blood samples were obtained after an overnight fast; they were coded, shipped to a central laboratory and frozen at -80°C until analysis. Analyses

Resultats

determined in frozen samples from the participants of whole serum or plasma as appropriate were blood glucose level by the glucose-oxidase method; serum insulin level by radioimmunoassay, cholesterol and triglyceride level by enzymatic procedures; HDL cholesterol level after precipitation with phosphotungstic acid and magnesium chloride. We performed all the analysis by duplicate. Analysis of total polyphenols and creatinine in urine samples were performed following the procedure described by Medina-Remón *et al* [28]; total phenols excreted (TPE) was expressed as mg gallic acid equivalent (GAE)/ g of creatinine.

Total polyphenol consumption from plant food and beverages (mg/ g fresh matter) was quantified according to Saura-Calixto F *et al*. [29] and Brat P *et al*. [30], from the data of FFQ. Creatinine-estimated glomerular filtration rate (GFR) was calculated using the Modification of Diet in Renal Disease (MDRD) formula in all participants [31]. Energy and nutrients intake were derived from Spanish food composition tables [32].

For creatinine, 3 μ L of urine was mixed with 60 μ L of aqueous picric acid solution (1%) and 5 μ L of sodium hydroxide (10%) in the thermo microtiter 96-well plate. After shaking, the mixture was left for 15 min in dark at room temperature; after the reaction time, 232 μ L of Milli-Q water was added and the absorbance was measured at 500 nm in the UV/VIS spectrophotometers.

Statistical analysis.

Analyses were performed using SPSS software v16.0 (Chicago, USA). Baseline characteristics of the participants were expressed as means or percentages and standard deviations (SD). Variables were examined for normality and skewness (Kolmogorov and Levene tests). At the baseline ANOVA-one factor was used for continuous variables and χ^2 -test for categorical variables. For baseline level and 1-year changes, no-parametric Wilcoxon test were used. For the comparison of changes in risk factors, the average of two baseline measurements was used as the baseline value and the average of the two one-year measurements was used as the final variable. We used the General Linear Model (GLM) approach to ANCOVA to determine the effects of both interventions with Mediterranean diet (the factors) compared with control diet, on the systolic and diastolic BP, using the baseline measures as covariates and others as additional covariates. Model 1 was unadjusted; Model 2, adjusted by sex, age, weight, smoking status, physical activity, and educational level at baseline; Model 3 was adjusted as in Model 2 plus medication intake: ACE inhibitor, diuretics, statins (hypolipidemic drugs), insulin, oral hypoglycemic drugs, aspirin or other antiplatelet drug supplements taken in the last month, sodium and potassium intake and GFR. *P*-values<0.05 (two-tailed) were considered to be significant. Confidence intervals (CI) are expressed as 95% CI. Diagnostics for the detection of outliers, multicollinearity, homoscedasticity, and the normality and independence of errors were assessed when it was appropriate.

Results

Participant characteristics

We excluded 31 of 1170 eligible participants before randomization for different reasons: no meeting inclusion criteria (n=9), not accepting to change their

dietary habits (n=6), food allergies (n=5) or refusing to participate (n=11). Baseline characteristics of the tree group are shown in **Table 1** (511 men and 628 women); most of them were overweight or obese (>90%), the means of systolic and diastolic blood pressure were high and very similar in the tree groups. More than the 75 % of the patients had hypertension; more than 42% are diabetics, more than 63% had dyslipidemia and more than 15% are current smokers and had family history of cardiovascular disease. Most of them taken angiotensin-converting enzyme (ACE) and diuretic and statins (hypolipidemic drugs); more than one-quarter taken oral hypoglycemic drugs and aspirin or other antiplatelet drugs. Subsequent data refer only to the 1139 participants who completed the study.

Food, energy and nutrient intake, Mediterranean diet score.

The main dietary changes were the high increase in consumption of virgin olive oil and total nuts in the Med-VOO and Med-nut groups respectively (**Table 2**), because they were provided of this type of foods. **Table 3** shows the average daily intake of energy and nutrients provided by VOO and mixed nuts. The significant decrease of refined olive oil in Med-VOO and control-diet groups indicated that participants replaced this oil by the VOO recommended. Total nuts were significantly reduced in the control-diet. Participants in the tree group increased significantly the intake of fruit, and decreased the intake of meat or meat products and pastries, cakes or sweets. Participant from the two Mediterranean diet groups increased significantly the intake of vegetables, legumes, fish or seafood; the others food groups remained unchanged. In all groups the Mediterranean diet score increased significantly.

Total energy intake was increased in the two Mediterranean groups (**Table 4**) and it was reduced in the control-diet; fundamentally due to the changes in the oils and nuts intake from baseline. In both Mediterranean diets we observed an significantly increase in the fibre, total fat, monounsaturated fat acids (MUFA), polyunsaturated fat acids (PUFA), linoleic, linolenic and marine n-3 fatty acids intake; magnesium, potassium and vitamins A, C and E also increased from baseline. Saturated fat acid (SFA), total cholesterol and sodium intake were significantly reduced in all groups; this last nutrient reduction was nonsignificantly in the Med-nuts group but total energy intake, protein, total fat, SFA, PUFA, cholesterol, linolenic, sodium and vitamin E were significantly decreased from baseline; estimated energy expenditure from physical activity was significantly increase in this group.

Urinary polyphenol analysis and cardiovascular risk factors

Table 5 shows the changes from baseline, in total polyphenols, cardiovascular risk factors and anthropometric measurements. Urinary total polyphenols excreted, expressed as mg GAE/ g creatinine, were statistically significant increased in two Mediterranean diets; in the control diet the increase was not statistically significant (**figure 1**). The weight and BMI have not differences from baseline in any group or between groups. Participants in both Mediterranean diets had decreased systolic and diastolic blood pressure (**figure 1**), blood glucose levels, total cholesterol, cholesterol-LDL and triglyceride and increased cholesterol-HDL levels, some of these changes from baseline were statistically significant and other were not, as is possible to see in these table. Participants

Resultats

in the control diet have not differences from baseline statistically significant, except for the decreased waists measured.

It was measured the covariate analysis for the differences in TPs excreted at one-year respect to baseline, delta excretion as the dependent variables, intervention groups (Med-VOO, Med-nuts and control diet) as the fixed factors and other additional covariates. After the intervention with Med-VOO and Med-nuts and adjusting by different covariates these participants had an increases statistically significant of 24.673 (CI: 11.623 to 37.722; $P<0.001$) and 22,054 (CI: 8.890 to 35.217; $P=0.001$) respectively, compared with Control diet.

In the covariate analysis with systolic and diastolic BP at one-year as the dependent variables, intervention groups (Med-VOO, Med-nuts and control diet) as the fixed factors, baseline systolic and diastolic BP as covariates and other additional covariates (**Table 6**); we observed the effects of different interventions with Mediterranean diet respect to the control diet. In this table Non-standardized coefficient (B) represent the differences of the Medi-diet interventions with the control diet. In the model 3, after adjusted by all possible covariates, participants with the same systolic BP at baseline, after the intervention with Med-VOO and Med-nuts had a reduction statistically significant of 3.429 and 2.155 respectively, compared with Control diet. In this model, participants with the same diastolic BP at baseline, after the intervention with Med-VOO and Med-nuts have a reduction of 1.382 and 0.696 respectively, compared with control diet, being statistically significant only in the first comparison.

Discussion

In this cohort of elderly participants, we observed statistically significant increases in total polyphenols excreted in spot urine samples and statistically significant decrease in systolic and diastolic BP, after the intervention with traditional Mediterranean diet supplemented with both, VOO and nuts, compared with the control diet, after adjustment for potential confounders. In addition, other cardiovascular risk factors like LDL-cholesterol, total cholesterol, total triglycerides and fasting glucose were decreased in both TMD; as well as total HDL-cholesterol were increased.

Polyphenols are the most abundant antioxidants in human diets. They are secondary metabolites of plants. F&V and their derivates food and beverages, constitute the main sources of polyphenols [33]. VOO contains considerable amount of polyphenols that have a great effect on the stability and nutritional characteristics of the product. Some of the most representative simple phenols are hydroxytyrosol and tyrosol. However, phenolic compounds are removed when the oil is refined [34]. The phenolic content of VOO is influenced by the variety, location, degree of ripeness and the type of oil extraction procedure used [35; 36]. Nuts contain bioactive compounds that explain their multiple cardiovascular benefits, phytochemicals such as phytosterols, phenolic compounds, and arginine [37]. However, the biological effects of these compounds depends on their bioavailability, their kinetics and exposure time [38].

The most common polyphenols in the human diet are not necessarily the most active *in vivo* [39], most dietary polyphenols (75–99%) are not found in urine, and the quantities detected intact vary from one phenolic compound to another

[40]. This fact may be due to their reduced absorption through the gut barrier, their excretion to the bile or their metabolism by the colonic microflora or our own tissues. To acquire high plasma concentrations requires repeated ingestion of polyphenol-rich foods [41].

Remarkably, in all nuts, most of the antioxidants are located in the pellicle or outer soft shell, and more than 50% of them are lost when the skin is removed [42]. This fact, rarely taken into consideration in prior feeding trials with nuts, should not be overlooked in future studies. Walnuts are an exception, because they are almost always consumed as the raw product with skins. Recent studies have shown that almond [43] and peanut [44] skins are very high in antioxidants. A pooled analysis of 4 U.S. epidemiologic studies showed that subjects in the highest intake group for nut consumption had an approximately 35% reduced risk of CHD incidence, and the reduction in total CHD death was due primarily to a decrease in sudden cardiac death. Evidence from others clinical studies consistently shows a beneficial effect on CHD risk factors.

Data from the Nurses' Health Study indicate that frequent nuts consumption is associated with a reduced risk of developing cardiovascular disease. Epidemiologic and clinical trial evidence has demonstrated consistent benefits of nuts and peanut consumption on CHD risk and associated risk factors [37]. Nuts are a rich source of many antioxidants that protect the PUFA in vivo against oxidative modification, it have been examined in several clinical trials. Berry *et al.* [45] showed that oxidation of plasma and LDL lipids in healthy volunteers were less after an almond diet compared with a low-fat diet. Jenkins *et al.* [46], in a dose-response study comparing 2 doses of almonds with a low-fat diet in hyperlipidemic subjects. The full-dose almonds produced the greatest reduction in levels of blood lipids. Significant reductions from baseline were seen on both half- and full-dose almonds for LDL cholesterol ($4.4 \pm 1.7\%$, $P=0.018$, and $9.4 \pm 1.9\%$, $P<0.001$, respectively) and LDL:HDL cholesterol ($7.8 \pm 2.2\%$, $P=0.001$, and $12.0 \pm 2.1\%$, $P<0.001$, respectively) and on full-dose almonds alone for lipoprotein ($7.8 \pm 3.5\%$, $P=0.034$) and oxidized LDL concentrations ($14.0 \pm 3.8\%$, $P<0.001$), with no significant reductions on the control diet.

However, Hyson *et al.* [47] conducted a study to compare the effects of whole-almond vs. almond oil consumption on plasma lipids and LDL oxidation in healthy men and women, for 6 week periods. The effects of whole almonds and almond oil on plasma lipids did not differ compared with baseline; plasma triglyceride, total and LDL cholesterol significantly decreased, 14, 4 and 6% respectively, whereas HDL cholesterol increased 6%. Nuts, including peanuts, have been recognized as having the potential to improve the blood lipid profile, and, in cohort studies, nuts consumption has been associated with a reduced risk of CHD [48].

The concentrations of oleic acid and antioxidants (phenolic compounds and vitamin E) in plasma and LDL were compared in thirty healthy volunteers after consumption of three similar olive oils, but with differences in their phenolic content. After the administration of the oils during three periods of 3 weeks separated by a 2-week washout period, all three olive oils caused an increase in plasma and LDL oleic acid content. The concentration of phenolic compounds in LDL was directly correlated with the phenolic concentration in the olive oils. The increase in the phenolic content of LDL could account for the increase of

Resultats

the resistance of LDL to oxidation, and the decrease of the in vivo oxidized LDL [35].

In similarity to our findings, others sub-study [49] within the PREDIMED study have found that the Mediterranean diet enriched with nuts or VOO, after 3-month interventions decreased oxidized LDL level $[-7.3 \text{ U/L (95\% CI, } -11.2 \text{ to } -3.3)]$ or $[-10.6 \text{ U/L (95\% CI, } -14.2 \text{ to } -6.1)]$ respectively, compared with a control diet with not significantly changes $[-2.9 \text{ U/L (95\% CI, } -7.3 \text{ to } 1.5)]$. In other sub-study [50] it was compared the 1-year effect of 2 behavioural interventions to implement the Mediterranean diet vs. advice on a low-fat diet on metabolic syndrome status in 1224 participants. One-year prevalence of metabolic syndrome was reduced by 6.7%, 13.7%, and 2.0% in the Med-VOO, Med-nuts, and control diet groups, respectively. After adjustment for different factors, the odds ratios for reversion of metabolic syndrome were 1.3 (95% CI, 0.8-2.1) for the Med -VOO group and 1.7 (1.1-2.6) for the Med-nuts group compared with the control diet group.

Estruch *et al.* [2] compared the short-term effects of these Mediterranean diets, after 3 months of intervention. Compared with the low-fat diet, the mean changes in the Med-VOO and the Med-nuts group were $-0.39 \text{ mmol/L (95\% CI, } -0.70 \text{ to } -0.07 \text{ mmol/L)}$ and $-0.30 \text{ mmol/L (CI, } -0.58 \text{ to } -0.01 \text{ mmol/L)}$, respectively, for plasma glucose levels; $-5.9 \text{ mm Hg (CI, } -8.7 \text{ to } -3.1 \text{ mm Hg)}$ and $-7.1 \text{ mm Hg (CI, } -10.0 \text{ to } -4.1 \text{ mm Hg)}$, respectively, for systolic BP; and $-0.38 \text{ (CI, } -0.55 \text{ to } -0.22)$ and $-0.26 \text{ (CI, } -0.42 \text{ to } -0.10)$, respectively, for the cholesterol-high-density lipoprotein cholesterol ratio. Mediterranean diet, especially rich in VOO, is associated with higher levels of plasma antioxidant capacity. Plasma TAC is related to a reduction in body weight after 3 years of intervention in a high cardiovascular risk population with a Mediterranean-style diet rich in VOO. Plasma TAC in the control, VOO and nuts groups was 2.01 ± 0.15 , 3.51 ± 0.14 and $3.02 \pm 0.14 \text{ mM Trolox}$, respectively [51]. Consistently with these previous studies, we have observed that adherence to a traditional Med-diet may be able to reduce the cardiovascular risk factors.

Finally, the relationship between overall food patterns rich in polyphenols, such as DASH and Mediterranean diets, and BP has been also examined. The DASH diet is widely promoted in the USA for the prevention and treatment of hypertension [18]. This dietary pattern has been assessed in a free-living UK population [52] and systolic and diastolic BP decreased significantly ($P < 0.05$) by 4.6 and 3.9 mmHg, respectively, in those who followed a DASH-style diet. After 3 weeks on the DASH diet, systolic and diastolic BP in obese hypertensives was lower than on the usual diet ($-7.6 \pm 1.4/ -5.3 \pm 1.4 \text{ mmHg}$, $P < 0.001/0.02$) and the usual diet supplemented with potassium, magnesium and fibre ($-6.2 \pm 1.4/ -3.7 \pm 1.4 \text{ mmHg}$, $P < 0.005/0.06$), whereas BP was not significantly different in the usual and supplemented diets, BP values were not different among these three diets in lean normotensives [53]. In another study [54] with 27 men and women who followed a DASH or a control diet, the DASH-diet group showed a significant reduction in systolic ($P < 0.001$) and diastolic ($P = 0.005$) BP.

Some epidemiological evidence supports that a diet rich in F&V may help prevent BP from increasing and help decrease elevated BP levels for those with high normal BP or hypertension [55]. In the SUN study [5], the prevalence of non-previously diagnosed hypertension was inversely associated with F&V

consumption in a Mediterranean population with a very high intake of both fat- and plant-derived foods. In the Nurses' Health Study, intake of F&V was also inversely associated with systolic and diastolic BP [56]. In other types of foods, flavonoid-rich juice intake [57] and flavonoid-rich dark chocolate significantly reduced BP in a well-designed double-blind cross-over trial [11]. The intake of consecutive daily doses of 100 g of polyphenol-rich dark chocolate (500 mg of polyphenols) decreased both systolic and diastolic BP [58]. A reduction in total and saturated fatty acid intake and interventions with extra-virgin olive oil favourably affected BP in hypertensive patients [59].

All these epidemiological evidence support the view that polyphenol-rich diets may prevent BP from increasing and may help to decrease elevated BP levels in hypertensive subjects. However, our previous study [21] has attempted to correlate the biomarker of TP intake, determined in spot urine samples, with BP measurements or with the prevalence of hypertension. Taking into account that greater excretion of polyphenols in urine is determined by high TP consumption, it was suggested that the inverse association observed between the objectively measured TPE in urine samples with BP may be related to a favourable effect of TP intake on preventing raised BP levels. In the current study performed in a Spanish high-risk population, we observed a similar effect with a Med-diet supplemented with polyphenol-rich foods: VOO or nuts; our results also add to the increasing evidence that polyphenol-rich diets decrease BP.

Our study has several limitations. First, because our subjects were elderly peoples at high risk for cardiovascular disease, our results may not be generalizable to other populations who may otherwise be at risk for cardiovascular disease. However, since most were hypertensive subjects, our results confirm the usefulness of polyphenol-rich diets in the management of hypertension. A second limitation of our study is the study population, it is limited by a relatively smaller sample size in comparison to other published reports, hence it is possible that this lack of significance reflects inadequate statistical power, rather than a true negative result.

In conclusion, these results suggest that a Mediterranean diet supplemented with VOO or nuts are positively correlated with an increase of total polyphenols excreted in spot urine samples. These statistically significant increases in TPE in spot urine were negatively associated with systolic and diastolic BP levels. Thus, a high intake of polyphenol-rich diet may help to decrease BP in elderly hypertensive populations in order to lower their cardiovascular risk. We conclude that there is justification to consider the inclusion of nuts and VOO in the diets of individuals with high cardiovascular risk in order to reduce this risk. Further studies are needed to clarify whether antioxidants contribute to this apparent beneficial health effect of Mediterranean diet with nuts and VOO.

Acknowledgments

We would like to thank all of the volunteers involved in the PREDIMED study for their valuable cooperation. The authors express their gratitude for financial support from CICYT's (AGL2005-05597; AGL2006-14228-C03-02; AGL2007-66638-C02-01 and AGL2009-13906-C02-01), RETICS RD06/0045/0003 from the Spanish Ministry of Science and Innovation (MICINN) and grant PI070240 from Instituto de Salud Carlos III, Spain. The CIBERObn CB06/03 is an initiative from the Instituto de Salud Carlos III, Spain. This work has been funded in part

Resultats

by the CONSOLIDER INGENIO 2010 Programme, FUN-C-FOOD CSD2007-063 and also from MICINN. None of the funding sources played a role in the design, collection, analysis and interpretation of data; in the writing of the report or in the decision to submit the paper for publication. None of the authors have any conflict of interest. A. M-R received support from the Generalitat of Catalonia for training of researchers.

Reference List

- [1] C.M.Lawes, H.S.Vander, and A.Rodgers, Global burden of blood-pressure-related disease, 2001, *Lancet* 371 (2008) 1513-1518.
- [2] R.Estruch, M.A.Martinez-Gonzalez, D.Corella, J.Salas-Salvado, V.Ruiz-Gutierrez, M.I.Covas, M.Fiol, E.Gomez-Gracia, M.C.Lopez-Sabater, E.Vinyoles, F.Aros, M.Conde, C.Lahoz, J.Lapetra, G.Saez, and E.Ros, Effects of a Mediterranean-style diet on cardiovascular risk factors: a randomized trial, *Ann.Intern.Med.* 145 (2006) 1-11.
- [3] L.J.Appel, T.J.Moore, E.Obarzanek, W.M.Vollmer, L.P.Svetkey, F.M.Sacks, G.A.Bray, T.M.Vogt, J.A.Cutler, M.M.Windhauser, P.H.Lin, N.Karanja, D.Simons-Morton, M.McCullough, J.Swain, P.Steele, M.A.Evans, E.R.Miller, D.W.Harsha, and The DASH Collaborative Research Group, A Clinical Trial of the Effects of Dietary Patterns on Blood Pressure, *N Engl J Med* 336 (1997) 1117-1124.
- [4] G.Mancia, B.G.De, A.Dominiczak, R.Cifkova, R.Fagard, G.Germano, G.Grassi, A.M.Heagerty, S.E.Kjeldsen, S.Laurent, K.Narkiewicz, L.Ruilope, A.Rynkiewicz, R.E.Schmieder, H.A.Boudier, A.Zanchetti, A.Vahanian, J.Camm, C.R.De, V.Dean, K.Dickstein, G.Filippatos, C.Funck-Brentano, I.Hellemans, S.D.Kristensen, K.McGregor, U.Sechtem, S.Silber, M.Tendera, P.Widimsky, J.L.Zamorano, S.Erdine, W.Kiowski, E.gabiti-Rosei, E.Ambrosioni, L.H.Lindholm, M.Viigimaa, S.Adamopoulos, E.gabiti-Rosei, E.Ambrosioni, V.Bertomeu, D.Clement, S.Erdine, C.Farsang, D.Gaita, G.Lip, J.M.Mallion, A.J.Manolis, P.M.Nilsson, E.O'Brien, P.Ponikowski, J.Redon, F.Ruschitzka, J.Tamargo, Z.P.van, B.Waeber, and B.Williams, 2007 Guidelines for the Management of Arterial Hypertension: The Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC), *J.Hypertens.* 25 (2007) 1105-1187.
- [5] A.Alonso, F.C.de la, A.M.Martin-Arnau, I.J.de, J.A.Martinez, and M.A.Martinez-Gonzalez, Fruit and vegetable consumption is inversely associated with blood pressure in a Mediterranean population with a high vegetable-fat intake: the Seguimiento Universidad de Navarra (SUN) Study, *Br.J.Nutr.* 92 (2004) 311-319.
- [6] K.E.Harnden, K.N.Frayn, and L.Hodson, Dietary Approaches to Stop Hypertension (DASH) diet: applicability and acceptability to a UK population, *J.Hum.Nutr.Diet.* 23 (2010) 3-10.
- [7] A.Trichopoulou, T.Costacou, C.Bamia, and D.Trichopoulos, Adherence to a Mediterranean diet and survival in a Greek population, *N.Engl.J.Med.* 348 (2003) 2599-2608.

- [8] A.Agudo, L.Cabrera, P.Amiano, E.Ardanaz, A.Barricarte, T.Berenguer, M.D.Chirlaque, M.Dorronsoro, P.Jakszyn, N.Larranaga, C.Martinez, C.Navarro, J.R.Quiros, M.J.Sanchez, M.J.Tormo, and C.A.Gonzalez, Fruit and vegetable intakes, dietary antioxidant nutrients, and total mortality in Spanish adults: findings from the Spanish cohort of the European Prospective Investigation into Cancer and Nutrition (EPIC-Spain), *Am.J.Clin.Nutr.* 85 (2007) 1634-1642.
- [9] M.I.Covas, M.Fito, J.Marrugat, E.Miro, M.Farre, T.R.de la, E.Gimeno, M.C.Lopez-Sabater, R.Lamuela-Raventos, and de la Torre-Boronat MC, [Coronary disease protective factors: antioxidant effect of olive oil], *Therapie* 56 (2001) 607-611.
- [10] M.I.Covas, K.Nyyssonen, H.E.Poulsen, J.Kaikkonen, H.J.Zunft, H.Kiesewetter, A.Gaddi, T.R.de la, J.Mursu, H.Baumler, S.Nascetti, J.T.Salonen, M.Fito, J.Virtanen, J.Marrugat, and EUROLIVE Study Group, The effect of polyphenols in olive oil on heart disease risk factors: a randomized trial, *Ann.Intern.Med.* 145 (2006) 333-341.
- [11] D.Grassi, S.Necozione, C.Lippi, G.Croce, L.Valeri, P.Pasqualetti, G.Desideri, J.B.Blumberg, and C.Ferri, Cocoa reduces blood pressure and insulin resistance and improves endothelium-dependent vasodilation in hypertensives, *Hypertension* 46 (2005) 398-405.
- [12] C.Manach, A.Mazur, and A.Scalbert, Polyphenols and prevention of cardiovascular diseases, *Curr.Opin.Lipidol.* 16 (2005) 77-84.
- [13] L.Wang, J.E.Manson, J.E.Buring, I.M.Lee, and H.D.Sesso, Dietary intake of dairy products, calcium, and vitamin D and the risk of hypertension in middle-aged and older women, *Hypertension* 51 (2008) 1073-1079.
- [14] A.Alonso, J.J.Beunza, M.Delgado-Rodriguez, J.A.Martinez, and M.A.Martinez-Gonzalez, Low-fat dairy consumption and reduced risk of hypertension: the Seguimiento Universidad de Navarra (SUN) cohort, *Am.J Clin.Nutr.* 82 (2005) 972-979.
- [15] D.Q.Bao, T.A.Mori, V.Burke, I.B.Puddey, and L.J.Beilin, Effects of dietary fish and weight reduction on ambulatory blood pressure in overweight hypertensives, *Hypertension* 32 (1998) 710-717.
- [16] T.A.Mori, D.Q.Bao, V.Burke, I.B.Puddey, G.F.Watts, and L.J.Beilin, Dietary fish as a major component of a weight-loss diet: effect on serum lipids, glucose, and insulin metabolism in overweight hypertensive subjects, *Am.J Clin.Nutr.* 70 (1999) 817-825.
- [17] M.F.Engberink, M.A.Hendriksen, E.G.Schouten, F.J.van Rooij, A.Hofman, J.C.Witteman, and J.M.Geleijnse, Inverse association between dairy intake and hypertension: the Rotterdam Study, *Am.J Clin.Nutr.* 89 (2009) 1877-1883.
- [18] L.J.Appel, T.J.Moore, E.Obarzanek, W.M.Vollmer, L.P.Svetkey, F.M.Sacks, G.A.Bray, T.M.Vogt, J.A.Cutler, M.M.Windhauser, P.H.Lin, N.Karanja, D.Simons-Morton, M.McCullough, J.Swain, P.Steele, M.A.Evans, E.R.Miller, D.W.Harsha, and The DASH Collaborative Research Group, A Clinical Trial of the Effects of Dietary Patterns on Blood Pressure, *N Engl J Med* 336 (1997) 1117-1124.

Resultats

- [19] C.de la Torre-Carbot, O.Jauregui, E.Gimeno, A.I.Castellote, R.M.Lamuela-Raventos, and M.C.Lopez-Sabater, Characterization and quantification of phenolic compounds in olive oils by solid-phase extraction, HPLC-DAD, and HPLC-MS/MS, *J.Agric.Food Chem.* 53 (2005) 4331-4340.
- [20] P.M.Kris-Etherton, G.Zhao, A.E.Binkoski, S.M.Coval, and T.D.Etherton, The effects of nuts on coronary heart disease risk, *Nutr.Rev.* 59 (2001) 103-111.
- [21] A.Medina-Remón, R.Zamora-Ros, M.Rotchés-Ribalta, C.Andres-Lacueva, M.A.Martínez-González, M.I.Covas, D.Corella, J.Salas-Salvadó, E.Gómez-Gracia, V.Ruiz-Gutiérrez, F.J.García de la Corte, M.Fiol, M.A.Pena, G.T.Saez, E.Ros, L.Serra-Majem, X.Pinto, J.Warnberg, R.Estruch, and R.M.Lamuela-Raventos, Total polyphenol excretion and blood pressure in subjects at high cardiovascular risk, *Nutrition, Metabolism and Cardiovascular Diseases* In Press, Corrected Proof (2010).
- [22] C.R.Nigg, P.M.Burbank, C.Padula, R.Dufresne, J.S.Rossi, W.F.Velicer, R.G.Laforge, and J.O.Prochaska, Stages of change across ten health risk behaviors for older adults, *Gerontologist* 39 (1999) 473-482.
- [23] J.M.Martin-Moreno, P.Boyle, L.Gorgojo, P.Maisonneuve, J.C.Fernandez-Rodriguez, S.Salvini, and W.C.Willett, Development and validation of a food frequency questionnaire in Spain, *Int.J.Epidemiol.* 22 (1993) 512-519.
- [24] R.Elosua, J.Marrugat, L.Molina, S.Pons, and E.Pujol, Validation of the Minnesota Leisure Time Physical Activity Questionnaire in Spanish men. The MARATHOM Investigators, *Am.J.Epidemiol.* 139 (1994) 1197-1209.
- [25] R.M.Krauss, R.H.Eckel, B.Howard, L.J.Appel, S.R.Daniels, R.J.Deckelbaum, J.W.Erdman, Jr., P.Kris-Etherton, I.J.Goldberg, T.A.Kotchen, A.H.Lichtenstein, W.E.Mitch, R.Mullis, K.Robinson, J.Wylie-Rosett, J.S.St, J.Suttie, D.L.Tribble, and T.L.Bazzarre, AHA Dietary Guidelines: revision 2000: A statement for healthcare professionals from the Nutrition Committee of the American Heart Association, *Circulation* 102 (2000) 2284-2299.
- [26] I.Zazpe, A.Sanchez-Tainta, R.Estruch, R.M.Lamuela-Raventos, H.Schroder, J.Salas-Salvado, D.Corella, M.Fiol, E.Gomez-Gracia, F.Aros, E.Ros, V.Ruiz-Gutierrez, P.Iglesias, M.Conde-Herrera, and M.A.Martinez-Gonzalez, A large randomized individual and group intervention conducted by registered dietitians increased adherence to Mediterranean-type diets: the PREDIMED study, *J Am.Diet.Assoc.* 108 (2008) 1134-1144.
- [27] P.Iglesias-Bonilla, E.Mayoral-Sánchez, J.Lapetra-Peralta, M.Iborra-Oquendo, F.Villalba-Alcalá, and A.Cayuela-Domínguez, Validación de dos sistemas de automedida de presión arterial, modelos OMRON HEM 705 CP y OMRON MI (HEM 422C2-E), *Atención Primaria* 30 (2002) 22-28.

- [28] A.Medina-Remón, A.Barrionuevo-González, R.Zamora-Ros, C.Andres-Lacueva, R.Estruch, M.A.Martínez-González, J.Diez-Espino, and R.M.Lamuela-Raventos, Rapid Folin-Ciocalteu method using microtiter 96-well plate cartridges for solid phase extraction to assess urinary total phenolic compounds, as a biomarker of total polyphenols intake, *Analytica Chimica Acta* 634 (2009) 54-60.
- [29] F.Saura-Calixto and I.Goni, Antioxidant capacity of the Spanish Mediterranean diet, *Food Chemistry* 94 (2006) 442-447.
- [30] P.Brat, S.George, A.Bellamy, C.L.Du, A.Scalbert, L.Mennen, N.Arnault, and M.J.Amiot, Daily polyphenol intake in France from fruit and vegetables, *J.Nutr.* 136 (2006) 2368-2373.
- [31] A.S.Levey, J.Coresh, T.Greene, L.A.Stevens, Y.L.Zhang, S.Hendriksen, J.W.Kusek, and L.F.Van, Using standardized serum creatinine values in the modification of diet in renal disease study equation for estimating glomerular filtration rate, *Ann.Intern Med* 145 (2006) 247-254.
- [32] J.Mataix. Tabla de composición de alimentos (Food composition tables). 2003. Universidad de Granada: Granada, Spain. Ref Type: Generic
- [33] C.Manach, A.Scalbert, C.Morand, C.Remesy, and L.Jimenez, Polyphenols: food sources and bioavailability, *Am.J Clin.Nutr.* 79 (2004) 727-747.
- [34] M.J.Tovar, M.J.Motilva, and M.P.Romero, Changes in the Phenolic Composition of Virgin Olive Oil from Young Trees (*Olea europaea* L. cv. Arbequina) Grown under Linear Irrigation Strategies, *Journal of Agricultural and Food Chemistry* 49 (2001) 5502-5508.
- [35] E.Gimeno, I.T.-C.de, R.M.Lamuela-Raventos, A.I.Castellote, M.Fito, T.R.de la, M.I.Covas, and M.C.Lopez-Sabater, Changes in the phenolic content of low density lipoprotein after olive oil consumption in men. A randomized crossover controlled trial, *Br.J Nutr* 98 (2007) 1243-1250.
- [36] R.W.Owen, W.Mier, A.Giacosa, W.E.Hull, B.Spiegelhalder, and H.Bartsch, Phenolic compounds and squalene in olive oils: the concentration and antioxidant potential of total phenols, simple phenols, secoiridoids, lignans and squalene, *Food Chem.Toxicol.* 38 (2000) 647-659.
- [37] P.M.Kris-Etherton, F.B.Hu, E.Ros, and J.Sabate, The role of tree nuts and peanuts in the prevention of coronary heart disease: multiple potential mechanisms, *J Nutr.* 138 (2008) 1746S-1751S.
- [38] E.Roura, M.P.Almajano, M.L.Bilbao, C.Andres-Lacueva, R.Estruch, and R.M.Lamuela-Raventos, Human urine: epicatechin metabolites and antioxidant activity after cocoa beverage intake, *Free Radic.Res* 41 (2007) 943-949.
- [39] C.Manach, A.Scalbert, C.Morand, C.Remesy, and L.Jimenez, Polyphenols: food sources and bioavailability, *Am.J Clin.Nutr.* 79 (2004) 727-747.

Resultats

- [40] A.Scalbert and G.Williamson, Dietary intake and bioavailability of polyphenols, *J Nutr.* 130 (2000) 2073S-2085S.
- [41] K.H.van het Hof, S.A.Wiseman, C.S.Yang, and L.B.Tijburg, Plasma and lipoprotein levels of tea catechins following repeated tea consumption, *Proc.Soc.Exp.Biol.Med* 220 (1999) 203-209.
- [42] R.Blomhoff, M.H.Carlsen, L.F.Andersen, and D.R.Jacobs, Jr., Health benefits of nuts: potential role of antioxidants, *Br.J.Nutr.* 96 Suppl 2 (2006) S52-S60.
- [43] M.Urpi-Sarda, I.Garrido, M.Monagas, C.Gomez-Cordoves, A.Medina-Remón, C.Andres-Lacueva, and B.Bartolome, Profile of plasma and urine metabolites after the intake of almond [*Prunus dulcis* (Mill.) D.A. Webb] polyphenols in humans, *J Agric.Food Chem.* 57 (2009) 10134-10142.
- [44] H.Lou, H.Yuan, B.Ma, D.Ren, M.Ji, and S.Oka, Polyphenols from peanut skins and their free radical-scavenging effects, *Phytochemistry* 65 (2004) 2391-2399.
- [45] E.M.Berry, S.Eisenberg, Y.Friedlander, D.Harats, N.A.Kaufmann, Y.Norman, and Y.Stein, Effects of diets rich in monounsaturated fatty acids on plasma lipoproteins--the Jerusalem Nutrition Study. II. Monounsaturated fatty acids vs carbohydrates, *Am J Clin Nutr* 56 (1992) 394-403.
- [46] D.J.Jenkins, C.W.Kendall, A.Marchie, T.L.Parker, P.W.Connelly, W.Qian, J.S.Haight, D.Faulkner, E.Vidgen, K.G.Lapsley, and G.A.Spiller, Dose response of almonds on coronary heart disease risk factors: blood lipids, oxidized low-density lipoproteins, lipoprotein(a), homocysteine, and pulmonary nitric oxide: a randomized, controlled, crossover trial, *Circulation* 106 (2002) 1327-1332.
- [47] D.A.Hyson, B.O.Schneeman, and P.A.Davis, Almonds and Almond Oil Have Similar Effects on Plasma Lipids and LDL Oxidation in Healthy Men and Women, *Journal of Nutrition* 132 (2002) 703-707.
- [48] D.J.Jenkins, F.B.Hu, L.C.Tapsell, A.R.Josse, and C.W.Kendall, Possible benefit of nuts in type 2 diabetes, *J.Nutr.* 138 (2008) 1752S-1756S.
- [49] M.Fito, M.Guxens, D.Corella, G.Saez, R.Estruch, R.de la Torre, F.Frances, C.Cabezas, M.d.C.Lopez-Sabater, J.Marrugat, A.Garcia-Arellano, F.Aros, V.Ruiz-Gutierrez, E.Ros, J.Salas-Salvado, M.Fiol, R.Sola, M.I.Covas, and for the PREDIMED Study Investigators, Effect of a Traditional Mediterranean Diet on Lipoprotein Oxidation: A Randomized Controlled Trial, *Arch Intern Med* 167 (2007) 1195-1203.
- [50] J.Salas-Salvado, J.Fernandez-Ballart, E.Ros, M.A.Martinez-Gonzalez, M.Fito, R.Estruch, D.Corella, M.Fiol, E.Gomez-Gracia, F.Aros, G.Flores, J.Lapetra, R.Lamuela-Raventos, V.Ruiz-Gutierrez, M.Bullo, J.Basora, M.I.Covas, and for the PREDIMED Study Investigators, Effect of a Mediterranean Diet Supplemented With Nuts on Metabolic Syndrome Status: One-Year Results of the PREDIMED Randomized Trial, *Arch Intern Med* 168 (2008) 2449-2458.

- [51] C.Razquin, J.A.Martinez, M.A.Martinez-Gonzalez, M.T.Mitjavila, R.Estruch, and A.Marti, A 3 years follow-up of a Mediterranean diet rich in virgin olive oil is associated with high plasma antioxidant capacity and reduced body weight gain, *Eur J Clin Nutr* 63 (2009) 1387-1393.
- [52] K.E.Harnden, K.N.Frayn, and L.Hodson, Dietary Approaches to Stop Hypertension (DASH) diet: applicability and acceptability to a UK population, *J.Hum.Nutr.Diet.* 23 (2010) 3-10.
- [53] Y.Al-Solaiman, A.Jesri, W.K.Mountford, D.T.Lackland, Y.Zhao, and B.M.Egan, DASH lowers blood pressure in obese hypertensives beyond potassium, magnesium and fibre, *J Hum Hypertens* (2009).
- [54] L.Hodson, K.E.Harnden, R.Roberts, A.L.Dennis, and K.N.Frayn, Does the DASH diet lower blood pressure by altering peripheral vascular function[quest], *J Hum Hypertens* (2009).
- [55] P.K.Whelton, J.He, L.J.Appel, J.A.Cutler, S.Havas, T.A.Kotchen, E.J.Roccella, R.Stout, C.Vallbona, M.C.Winston, and J.Karimbakas, Primary prevention of hypertension: clinical and public health advisory from The National High Blood Pressure Education Program, *JAMA* 288 (2002) 1882-1888.
- [56] A.Ascherio, C.Hennekens, W.C.Willett, F.Sacks, B.Rosner, J.Manson, J.Wittelman, and M.J.Stampfer, Prospective study of nutritional factors, blood pressure, and hypertension among US women, *Hypertension* 27 (1996) 1065-1072.
- [57] N.Reshef, Y.Hayari, C.Goren, M.Boaz, Z.Madar, and H.Knobler, Antihypertensive effect of sweetie fruit in patients with stage I hypertension, *Am.J.Hypertens.* 18 (2005) 1360-1363.
- [58] D.Taubert, R.Berkels, R.Roesen, and W.Klaus, Chocolate and blood pressure in elderly individuals with isolated systolic hypertension, *JAMA* 290 (2003) 1029-1030.
- [59] L.A.Ferrara, A.S.Raimondi, L.d'Episcopo, L.Guida, A.lo Russo, and T.Marotta, Olive Oil and Reduced Need for Antihypertensive Medications, *Arch Intern Med* 160 (2000) 837-842.

Appendix 1: Other PREDIMED Investigators

Nutrition and Food Science Department, XaRTA, INSA. Pharmacy School, University of Barcelona, Barcelona, Spain: Mireia Urpi-Sarda, PhD; Rafael Llorach, PhD; Carmen López-Sabater, PhD.

Hospital Clinic, Institut d'Investigacions Biomèdiques August Pi Sunyer, Barcelona, Spain: Mercè Serra, RD; Ana Pérez-Heras, RD; Concha Viñas, RD; Emilio Sacanella, MD, PhD; Saul Romero, MD; Rosa Casas, BS.

University of Navarra, Primary Care Division, Pamplona, Spain: Estefania Toledo, MD, PhD; Pilar Buil-Cosiales, MD, and Javier Diez-Espino, MD.

University of Valencia, Valencia, Spain: Olga Portolés, PhD; José Vicente Sorlí, MD.

University Rovira i Virgili, Reus (Tarragona), Spain: Mónica Bulló, PhD; Josep Basora, MD; Nancy Babio, PhD;

Instituto de la Grasa, Consejo Superior de Investigaciones Científicas, Sevilla, Spain: Javier S. Perona, PhD

Municipal Institute for Medical Research, Barcelona, Spain: Montserrat Fitó, PhD; Jaime Marrugat, MD, PhD; Roberto Elosúa, MD, PhD.

University of Málaga, Málaga, Spain: Juan José Sánchez-Luque, MD, PhD; Virginia Velasco-García, MD.

University Institute for Health Sciences Investigation, Palma de Mallorca, Spain: Fernando Rigo, MD; Guillem Frontera, MD.

Primary Care Division, Catalan Institute of Health, Barcelona, Spain: Carmen Cabezas, MD.

Hospital Txagorritxu, Vitoria, Spain: E. Sanz MD.

Hospital Universitario Virgen del Rocío, Sevilla, Spain: Adoración Nieto, MD, PhD.

Department of Family Medicine, Primary Care Division of Sevilla, San Pablo Health Center, Sevilla: José Lapetra, MD, PhD; José Manuel Santos, MD, PhD; Pablo Iglesias, MD, PhD; Manuel Ortega, MD, PhD.

Table 1: Baseline of the study participants completing 1 year of follow-up.

	DMed+VOO	DMed+Nuts	Control Diet	P for trend ^a
No. of subjects	394	366	379	
Age, (y) mean (SD)	67.2 (6.1)	67.2 (6.0)	68.3 (5.9)	0.025
Women, n (%)	219 (55.6)	181 (49.5)	228 (60.2)	0.013
Weight (Kg) mean (SD)	74.8 (10.7)	75.3 (11.37)	75.2 (11.2)	0.844
BMI, (kg/m ²) mean (SD)	29.2 (3.2)	29.3 (3.4)	29.7 (3.5)	0.128
Overweight or obese (BMI ≥25 Kg/m ²), n (%)	356 (90.4)	332 (90.7)	340 (89.7)	0.896
Systolic BP (mm Hg), mean (SD)	150.4 (17.5)	152.2 (19.2)	152.5 (17.8)	0.230
Diastolic BP (mmHg), mean (SD)	83.9 (9.7)	85.3 (10.7)	84.0 (9.9)	0.145
Heart rate (beats/min), mean (SD)	69.5 (10.4)	69.3 (10.4)	70.9 (10.6)	0.072
Hypertension, n (%)	302 (76.6)	285 (77.9)	314 (82.8)	0.082
Diabetes, n (%)	168 (42.6)	161(44.0)	176 (46.4)	0.561
Dyslipidemia, n (%)	256 (65.0)	242 (66.1)	242 (63.9)	0. 678
Current smoker, n (%)	64 (16.2)	58 (15.8)	64 (16.9)	0.927
Family history of CHD, n (%)	76 (19.3)	64 (17.5)	64 (17.0)	0.875
Medication, n (%)				
ACE inhibitors	268 (71.1)	260 (73.2)	287 (77.4)	0.140
Diuretics	273 (69.5)	264 (72.3)	290 (76.5)	0.087
Statins (hypolipidemic drugs)	169 (43.3)	155 (42.5)	158 (41.7)	0.899
Insulin	21 (5.4)	20 (5.5)	21 (5.5)	0.995
Oral hypoglycemic drugs	99 (25.3)	89 (24.5)	130 (34.3)	0.004
Aspirin or other antiplatelet drugs	170 (44.7)	163 (45.8)	186 (50.0)	0.313
Educational level, n (%)				
Primary school	281 (71.3)	258 (70.5)	295 (77.8)	0.044
High school	62 (15.7)	65 (17.8)	46 (12.1)	0.095
University	35 (8.9)	32 (8.7)	26 (6.9)	0.523
Energy expenditure in physical activity (kcal/d), mean (SD)	311.9 (241.3)	289.1 (212.3)	240.7 (187.3)	<0.001
Glomerular filtration rate, mL/min	75.36 (15.94)	76.18 (17.66)	74.54 (14.21)	0.378
Any supplement in the last month	35 (9.2)	35 (9.8)	42 (11.3)	0.611

^aANOVA-one factor was used for continuous variables and χ^2 -test for categorical variables. BMI: body mass index (calculated as weight in kilograms divided by height in square meters); ACE: angiotensin-converting enzyme; CHD: coronary heart disease; GAE: gallic acid equivalent; BP: blood pressure; DMed: Mediterranean diet, VOO: virgin olive oil.

Resultats

Table 2: Baseline level and 1-year changes in daily intake of selected foods and Mediterranean diet score.^a

		DMed+VOO	<i>P</i> Value ^b	DMed+Nuts	<i>P</i> Value ^b	Control Diet	<i>P</i> Value ^b	<i>P</i> Value ^c
Total nuts (g)								
	Baseline	10.0 (12.2)		12.2 (13.9)		7.9 (11.2)	0.040	
	1 year	13.7 (13.0)	< 0.001	32.3 (16.6)	< 0.001	7.1 (10.2)		< 0.001 ^{d,e,f}
VOO (g)								
	Baseline	19.8 (22.9)		23.1 (24.5)		13.8 (20.1)	< 0.001	
	1 year	54.9 (16.5)	< 0.001	29.4 (28.8)	< 0.001	18.0 (22.7)		< 0.001 ^{d,e,f}
Refined OO (g)								
	Baseline	22.6 (21.3)		20.4 (21.9)	0.134	25.0 (19.6)	< 0.001	< 0.001 ^f
	1 year	1.3 (6.7)	< 0.001	23.1 (25.7)		21.1 (19.7)		
Fruits (g)								
	Baseline	385.1 (206.4)		384.8 (197.2)	< 0.001	356.7 (205.1)	< 0.001	< 0.001 ^{d,e}
	1 year	463.4 (195.6)	< 0.001	452.5 (182.4)		405.3 (209.4)		
Vegetables (g)								
	Baseline	315.3 (138.3)		305.1 (107.2)	< 0.001	302.2 (156.6)	0.858	< 0.001 ^{d,e}
	1 year	356.5 (156.5)	< 0.001	333.1 (110.7)		290.2 (121.7)		
Legumes (g)								
	Baseline	18.6 (8.5)		19.8 (18.6)	< 0.001	17.9 (9.2)	0.581	< 0.001 ^{d,e}
	1 year	22.5 (9.3)	< 0.001	21.6 (7.5)		17.6 (7.9)		
Fish or seafood (g)								
	Baseline	95.7 (42.6)	0.003	97.3 (39.8)	0.047	95.3 (42.8)	0.273	0.001 ^{d,e}
	1 year	100.6 (37.8)		101.3 (40.2)		90.4 (37.4)		
Meat or meat products (g)								
	Baseline	136.6 (51.8)	0.008	140.1 (50.7)	< 0.001	144.7 (51.7)	< 0.001	0.695
	1 year	128.0 (43.3)		127.9 (44.6)		127.2 (44.9)		
Cereals (g)								
	Baseline	255.5 (116.4)	0.162	242.1 (102.0)	0.665	238.9 (109.3)	0.065	0.032 ^e
	1 year	237.0 (90.0)		239.8 (93.2)		223.2 (97.6)		
Milk and dairy products (g)								
	Baseline	372.5 (211.8)	0.153	379.1 (212.2)	0.168	366.0 (228.6)	0.910	0.064 ^d
	1 year	381.2 (207.1)		368.6 (222.3)		364.9 (215.2)		
Pastries, cakes or sweets (g)								
	Baseline	22.6 (23.9)	< 0.001	24.9 (31.9)	< 0.001	23.9 (29.1)	0.026	0.053 ^e
	1 year	17.6 (21.1)		17.5 (23.5)		19.4 (22.2)		
Wine (g)								
	Baseline	89.4 (140.9)	0.433	97.3 (152.5)	0.926	76.3 (134.4)	0.537	<0.014 ^{d,e}
	1 year	92.8 (139.7)		94.2 (142.4)		74.3 (123.5)		
Alcohol (g)								
	Baseline	11.6 (17.0)	0.706	12.5 (18.5)	0.232	9.9 (16.0)	0.209	<0.036 ^{d,e}
	1 year	11.4 (16.1)		11.4 (16.2)		9.4 (15.0)		
Tee (mL)								
	Baseline	6.1 (21.4)	0.646	3.3 (11.7)	0.050	4.4 (15.1)	0.515	< 0.002 ^f
	1 year	5.7 (21.8)		2.7 (12.6)		3.7 (13.2)		

Resultats

Coffee (mL)								
	Baseline	32.6 (50.0)	0.743	31.3 (47.2)	0.600	27.9 (45.5)	0.544	0.496 ^d
	1 year	32.3 (50.7)		33.5 (52.1)		30.1 (47.2)		
Med Diet score								
	Baseline	8.8 (2.1)	<0.001	8.7 (2.1)	<0.001	8.8 (1.6)	0.016	< 0.005 ^{d,e,f}
	1 year	10.8 (1.7)		11.2 (1.6)		9.1 (1.7)		

^a Data are given as means (S.D.); ^b Differences from baseline by no-parametric Wilcoxon test; $P < 0.05$ indicates statistical significance. DMed: Mediterranean diet; VOO: virgin olive oil; OO: olive oil.

^c P values for differences among diets at 1 year; ^d Between DMed+VOO and Control diet.

^e Between DMed+Nuts and Control diet; ^f Between DMed+VOO and DMed+Nuts.

Resultats

Table 3: Average daily intake of energy and nutrients provided by VOO and mixed nuts^a

Variable	VOO, 50 g/d	Nuts, 30 g/d
Energy, kcal	442	189
Total protein, g	0	5.0
Arginine, g	0	0.7
Total carbohydrates, g	0	4.8
Fiber, g	0	2.6
Total fat, g	50.0	17.2
Oleic acid, g	37.5	6.5
Linoleic acid, g	3.4	2.3
α - Linolenic acid, g	0.2	1.3
Total plant sterols, mg	77.9	59.7
α - tocopherol, mg	7.3	7.2
β - tocopherol, mg	0.2	1.4
δ - tocopherol, mg	2.1	9.4
Calcium, mg	0.5	42.9
Magnesium, mg	0	57.3
Sodium, mg	1.0	0.4
Potassium	0.5	172.0

PREDIMED: Prevención con Dieta Mediterránea; VOO: virgin olive oil.

^a Data on nutrients are from previous report supplied in the PREDIMED study [50]

Table 4: Baseline level and 1-year changes in daily intake of nutrient intake ^a

	DMed+VOO	<i>P</i> Value ^b	DMed+Nuts	<i>P</i> Value ^b	Control Diet	<i>P</i> Value ^b	<i>P</i> Value ^c
Total energy, Kcal/d							
Baseline	2318.3 (565.2)	< 0.001	2351.2 (584.2)	< 0.001	2194.8 (616.5)	0.001	< 0.001 ^{d,e}
1 year	2367.0 (487.4)		2428.7 (505.4)		2079.8 (468.4)		
Total protein							
Baseline	92.3 (20.9)	0.077	93.2 (19.2)	0.137	91.2 (23.3)	< 0.001	< 0.001 ^{d,e}
1 year	92.7 (17.3)		94.6 (19.0)		84.9 (17.8)		
Total Carbohydrate							
Baseline	245.9 (77.4)	0.901	242.2 (80.5)	0.883	229.9 (80.6)	0.161	<0.001 ^{d,e}
1 year	240.1 (66.1)		236.8 (67.8)		219.7 (63.8)		
Fibre (g)							
Baseline	24.9 (8.9)	< 0.001	25.1 (7.9)	< 0.001	23.9 (8.0)	0.916	<0.014 ^{d,e,f}
1 year	26.9 (7.8)		28.2 (7.6)		23.3 (7.2)		
Total fat							
Baseline	98.2 (27.5)	< 0.001	102.5 (27.1)	< 0.001	93.4 (28.9)	0.007	< 0.001 ^{d,e,f}
1 year	106.2 (24.0)		113.7 (23.7)		88.3 (22.5)		
SFA							
Baseline	24.6 (8.1)	0.216	25.7 (8.4)	0.036	24.2 (9.0)	< 0.001	<0.001 ^{d,e}
1 year	24.0 (7.2)		24.7 (7.0)		21.8 (6.8)		
MUFA							
Baseline	49.6 (14.8)	< 0.001	51.5 (14.3)	< 0.001	46.5 (15.0)	0.113	< 0.044 ^{d,e,f}
1 year	56.6 (12.7)		58.6 (12.5)		44.7 (12.1)		
PUFA							
Baseline	15.3 (6.1)	< 0.001	16.5 (6.9)	< 0.001	14.6 (6.6)	0.028	< 0.001 ^{d,e,f}
1 year	16.5 (5.0)		21.0 (6.5)		13.9 (5.5)		
Cholesterol (g)							
Baseline	348.9 (107.5)	0.232	365.4 (121.4)	< 0.001	358.9 (114.8)	< 0.001	0.110
1 year	340.7 (92.9)		340.7 (109.5)		334.4 (120.2)		
Linoleic acid (g)							
Baseline	12.8 (5.6)	< 0.001	13.9 (6.4)	< 0.001	12.1 (6.1)	0.159	< 0.001 ^{d,e,f}
1 year	13.6 (4.6)		17.7 (5.9)		11.6 (5.1)		
Linolenic acid (g)							
Baseline	1.4 (0.7)	< 0.001	1.6 (0.8)	< 0.001	1.3 (0.7)	0.003	< 0.001 ^{d,e,f}
1 year	1.6 (0.6)		2.1 (0.7)		1.2 (0.6)		
Marine n-3 fatty acid (g)							
Baseline	0.7 (0.4)	< 0.001	0.8 (0.4)	< 0.001	0.7 (0.5)	0.318	< 0.001 ^{d,e}
1 year	0.8 (0.4)		0.9 (0.4)		0.7 (0.4)		
Calcium (mg)							
Baseline	1001.1 (332.9)	0.030	998.3 (324.7)		939.9 (364.2)		
1 year	1034.2 (322.5)		1019.3 (358.7)		935.1 (316.9)		
Magnesium (mg)							
Baseline	369.8 (101.8)	< 0.001	374.5 (93.6)	< 0.001	351.9 (99.9)	0.124	<0.001 ^{d,e,f}
1 year	387.4 (90.4)		417.2 (94.8)		342.6 (87.0)		
Potassium(mg)							
Baseline	4325.2 (1050.4)	< 0.001	4380.2 (989.1)	< 0.001	4108.3 (1039.8)	0.785	< 0.001 ^{d,e}
1 year	4606.0 (1028.5)		4635.5 (903.1)		4085.6 (865.2)		

Resultats

Sodium (mg)								
Baseline	2369.2 (873.7)	0.019	2323.2 (825.8)	0.138	2224.1 (944.9)	0.006	<0.005 ^{d,e}	
1 year	2238.5 (724.9)		2260.7 (776.3)		2090.7 (736.2)			
Vitamin A (mg)								
Baseline	1188.1 (724.5)	< 0.001	1284.0 (1933.5)	0.015	1095.6 (559.2)	0.460	<0.001 ^{d,e}	
1 year	1283.1 (641.9)		1233.4 (673.5)		1068.5 (517.7)			
Vitamin C (mg)								
Baseline	191.2 (82.6)	< 0.001	188.7 (72.1)	< 0.001	180.9 (97.8)	0.061	< 0.001 ^{d,e}	
1 year	215.9 (86.4)		210.8 (67.3)		181.6 (74.3)			
Vitamin E (mg)								
Baseline	9.4 (3.5)	< 0.001	9.7 (4.1)	< 0.001	9.5 (4.4)	0.026	< 0.001 ^{d,e,f}	
1 year	9.7 (2.4)		11.2 (3.3)		8.8 (3.6)			
Energy expenditure in physical activity (kcal/d), mean (SD)								
Baseline	311.9 (241.3)	0.129	289.1 (212.3)	0.023	240.7 (187.3)	0.139	< 0.001 ^{d,e}	
1 year	316.5 (222.4)		304.7 (198.0)		260.4 (220.5)			

^a Data are given as means (S.D.); ^b Differences from baseline by no-parametric Wilcoxon test; $P < 0.05$ indicates statistical significance. DMed: Mediterranean diet; VOO: virgin olive oil; OO: olive oil; MUFA: monounsaturated fat acids; PUFA, polyunsaturated fat acids and SFA: saturated fat acid.

^c P values for differences among diets. ^d Between DMed+VOO and Control diet.

^e Between DMed+Nuts and Control diet. ^f Between DMed+VOO and DMed+Nuts.

Table 5: Baseline level and 1-year changes in lipid profiles, blood pressure and total polyphenol excreted.^a

	DMed+VOO	<i>P</i> Value ^b	DMed+Nuts	<i>P</i> Value ^b	Control Diet	<i>P</i> Value ^b	<i>P</i> Value ^c
N	394		366		379		
Urine total polyphenol (mg GAE/ g creatinine)							
Baseline	110.8 (47.1)	< 0.001	127.5 (66.5)	< 0.001	138.2 (81.3)	0.903	<0.027 ^{e,f}
1 year	133.5 (74.7)		149.3 (85.6)		139.7 (87.8)		
Weight (kg)							
Baseline	74.8 (10.7)	0.392	75.3 (11.3)	0.654	75.2 (11.2)	0.195	0.330 ^d
1 year	74.7 (10.9)		74.9 (11.3)		78.8 (49.9)		
BMI							
Baseline	29.2 (3.2)	0.384	29.3 (3.4)	0.601	29.7 (3.5)	0.208	0.043 ^d
1 year	29.2 (3.3)		29.1 (3.5)		31.3 (22.2)		
Waist, cm							
Baseline	97.2 (9.7)	0.022	97.5 (10.3)	< 0.001	97.9 (10.4)	0.001	0.435 ^d
1 year	96.5 (9.6)		96.3 (10.2)		97.2 (10.9)		
Systolic BP (mm Hg), mean (SD)							
Baseline	150.4 (17.5)	0.003	152.2 (19.2)	0.008	152.5 (17.8)	0.071	0.001 ^d
1 year	147.5 (16.9)		149.2 (18.6)		151.9 (18.2)		
Diastolic BP (mmHg), mean (SD)							
Baseline	83.9 (9.7)	0.002	85.3 (10.7)	0.021	84.0 (9.9)	0.370	0.056 ^f
1 year	82.6 (9.7)		84.1 (10.1)		84.0 (9.5)		
Fasting glucose, mg/dl							
Baseline	117.8 (33.1)	0.116	120.9 (42.9)	0.496	128.9 (41.6)	0.665	0.073 ^e
1 year	114.3 (28.4)		115.6 (33.5)		124.8 (39.9)		
Total cholesterol, mg/dl							
Baseline	216.8 (34.6)	0.037	220.4 (36.8)	0.000	205.9 (30.5)	0.613	0.294 ^e
1 year	210.8 (33.7)		207.1 (30.3)		205.7 (36.7)		
LDL cholesterol, mg/dl							
Baseline	137.8 (27.7)	0.471	143.7 (29.0)	0.048	120.4 (28.6)	0.630	<0.01 ^{d,e}
1 year	134.7 (28.6)		135.1 (28.8)		117.7 (29.7)		
HDL cholesterol, mg/dl							
Baseline	54.1 (14.1)	0.529	52.0 (11.8)	0.782	51.4 (10.9)	0.061	0.019 ^d
1 year	54.7 (13.4)		52.7 (11.3)		51.1 (10.8)		
Triglyceride, mg/dl							
Baseline	128.5 (61.1)	0.011	129.1 (68.7)	0.078	131.1 (70.9)	0.578	0.083 ^d
1 year	117.1 (49.2)		121.4 (54.6)		126.6 (50.6)		

^a Data are given as means (S.D.); ^b Differences from baseline by no-parametric Wilcoxon test; *P*<0.05 indicates statistical significance. DMed: Mediterranean diet; VOO: virgin olive oil; GAE: gallic acid equivalent; OxLDL: oxidized low-density lipoprotein.

^c *P* values for differences among diets; ^d Between DMed+VOO and Control diet.

^e Between DMed+Nuts and Control diet; ^f Between DMed+VOO and DMed+Nuts.

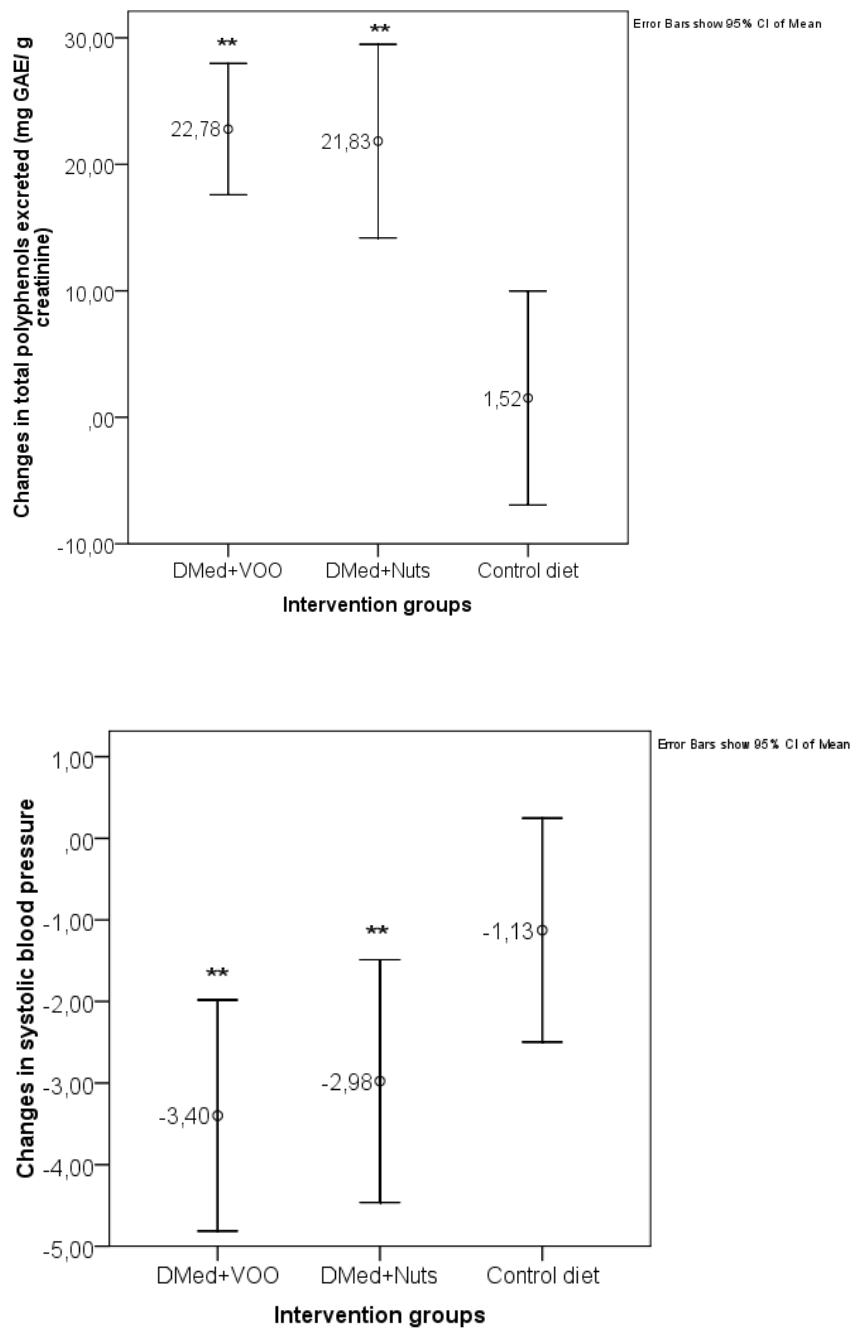
Resultats

Table 6: Covariate analysis with systolic blood pressure and diastolic blood pressure at one-year as the dependent variables; intervention groups as the fixed factor and baseline measures as covariates.

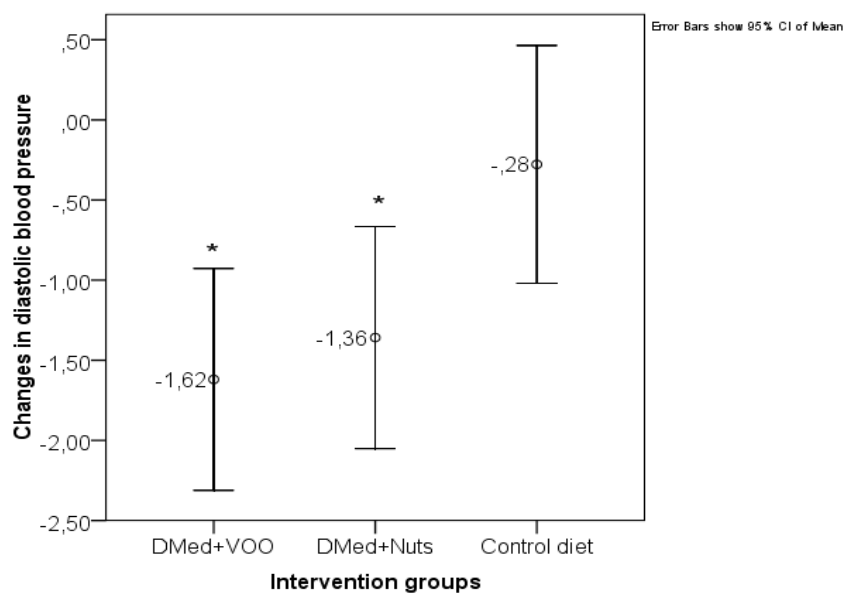
	Model	B	P	95 % CI
Systolic blood pressure	<i>Model 1</i>			
	DMed-VOO vs. Control diet	-3,377	0,001	-5,440 to -1,313
	DMed-Nuts vs. Control diet	-2,500	0,020	-4,598 to -0,401
	<i>Model 2</i>			
	DMed-VOO vs. Control diet	-3,342	0,002	-5,465 to -1,218
	DMed-Nuts vs. Control diet	-2,433	0,026	-4,580 to -0,286
	<i>Model 3</i>			
	DMed-VOO vs. Control diet	-3,429	0,002	-5,598 to -1,261
	DMed-Nuts vs. Control diet	-2,268	0,042	-4,450 to -0,086
Diastolic blood pressure	<i>Model 1</i>			
	DMed-VOO vs. Control diet	-1,311	0,014	-2,361 to -0,262
	DMed-Nuts vs. Control diet	-0,662	0,225	-1,731 to 0,407
	<i>Model 2</i>			
	DMed-VOO vs. Control diet	-1,452	0,008	-2,528 to -0,377
	DMed-Nuts vs. Control diet	-0,737	0,185	-1,827 to 0,353
	<i>Model 3</i>			
	DMed-VOO vs. Control diet	-1,382	0,014	-2,479 to -0,285
	DMed-Nuts vs. Control diet	-0,696	0,217	-1,803 to 0,410

B: Non-standardized coefficient; CI: Confidence interval; P: two-sided test of significance; Model 1: unadjusted; Model 2: adjusted by sex, age, weight, smoking status, physical activity, and educational level at baseline; Model 3 was adjusted as in Model 2 plus medication intake: ACE inhibitor, diuretics, statins (hypolipidemic drugs), insulin, oral hypoglycemic drugs, aspirin or other antiplatelet drug supplements taken in the last month, sodium and potassium intake and glomerular filtration rate (GFR); DMed: Mediterranean diet; VOO: virgin olive oil.

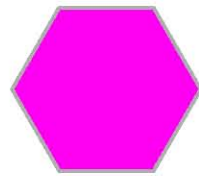
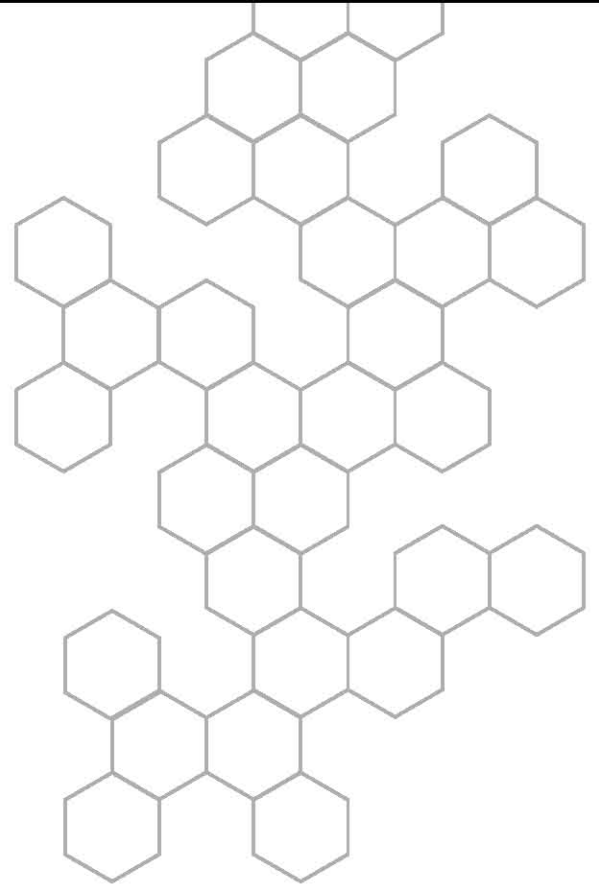
Figure 1: Mean $\pm$ SD changes in total polyphenols excreted in spot urine samples, systolic and diastolic blood pressure after 1-year with different intervention.



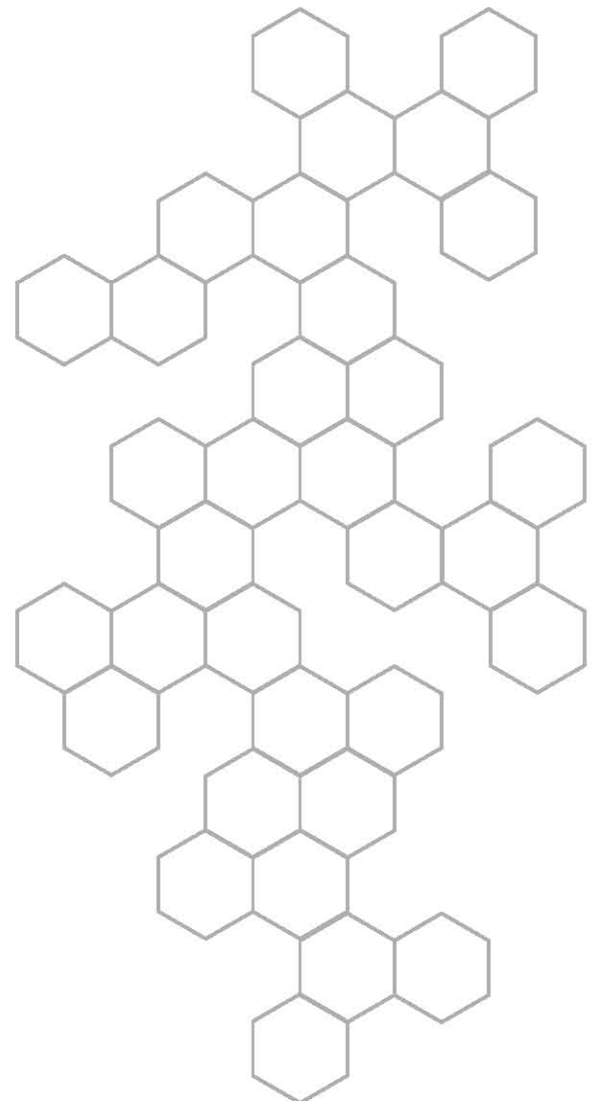
Resultats



Med Diet: Mediterranean diet; VOO: virgin olive oil; GAE: gallic acid equivalent; ** $P < 0.01$, * $P < 0.05$ indicates statistical significance between the baseline and 1-year of intervention period with a confidence interval of 95%.



DISCUSSIÓ GLOBAL



5. Discussió Global

La discussió general dels resultats derivats d'aquesta tesis es presenta a continuació estructurada en base als articles anteriorment descrits durant el treball d'investigació. En primer lloc es va posar a punt i es va validar un mètode per a la determinació de polifenols totals en mostres d'orina, utilitzant el mètode de Folin-Ciocalteu, com a biomarcador del consum de polifenols totals (publicació 1). Aquesta metodologia va requerir d'una neteja prèvia de les mostres, per eliminar les possibles interferències de substàncies reductores que reaccionen amb el reactiu de Folin-Ciocalteu. Es van estudiar les recuperacions en varis cartutxos de SPE, amb el format de plaques de 96 cartutxos, utilitzant deu polifenols de diferents polaritats, i a diferents concentracions de 5, 10, 25 i 50 mg L⁻¹ (n=6). La metodologia va ser optimitzada i els cartutxos Oasis® MAX, 30mg (Mixed-Mode Anion-eXchange and Reversed-Phase Solvent) van ser seleccionats, per obtenir amb ells millor recuperació en comparació amb la resta de cartutxos de SPE, degut a que amb la barreja del mode d'intercanvi aniònic i el solvent de fase reversa, hi ha una alta selectivitat y sensibilitat pels compostos fenòlics provats.

Amb aquesta metodologia es va incrementar la sensibilitat i la recuperació de polifenols amb un ampli rang de polaritat, els límits de detecció i quantificació van ser significativament reduïts, respecte a la metodologia prèvia d'anàlisi d'aquest biomarcador en orines (Roura et al. 2006). La precisió y exactitud es va calcular a diferents concentracions; els valors de precisió obtinguts van estar dins dels valors acceptats per la *AOAC INTERNATIONAL* (Horwitz 2003), al igual que els valors d'exactitud obtinguts entre el 82.5% i 105.7%. A més va ser determinada l'estabilitat a curt i llarg termini, així com després de varis cicles de congelació i descongelació a -20 i -80°C. Els compostos fenòlics en les solucions patró i en les mostres d'orina van ser estables sota les condicions de manipulació o emmagatzematge utilitzades per aquesta metodologia.

Amb aquesta metodologia s'aconsegueix l'anàlisi de 96 mostres d'orines en només 3 hores, utilitzant els cartutxos Oasis® MAX en plaques de 96 unitats, a diferència d'altres mètodes que requereixen aproximadament 12.5 hores (Roura et al. 2006; Saura-Calixto and Goñi 2006; Singleton et al. 1999). Una

vegada eliminades les interferències de les mostres d'orina, la reacció per la determinació de polifenols totals i creatinina van ser realitzades en plaques de lectura de 96 pouets, amb un volum màxim de 300 µL; d'aquesta manera es va evitar una laboriosa manipulació de la mostra. Les plaques de 96 cartutxos fan la metodologia més pràctica en l'anàlisi d'un gran nombre de mostres, per exemple en estudis clínics i epidemiològics, en els quals els subjectes ingereixen una ampla varietat de polifenols en la seva dieta habitual.

El mètode validat es va aplicar a tres estudis clínics amb diferents voluntaris: un estudi prospectiu aleatoritzat creuat amb 12 voluntaris sans i un estudi transversal amb 60 voluntaris de l'estudi PREDIMED (publicació 1); un estudi d'intervenció amb 49 homes joves egipcis d'entre 7 i 14 anys d'edat (publicació 2), un sub-estudi transversal dins de l'estudi PREDIMED amb 263 homes i 326 dones amb edats entre 55-80 (publicació 3) i un sub-estudi d'intervenció amb 1139 participants d'alt risc cardiovascular dins de l'estudi PREDIMED (publicació 5).

A la publicació 1, els polifenols totals ingerits es van correlacionar positivament amb els polifenols totals excretats en l'orina puntual del matí. En l'estudi clínic descrit, els polifenols totals excretats en l'orina, expressats com a mg GAE/ g creatinina, es van correlacionar positivament amb els polifenols totals ingerits ($P<0.01$), amb el consum total de polifenols de les fruites ($P<0.05$), verdures i hortalisses ($P<0.01$) i fruita-verdures-hortalisses ($P<0.01$). En l'estudi transversal, amb la sub-mostra del PREDIMED l'excreció de polifenols totals es va correlacionar també amb la ingesta total de polifenols ($r=0.257$, $P=0.04$), així com amb la ingesta total de fruita-verdures-hortalisses ($r=0.339$; $P=0.008$).

Respecte a estudis previs (Krogholm et al. 2004; Mennen et al. 2006) en els que es van estudiar la correlació entre el consum d'aliments i begudes riques en polifenols y la concentració de polifenols en les mostres de l'orina determinades per LC-MS/MS, es van mostrar resultats que suggereixen que els polifenols individuals mesurats en mostres d'orina es poden utilitzar com a biomarcador de la ingesta d'aliments rics en polifenols. La majoria de les correlacions observades entre l'excreció urinària i la ingesta de polifenols van ser realitzades quantificant els polifenols individuals en els aliments (Manach et al. 2004) encara que es va requerir d'equips instrumentals especialitzats. El

mètode desenvolupat per a la determinació de polifenols totals en mostres d'orina com a biomarcador del consum d'aliments rics en polifenols, és molt més respectuós amb el medi ambient i més simple que altres mètodes descrits (Krogholm et al. 2004; Nielsen et al. 2002) els quals requereixen d'instrumentació costosa per LC-MS.

La quantificació d'un nombre de metabòlits en les mostres d'orines de 24h pot ser un apropament convenient a estudis d'intervenció a petita escala, però no una possibilitat realista en els grans estudis epidemiològics, degut als problemes de recollida d'orina durant 24 h en estudis de grans poblacions.

En la publicació 2, després de la intervenció amb 240 g de suc de tomàquet durant tres setmanes en 49 voluntaris se'ls va analitzar els polifenols totals en les mostres d'orina, recollides de forma puntual i durant les 24 hores consecutives, després d'acabar l'estudi. Els polifenols totals es van expressar en mg GAE/g creatinina o en mg GAE/24 hores. Les mitjanes de polifenols totals urinaris excretats, incrementen significativament ($P < 0.05$) seguida per la intervenció amb suc de tomàquet (287.4 ± 64.3 mg GAE /g creatinina) comparat amb les respectives basals. Els nivells mitjans d'excreció de polifenols totals, van ser similars als obtinguts prèviament (Roura et al. 2006). Les quantitats de suc de tomàquet es van mantenir de forma raonable tal com es consumeix en una dieta normal. Els 240 g servits durant la intervenció van reflectir un consum de tres tomàquets de mida mitjana, equivalent a 2.3 g de pell proporcionant diàriament 25.6 ± 1.27 mg de polifenols GAE.

En la publicació 3 es va avaluar l'efecte del consum dels polifenols sobre la hipertensió arterial en una població adulta amb alt risc cardiovascular. Com a paràmetre per determinar la ingesta de polifenols totals s'ha utilitzat l'excreció de polifenols totals com biomarcador de la ingesta de polifenols. Tenint en compte la correlació de Pearson, els polifenols totals ingerits mesurats pel qüestionari de freqüència de consum, es van correlacionar positivament amb els polifenols totals excretats, mesurats en l'orina. La ingesta d'una porció de 100 g de F&V, va tenir una major contribució sobre l'excreció de polifenols totals que una porció de 100 mL de cafè o 100 mL de vi. L'anàlisi de regressió logística mostrava una associació inversa entre els quartils d'excreció de polifenols totals i la prevalència d'hipertensió. Aquells participants del quartil

més alt d'excreció de polifenols totals van tenir una reducció de la prevalència d'hipertensió en comparació amb el quartil més baix (OR = 0.64; $P = 0.16$). En l'anàlisi de regressió lineal múltiple la PA sistòlica ($\beta = -1.731$; $P = 0.024$) i diastòlica ($\beta = -1.264$; $P = 0.003$) es van associar inversament amb l'excreció de polifenols totals després d'ajustar per possibles factors de confusió.

Els efectes de las dietes riques en polifenols sobre la pressió arterial, en subjectes hipertensos, ha sigut fonamentalment atribuït a la presència de múltiples antioxidants en aquests aliments (Grassi et al. 2005; Reshef et al. 2005; Shimada et al. 1999; Taubert et al. 2003). Les F&V semblen ser un punt essencial en la prevenció de malalties cardiovasculars. Alguns estudis epidemiològics suposen l'evidència de que una dieta rica en F&V podria ajudar a prevenir increments en la PA, i ajudar a disminuir valors elevats de PA en persones hipertenses (Whelton et al. 2002).

En l'estudi SUN (Alonso et al. 2005), la prevalència d'hipertensió no diagnosticada prèviament va ser inversament associada amb el consum de F&V en una població Mediterrània. En l'Estudi de les infermeres (Ascherio et al. 1996), el consum de F&V va ser inversament associat amb la PA sistòlica i diastòlica, no obstant, el consum de cereals i carn va ser directament associat amb la PA diastòlica. En tots els estudis es correlaciona un alt consum de F&V amb una disminució en els nivells de PA. En el nostre estudi, realitzat en una població espanyola d'alt risc cardiovascular (PREDIMED), es va observar un efecte similar quan es va incrementar el consum de F&V i per tant l'excreció de polifenols totals. Malgrat això, fins ara, cap estudi ha intentat correlacionar el biomarcador de la ingesta de polifenols totals, determinat en mostres d'orina puntual, amb les mesures de PA, o amb la prevalència de la hipertensió. Tenint en compte que una major excreció de polifenols en l'orina es determina per l'alt consum de polifenols totals, suggerim que l'associació independent inversa observada dels polifenols totals excretats mesurats en mostres d'orina amb PA està molt probablement relacionada amb un efecte favorable de la ingesta de polifenols totals, prevenint un increment dels nivells de PA.

En la publicació 5 es va avaluar l'efecte, durant un any, de dos intervencions amb una dieta tradicional mediterrània suplementada amb oli d'oliva verge o nous, sobre l'excreció de polifenols totals en mostres d'orina preses de forma

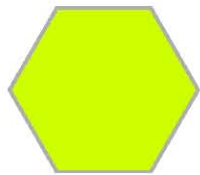
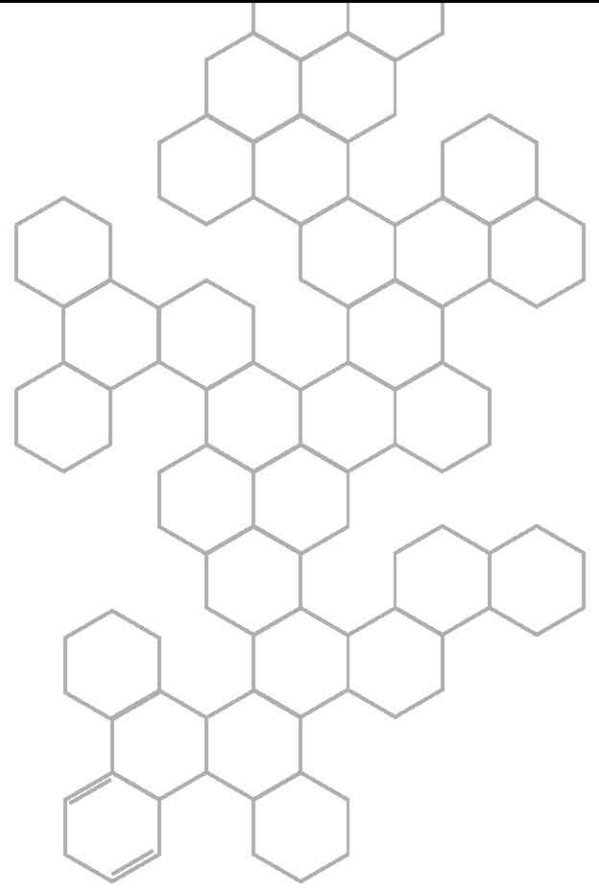
puntual; a més de com influeix aquest canvi sobre les variacions de determinats marcadors de risc cardiovascular. 1139 participants d'alt risc cardiovascular, dins de l'estudi PREDIMED, es van assignar aleatòriament a una dieta baixa en greixos (dieta control), $n=379$ o a dues dietes estil mediterrània, una suplementada amb VOO, $n=394$ i l'altra amb fruits secs, $n=366$. Després de la intervenció amb VOO i fruits secs, i ajustar per diferents covariables els participants van mostrar un increment significatiu dels polifenols totals excretats, així com una reducció estadísticament significativa de PA sistòlica i diastòlica. A més, altres factors de riscos cardiovasculars com LDL-colesterol, colesterol total, triglicèrids totals i la glucosa van disminuir en ambdues dietes mediterrànies suplementades; així com van incrementar els nivells totals de HDL-colesterol.

Semblant amb els nostres resultats, altres sub-estudis amb 1224 participants (Salas-Salvado et al. 2008) va comparar l'efecte, durant 1 any, de 2 de les intervencions de dieta mediterrània suplementada amb nous i oli d'oliva verge sobre la prevalència de síndrome metabòlic, aquesta es va reduir en un 6.7%, 13.7% i 2.0% en el grup de Med-VOO, Med-fruits secs, i dieta control, respectivament. Després d'ajustar per factors diferents, la odds ratio per la reversió de la síndrome metabòlica va ser d'1.3 (IC 95%: 0.8-2.1) pel grup Med-VOO i 1.7 (1.1-2.6) pel grup Med-fruits secs en comparació amb el grup de dieta control. En un altre estudi similar (Estruch et al. 2006) es van comparar els efectes a curt termini d'aquestes dietes mediterrànies, després de 3 mesos d'intervenció. En comparació amb la dieta baixa en greixos, la Med-VOO i la Med-fruits secs disminueix -5.9 mm Hg (IC, -8.7 a -3.1 mm Hg) i -7.1 mm Hg (IC, -10.0 a -4.1 mm Hg) respectivament, la PA sistòlica. En consonància amb els estudis anteriors, hem observat que l'adhesió a una dieta mediterrània tradicional, pot ser capaç de reduir els factors de risc cardiovascular.

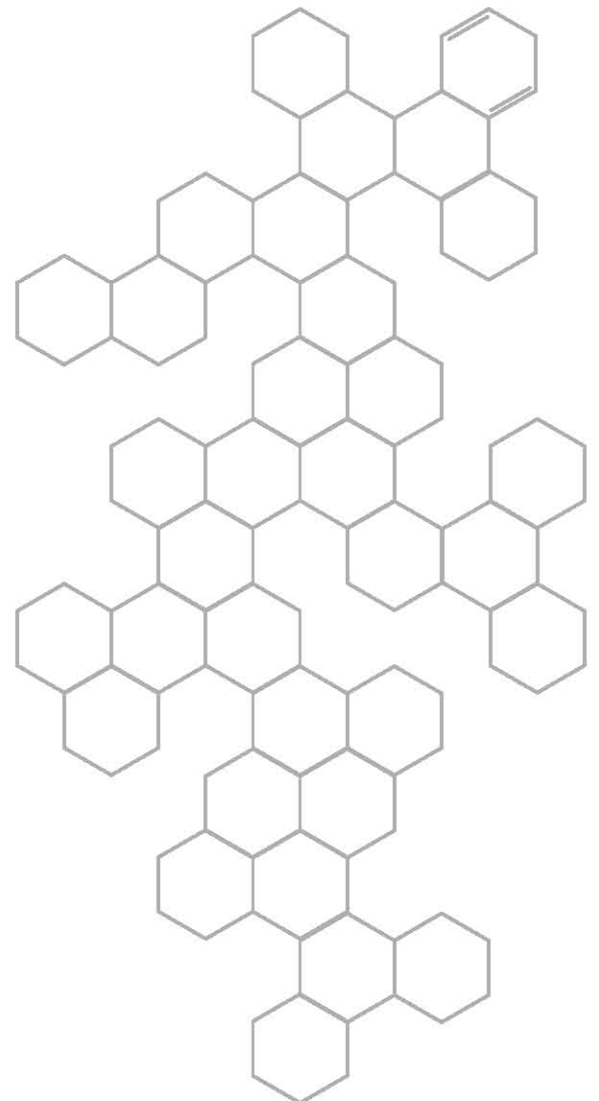
La dieta DASH està àmpliament promoguda als EE.UU. per la prevenció i el tractament de la hipertensió (Appel et al. 1997). Aquest patró de dieta ha sigut avaluat en una població del Regne Unit (Harnden et al. 2010), on PA sistòlica i diastòlica van disminuir significativament en -4.6 i -3.9 mm Hg, respectivament, en els que segueixen un estil de dieta DASH. Després de 3 setmanes amb la dieta DASH, la PA sistòlica y diastòlica en pacients hipertensos obesos era

inferior que els que seguien una dieta habitual. (Al-Solaiman et al. 2010). En un altre estudi amb 27 homes i dones que van seguir una dieta DASH o una dieta control (Hodson et al. 2009) el grup de la dieta DASH va mostrar una reducció significativa en PA sistòlica i diastòlica. Recentment, en l'estudi SUN (Núñez-Córdoba et al. 2009), l'adherència a la dieta mediterrània es va associar amb canvis en la reducció dels nivells mitjans de PA sistòlica (adherència moderada a -2.4 mmHg, alta adherència, -3.1 mmHg) i PA diastòlica (adherència moderada, -1.3 mmHg, alta adherència, -1.9 mm Hg) després de 6 anys de seguiment, el que suggereix que l'adhesió a una dieta de tipus mediterrani podria contribuir a la prevenció dels canvis en la PA relacionats amb l'edat.

En aquest últim estudi d'intervenció amb dieta mediterrània, realitzat en una població espanyola d'alt risc, es va observar un efecte similar amb una dieta mediterrània suplementada amb aliments rics en polifenols; els nostres resultats també es sumen a la creixent evidència de que les dietes riques en polifenols redueixen la PA.



CONCLUSIONS



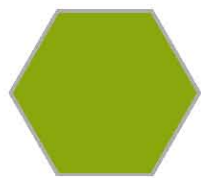
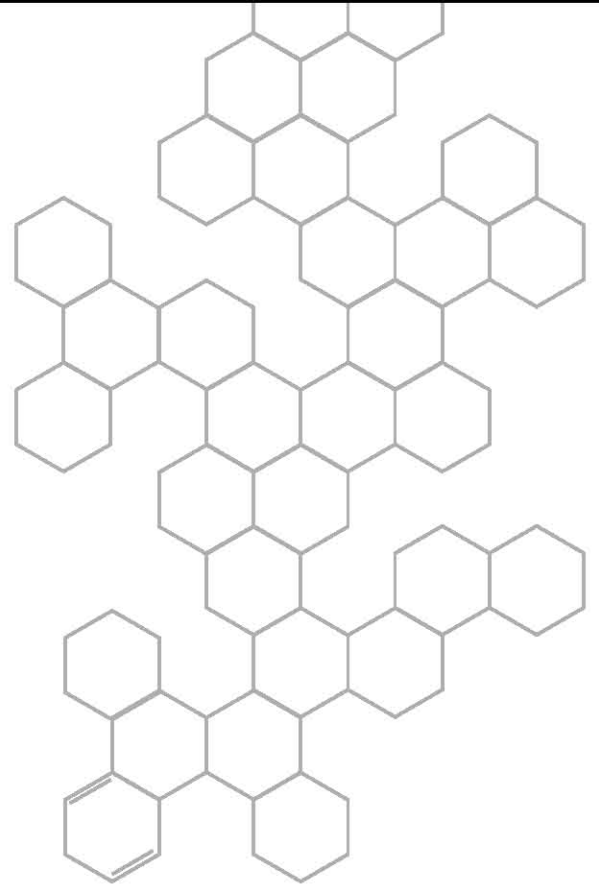
6. Conclusions

D'aquest treball de tesis doctoral s'obtenen les següents conclusions:

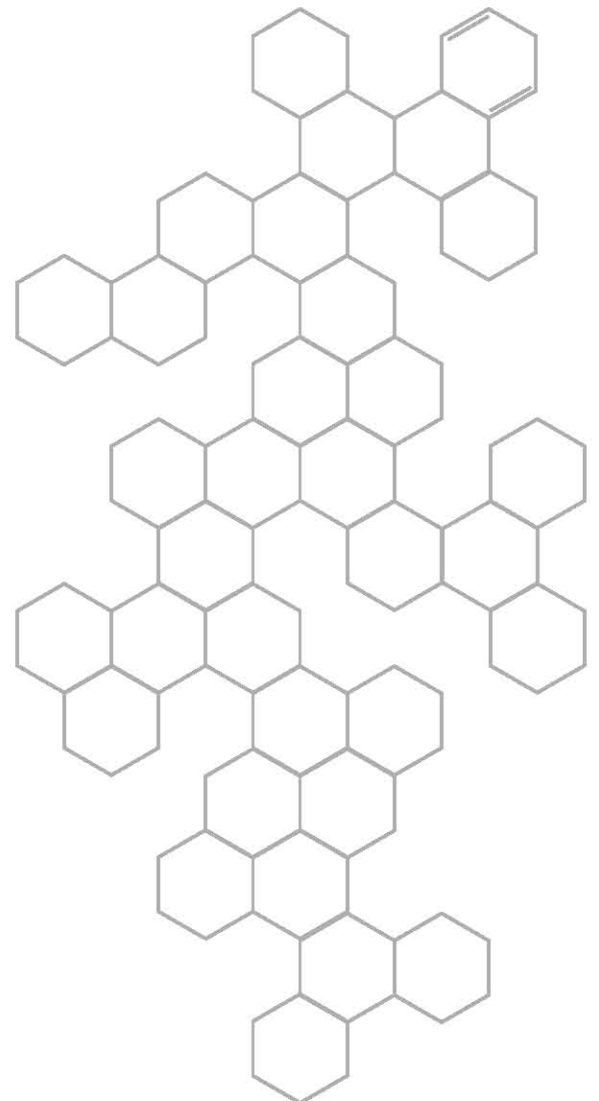
- S'ha desenvolupat un mètode sensible, específic i ràpid per la quantificació de polifenols totals en un gran nombre de mostres d'orina obtinguda de forma puntual, amb un tractament de mostra molt senzill. Aquest mètode és més econòmic, a part de respectuós amb el medi ambient a l'utilitzar menys reactius.
- La validació del mètode es va basar en l'anàlisi dels resultats obtinguts a partir d'un estudi clínic prospectiu aleatoritzat, creuat, amb diferents períodes d'intervenció i es va corroborar en un estudi transversal amb 60 voluntaris amb una població *free-living*. En ambdós tipus d'estudis s'ha trobat una associació positiva entre la quantitat de polifenols totals excretats en l'orina del matí, i el consum de polifenols totals i el consum total de fruites, verdures i hortalisses mesurat mitjançant enquestes de consum d'aliments.
- La ingesta de polifenols totals avaluats a través de l'excreció de polifenols totals en orina puntual del matí es va associar negativament amb els nivells de pressió arterial sistòlica i diastòlica en una població mediterrània d'avançada edat amb alt risc cardiovascular. Per tant, una alta ingesta de dieta rica en polifenols, pot ajudar a disminuir la pressió arterial en poblacions d'hipertensos d'avançada edat, amb el fi de reduir el seu risc cardiovascular.
- En els individus en el quartil més alt d'excreció de polifenols totals el risc d'hipertensió disminueix en un 36% en comparació amb el quartil més baix d'excreció de polifenols totals.
- Una intervenció dietètica dirigida a incrementar la ingesta d'aliments rics en polifenols podria ser eficient en la prevenció i el tractament de la hipertensió. Una dieta mediterrània dirigida a incrementar la ingesta d'aliments rics en polifenols, pot ajudar a disminuir la pressió arterial en

Conclusions

poblacions d'hipertensos d'edat avançada, amb la finalitat de reduir el seu risc cardiovascular.



BIBLIOGRAFIA



7. Bibliografía

Agudo A, Cabrera L, Amiano P, Ardanaz E, Barricarte A, Berenguer T, Chirlaque MD, Dorronsoro M, Jakszyn P, Larranaga N, Martinez C, Navarro C, Quiros JR, Sanchez MJ, Tormo MJ, Gonzalez CA. 2007. Fruit and vegetable intakes, dietary antioxidant nutrients, and total mortality in Spanish adults: findings from the Spanish cohort of the European Prospective Investigation into Cancer and Nutrition (EPIC-Spain). *Am J Clin Nutr* 85:1634-1642.

Al-Solaiman Y, Jesri A, Mountford WK, Lackland DT, Zhao Y, Egan BM. 2010. DASH lowers blood pressure in obese hypertensives beyond potassium, magnesium and fibre. *J Hum Hypertens* 24:237-246.

Alonso A, Beunza JJ, Delgado-Rodriguez M, Martinez JA, Martinez-Gonzalez MA. 2005. Low-fat dairy consumption and reduced risk of hypertension: the Seguimiento Universidad de Navarra (SUN) cohort. *Am J Clin Nutr* 82:972-979.

Alonso A, de la Fuente C, Martin-Arnau AM, de Irala J, Martinez JA, Martinez-Gonzalez MA. 2004. Fruit and vegetable consumption is inversely associated with blood pressure in a Mediterranean population with a high vegetable-fat intake: the Seguimiento Universidad de Navarra (SUN) Study. *Br J Nutr* 92:311-319.

Alonso A, Ruiz-Gutierrez V, Martinez-Gonzalez MA. 2006. Monounsaturated fatty acids, olive oil and blood pressure: epidemiological, clinical and experimental evidence. *Public Health Nutr* 9:251-257.

Andlauer W, Stumpf C, Hubert M, Rings A, Fürst P. 2003. Influence of Cooking Process on Phenolic Marker Compounds of Vegetables. *International Journal for Vitamin and Nutrition Research* 73:152-159.

Andrikopoulos NK, Dedoussis GV, Falirea A, Kalogeropoulos N, Hatzinikola HS. 2002. Deterioration of natural antioxidant species of vegetable edible oils during the domestic deep-frying and pan-frying of potatoes. *Int J Food Sci Nutr* 53:351-363.

Appel LJ, Moore TJ, Obarzanek E, Vollmer WM, Svetkey LP, Sacks FM, Bray GA, Vogt TM, Cutler JA, Windhauser MM, Lin PH, Karanja N, Simons-Morton D, McCullough M, Swain J, Steele P, Evans MA, Miller ER, Harsha DW, The DASH Collaborative Research Group. 1997. A Clinical Trial of the Effects of Dietary Patterns on Blood Pressure. *N Engl J Med* 336:1117-1124.

Ascherio A, Hennekens C, Willett WC, Sacks F, Rosner B, Manson J, Witteman J, Stampfer MJ. 1996. Prospective study of nutritional factors, blood pressure, and hypertension among US women. *Hypertension* 27:1065-1072.

Azuma K, Ippoushi K, Ito H, Higashio H, Terao J. 2002. Combination of lipids and emulsifiers enhances the absorption of orally administered quercetin in rats. *J Agric Food Chem* 50:1706-1712.

Bibliografia

Badimon JJ, Zaman A, Helft G, Fayad Z, Fuster V. 1999. Acute coronary syndromes: pathophysiology and preventive priorities. *Thromb Haemost* 82:997-1004.

Badimon L, Vilahur G, Padro T. 2009. Lipoproteins, platelets and atherothrombosis. *Rev Esp Cardiol* 62:1161-1178.

Bao DQ, Mori TA, Burke V, Puddey IB, Beilin LJ. 1998. Effects of dietary fish and weight reduction on ambulatory blood pressure in overweight hypertensives. *Hypertension* 32:710-717.

Beaton GH, Burema J, Ritenbaugh C. 1997. Errors in the interpretation of dietary assessments. *Am J Clin Nutr* 65:1100S-1107S.

Beckman KB, Ames BN. 1998. The free radical theory of aging matures. *Physiol Rev* 78:547-581.

Bergamini E, Cavallini G, Donati A, Gori Z. 2003. The anti-ageing effects of caloric restriction may involve stimulation of macroautophagy and lysosomal degradation, and can be intensified pharmacologically. *Biomed Pharmacother* 57:203-208.

Berry EM, Eisenberg S, Friedlander Y, Harats D, Kaufmann NA, Norman Y, Stein Y. 1992. Effects of diets rich in monounsaturated fatty acids on plasma lipoproteins--the Jerusalem Nutrition Study. II. Monounsaturated fatty acids vs carbohydrates. *Am J Clin Nutr* 56:394-403.

Bevilacqua MP, Pober JS, Majeau GR, Fiers W, Cotran RS, Gimbrone MA, Jr. 1986. Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin 1. *Proc Natl Acad Sci U S A* 83:4533-4537.

Blomhoff R, Carlsen MH, Andersen LF, Jacobs DR, Jr. 2006. Health benefits of nuts: potential role of antioxidants. *Br J Nutr* 96 Suppl 2:S52-S60.

Buckland G, Bach A, Serra-Majem L. 2008. Obesity and the Mediterranean diet: a systematic review of observational and intervention studies. *Obes Rev* 9:582-593.

Bunea A, Andjelkovic M, Socaciu C, Bobis O, Neacsu M, Verhé R, Camp JV. 2008. Total and individual carotenoids and phenolic acids content in fresh, refrigerated and processed spinach (*Spinacia oleracea* L.). *Food Chemistry* 108:649-656.

Cassidy A, Brown JE, Hawdon A, Faughnan MS, King LJ, Millward J, Zimmer-Nechemias L, Wolfe B, Setchell KD. 2006. Factors affecting the bioavailability of soy isoflavones in humans after ingestion of physiologically relevant levels from different soy foods. *J Nutr* 136:45-51.

Chadjichristos CE, Kwak BR. 2007. Connexins: new genes in atherosclerosis. *Ann Med* 39:402-411.

- Corella D, Ordovas JM. 2009. Nutrigenomics in cardiovascular medicine. *Circ Cardiovasc Genet* 2:637-651.
- Covas MI, Fito M, Marrugat J, Miro E, Farre M, de laTorre R, Gimeno E, Lopez-Sabater MC, Lamuela-Raventos R, de la Torre-Boronat M. 2001. [Coronary disease protective factors: antioxidant effect of olive oil]. *Therapie* 56:607-611.
- Covas MI, Nyyssonen K, Poulsen HE, Kaikkonen J, Zunft HJ, Kiesewetter H, Gaddi A, de la Torre R, Mursu J, Baumler H, Nascetti S, Salonen JT, Fito M, Virtanen J, Marrugat J, EUROLIVE Study Group. 2006. The effect of polyphenols in olive oil on heart disease risk factors: a randomized trial. *Ann Intern Med* 145:333-341.
- Crespy V, Morand C, Besson C, Cotellet N, Vezin H, Demigne C, Remesy C. 2003. The splanchnic metabolism of flavonoids highly differed according to the nature of the compound. *Am J Physiol Gastrointest Liver Physiol* 284:G980-G988.
- Cui T, Li JZ, Kayahara H, Ma L, Wu LX, Nakamura K. 2006. Quantification of the polyphenols and triterpene acids in chinese hawthorn fruit by high-performance liquid chromatography. *J Agric Food Chem* 54:4574-4581.
- de Caterina R. 1999. Inflammation and early events in atherogenesis. *Cardiologia* 44 Suppl 1:109-115.
- Delgado M., Llorca J., and Doménech JM. 2008. Investigación científica: Fundamentos metodológicos y estadísticos.
- Elosua R, Marrugat J, Molina L, Pons S, Pujol E. 1994. Validation of the Minnesota Leisure Time Physical Activity Questionnaire in Spanish men. The MARATHOM Investigators. *Am J Epidemiol* 139:1197-1209.
- Engberink MF, Hendriksen MA, Schouten EG, van Rooij FJ, Hofman A, Witteman JC, Geleijnse JM. 2009. Inverse association between dairy intake and hypertension: the Rotterdam Study. *Am J Clin Nutr* 89:1877-1883.
- Erdman JW, Jr., Balentine D, Arab L, Beecher G, Dwyer JT, Folts J, Harnly J, Hollman P, Keen CL, Mazza G, Messina M, Scalbert A, Vita J, Williamson G, Burrowes J. 2007. Flavonoids and heart health: proceedings of the ILSI North America Flavonoids Workshop, May 31-June 1, 2005, Washington, DC. *J Nutr* 137:718S-737S.
- Esti M, Cinquanta L, La Notte E. 1998. Phenolic Compounds in Different Olive Varieties. *J Agric Food Chem* 46:32-35.
- Estruch R, Martinez-Gonzalez MA, Corella D, Salas-Salvado J, Ruiz-Gutierrez V, Covas MI, Fiol M, Gomez-Gracia E, Lopez-Sabater MC, Vinyoles E, Aros F, Conde M, Lahoz C, Lapetra J, Saez G, Ros E. 2006. Effects of a Mediterranean-style diet on cardiovascular risk factors: a randomized trial. *Ann Intern Med* 145:1-11.

Bibliografia

Ferrara LA, Raimondi AS, d'Episcopo L, Guida L, Io Russo A, Marotta T. 2000. Olive Oil and Reduced Need for Antihypertensive Medications. *Arch Intern Med* 160:837-842.

Fisher MB, Paine MF, Strelevitz TJ, Wrighton SA. 2001. The role of hepatic and extrahepatic UDP-glucuronosyltransferases in human drug metabolism. *Drug Metab Rev* 33:273-297.

Fito M, Covas MI, Lamuela-Raventos RM, Vila J, Torrents L, de la Torre C, Marrugat J. 2000. Protective effect of olive oil and its phenolic compounds against low density lipoprotein oxidation. *Lipids* 35:633-638.

Fito M, Guxens M, Corella D, Saez G, Estruch R, de la Torre R, Frances F, Cabezas C, Lopez-Sabater MdC, Marrugat J, Garcia-Arellano A, Aros F, Ruiz-Gutierrez V, Ros E, Salas-Salvado J, Fiol M, Sola R, Covas MI, for the PREDIMED Study Investigators. 2007. Effect of a Traditional Mediterranean Diet on Lipoprotein Oxidation: A Randomized Controlled Trial. *Arch Intern Med* 167:1195-1203.

Fraser GE, Sabate J, Beeson WL, Strahan TM. 1992. A possible protective effect of nut consumption on risk of coronary heart disease. The Adventist Health Study. *Arch Intern Med* 152:1416-1424.

Fuster V, Badimon JJ, Badimon L. 1992. Clinical-pathological correlations of coronary disease progression and regression. *Circulation* 86:III1-11.

Gimeno E, de la Torre-Carbot C, Lamuela-Raventos RM, Castellote AI, Fito M, de la Torre R, Covas MI, Lopez-Sabater MC. 2007. Changes in the phenolic content of low density lipoprotein after olive oil consumption in men. A randomized crossover controlled trial. *Br J Nutr* 98:1243-1250.

Gleissner CA, Leitinger N, Ley K. 2007. Effects of native and modified low-density lipoproteins on monocyte recruitment in atherosclerosis. *Hypertension* 50:276-283.

Goldberg DM, Yan J, Soleas GJ. 2003. Absorption of three wine-related polyphenols in three different matrices by healthy subjects. *Clin Biochem* 36:79-87.

Grassi D, Necozione S, Lippi C, Croce G, Valeri L, Pasqualetti P, Desideri G, Blumberg JB, Ferri C. 2005. Cocoa reduces blood pressure and insulin resistance and improves endothelium-dependent vasodilation in hypertensives. *Hypertension* 46:398-405.

Grau M, Subirana I, Elosua R, Fito M, Covas MI, Sala J, Masia R, Ramos R, Solanas P, Cordon F, Nieto FJ, Marrugat J. 2010. Why should population attributable fractions be periodically recalculated? An example from cardiovascular risk estimation in southern Europe. *Prev Med*.

Gutteridge JM, Halliwell B. 2000. Free radicals and antioxidants in the year 2000. A historical look to the future. *Ann N Y Acad Sci* 899:136-147.

- Guxens M, Fito M, Martinez-Gonzalez MA, Salas-Salvado J, Estruch R, Vinyoles E, Fiol M, Corella D, Aros F, Gomez-Gracia E, Ruiz-Gutierrez V, Lapetra J, Ros E, Vila J, Covas MI. 2009. Hypertensive status and lipoprotein oxidation in an elderly population at high cardiovascular risk. *Am J Hypertens* 22:68-73.
- Halliwel B. 1996. Antioxidants in human health and disease. *Annu Rev Nutr* 16:33-50.
- Harnden KE, Frayn KN, Hodson L. 2010. Dietary Approaches to Stop Hypertension (DASH) diet: applicability and acceptability to a UK population. *J Hum Nutr Diet* 23:3-10.
- Hashimoto M, Kim S, Eto M, Iijima K, Ako J, Yoshizumi M, Akishita M, Kondo K, Itakura H, Hosoda K, Toba K, Ouchi Y. 2001. Effect of acute intake of red wine on flow-mediated vasodilatation of the brachial artery. *Am J Cardiol* 88:1457-60, A9.
- Hertog MG, Kromhout D, Aravanis C, Blackburn H, Buzina R, Fidanza F, Giampaoli S, Jansen A, Menotti A, Nedeljkovic S, . 1995. Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. *Arch Intern Med* 155:381-386.
- Hodgson JM, Chan SY, Puddey IB, Devine A, Wattanapenpaiboon N, Wahlqvist ML, Lukito W, Burke V, Ward NC, Prince RL, Croft KD. 2004. Phenolic acid metabolites as biomarkers for tea- and coffee-derived polyphenol exposure in human subjects. *British Journal of Nutrition* 91:301-305.
- Hodson L, Harnden KE, Roberts R, Dennis AL, Frayn KN. 2009. Does the DASH diet lower blood pressure by altering peripheral vascular function[quest]. *J Hum Hypertens*.
- Hong YJ, Mitchell AE. 2006. Identification of glutathione-related quercetin metabolites in humans. *Chem Res Toxicol* 19:1525-1532.
- Horwitz W. 2003. Official Method of Analysis of AOAC INTERNATIONAL (Appendix D and E).
- Hu FB, Stampfer MJ, Manson JE, Rimm EB, Colditz GA, Rosner BA, Speizer FE, Hennekens CH, Willett WC. 1998. Frequent nut consumption and risk of coronary heart disease in women: prospective cohort study. *BMJ* 317:1341-1345.
- Hung HC, Merchant A, Willett W, Ascherio A, Rosner BA, Rimm E, Joshipura KJ. 2003. The association between fruit and vegetable consumption and peripheral arterial disease. *Epidemiology* 14:659-665.
- Hyson DA, Schneeman BO, Davis PA. 2002. Almonds and Almond Oil Have Similar Effects on Plasma Lipids and LDL Oxidation in Healthy Men and Women. *Journal of Nutrition* 132:703-707.

Bibliografía

Iglesias-Bonilla P, Mayoral-Sánchez E, Lapetra-Peralta J, Iborra-Oquendo M, Villalba-Alcalá F, Cayuela-Domínguez A. 2002. Validación de dos sistemas de automedida de presión arterial, modelos OMRON HEM 705 CP y OMRON MI (HEM 422C2-E). *Atención Primaria* 30:22-28.

Ingram DK, Anson RM, de CR, Mamczarz J, Zhu M, Mattison J, Lane MA, Roth GS. 2004. Development of calorie restriction mimetics as a longevity strategy. *Ann N Y Acad Sci* 1019:412-423.

Jenkins DJ, Hu FB, Tapsell LC, Josse AR, Kendall CW. 2008. Possible benefit of nuts in type 2 diabetes. *J Nutr* 138:1752S-1756S.

Jenkins DJ, Kendall CW, Marchie A, Parker TL, Connelly PW, Qian W, Haight JS, Faulkner D, Vidgen E, Lapsley KG, Spiller GA. 2002. Dose response of almonds on coronary heart disease risk factors: blood lipids, oxidized low-density lipoproteins, lipoprotein(a), homocysteine, and pulmonary nitric oxide: a randomized, controlled, crossover trial. *Circulation* 106:1327-1332.

Jimenez-Monreal AM, Garcia-Diz L, Martinez-Tome M, Mariscal M, Murcia MA. 2009. Influence of cooking methods on antioxidant activity of vegetables. *J Food Sci* 74:H97-H103.

Kalogeropoulos N, Chiou A, Mylona A, Ioannou MS, Andrikopoulos NK. 2007. Recovery and distribution of natural antioxidants ([alpha]-tocopherol, polyphenols and terpenic acids) after pan-frying of Mediterranean finfish in virgin olive oil. *Food Chemistry* 100:509-517.

Kato H. 2002. Regulation of functions of vascular wall cells by tissue factor pathway inhibitor: basic and clinical aspects. *Arterioscler Thromb Vasc Biol* 22:539-548.

Kelemen LE. 2006. Food frequency questionnaires: not irrelevant yet. *Cancer Epidemiol Biomarkers Prev* 15:1054.

Khazaei M, Moien-Afshari F, Laher I. 2008. Vascular endothelial function in health and diseases. *Pathophysiology* 15:49-67.

Knekt P, Kumpulainen J, Jarvinen R, Rissanen H, Heliovaara M, Reunanen A, Hakulinen T, Aromaa A. 2002. Flavonoid intake and risk of chronic diseases. *Am J Clin Nutr* 76:560-568.

Koster H, Halsema I, Scholtens E, Knippers M, Mulder GJ. 1981. Dose-dependent shifts in the sulfation and glucuronidation of phenolic compounds in the rat in vivo and in isolated hepatocytes. The role of saturation of phenolsulfotransferase. *Biochem Pharmacol* 30:2569-2575.

Krauss RM, Eckel RH, Howard B, Appel LJ, Daniels SR, Deckelbaum RJ, Erdman JW, Jr., Kris-Etherton P, Goldberg IJ, Kotchen TA, Lichtenstein AH, Mitch WE, Mullis R, Robinson K, Wylie-Rosett J, St JS, Suttie J, Tribble DL, Bazzarre TL. 2000. AHA Dietary Guidelines: revision 2000: A statement for

healthcare professionals from the Nutrition Committee of the American Heart Association. *Circulation* 102:2284-2299.

Kris-Etherton PM, Hecker KD, Bonanome A, Coval SM, Binkoski AE, Hilpert KF, Griel AE, Etherton TD. 2002. Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. *Am J Med* 113 Suppl 9B:71S-88S.

Kris-Etherton PM, Hu FB, Ros E, Sabate J. 2008. The role of tree nuts and peanuts in the prevention of coronary heart disease: multiple potential mechanisms. *J Nutr* 138:1746S-1751S.

Kristal AR, Peters U, Potter JD. 2005. Is it time to abandon the food frequency questionnaire? *Cancer Epidemiol Biomarkers Prev* 14:2826-2828.

Krogholm KS, Haraldsdottir J, Knuthsen P, Rasmussen SE. 2004. Urinary total flavonoid excretion but not 4-pyridoxic acid or potassium can be used as a biomarker for the intake of fruits and vegetables. *Journal of Nutrition* 134:445-451.

Kushi LH, Folsom AR, Prineas RJ, Mink PJ, Wu Y, Bostick RM. 1996. Dietary antioxidant vitamins and death from coronary heart disease in postmenopausal women. *N Engl J Med* 334:1156-1162.

Lapidot T, Harel S, Granit R, Kanner J. 1998. Bioavailability of red wine anthocyanins as detected in human urine. *Journal of Agricultural and Food Chemistry* 46:4297-4302.

Lawes CM, Vander HS, Rodgers A. 2008. Global burden of blood-pressure-related disease, 2001. *Lancet* 371:1513-1518.

Lesser S, Cermak R, Wolffram S. 2006. The fatty acid pattern of dietary fat influences the oral bioavailability of the flavonol quercetin in pigs. *Br J Nutr* 96:1047-1052.

Lindsay DG, Astley SB. 2002. European research on the functional effects of dietary antioxidants - EUROFEDA. *Mol Aspects Med* 23:1-38.

Liu DQ, Xia YQ, Bakhtiar R. 2002. Use of a liquid chromatography/ion trap mass spectrometry/triple quadrupole mass spectrometry system for metabolite identification. *Rapid Commun Mass Spectrom* 16:1330-1336.

Llorente-Cortes V, Martinez-Gonzalez J, Badimon L. 2000. LDL receptor-related protein mediates uptake of aggregated LDL in human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 20:1572-1579.

Lou H, Yuan H, Ma B, Ren D, Ji M, Oka S. 2004. Polyphenols from peanut skins and their free radical-scavenging effects. *Phytochemistry* 65:2391-2399.

Manach C, Donovan JL. 2004. Pharmacokinetics and metabolism of dietary flavonoids in humans. *Free Radic Res* 38:771-785.

Bibliografia

Manach C, Mazur A, Scalbert A. 2005a. Polyphenols and prevention of cardiovascular diseases. *Curr Opin Lipidol* 16:77-84.

Manach C, Morand C, Gil-Izquierdo A, Bouteloup-Demange C, Remesy C. 2003. Bioavailability in humans of the flavanones hesperidin and narirutin after the ingestion of two doses of orange juice. *European Journal of Clinical Nutrition* 57:235-242.

Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. 2004. Polyphenols: food sources and bioavailability. *Am J Clin Nutr* 79:727-747.

Manach C, Williamson G, Morand C, Scalbert A, Remesy C. 2005b. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am J Clin Nutr* 81:230S-242S.

Mancia G, de Backer G, Dominiczak A, Cifkova R, Fagard R, Germano G, Grassi G, Heagerty AM, Kjeldsen SE, Laurent S, Narkiewicz K, Ruilope L, Rynkiewicz A, Schmieder RE, Boudier HA, Zanchetti A, Vahanian A, Camm J, de Caterina R, Dean V, Dickstein K, Filippatos G, Funck-Brentano C, Hellemans I, Kristensen SD, McGregor K, Sechtem U, Silber S, Tendera M, Widimsky P, Zamorano JL, Erdine S, Kiowski W, gabiti-Rosei E, Ambrosioni E, Lindholm LH, Viigimaa M, Adamopoulos S, Bertomeu V, Clement D, Erdine S, Farsang C, Gaita D, Lip G, Mallion JM, Manolis AJ, Nilsson PM, O'Brien E, Ponikowski P, Redon J, Ruschitzka F, Tamargo J, van Zwieten P., Waeber B, Williams B. 2007. 2007 Guidelines for the Management of Arterial Hypertension: The Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). *J Hypertens* 25:1105-1187.

Marshall JR. 2003. Methodologic and statistical considerations regarding use of biomarkers of nutritional exposure in epidemiology. *J Nutr* 133 Suppl 3:881S-887S.

Martin-Moreno JM, Boyle P, Gorgojo L, Maisonneuve P, Fernandez-Rodriguez JC, Salvini S, Willett WC. 1993. Development and validation of a food frequency questionnaire in Spain. *Int J Epidemiol* 22:512-519.

Matsumoto H, Inaba H, Kishi M, Tominaga S, Hirayama M, Tsuda T. 2001. Orally administered delphinidin 3-rutinoside and cyanidin 3-rutinoside are directly absorbed in rats and humans and appear in the blood as the intact forms. *J Agric Food Chem* 49:1546-1551.

McCord JM. 2000. The evolution of free radicals and oxidative stress. *Am J Med* 108:652-659.

Mennen LI, Sapinho D, Ito H, Bertrais S, Galan P, Hercberg S, Scalbert A. 2006. Urinary flavonoids and phenolic acids as biomarkers of intake for polyphenol-rich foods. *Br J Nutr* 96:191-198.

Meydani M. 2005. A Mediterranean-style diet and metabolic syndrome. *Nutr Rev* 63:312-314.

- Mirie W. 1997. Ageing and nutritional needs. *East Afr Med J* 74:622-624.
- Muñoz MA, Fito M, Marrugat J, Covas MI, Schroder H. 2009. Adherence to the Mediterranean diet is associated with better mental and physical health. *Br J Nutr* 101:1821-1827.
- Nielsen SE, Freese R, Kleemola P, Mutanen M. 2002. Flavonoids in human urine as biomarkers for intake of fruits and vegetables. *Cancer Epidemiol Biomarkers Prev* 11:459-466.
- Noroozi M, Burns J, Crozier A, Kelly IE, Lean ME. 2000. Prediction of dietary flavonol consumption from fasting plasma concentration or urinary excretion. *Eur J Clin Nutr* 54:143-149.
- Núñez-Córdoba JM, Valencia-Serrano F, Toledo E, Alonso A, Martínez-González MA. 2009. The Mediterranean diet and incidence of hypertension: the Seguimiento Universidad de Navarra (SUN) Study. *Am J Epidemiol* 169:339-346.
- Orr AW, Stockton R, Simmers MB, Sanders JM, Sarembok IJ, Blackman BR, Schwartz MA. 2007. Matrix-specific p21-activated kinase activation regulates vascular permeability in atherogenesis. *J Cell Biol* 176:719-727.
- Perona JS, Canizares J, Montero E, Sanchez-Dominguez JM, Catala A, Ruiz-Gutierrez V. 2004. Virgin olive oil reduces blood pressure in hypertensive elderly subjects. *Clin Nutr* 23:1113-1121.
- Pietta PG. 2000. Flavonoids as antioxidants. *J Nat Prod* 63:1035-1042.
- Piskula MK. 2000. Soy isoflavone conjugation differs in fed and food-deprived rats. *J Nutr* 130:1766-1771.
- Piskula MK, Terao J. 1998. Accumulation of (-)-epicatechin metabolites in rat plasma after oral administration and distribution of conjugation enzymes in rat tissues. *J Nutr* 128:1172-1178.
- Potischman N. 2003. Biologic and methodologic issues for nutritional biomarkers. *J Nutr* 133 Suppl 3:875S-880S.
- Radhika G, Sudha V, Mohan Sathya R, Ganesan A, Mohan V. 2008. Association of fruit and vegetable intake with cardiovascular risk factors in urban south Indians. *British Journal of Nutrition* 99:398-405.
- Radtke J, Linseisen J, Wolfram G. 2002. Fasting plasma concentrations of selected flavonoids as markers of their ordinary dietary intake. *Eur J Nutr* 41:203-209.
- Razquin C, Martinez JA, Martinez-Gonzalez MA, Mitjavila MT, Estruch R, Marti A. 2009. A 3 years follow-up of a Mediterranean diet rich in virgin olive oil is associated with high plasma antioxidant capacity and reduced body weight gain. *Eur J Clin Nutr* 63:1387-1393.

Bibliografia

Rechner AR, Kuhnle G, Bremner P, Hubbard GP, Moore KP, Rice-Evans CA. 2002. The metabolic fate of dietary polyphenols in humans. *Free Radic Biol Med* 33:220-235.

Reshef N, Hayari Y, Goren C, Boaz M, Madar Z, Knobler H. 2005. Antihypertensive effect of sweetie fruit in patients with stage I hypertension. *Am J Hypertens* 18:1360-1363.

Rice-Evans C, Miller N. 1997. Measurement of the antioxidant status of dietary constituents, low density lipoproteins and plasma. *Prostaglandins Leukot Essent Fatty Acids* 57:499-505.

Rissanen TH, Voutilainen S, Virtanen JK, Venho B, Vanharanta M, Mursu J, Salonen JT. 2003. Low intake of fruits, berries and vegetables is associated with excess mortality in men: the Kuopio Ischaemic Heart Disease Risk Factor (KIHD) Study. *J Nutr* 133:199-204.

Rodrigo R, Prat H, Passalacqua W, Araya J, Bachler JP. 2008. Decrease in oxidative stress through supplementation of vitamins C and E is associated with a reduction in blood pressure in patients with essential hypertension. *Clin Sci (Lond)* 114:625-634.

Roura E, Almajano MP, Bilbao ML, Andres-Lacueva C, Estruch R, Lamuela-Raventos RM. 2007a. Human urine: epicatechin metabolites and antioxidant activity after cocoa beverage intake. *Free Radic Res* 41:943-949.

Roura E, Andres-Lacueva C, Estruch R, Lamuela-Raventos RM. 2006. Total polyphenol intake estimated by a modified Folin-Ciocalteu assay of urine. *Clin Chem* 52:749-752.

Roura E, Andres-Lacueva C, Estruch R, Lourdes Mata BM, Izquierdo-Pulido M, Lamuela-Raventos RM. 2008. The effects of milk as a food matrix for polyphenols on the excretion profile of cocoa (-)-epicatechin metabolites in healthy human subjects. *Br J Nutr* 100:846-851.

Roura E, Andres-Lacueva C, Estruch R, Mata-Bilbao ML, Izquierdo-Pulido M, Waterhouse AL, Lamuela-Raventos RM. 2007b. Milk does not affect the bioavailability of cocoa powder flavonoid in healthy human. *Ann Nutr Metab* 51:493-498.

Sahlin E, Savage GP, Lister CE. 2004. Investigation of the antioxidant properties of tomatoes after processing. *Journal of Food Composition and Analysis* 17:635-647.

Salas-Salvado J, Fernandez-Ballart J, Ros E, Martinez-Gonzalez MA, Fito M, Estruch R, Corella D, Fiol M, Gomez-Gracia E, Aros F, Flores G, Lapetra J, Lamuela-Raventos R, Ruiz-Gutierrez V, Bullo M, Basora J, Covas MI, for the PREDIMED Study Investigators. 2008. Effect of a Mediterranean Diet Supplemented With Nuts on Metabolic Syndrome Status: One-Year Results of the PREDIMED Randomized Trial. *Arch Intern Med* 168:2449-2458.

- Saura-Calixto F, Goñi I. 2006. Antioxidant capacity of the Spanish Mediterranean diet. *Food Chemistry* 94:442-447.
- Scalbert A, Williamson G. 2000. Dietary intake and bioavailability of polyphenols. *J Nutr* 130:2073S-2085S.
- Scholz S, Williamson G. 2007. Interactions affecting the bioavailability of dietary polyphenols in vivo. *Int J Vitam Nutr Res* 77:224-235.
- Serafini M, Ghiselli A, Ferro-Luzzi A. 1996. In vivo antioxidant effect of green and black tea in man. *Eur J Clin Nutr* 50:28-32.
- Serra-Majem L, Bes-Rastrollo M, Roman-Vinas B, Pfrimer K, Sanchez-Villegas A, Martinez-Gonzalez MA. 2009. Dietary patterns and nutritional adequacy in a Mediterranean country. *Br J Nutr* 101 Suppl 2:S21-S28.
- Serra-Majem L, Roman B, Estruch R. 2006. Scientific evidence of interventions using the Mediterranean diet: a systematic review. *Nutr Rev* 64:S27-S47.
- Sesso HD, Gaziano JM, Liu S, Buring JE. 2003. Flavonoid intake and the risk of cardiovascular disease in women. *Am J Clin Nutr* 77:1400-1408.
- Shimada K, Watanabe H, Hosoda K, Takeuchi K, Yoshikawa J. 1999. Effect of red wine on coronary flow-velocity reserve. *Lancet* 354:1002.
- Silberberg M, Morand C, Mathevon T, Besson C, Manach C, Scalbert A, Remesy C. 2006. The bioavailability of polyphenols is highly governed by the capacity of the intestine and of the liver to secrete conjugated metabolites. *Eur J Nutr* 45:88-96.
- Silva MM, Santos MR, Caroco G, Rocha R, Justino G, Mira L. 2002. Structure-antioxidant activity relationships of flavonoids: a re-examination. *Free Radic Res* 36:1219-1227.
- Sinclair DA. 2005. Toward a unified theory of caloric restriction and longevity regulation. *Mech Ageing Dev* 126:987-1002.
- Singleton VL, Orthofer R, Lamuela-Raventos RM. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Oxidants and Antioxidants, Pt A* 299:152-178.
- Sofi F, Cesari F, Abbate R, Gensini GF, Casini A. 2008. Adherence to Mediterranean diet and health status: meta-analysis. *BMJ* 337:a1344.
- Spencer JP. 2003. Metabolism of tea flavonoids in the gastrointestinal tract. *J Nutr* 133:3255S-3261S.
- Spencer JP, Abd El Mohsen MM, Minihaane AM, Mathers JC. 2008. Biomarkers of the intake of dietary polyphenols: strengths, limitations and application in nutrition research. *Br J Nutr* 99:12-22.

Bibliografía

Stamler J, Liu K, Ruth KJ, Pryer J, Greenland P. 2002. Eight-year blood pressure change in middle-aged men: relationship to multiple nutrients. *Hypertension* 39:1000-1006.

Taubert D, Berkels R, Roesen R, Klaus W. 2003. Chocolate and blood pressure in elderly individuals with isolated systolic hypertension. *JAMA* 290:1029-1030.

Tomás-Barberán FA. 2003. Los polifenoles de los alimentos y la salud. *Alimentación, Nutrición y Salud*. Instituto Danone.p 41-53.

Tovar MJ, Motilva MJ, Romero MP. 2001. Changes in the Phenolic Composition of Virgin Olive Oil from Young Trees (*Olea europaea* L. cv. Arbequina) Grown under Linear Irrigation Strategies. *Journal of Agricultural and Food Chemistry* 49:5502-5508.

Trichopoulou A, Lagiou P, Kuper H, Trichopoulos D. 2000. Cancer and Mediterranean dietary traditions. *Cancer Epidemiol Biomarkers Prev* 9:869-873.

Trichopoulou A, Naska A, Orfanos P, Trichopoulos D. 2005. Mediterranean diet in relation to body mass index and waist-to-hip ratio: the Greek European Prospective Investigation into Cancer and Nutrition Study. *Am J Clin Nutr* 82:935-940.

Turkmen N, Sari F, Velioglu YS. 2005. The effect of cooking methods on total phenolics and antioxidant activity of selected green vegetables. *Food Chemistry* 93:713-718.

Urpi-Sarda M, Garrido I, Monagas M, Gomez-Cordoves C, Medina-Remón A, Andres-Lacueva C, Bartolome B. 2009a. Profile of plasma and urine metabolites after the intake of almond [*Prunus dulcis* (Mill.) D.A. Webb] polyphenols in humans. *J Agric Food Chem* 57:10134-10142.

Urpi-Sarda M, Jauregui O, Lamuela-Raventos RM, Jaeger W, Miksits M, Covas MI, Andres-Lacueva C. 2005. Uptake of diet resveratrol into the human low-density lipoprotein. Identification and quantification of resveratrol metabolites by liquid chromatography coupled with tandem mass spectrometry. *Anal Chem* 77:3149-3155.

Urpi-Sarda M, Monagas M, Khan N, Lamuela-Raventos RM, Santos-Buelga C, Sacanella E, Castell M, Permanyer J, Andres-Lacueva C. 2009b. Epicatechin, procyanidins, and phenolic microbial metabolites after cocoa intake in humans and rats. *Anal Bioanal Chem* 394:1545-1556.

Urpi-Sarda M, Zamora-Ros R, Lamuela-Raventos R, Cherubini A, Jauregui O, de la Torre R, Covas MI, Estruch R, Jaeger W, Andres-Lacueva C. 2007. HPLC-tandem mass spectrometric method to characterize resveratrol metabolism in humans. *Clin Chem* 53:292-299.

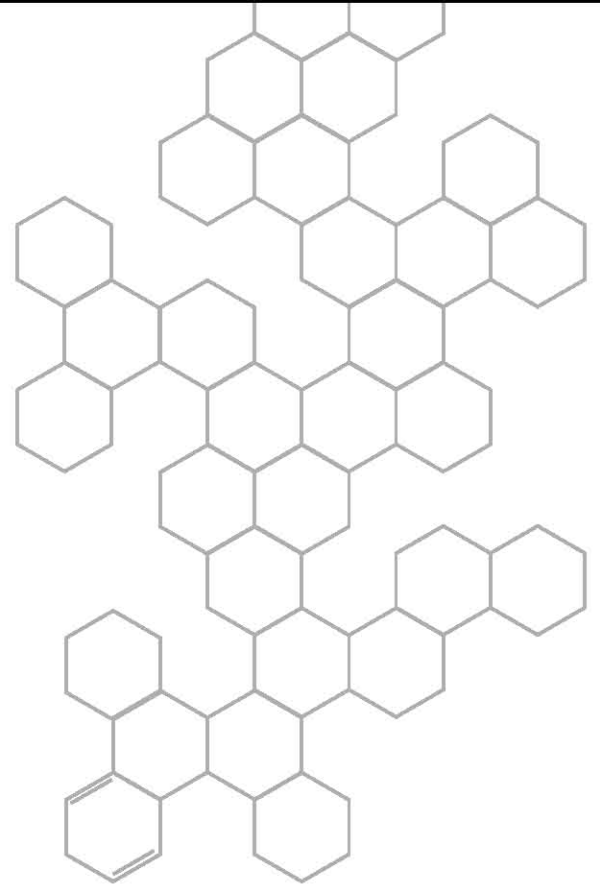
van het Hof KH, de Boer BC, Tijburg LB, Lucius BR, Zijp I, West CE, Hautvast JG, Weststrate JA. 2000a. Carotenoid bioavailability in humans from tomatoes processed in different ways determined from the carotenoid response in the

- triglyceride-rich lipoprotein fraction of plasma after a single consumption and in plasma after four days of consumption. *J Nutr* 130:1189-1196.
- van het Hof KH, West CE, Weststrate JA, Hautvast JG. 2000b. Dietary factors that affect the bioavailability of carotenoids. *J Nutr* 130:503-506.
- van het Hof KH, Wiseman SA, Yang CS, Tijburg LB. 1999. Plasma and lipoprotein levels of tea catechins following repeated tea consumption. *Proc Soc Exp Biol Med* 220:203-209.
- Visioli F, Galli C, Grande S, Colonnelli K, Patelli C, Galli G, Caruso D. 2003. Hydroxytyrosol excretion differs between rats and humans and depends on the vehicle of administration. *J Nutr* 133:2612-2615.
- Whelton PK, He J, Appel LJ, Cutler JA, Havas S, Kotchen TA, Roccella EJ, Stout R, Vallbona C, Winston MC, Karimbakas J. 2002. Primary prevention of hypertension: clinical and public health advisory from The National High Blood Pressure Education Program. *JAMA* 288:1882-1888.
- Willcox BJ, Willcox DC, Ferrucci L. 2008. Secrets of healthy aging and longevity from exceptional survivors around the globe: lessons from octogenarians to supercentenarians. *J Gerontol A Biol Sci Med Sci* 63:1181-1185.
- Willcox BJ, Willcox DC, Todoriki H, Fujiyoshi A, Yano K, He Q, Curb JD, Suzuki M. 2007a. Caloric restriction, the traditional Okinawan diet, and healthy aging: the diet of the world's longest-lived people and its potential impact on morbidity and life span. *Ann N Y Acad Sci* 1114:434-455.
- Willcox DC, Willcox BJ, Shimajiri S, Kurechi S, Suzuki M. 2007b. Aging gracefully: a retrospective analysis of functional status in Okinawan centenarians. *Am J Geriatr Psychiatry* 15:252-256.
- World Health Organization. 2002. Nutrition and Health in Europe . ISBN:9289013648:14-15.
- Wu X, Cao G, Prior RL. 2002. Absorption and metabolism of anthocyanins in elderly women after consumption of elderberry or blueberry. *J Nutr* 132:1865-1871.
- Yamaguchi T, Mizobuchi T, Kajikawa R, Kawashima H, Miyabe F, Terao J, Takamura H, Matoba T. 2001. Radical scavenging activity of vegetables and the effect of cooking on their activity. *Food Sci Technol Res* 7:250-257.
- Yamashita S, Sakane T, Harada M, Sugiura N, Koda H, Kiso Y, Sezaki H. 2002. Absorption and metabolism of antioxidative polyphenolic compounds in red wine. *Ann N Y Acad Sci* 957:325-328.
- Zamora-Ros R, Urpi-Sarda M, Lamuela-Raventos RM, Estruch R, Martinez-Gonzalez MA, Bullo M, Aros F, Cherubini A, Andres-Lacueva C. 2009. Resveratrol metabolites in urine as a biomarker of wine intake in free-living subjects: The PREDIMED Study. *Free Radic Biol Med* 46:1562-1566.

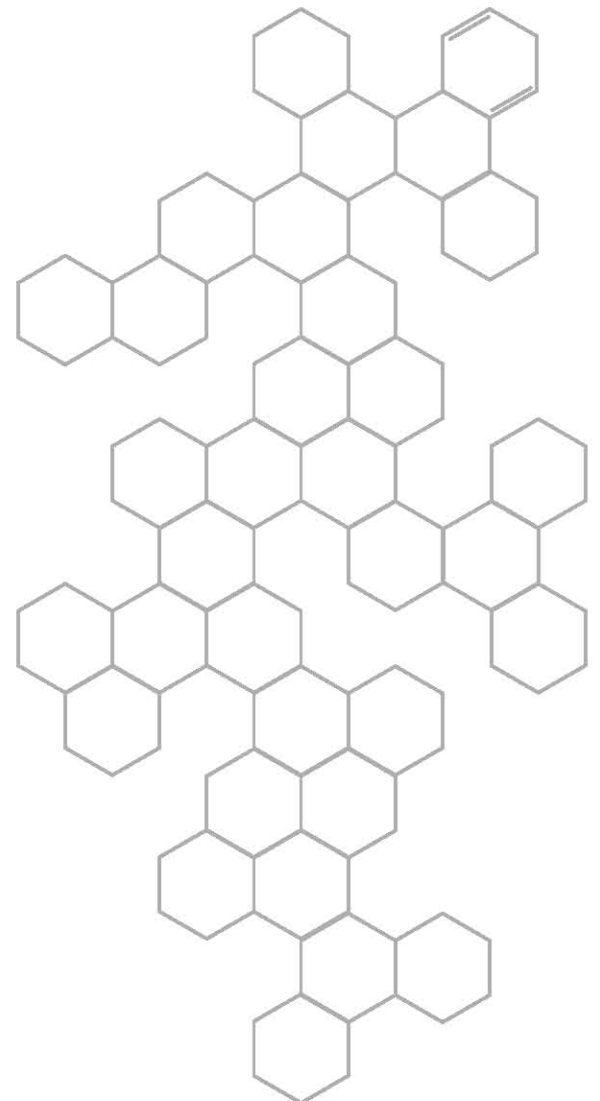
Bibliografia

Zamora-Ros R, Urpi-Sarda M, Lamuela-Raventos RM, Estruch R, Vazquez-Agell M, Serrano-Martinez M, Jaeger W, Andres-Lacueva C. 2006. Diagnostic performance of urinary resveratrol metabolites as a biomarker of moderate wine consumption. *Clin Chem* 52:1373-1380.

Zazpe I, Sanchez-Tainta A, Estruch R, Lamuela-Raventos RM, Schroder H, Salas-Salvado J, Corella D, Fiol M, Gomez-Gracia E, Aros F, Ros E, Ruiz-Gutierrez V, Iglesias P, Conde-Herrera M, Martinez-Gonzalez MA. 2008. A large randomized individual and group intervention conducted by registered dietitians increased adherence to Mediterranean-type diets: the PREDIMED study. *J Am Diet Assoc* 108:1134-1144.



ANNEXES



8. ANNEXES

8.1. ALTRES PUBLICACIONS EN REVISTES

En aquest annex s'inclouen altres publicacions, no incloses com a treball de tesis doctoral, però en les que també he col·laborat i es pot aplicar la metodologia desenvolupada en el primer article.

Publicació 6: Perfil dels metabòlits del plasma i orina en humans després del consum de polifenols de l'ametlla [*Prunus dulcis* (Mill.) D.A. Webb]

Mireia Urpi-Sarda; Ignacio Garrido; María Monagas; Carmen Gómez-Cordovés; Alexander Medina-Remón; Cristina Andres-Lacueva; Begoña Bartolomé.

J Agric.Food Chem. 2010, 57 (21): 10134-10142

Erratum in: J Agric Food Chem. 2009 Dec 23;57(24):11581-2.

Profile of Plasma and Urine Metabolites after the Intake
of Almond [*Prunus dulcis* (Mill.) D.A. Webb] Polyphenols
in HumansMIREIA URPI-SARDA,[†] IGNACIO GARRIDO,[‡] MARÍA MONAGAS,[‡]
CARMEN GÓMEZ-CORDOVÉS,[‡] ALEXANDER MEDINA-REMÓN,[†]
CRISTINA ANDRES-LACUEVA,^{*,†} AND BEGOÑA BARTOLOME[‡][†]Nutrition and Food Science Department, XaRTA, INSA, CONSOLIDER INGENIO 2010
Program (FUN-C-FOOD CSD2007-063), Pharmacy Faculty, University of Barcelona, Avinyuda Joan
XXIII s/n, 08028 Barcelona, Spain, and [‡]Instituto de Fermentaciones Industriales (CSIC),
28006 Madrid, Spain

Nut skins are considered to be a rich source of polyphenols and may be partially responsible for the numerous health effects associated with nut consumption. However, more bioavailability studies of nut skin polyphenols are needed to understand the health effects derived from nut consumption. The aim of the present study was to determine the profiles of both phase II and microbial-derived phenolic metabolites in plasma and urine samples before and after the intake of almond skin polyphenols by healthy human subjects ($n = 2$). Glucuronide, *O*-methyl glucuronide, sulfate, and *O*-methyl sulfate derivatives of (epi)catechin, as well as the glucuronide conjugates of naringenin and glucuronide and sulfate conjugates of isorhamnetin, were detected in plasma and urine samples after consumption of almond skin polyphenols. The main microbial-derived metabolites of flavanols, such as 5-(dihydroxyphenyl)- γ -valerolactone and 5-(hydroxymethoxyphenyl)- γ -valerolactone, were also detected in their glucuronide and sulfate forms. In addition, numerous metabolites derived from further microbial degradation of hydroxyphenylvalerolactones, including hydroxyphenylpropionic, hydroxyphenylacetic, hydroxycinnamic, hydroxybenzoic, and hydroxyhippuric acids, registered major changes in urine after the consumption of almond skin polyphenols. The urinary excretion of these microbial metabolites was estimated to account for a larger proportion of the total polyphenol ingested than phase II metabolites of (epi)catechin, indicating the important role of intestinal bacteria in the metabolism of highly polymerized almond skin polyphenols. To the authors' knowledge this study constitutes the most complete report of the absorption of almond skin polyphenols in humans.

KEYWORDS: Almond; polyphenols; metabolites; microflora; hydroxyphenylvalerolactone

INTRODUCTION

Polyphenols are among the most abundant bioactive compounds of the Mediterranean diet and may play a key role in the prevention of cardiovascular and neurodegenerative diseases and cancer. Among the foods traditionally associated with the Mediterranean diet, the consumption of almonds [*Prunus dulcis* (Mill.) D.A. Webb] has been associated with beneficial protection against cardiovascular and obesity-related diseases (1). Moreover, Jenkins et al. (2,3) have also demonstrated that consumption of whole almonds decreases postprandial glycemia, insulinemia, and oxidative protein damage in humans. In fact, results from in vitro studies have revealed that almond skin polyphenols act in synergy with vitamins C and E to protect low-density lipoprotein (LDL) from oxidation (4,5) and enhance the antioxidant defense in other physiological environments (6).

*Corresponding author (telephone +34-93-4034840; fax +34-93-4035931; e-mail candres@ub.edu).

Besides their role in the prevention of cardiovascular and obesity-related diseases, results from in vitro studies have revealed that ground almonds significantly increased the populations of bifidobacteria and *Eubacterium rectale*, resulting in a higher prebiotic index (4.43) than for commercial prebiotic fructooligosaccharides (4.08) (7), indicating a potential prebiotic effect of almond seeds.

Almond skins are considered to be a very rich source of polyphenols. Non-flavonoid compounds include protocatechuic aldehyde and protocatechuic, vanillic, *p*-hydroxybenzoic, *trans-p*-coumaric, and chlorogenic acids (8–10). Flavonoid compounds identified in almond skins include flavanols, flavonols, dihydroflavonols, and flavanones. The monomers (+)-catechin and (–)-epicatechin, as well as procyanidins B1, B2, B3, and B4, have been identified in almond skins (11, 12). Although it was originally thought that almond skin proanthocyanidins were exclusively constituted of (+)-catechin and (–)-epicatechin units linked by B-type bonds (11, 13), later studies revealed the presence of delphinidin after *n*-butanol–HCl hydrolysis of almond seed

Article

J. Agric. Food Chem., Vol. 57, No. 21, 2009 10135

crude extracts, indicating the additional occurrence of prodelphinidins in almonds (12). More recently, Monagas et al. (10) have confirmed the presence of A-type procyanidins and both A- and B-type propylgallate and prodelphinidin polymers up to decamers for the first time in almond skins. Concerning flavonols, the -3-O-glucosides, -3-O-galactosides, and -3-O-rutinosides of quercetin, kaempferol, and isorhamnetin, as well as their corresponding aglycones, have been identified in almond skins (8–10, 14–16). Recently, morin (16), dihydrokaempferol (9), and dihydroquercetin (taxifolin) (10) have also been reported. Finally, the flavanones identified in almond skins include naringenin-7-O-glucoside, eriodictyol-7-O-glucoside, eriodictyol-7-O-galactoside, and their corresponding aglycones (8–10).

The *in vivo* physiological importance derived from the consumption of almond polyphenols would depend on their bioavailability. However, studies concerning the bioavailability of almond polyphenols are scarce. Polyphenols bioavailability is strongly influenced by their chemical structure (17, 18). Nonglycosylated polyphenols contained in almond skins, such as monomeric flavanols and dimeric procyanidins (to a lower extent), would be directly absorbed in the small intestine, where they would be first conjugated and later metabolized in the liver into methyl, glucuronide, and sulfate derivatives by phase II enzymes. However, oligomers presenting a mean degree of polymerization (mDP) of ≥ 3 and polymeric flavanols (proanthocyanidins) are not absorbed and reach the colon, where they are metabolized by the intestinal microbiota into hydroxyphenylvalerolactones and various phenolic acids, including phenylpropionic, phenylacetic, and benzoic acid derivatives, that can be further absorbed and then conjugated in the liver (19, 20). Conversely, glycosylated polyphenols such as flavonol glycosides must be first hydrolyzed by intestinal β -glucosidases before they can be absorbed, although the type of sugar that is attached to the flavonoid limits the absorption in the small intestine (18). Almond skin flavonols containing rhamnose, such as rutinosides, would be poorly absorbed and transferred to the large intestine, where they would be degraded by the rhamnosidases of the intestinal microbiota (21). In this sense, a large amount of polyphenols contained in almond skins would be exclusively metabolized by the intestinal microbiota.

To our knowledge, only Blumberg and his colleagues have carried out bioavailability studies in plasma after the ingestion of almond polyphenols in hamsters (4) and in humans (22). In both cases, plasma samples were subjected to hydrolysis prior to LC analysis, and only total levels of flavonoid aglycones were quantified. No phenolic metabolites in their actual conjugate forms, or phenolic metabolites derived from the microbiota activity, have been reported so far in human fluids after the intake of almond polyphenols. Taking these facts into consideration, as well as the previously described potential prebiotic effect derived from almond consumption, a pilot study was conducted with healthy human volunteers to determine the profile of both phase II and microbial-derived phenolic metabolites in plasma and urine samples after the intake of almond skin polyphenols.

MATERIALS AND METHODS

Standards and Reagents. Phenylacetic acid, 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, 4-hydroxy-3-methoxyphenylacetic acid, 3-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylpropionic acid, *p*-coumaric acid, caffeic acid, ferulic acid, 3-hydroxy-4-methoxycinnamic acid, protocatechuic acid, 3- and 4-hydroxybenzoic acids, ethyl gallate, (–)-epicatechin, (+)-catechin, procyanidin B2, and β -glucuronidase/sulfatase (from *Helix pomatia*) were purchased from Sigma-Aldrich (St. Louis, MO). 4-Hydroxyhippuric acid was purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). Procyanidin B3 and

B1, vanillic acid, *m*-coumaric acid, eriodictyol-7-O-glucoside, eriodictyol, kaempferol-3-O-rutinoside, kaempferol-3-O-glucoside, kaempferol-3-O-galactoside, dihydroquercetin, isorhamnetin-3-O-rutinoside, isorhamnetin-3-O-glucoside, isorhamnetin-3-O-galactoside, isorhamnetin, naringenin-7-O-glucoside, naringenin, and kaempferol were purchased from Extrasynthèse (Genay, France). LC grade solvents methanol, acetonitrile, glacial acetic acid, and formic acid were purchased from Scharlau (Barcelona, Spain). Hydrochloric acid was purchased from Panreac (Barcelona, Spain).

Almond Extract. A commercial almond skin extract (Amanda), kindly supplied by Puleva Biotech, S.A. (Granada, Spain), was used for this study. The extract was encapsulated (350 mg of extract + 50 mg of cellulose per capsule) by the supplier and presented a total polyphenol content of 221 mg of gallic acid/g of encapsulated extract, a total proanthocyanidin content of 314.8 mg of cyanidin/g, and an antioxidant capacity (ORAC value) of 3.10 mmol of Trolox/g.

Analysis of Phenolic Compounds of the Encapsulated Almond Skin Extract by LC-DAD–Fluorescence and LC-DAD/ESI-MS. An amount of 0.025 g of the almond skin extract contained in capsules was dissolved in 50 mL of water/HCl (1000:1, v/v) and sonicated for 15 min. An aliquot (40 mL) was extracted with ethyl acetate (40 mL $\times$ 4). The organic phases were combined and dried with anhydrous Na_2SO_4 for 20 min. The extract was then taken to dryness under vacuum, dissolved in 2 mL of methanol/ H_2O (50:50, v/v), and finally filtered (0.45 μm). The extractions were performed in duplicate.

A Waters (Milford, MA) liquid chromatography system, equipped with a 2695 separation module, a 2996 photodiode array detector (DAD), and a 2475 multi λ fluorescence detector coupled to the Waters Empower (version 5.0) for data acquisition and processing, was used. Separation was performed on a 150 $\times$ 4.6 mm i.d., 4 μm reversed-phase ACE 3 C₁₈ (Advanced Chromatography Technologies, Aberdeen, Scotland) column at room temperature. A gradient consisting of solvent A (water/acetic acid, 98:2, v/v) and solvent B (water/acetonitrile/acetic acid, 73:25:2, v/v/v) was applied at a flow rate of 1.0 mL/min as follows (10): 0–80% B linear from 0 to 55 min, 80–90% B linear from 55 to 57 min, 90% B isocratic from 57 to 70 min, 90–95% B linear from 70 to 80 min, 95–100% B from 80 to 90 min, followed by washing (methanol) and re-equilibration of the column from 90 to 120 min. A 75 μL volume sample was injected into the column. The detection conditions were 210–360 nm (DAD) and 280 and 310 nm for the emission and excitation filters, respectively (fluorescence detector). Quantification was carried out by external standard calibration curves. All of the phenolic compounds were quantified at 280 nm, except flavanols, which were quantified by their fluorescence response. Due to the lack of commercial standards, oligomeric flavanols were quantified using the (–)-epicatechin calibration curve.

In addition to their UV spectra, the identification of phenolic compounds was also carried out by mass spectrometry coupled to LC. A Hewlett-Packard series 1100 (Palo Alto, CA) chromatography system, equipped with a DAD and a quadrupole mass spectrometer (Hewlett-Packard series 1100 MSD) with an electrospray interface, was used. Separation conditions were the same as described above except for the flow rate, which was set to 0.7 mL/min. The ESI source was operated in negative mode using the following parameters: drying gas (N_2) flow and temperature, 10 L/min and 350 $^\circ\text{C}$, respectively; nebulizer pressure, 55 psi; and capillary voltage, 4000 V. Mass spectra were obtained using in-source collision-induced dissociation mass spectrometry, scanning negative ions from m/z 100 to 2000 using the following fragmentation program: from m/z 0 to 200 (150 V) and from m/z 200 to 2000 (300 V).

Human Experimental Design. Two healthy and non-medication-receiving volunteers (ages 41 and 29 years) were recruited for the study. Volunteers were advised to avoid alcohol and vegetable-containing foods and beverages for 48 h before the study. In the morning (9:00 a.m.), after overnight fasting, the volunteers ingested 10 capsules containing the almond skin extract (884.4 mg of total polyphenols/dose), which was ~ 8 times higher than the estimated dietary intake (102–121 mg/person/day) of nut polyphenols in the Spanish diet (23). The volunteers did not consume any food before or with the almond skin extract. At 2:00 p.m., volunteers had a light lunch meal composed of pasta and meat, and at 8:00 p.m. they had a dinner meal composed of jam and bread. Venous blood samples were collected into vacuum tubes containing EDTA before and after ingestion (2.5 h for both volunteers and 4.5 h for volunteer 2).

After sampling, blood was placed on ice, and plasma was separated by centrifugation for 20 min at 1500g at 4 °C and stored at -80 °C. Urine samples were also collected at the following intervals: from 2 h before the time of ingestion (-2 to 0 h, before extract intake) and from the time of ingestion to 24 h after ingestion (0–24 h, after extract intake). Urine samples were acidified to 0.2 M HCl and were stored at -80 °C. The study protocols were approved by the Ethics Committee of Clinical Investigation of the University of Barcelona (Spain), and informed consent was obtained from all participants.

Enzymatic Hydrolysis of Urine and Plasma Samples. Urine and plasma samples were subjected to enzymatic hydrolysis as previously described (24) with the aim of releasing the phenolic acid/aglycone from conjugated microbial-derived phenolic metabolites and allowing their identification and total quantification in their free form. Briefly, urine samples (1 mL) were taken to pH 4.9 with 200 μ L of 2 M sodium acetate, and plasma samples (1 mL) were acidified with 50 μ L of 0.58 M acetic acid. Urine and plasma samples were incubated with β -glucuronidase/sulfatase at 37 °C for 45 min. Straight afterward, samples were acidified to pH 2 with 6 M HCl.

Nonhydrolyzed samples were also studied in plasma and urine samples to allow identification of phenolic metabolites in their actual conjugate forms.

Extraction of Phenolic Metabolites from Urine and Plasma Samples. Extraction of phenolic metabolites from hydrolyzed samples was performed using solid-phase extraction (SPE) Oasis MCX 96-well plates (Waters), a vacuum manifold, and a vacuum source as described by Urpi-Sarda et al. (24). Briefly, the plate was conditioned with 1 mL of methanol followed by 1 mL of 2% formic acid. The hydrolyzed plasma/urine samples were then loaded onto the plate with 100 μ L of 2 μ M ethyl gallate used as internal standard (IS). The plate was washed with 1 mL of 2% formic acid. Analytes were then eluted with methanol (0.5 mL $\times$ 3), and the eluates were evaporated to dryness under a stream of nitrogen. Residues were reconstituted with 100 μ L of mobile phase.

The nonhydrolyzed samples were also fractionated by SPE, but using Oasis HBL 96-well plates (30 mg) (Waters). The plate was conditioned with 1 mL of methanol followed by 1 mL of 1.5 M formic acid. One milliliter of the nonhydrolyzed plasma/urine samples was then loaded onto the plate with 100 μ L of 2 μ M ethyl gallate used as IS. The plate was washed with 1 mL of 1.5 M formic acid and 1 mL of 5% methanol. The plates were thoroughly dried by vacuum. Analytes were then eluted with 2 mL of methanol containing 0.1% formic acid and 2 mL of methanol containing NH₃ (pH 5), and the eluates were evaporated to dryness under a stream of nitrogen. Residues were reconstituted with 100 μ L of mobile phase.

Analysis of Phenolic Metabolites in Urine and Plasma Samples by LC-ESI-MS/MS. The analyses of phenolic metabolites in urine and plasma samples were carried out by LC-ESI-MS/MS on an Agilent 1200 system equipped with a quaternary pump and a refrigerated autosampler plate (Waldbronn, Germany) and coupled to an Applied Biosystems API 3000 triple-quadrupole mass spectrometer (PE Sciex, Concord, ON, Canada) equipped with a turbo ion spray ionizing in negative mode. A Phenomenex Luna C18 analytical column [50 $\times$ 2.0 mm i.d., 5 μ m] (Torrance, CA) with mobile phases A (100% water and 0.1% formic acid) and B (100% acetonitrile and 0.1% formic acid) was used. For the nonhydrolyzed samples, the gradient was linear of 4–100% of mobile phase B from 0 to 8 min, followed by washing and re-equilibration of the column for 2 min, at a flow rate of 500 μ L/min. For the hydrolyzed samples, the gradient was linear of 8–100% of mobile phase B from 0 to 7 min, followed by washing and re-equilibration of the column for 3 min, at a flow rate of 400 μ L/min. The volume injected was 15 μ L. MS/MS parameters used were as follows: capillary voltage, -3700 V; focusing potential, -200 V; entrance potential, -10 V; declustering potential, -50 V; nebulizer gas, 10 (arbitrary units); curtain gas, 12 (arbitrary units); collision gas, 5 (arbitrary units); auxiliary gas temperature, 400 °C; auxiliary gas flow rate, 6000 cm³/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. Phenolic standard curves were constructed with standard solutions subjected to the same SPE procedure as the samples. Conjugated metabolites of (epi)catechin, naringenin, and isorhamnetin

Table 1. Concentration of Individual Phenolic Compounds in the Encapsulated Almond Skin Extract

	mean $\pm$ SD* (μ g/g)
non-flavonoids	
protocatechuic acid	134.86 $\pm$ 20.77
vanillic acid	372.82 $\pm$ 41.16
ellagic acid	9.00 $\pm$ 1.34
flavonoids	
kaempferol-3-O-galactoside	22.24 $\pm$ 1.31
kaempferol-3-O-rutinoside	235.63 $\pm$ 23.50
kaempferol-3-O-glucoside	32.45 $\pm$ 3.86
isorhamnetin-3-O-galactoside	289.51 $\pm$ 32.34
isorhamnetin-3-O-rutinoside	1253.06 $\pm$ 130.76
isorhamnetin-3-O-glucoside	185.85 $\pm$ 20.11
kaempferol	4.28 $\pm$ 0.34
isorhamnetin	24.21 $\pm$ 3.53
naringenin-7-O-glucoside	55.19 $\pm$ 6.31
eriodictyol-7-O-glucoside	5.17 $\pm$ 0.69
naringenin	54.87 $\pm$ 8.72
eriodictyol	7.82 $\pm$ 1.67
dihydroquercetin	46.99 $\pm$ 8.13
(+)-catechin	682.83 $\pm$ 55.46
(-)-epicatechin	292.48 $\pm$ 34.46
procyanidin dimers B3 + B1	269.45 $\pm$ 41.69
procyanidin dimer B2	355.34 $\pm$ 7.18
procyanidin dimer B7	112.63 $\pm$ 8.29
unknown A-type procyanidin dimer (A-1)	99.37 $\pm$ 7.14
unknown A-type procyanidin dimer (A-2)	33.54 $\pm$ 2.97
unknown A-type procyanidin dimer (A-3)	69.03 $\pm$ 7.49
unknown B-type propelargonidin dimer	117.91 $\pm$ 15.76
unknown A-type propelargonidin dimer	50.44 $\pm$ 0.44
procyanidin trimer C1	173.97 $\pm$ 10.27

* Mean ($n = 2$) $\pm$ standard deviation (SD).

were quantified using the calibration curves of (-)-epicatechin, naringenin, and isorhamnetin, respectively. Hydroxyphenylvalerolactones and their conjugated metabolites were quantified as (-)-epicatechin. The mean recovery of analytes ranged from 87 to 109% (24, 25). Accuracy ranged from 87.5 to 113.8%, and precision met acceptance criteria (<15% RSD). The LODs varied between 0.050 and 44.4 μ g/L (24, 25).

Determination of Total Polyphenols in Urine Samples. The analysis of total polyphenols in urine samples was performed using the Folin-Ciocalteu (F-C) reagent after SPE of phenolic compounds using Oasis MAX 96-well plates (Waters) (26). Urine samples were thawed on an ice bed for 3 h and then centrifuged for 10 min at 4 °C. One milliliter of supernatant was diluted with 1 mL of Milli-Q water. The MAX 96-well plate was conditioned with 1 mL of methanol followed by 1 mL of 50 mM sodium acetate (pH 7). The sample was then loaded onto the plate and washed with 50 mM sodium acetate (pH 7) and 5% (v/v) methanol. Polyphenols were then eluted with 2% formic acid in methanol (1.8 mL).

The determination of total polyphenols was performed by mixing 15 μ L of the eluted fraction with 170 μ L of Milli-Q water in the thermo microtiter 96-well plate (Nunc™, Roskilde, Denmark), followed by the addition of 12 μ L of F-C reagent and 30 μ L of sodium carbonate (200 g/L). The mixture was incubated for 1 h at room temperature in the dark. After the reaction period, 73 μ L of Milli-Q water was added with the multichannel pipet. Absorbance was measured at 765 nm in UV-vis Thermo Multiskan Spectrum spectrophotometers (Vantaa, Finland), and the results were expressed as micrograms of gallic acid equivalent per milliliter of urine.

RESULTS AND DISCUSSION

Phenolic Composition of the Encapsulated Almond Skin Extract.

The analysis of the encapsulated almond skin extract by LC-DAD-fluorescence and LC-DAD-ESI-MS revealed the presence of both non-flavonoid and flavonoid phenolic compounds (Table 1). Non-flavonoid compounds mainly include protocatechuic acid, ellagic acid, and vanillic acid, the latter present in the largest concentration. Flavonoid compounds include flavanols

Table 2. Concentrations of Conjugated Phenolic Metabolites Detected in Nonhydrolyzed Human Plasma and Urine before and after Consumption of Almond Skin Extract

metabolite	MRM transition	compound	plasma ^a (nM)				urine ^b (nM)			
			before		after		before		after	
			mean	SD	mean	SD	mean	SD	mean	SD
flavan-3-ols										
(epi)catechin-O-glucuronide 1	465→289	1	nd ^c		nd		0.55	0.78	6.95	3.29
(epi)catechin-O-glucuronide 2	465→289	2	nd		nd		2.25	0.05	6.39	0.89
(epi)catechin-O-glucuronide 3	465→289	3	nd		19.51	3.99	3.08	0.17	21.35	3.91
O-methyl (epi)catechin-O-glucuronide 1	479→303	4	nd		8.54	3.47	1.93	0.70	25.30	9.74
O-methyl (epi)catechin-O-glucuronide 2	479→303	5	nd		7.94	3.98	2.28	0.62	46.92	7.28
(epi)catechin sulfate 1	369→289	6	nd		5.59 ^d		5.33	1.77	972.70	344.64
(epi)catechin sulfate 2	369→289	7	nd		14.71	9.02	10.62	5.79	200.40	28.55
O-methyl (epi)catechin sulfate 1	383→303	8	nd		41.82	±17.17	8.24	4.04	586.31	±90.34
O-methyl (epi)catechin sulfate 2	383→303	9	nd		17.83 ^d		7.14	1.83	537.34	132.70
O-methyl (epi)catechin sulfate 3	383→303	10	nd		nd		2.90	0.57	87.52	10.37
hydroxyphenylvalerolactones										
5-(dihydroxyphenyl)- γ -valerolactone glucuronide derivative 1	383→207	11	nd		nd		18.03	3.95	2939.65	501.22
5-(dihydroxyphenyl)- γ -valerolactone glucuronide derivative 2	383→207	12	nd		nd		35.28	±17.30	7682.78	1942.14
5-(dihydroxyphenyl)- γ -valerolactone sulfate derivative	287→207	13	278.81	160.36	368.23	224.37	3969.68	283.23	449558.92	146817.62
5-(hydroxymethoxyphenyl)- γ -valerolactone glucuronide derivative 1	397→221	14	7.62	6.11	5.94	4.84	362.61	97.5	832.89	291.5
5-(hydroxymethoxyphenyl)- γ -valerolactone glucuronide derivative 2	397→221	15	nd		nd		6.18	1.68	117.89	41.19
5-(hydroxymethoxyphenyl)- γ -valerolactone sulfate derivative 1	301→221	16	nd		nd		57.26	50.50	1495.68	828.12
5-(hydroxymethoxyphenyl)- γ -valerolactone sulfate derivative 2	301→221	17	nd		nd		14.36	7.15	704.80	266.53
flavanones										
naringenin-O-glucuronide 1	447→271	18	0.84	0.15	3.35	0.17	2.81	1.44	14.97	6.41
naringenin-O-glucuronide 2	447→271	19	0.61	0.05	1.27	0.17	8.15	8.35	15.43	5.61
flavanols										
isorhamnetin-O-glucuronide 1	491→315	20	1.68	0.03	2.40	1.46	5.08	2.74	6.71	0.55
isorhamnetin-O-glucuronide 2	491→315	21	1.14	0.81	1.49	0.54	4.54	1.38	4.89	1.72
isorhamnetin sulfate	395→315	22	nd		nd		37.39	4.54	62.75	29.13

^aMean ($n = 2$) $\pm$ standard deviation (SD). Before (at 0 h); after (at 2.5 h). ^bMean ($n = 2$) $\pm$ standard deviation (SD). Before (from -2 to 0 h); after (from 0 to 24 h). ^cnd, not detected. ^dDetected in only one volunteer.

(50.4% of total flavonoids), flavanols (45.8%), flavanones (2.8%), and dihydroflavonols (1.1%). Isorhamnetin glycosides were more abundant than the corresponding glycosides of kaempferol, and naringenin glucoside was more abundant than the corresponding eriodictyol glucoside. However, the rutinoside derivatives were present in the largest concentration independent of the flavanol aglycone. Among flavanols, (+)-catechin was the predominant monomeric form, whereas procyanidin B2 was the most abundant oligomeric form. In addition, several mass signals were detected at m/z 591 corresponding to A-type prodelphinidin dimers, but UV peaks were not well resolved for quantification purposes (data not shown). These results are in agreement with previous reports describing the phenolic composition of almond skins (10).

Identification of Phase II and Microbial-Derived Phenolic Metabolites in Human Plasma and Urine. The concentration of phenolic metabolites detected in nonhydrolyzed and hydrolyzed samples before and after consumption of the almond skin extract is presented in Tables 2 and 3, respectively. The presence of metabolites derived from the main phenolic compounds (i.e., flavanols, flavanols, and flavanones) identified in the almond skin extract (Table 1) was investigated in both fluids. With regard to flavanols, three mass signals were detected at m/z 465/289 and two at m/z 369/289, indicating the possible presence of glucuronide (+176 amu) and sulfate (+80 amu) derivatives of (epi)catechin (Figure 1; Table 2). In addition, two signals were detected at m/z 479/303 and three at m/z 383/303, suggesting they were possible glucuronide and sulfate derivatives of O-methyl(epi)catechin, respectively (Figure 1; Table 2). These conjugated metabolites of (epi)catechin were detected in plasma and urine samples, with

some exceptions in the plasma samples (Table 2). Different signals detected at the same m/z may belong to different regioisomers resulting from conjugation at different OH groups of the flavanol molecule. In humans, methylation of (-)-epicatechin has been reported to occur at C-3' and at C-4' and glucuronidation at C-3', C-5, and C-7 (27). However, the active positions for flavanol sulfation have not been clearly identified. These metabolites may have been formed in the small intestine and/or further in the liver by phase II enzymes after absorption of (epi)catechin in the small intestine (18).

Conjugated metabolites, derived from the microbial degradation of flavanols, were also investigated. In particular, hydroxyphenylvalerolactones, originating from the C-ring-opening of flavanols by the colonic microflora followed by further lactonization, are considered to be main microbial metabolites derived from the biotransformation of flavanols (28–31). Bearing in mind that procyanidins are the most abundant type of proanthocyanidins in the almond extract (Table 1), conjugated metabolites of 5-(dihydroxyphenyl)- γ -valerolactone [i.e., 5-(3',4'-dihydroxyphenyl)- γ -valerolactone] were targeted (Figure 2; Table 2). Two mass signals were detected at m/z 383/207, indicating the possible occurrence of two different glucuronide derivatives of a 5-(dihydroxyphenyl)- γ -valerolactone molecule, probably originating from the esterification of glucuronic acid at the two different OH groups of the phenyl ring of 5-(3',4'-dihydroxyphenyl)- γ -valerolactone. One mass signal detected at m/z 287/207 was tentatively assigned to the corresponding sulfate conjugate (Figure 2). At m/z 397/221 two signals were detected, suggesting the presence of glucuronide derivatives of 5-(hydroxymethoxyphenyl)- γ -valerolactone. Similarly, two signals detected at m/z 301/221 were

Table 3. Concentration of Microbial-Derived Phenolic Metabolites Detected in Hydrolyzed Human Plasma and Urine before and after Consumption of Almond Skin Extract

metabolite	MRM transition	plasma ^a (nM)				urine ^b (nM)			
		before		after		before		after	
		mean	SD	mean	SD	mean	SD	mean	SD
hydroxyphenylvalerolactones									
5-(dihydroxyphenyl)- γ -valerolactone	207→163	nd ^c		nd		121.09	±11.70	23553.03	±1230.73
5-(hydroxyphenyl)- γ -valerolactone	191→147	nd		nd		48.81	10.12	229.21	54.93
hydroxyphenylpropionic acids									
3,4-dihydroxyphenylpropionic acid	181→137	2880.36	365.83	2787.25	14.53	2805.14	415.97	3618.37	787.73
3-hydroxyphenylpropionic acid	165→121	2038.17	367.60	1780.23	335.28	8.16 ^d		51.61 ^d	
hydroxyphenylacetic acids									
3,4-dihydroxyphenylacetic acid	167→123	406.28	112.62	385.83	30.04	1759.11	559.81	3122.01	161.09
3-hydroxyphenylacetic acid	151→107	986.95	143.93	939.50	52.28	2421.92	2366.71	7012.71	4401.08
4-hydroxy-3-methoxyphenylacetic acid	181→137	755.81	189.16	571.49	162.27	6327.50	2186.86	25212.61	10960.16
phenylacetic acid	135→91	8481.61	2343.50	8733.33	2384.11	5577.11	116.15	6716.28	921.82
hydroxycinnamic acids									
<i>m</i> -coumaric acid	163→119	17.94	9.58	22.93	4.98	15.54	8.91	29.35	17.15
<i>p</i> -coumaric acid	163→119	29.29	0.46	19.14	2.66	14.86	17.72	20.65	12.90
caffeic acid	179→135	509.99	95.05	484.26	24.04	809.06	318.26	1200.01	43.82
ferulic acid	193→134	46.50	2.68	25.38	12.68	111.13	17.70	365.45	258.27
3-hydroxy-4-methoxycinnamic acid	193→134	71.52	26.28	66.74	5.61	3034.48	128.10	4541.98	1705.02
hydroxybenzoic acids									
3-hydroxybenzoic acid	137→93	nd		nd		206.26	140.96	315.91	±10.28
4-hydroxybenzoic acid	137→93	6177.80	824.05	6527.09	±234.47	5529.69	1759.09	7034.03	240.27
protocatechuic acid	153→109	4553.05	792.78	4222.13	265.67	9012.44	1414.96	9451.31	1943.47
vanillic acid	167→152	2821.25	478.51	3087.02	273.06	218.39	111.27	1676.85	1183.74
hydroxyhippuric acids									
4-hydroxyhippuric acid	194→100	14.56	1.47	11.46	0.11	15475.48	13393.70	17909.39	12733.87

^a Mean ($n=2$) ± standard deviation (SD). Before (at 0 h); after (at 2.5 h). ^b Mean ($n=2$) ± standard deviation (SD). Before (from -2 to 0 h); after (from 0 to 24 h). ^c nd, not detected. ^d Quantified in only one volunteer.

tentatively assigned to the corresponding sulfate derivative of the same molecule. These metabolites were detected in both fluids, although with some exceptions in the plasma samples (Table 2), and have been previously reported in human urine after the consumption of tea catechins (32,33). The identification of these compounds was aided by the disappearance of the peaks after enzymatic hydrolysis with β -glucuronidase/sulfatase and by the appearance of peaks of corresponding aglycones, as will be described below.

With regard to other flavonoids, conjugated metabolites from main flavanones (naringenin) and flavonols (isorhamnetin) were studied (Figure 3; Table 2). At m/z 447/271 two signals were detected in both plasma and urine samples tentatively assigned to glucuronide derivatives of naringenin (m/z 447/271) originating from the metabolism of almond skin naringenin-7-*O*-glucoside and naringenin. In addition, two signals were detected at m/z 491/315 and one at m/z 395/315, indicating that they were possible glucuronide and sulfate derivatives of isorhamnetin (Figure 3; Table 2). These latter metabolites may have originated from the metabolism of isorhamnetin-3-*O*-galactoside and isorhamnetin-3-*O*-glucoside present in the almond skin extract (Table 1) because isorhamnetin-3-*O*-rutinoside needs to reach the colon to be metabolized by the microbiota (21).

Metabolites derived from the microbial degradation of almond skin flavanols were studied after enzymatic hydrolysis of plasma and urine samples. The targeted metabolites were classified into different groups according to their chemical structure (Table 3): hydroxyphenylvalerolactones and hydroxyphenylpropionic, hydroxyphenylacetic, hydroxycinnamic, hydroxybenzoic, and hydroxyhippuric acids. Among hydroxyphenylvalerolactones, besides 5-(dihydroxyphenyl)- γ -valerolactone (m/z 207/163), the transition corresponding to 5-(hydroxyphenyl)- γ -valerolactone (m/z 191/147) was additionally studied in hydrolyzed samples. In

plasma, no hydroxyphenylvalerolactone derivatives were detected, whereas in urine the two forms (i.e., mono- and dihydroxyphenyl- γ -valerolactones) were detected. The mass spectra of 5-(3',4'-dihydroxyphenyl)- γ -valerolactone (m/z 207) reported in a previous work (24) allowed the identification of dihydroxyphenyl- γ -valerolactone. The monohydroxylated form 5-(hydroxyphenyl)- γ -valerolactone could originate directly from (epi)afzelechin units of properlagonidins or more likely from dehydroxylation of (epi)catechin (i.e., procyanidins), which occur in larger concentrations than the former compounds in the almond skin extract (Table 1). Subsequent fission of the valerolactone ring leads to hydroxyphenylvaleric acids (34–37), which are further degraded to a series of phenolic acids. The targeted-studied phenolic acids (hydroxyphenylpropionic, hydroxyphenylacetic, hydroxycinnamic, hydroxybenzoic, and hydroxyhippuric acids) were identified in both plasma and urine samples, with the exception of 3-hydroxybenzoic acid in plasma (Table 3). Hydroxyphenylpropionic and hydroxybenzoic acids originate from the β -oxidation of hydroxyphenylvaleric acids (37). α -Oxidation of hydroxyphenylpropionic acids may also occur, leading to hydroxyphenylacetic acids (38). Phenolic acids hydroxylated at C-3 (i.e., 3-hydroxyphenylpropionic and 3-hydroxybenzoic acids) are likely to be produced via the selective dehydroxylation of the OH group of dihydroxylated compounds at C-4 (37), whereas C4-hydroxylated phenolic acids (i.e., 4-hydroxybenzoic acid) are the result of the dehydroxylation at C-3, although it seems to be characteristic of the degradation only of procyanidins and not of monomeric flavanols (19, 20, 35, 36). Other microbial metabolites targeted in the present study are derived from further metabolism in the liver and kidney, including 4-hydroxyhippuric acid, from conjugation of 4-hydroxybenzoic acid with glycine (39); *m*- and *p*-coumaric acids, from dehydrogenation of 3- and 4-hydroxypropionic acids, respectively; ferulic acid by further parahydroxylation

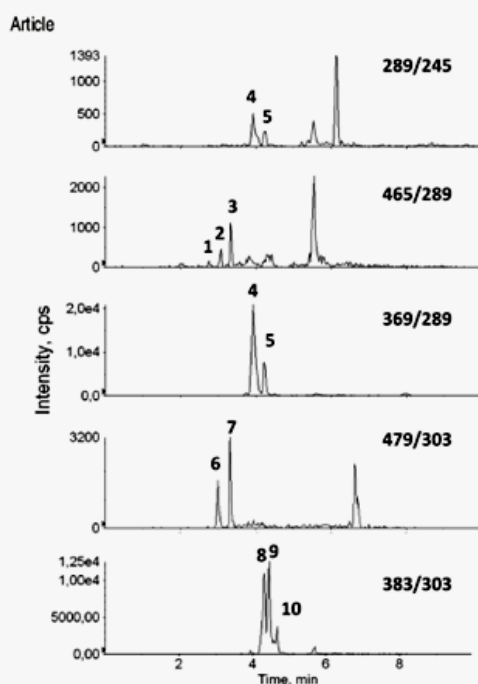


Figure 1. MRM trace chromatograms of (epi)catechin-O-glucuronides (465/289), (epi)catechin sulfates (369/289), O-methyl (epi)catechin-O-glucuronides (479/303), and O-methyl (epi)catechin sulfates (383/303) detected in urine samples after intake of the almond extract. The MRM trace chromatogram corresponding to (epi)catechin (289/245) is also shown to confirm metabolites.

and methylation of *m*-coumaric acid (20, 38); and vanillic and 4-hydroxy-3-methoxyphenylacetic acid by methylation of protocatechuic acid and 3,4-dihydroxyphenylacetic acid, respectively (20).

Microbial metabolites derived from flavanones and flavonols were also identified. Naringenin-7-*O*-rutinoside was degraded into 4-hydroxyphenylpropionic acid and phenylpropionic acid by human fecal microbiota *in vitro* (40). In the case of isorhamnetin glycosides, there is no previous report of microbial metabolites, but 4-hydroxy-3-methoxyphenylpropionic and phenylacetic acids, as well as vanillic acid, are expected to be formed from the degradation pathway characteristic of other flavonols. Among the possible metabolites derived from the microbial degradation of naringenin and isorhamnetin, only vanillic acid and 4-hydroxy-3-methoxyphenylacetic acid were targeted in the present study.

Changes in Conjugated Phenolic Metabolites in Human Plasma and Urine after the Consumption of the Almond Skin Extract. The concentration of conjugated phenolic metabolites (phase II and microbial-derived) detected in nonhydrolyzed human plasma and urine before and after consumption of almond skin extract is presented in Table 2. Among targeted compounds, conjugated metabolites of epicatechin [*O*-glucuronide (3), *O*-methyl glucuronides (1 and 2), sulfates (1 and 2), and *O*-methyl sulfates (1 and 2)] were detected in only plasma after the intake of the almond skin extract, indicating that they arise from the metabolism of almond skin polyphenols. Other metabolites, including some conjugated metabolites of hydroxyphenylvalerolactones [5-(dihydroxyphenyl)- γ -valerolactone sulfate], as well as glucuronides of

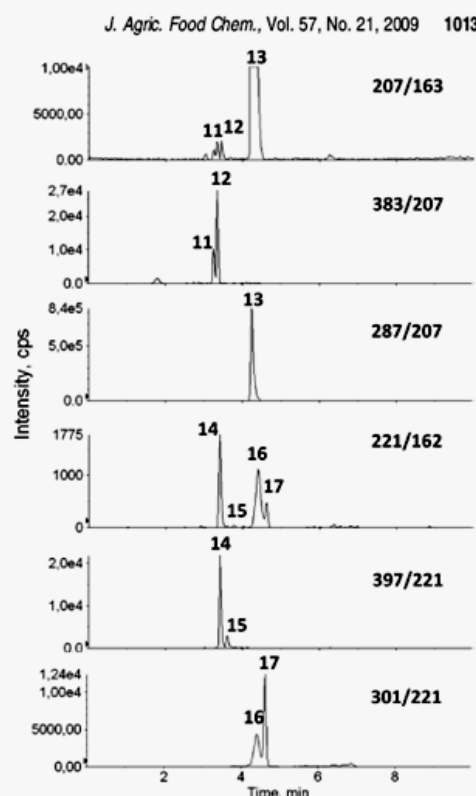


Figure 2. MRM trace chromatograms of 5-(dihydroxyphenyl)- γ -valerolactone glucuronide derivatives (383/207), 5-(dihydroxyphenyl)- γ -valerolactone sulfate derivatives (287/207), 5-(hydroxymethoxyphenyl)- γ -valerolactone glucuronide derivatives (397/221), and 5-(hydroxymethoxyphenyl)- γ -valerolactone sulfate derivatives (301/221). MRM trace chromatograms of aglycones, 5-(dihydroxyphenyl)- γ -valerolactone (207/163), and 5-(hydroxymethoxyphenyl)- γ -valerolactone (221/162) are also shown to confirm metabolites.

naringenin and isorhamnetin, were detected in plasma before consumption of almond skin extract, but their levels increased in all of them at 2.5 h after intake. Both (epi)catechin and hydroxyphenylvalerolactone derivatives have been quantified as (–)-epicatechin to estimate the changes in phenolic metabolite content after consumption of the almond skin extract. Taking this approach into account, the consumption of 3.5 g of almond skin extract resulted in a 57% increase in the total concentration of plasma conjugated metabolites at 2.5 h after intake. These findings seem to be in accordance with the pharmacokinetics of (epi)catechin in plasma ($t_{\max} \approx 2$ h), which indicates absorption in the small intestine (41). For naringenin, a $t_{\max} \approx 5$ h in plasma has been reported after the consumption of orange juice (42), although it could appear earlier. In the case of hydroxyphenylvalerolactones, pharmacokinetic data of these compounds in plasma are very limited. According to Li et al. (28), $t_{\max}$ of 5-(3',4'-dihydroxyphenyl)- γ -valerolactone occurred at 5–12 h after green tea consumption in humans. Meng et al. (29) reported the appearance of this compound in human plasma at approximately 5 h after the intake of green tea, achieving the maximum concentration at 12 h. Also in agreement with these results, Kohri et al. (30) reported that 5-(3',4'-dihydroxyphenyl)- γ -valerolactone

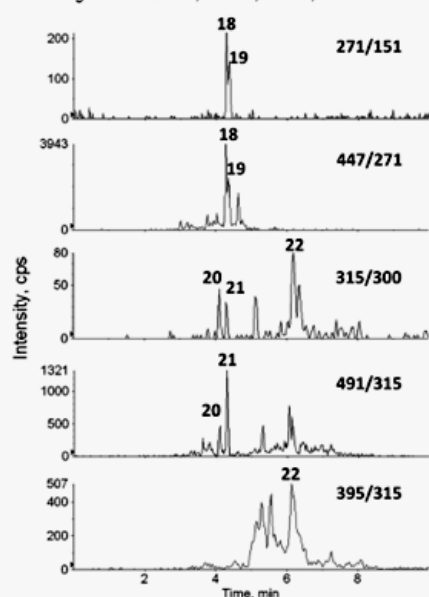


Figure 3. MRM trace chromatograms of naringenin-*O*-glucuronides (447/271), isorhamnetin-*O*-glucuronides (491/315), and isorhamnetin sulfate (395/315). MRM trace chromatograms of aglycones, naringenin (271/151), and isorhamnetin (315/300) are also shown to confirm metabolites.

appeared in plasma at 6 h after the administration of epicatechin gallate to rats, although in this case $t_{\max}$ was achieved at 24 h. These findings help to explain why only a small amount of hydroxyvalerolactone conjugated metabolites could be detected in the 2.5 h plasma sample of the present study. However, these metabolites increased at 4.5 h after the intake of the almond skin extract, and other new metabolites such as 5-(dihydroxyphenyl)- γ -valerolactone glucuronide derivatives (1 and 2) not detected at 2.5 h were also detected at 4.5 h after intake (results not shown), indicating that a longer time is required for the microbial biotransformation of almond polyphenols in the colon.

The analysis of urine revealed an increase in the urinary level of all targeted conjugated metabolites as a consequence of the consumption of the almond skin extract. Among these metabolites, 5-(dihydroxyphenyl)- γ -valerolactone glucuronides (1 and 2), 5-(dihydroxyphenyl)- γ -valerolactone sulfate, and (epi)catechin sulfate (1) presented the largest increases in concentration (163-, 218-, 113-, and 182-fold increases, respectively). Consumption of green tea catechins (28, 29) or cocoa (24) in humans has also resulted in an increase in the formation of hydroxyphenylvalerolactones. Maximum renal excretion of 5-(3',4'-dihydroxyphenyl)- γ -valerolactone in humans has been reported to have occurred at 3–6 h for pure (–)-epicatechin or at 8–24 h after green tea consumption, although it could vary greatly between subjects (28, 29). In our study, consumption of 3.5 g of almond skin extract resulted in a 102-fold increase in the total urinary concentration of conjugated metabolites 24 h after intake. It was also calculated that phase II metabolites of (epi)catechin, naringenin, and isorhamnetin accounted for 44, 53, and 1.4%, respectively, of the total monomeric flavanol [(+)-catechin + (–)-epicatechin], total naringenin (naringenin and its -7-*O*-glucoside derivative), and total isorhamnetin (isorhamnetin and its

-3-*O*-galactoside, -3-*O*-rutoside, and -3-*O*-glucoside derivatives), ingested in the almond skin extract (Table 1). Urinary excretion ranges of 2.1–55.0 and 1.1–30.2% have been reported for (epi)catechin and naringenin, respectively (41). However, there are no data currently available of the urinary excretion rates of isorhamnetin, although its pharmacokinetics in plasma seem to be similar to that of quercetin after the consumption of almond skins (4).

Changes in Microbial-Derived Phenolic Metabolites in Human Plasma and Urine after the Consumption of the Almond Skin Extract. The concentration of microbial-derived metabolites detected in hydrolyzed human plasma and urine before and after the consumption of almond skin extract is presented in Table 3. The plasmatic level of microbial-derived phenolic metabolites remained practically constant after consumption of almond skin extract. In fact, the plasmatic concentration of total microbial-derived phenolic metabolites accounted for 29.79 μ M before consumption and for 29.66 μ M after consumption of almond skin extract. According to Kohri et al. (30), subsequent microbial degradation of products of hydroxyvalerolactones, such as 3-hydroxyphenylpropionic acid and *m*-coumaric acid, appeared in rat plasma 6 h after consumption of (–)-epicatechin gallate, reaching maximum levels at 24 h. Therefore, the 2.5 h plasma sample may not reflect microbial metabolism because almond polyphenols need a longer time to reach the large intestine and be biotransformed.

In contrast, major changes were observed in the urinary levels of all microbial metabolites after consumption of almond skin extract. Hydroxyphenylvalerolactones, in particular 5-(dihydroxyphenyl)- γ -valerolactone, exhibited the largest increase in concentration (195-fold increase). Among the remaining compounds, the concentrations of vanillic acid and 3-hydroxyphenylpropionic acid also exhibited large changes (8- and 6-fold increases, respectively). These results seem to be consistent with those of Rios et al. (43), who reported a significant increase in the urinary excretion of these metabolites, among others, in healthy humans after acute consumption of a flavanol-rich chocolate. Most microbial-derived metabolites were found to increase from 6 to 48 h after consumption and registered maximum levels from 24 to 48 h (43). Also in line with our results, Ward et al. (44) reported an increased urinary excretion of 3-hydroxypropionic acid and 3-hydroxyphenylacetic acid after human consumption of grape seed polyphenols. As a consequence of the consumption of almond skin extract, the total urinary microbial metabolite concentration increased by 109%. The total polyphenol content of the 24 h urine sample (as determined by the F-C method) taken after the consumption of almond skin extract was 212.5 mg of gallic acid and accounted for 24% of the total polyphenol intake (884.4 mg of gallic acid). Microbial-derived phenolic metabolites (Table 3) represented 4.9% of the total polyphenol intake (884.4 mg of gallic acid), whereas (epi)catechin conjugates (Table 2) represented only 0.11% of this intake. These findings seem to be in line with the results reported by Gonthier et al. (19) for rats fed wine polyphenols, in which microbial metabolites and catechins [(+)-catechin + 3'-*O*-methylcatechin] represented 9.2 and 1.2% of the total polyphenol intake, respectively. Lower yields in the case of the almond skin extract could be due to the structural complexity (A- and B-type proanthocyanidins, procyanidins, and prodelphinidins) and the high mDP of the almond skin extract (total proanthocyanidins, 315 mg/g) in comparison to the wine powder used in the rat study (146 mg/g) (19).

In conclusion, to our knowledge this study constitutes the most complete report of the bioavailability of almond skin polyphenols in humans, including both phase II and microbial metabolism. Almond skin polyphenols were absorbed as phase II metabolites,

Article

J. Agric. Food Chem., Vol. 57, No. 21, 2009 10141

mainly including glucuronide, *O*-methyl glucuronide, sulfate, and *O*-methyl sulfate derivatives of (epi)catechin, which arise from the metabolism of monomeric flavanols, as well as glucuronide and sulfate derivatives of isorhamnetin and glucuronide derivatives of naringenin. Of particular importance was the detection of microbial-derived metabolites of flavanols, 5-(dihydroxyphenyl)- γ -valerolactone and 5-(hydroxymethoxyphenyl)- γ -valerolactone in their glucuronide and sulfate forms, which confirmed the absorption of both monomeric and polymeric flavanols from almond skins and subsequent conjugation in the liver. Numerous metabolites derived from the further microbial degradation of hydroxyvalerolactones were also detected. The urinary excretion of these metabolites accounted for a larger proportion of the total polyphenol ingested than phase II metabolites of monomeric flavanols, indicating the important role of intestinal bacteria in the metabolism of highly polymerized almond skin polyphenols.

ABBREVIATIONS USED

LDL, low-density lipoprotein; DAD, diode array detector; mDP, mean degree of polymerization; SPE, solid-phase extraction; IS, internal standard; MRM, multiple reaction monitoring; F-C, Folin–Ciocalteu.

SAFETY

Guidelines for work with organic solvents and acids were respected. Universal precautions for the handling of chemicals and fluids were applied.

ACKNOWLEDGMENT

We are grateful to Drs. Isidre Casals and Olga Jauregui from the Scientific and Technical Services (University of Barcelona, Barcelona, Spain) for technical support with the LC-ESI-MS/MS instrument.

LITERATURE CITED

- Chen, C. Y.; Lapsley, K.; Blumberg, J. A nutrition and health perspective on almonds. *J. Sci. Food Agric.* **2006**, *86* (14), 2245–2250.
- Jenkins, D. J. A.; Kendall, C. W. C.; Marchie, A.; Parker, T. L.; Connelly, P. W.; Qian, W.; Haight, J. S.; Faulkner, D.; Vidgen, E.; Lapsley, K. G.; Spiller, G. A. Dose response of almonds on coronary heart disease risk factors: blood lipids, oxidized low-density lipoproteins, lipoprotein(a), homocysteine, and pulmonary nitric oxide: A randomized, controlled, crossover trial. *Circulation* **2002**, *106* (11), 1327–1332.
- Jenkins, D. J. A.; Kendall, C. W. C.; Marchie, A.; Josse, A. R.; Nguyen, T. H.; Faulkner, D. A.; Lapsley, K. G.; Blumberg, J. Almonds reduce biomarkers of lipid peroxidation in older hyperlipidemic subjects. *J. Nutr.* **2008**, *138* (5), 908–913.
- Chen, C. Y.; Milbury, P. E.; Lapsley, K.; Blumberg, J. B. Flavonoids from almond skins are bioavailable and act synergistically with vitamins C and E to enhance hamster and human LDL resistance to oxidation. *J. Nutr.* **2005**, *135* (6), 1366–1373.
- Chen, C. Y.; Milbury, P. E.; Chung, S. K.; Blumberg, J. Effect of almond skin polyphenolics and quercetin on human LDL and apolipoprotein B-100 oxidation and conformation. *J. Nutr. Biochem.* **2007**, *18* (12), 785–794.
- Chen, C. Y. O.; Blumberg, J. B. In vitro activity of almond skin polyphenols for scavenging free radicals and inducing quinone reductase. *J. Agric. Food Chem.* **2008**, *56*, 4427–4434.
- Mandalari, G.; Nueno-Palop, C.; Bisignano, G.; Wickham, M. S. J.; Narbad, A. Potential prebiotic properties of almond (*Amygdalus communis* L.) seeds. *Appl. Environ. Microbiol.* **2008**, *74* (14), 4264–4270.
- Sang, S.; Lapsley, K.; Jeong, W. S.; Lachance, P. A.; Ho, C. T.; Rosen, R. T. Antioxidative phenolic compounds isolated from almond skins (*Prunus amygdalus* Batsch). *J. Agric. Food Chem.* **2002**, *50*, 2459–2463.
- Milbury, P. E.; Chen, C. Y.; Dolnikowski, G. G.; Blumberg, J. B. Determination of flavonoids and phenolics and their distribution in almonds. *J. Agric. Food Chem.* **2006**, *54*, 5027–5033.
- Monagas, M.; Garrido, I.; Lebrón-Aguilar, R.; Bartolome, B.; Gómez-Cordovés, C. Almond (*Prunus dulcis* (Mill.) D.A. Webb) skins as a potential source of bioactive polyphenols. *J. Agric. Food Chem.* **2007**, *55*, 8498–8507.
- Brieskorn, C. H.; Betz, R. Procyanidin polymers crucial to the structure of the almond seedcoat. *Z. Lebensm.-Unters.-Forsch.* **1988**, *187* (4), 347–353.
- Amarowicz, R.; Troszyńska, A.; Shahidi, F. Antioxidant activity of almond seed extract and its fractions. *J. Food Lipids* **2005**, *12* (4), 344–358.
- Lazarus, S. A.; Adamson, G. E.; Hammerstone, J. F.; Schmitz, H. H. High-performance liquid chromatography/mass spectrometry analysis of proanthocyanidins in foods and beverages. *J. Agric. Food Chem.* **1999**, *47*, 3693–3701.
- Frison, S.; Sporns, P. Variation in the flavonol glycoside composition of almond seedcoats as determined by MALDI-TOF mass spectrometry. *J. Agric. Food Chem.* **2002**, *50*, 6818–6822.
- Frison-Norrie, S.; Sporns, P. Identification and quantification of flavonol glycosides in almond seedcoats using MALDI-TOF MS. *J. Agric. Food Chem.* **2002**, *50*, 2782–2787.
- Wijeratne, S. S. K.; Abou-Zaid, M. M.; Shahidi, F. Antioxidant polyphenols in almond and its coproducts. *J. Agric. Food Chem.* **2006**, *54*, 312–318.
- Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **2000**, *130* (8 Suppl.), 2073S–2085S.
- Manach, C.; Scalbert, A.; Morand, C.; Remésy, C.; Jiménez, L. Polyphenols: food sources and bioavailability. *Am. J. Clin. Nutr.* **2004**, *79* (5), 727–747.
- Gonthier, M. P.; Cheynier, V.; Donovan, J. L.; Manach, C.; Morand, C.; Mila, I.; Lapiere, C.; Remésy, C.; Scalbert, A. Microbial aromatic acid metabolites formed in the gut account for a major fraction of the polyphenols excreted in urine of rats fed red wine polyphenols. *J. Nutr.* **2003**, *133* (2), 461–467.
- Gonthier, M. P.; Donovan, J. L.; Texier, O.; Felgines, C.; Remésy, C.; Scalbert, A. Metabolism of dietary procyanidins in rats. *Free Radical Biol. Med.* **2003**, *35* (8), 837–844.
- Hollman, P. C. H.; Van Trijp, J. M. P.; Mengelers, M. J. B.; De Vries, J. H. M.; Katan, M. B. Bioavailability of the dietary antioxidant flavonol quercetin in man. *Cancer Lett.* **1997**, *114* (1–2), 139–140.
- Milbury, P. E.; Chen, C. Y.; Kwak, H. K.; Blumberg, J. B. Almond polyphenolics feeding alters LDL oxidizability and glutathione metabolism without altering ORACpca in older adults. *Free Radical Biol. Med.* **2004**, *37*, 538.
- Saura-Calixto, F.; Serrano, J.; Goñi, I. Intake and bioaccessibility of total polyphenols in a whole diet. *Food Chem.* **2007**, *101*, 492–501.
- Urpi-Sarda, M.; Monagas, M.; Khan, N.; Lamuela-Raventos, R. M.; Santos-Buelga, C.; Sacanella, E.; Castell, M.; Permanyer, J.; Andres-Lacueva, C. Epicatechin, procyanidins and phenolic microbial metabolites after cocoa intake in humans and rats. *Anal. Bioanal. Chem.* **2009**, *394*, 1545–1556.
- Roura, E.; Andres-Lacueva, C.; Jauregui, O.; Badia, E.; Estruch, R.; Izquierdo-Pulido, M.; Lamuela-Raventos, R. M. Rapid liquid chromatography tandem mass spectrometry assay to quantify plasma (–)-epicatechin metabolites after ingestion of a standard portion of cocoa beverage in humans. *J. Agric. Food Chem.* **2005**, *53*, 6190–6194.
- Medina-Remón, A.; Barrionuevo-González, A.; Zamora-Ros, R.; Andres-Lacueva, C.; Estruch, R.; Martínez-González, M. A.; Díez-Espino, J.; Lamuela-Raventos, R. M. Rapid Folin–Ciocalteu method using microtiter 96-well plate cartridges for solid phase extraction to assess urinary total phenolic compounds, as a biomarker of total polyphenols intake. *Anal. Chim. Acta* **2009**, *634* (1), 54–60.
- Natsume, M.; Osakabe, N.; Oyama, M.; Sasaki, M.; Baba, S.; Nakamura, Y.; Osawa, T.; Terao, J. Structures of (–)-epicatechin glucuronide identified from plasma and urine after oral ingestion of (–)-epicatechin: differences between human and rat. *Free Radical Biol. Med.* **2003**, *34* (7), 840–849.

- (28) Li, C.; Lee, M. J.; Sheng, S.; Meng, X.; Prabhu, S.; Winnik, B.; Huang, B.; Chung, J. Y.; Yan, S.; Ho, C. T.; Yang, C. S. Structural identification of two metabolites of catechins and their kinetics in human urine and blood after tea ingestion. *Chem. Res. Toxicol.* **2000**, *13*, 177–184.
- (29) Meng, X.; Sang, S.; Zhu, N.; Lu, H.; Sheng, S.; Lee, M. J.; Ho, C. T.; Yang, C. S. Identification and characterization of methylated and ring-fission metabolites of tea catechins formed in humans, mice, and rats. *Chem. Res. Toxicol.* **2002**, *15*, 1042–1050.
- (30) Kohri, T.; Suzuki, M.; Nanjo, F. Identification of metabolites of (–)-epicatechin gallate and their metabolic fate in the rat. *J. Agric. Food Chem.* **2003**, *51*, 5561–5566.
- (31) Appeldoorn, M. M.; Vincken, J. P.; Aura, A. M.; Hollman, P. C. H.; Gruppen, H. Procyanidin dimers are metabolized by human microbiota with 2-(3,4-dihydroxyphenyl)acetic acid and 5-(3,4-dihydroxyphenyl)- γ -valerolactone as the major metabolites. *J. Agric. Food Chem.* **2009**, *57*, 1084–1092.
- (32) Li, C.; Meng, X.; Winnik, B.; Lee, M. J.; Lu, H.; Sheng, S.; Buckley, B.; Yang, C. S. Analysis of urinary metabolites of tea catechins by liquid chromatography/electrospray ionization mass spectrometry. *Chem. Res. Toxicol.* **2001**, *14*, 702–707.
- (33) Sang, S.; Lee, M. J.; Yang, I.; Buckley, B.; Yang, C. S. Human urinary metabolite profile of tea polyphenols analyzed by liquid chromatography/electrospray ionization tandem mass spectrometry with data-dependent acquisition. *Rapid Commun. Mass Spectrom.* **2008**, *22* (10), 1567–1578.
- (34) Das, N. P.; Griffiths, L. A. Studies on flavonoid metabolism. Metabolism of (+)-catechin in the guinea pig. *Biochem. J.* **1968**, *110* (3), 449–456.
- (35) Groenewoud, G.; Hundt, H. K. L. The microbial metabolism of (+)-catechin to two novel diarylpropan-2-ol metabolites in vitro. *Xenobiotica* **1984**, *14* (9), 711–717.
- (36) Groenewoud, G.; Hundt, H. K. L. The microbial metabolism of condensed (+)-catechins by rat-caecal microflora. *Xenobiotica* **1986**, *16* (2), 99–107.
- (37) Meselhy, M. R.; Nakamura, N.; Hattori, M. Biotransformation of (–)-epicatechin 3-O-gallate by human intestinal bacteria. *Chem. Pharm. Bull.* **1997**, *45* (5), 888–893.
- (38) Scheline, R. R. *Handbook of Mammalian Metabolism of Plant Compounds*; CRC Press: Boca Raton, FL, 1991.
- (39) Svardal, A. M.; Scheline, R. R. The aromatization of cyclohexanecarboxylic acid to hippuric acid: substrate specificity and species differences. *Mol. Cell. Biochem.* **1985**, *67* (2), 171–179.
- (40) Rechner, A. R.; Smith, M. A.; Kuhnle, G.; Gibson, G. R.; Debnam, E. S.; Srai, S. K. S.; Moore, K. P.; Rice-Evans, C. A. Colonic metabolism of dietary polyphenols: influence of structure on microbial fermentation products. *Free Radical Biol. Med.* **2004**, *36* (2), 212–225.
- (41) Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Rémésy, C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **2005**, *81* (1 Suppl.), 230S–242S.
- (42) Erlund, I.; Meririnne, E.; Alfthan, G.; Aro, A. Human nutrition and metabolism: Plasma kinetics and urinary excretion of the flavanones naringenin and hesperetin in humans after ingestion of orange juice and grapefruit juice. *J. Nutr.* **2001**, *131* (2), 235–241.
- (43) Rios, L. Y.; Gonthier, M. P.; Rémésy, C.; Mila, I.; Lapiere, C.; Lazarus, S. A.; Williamson, G.; Scalbert, A. Chocolate intake increases urinary excretion of polyphenol-derived phenolic acids in healthy human subjects. *Am. J. Clin. Nutr.* **2003**, *77* (4), 912–918.
- (44) Ward, N. C.; Croft, K. D.; Puddey, I. B.; Hodgson, J. M. Supplementation with grape seed polyphenols results in increased urinary excretion of 3-hydroxyphenylpropionic acid, an important metabolite of proanthocyanidins in humans. *J. Agric. Food Chem.* **2004**, *52*, 5545–5549.

Received April 30, 2009. Revised manuscript received September 16, 2009. Accepted September 25, 2009. I.G. was the recipient of a fellowship from the I3P Program funding by the European Social Fund. M.U.-S. and A.M.-R. gratefully acknowledge the FPI fellowship from MICINN and FI program for the training of researchers from the Generalitat of Catalonia, respectively. M.M. thanks the “Juan de la Cierva” postdoctoral program. This work has received financial support from AGL2004-07075-C02-02, AGL2004-08378-C02-01, AGL2006-14228-C03-02, and the CONSOLIDER INGENIO 2010 Program: FUN-C-FOOD CSD2007-063 from the Spanish Ministry of Science and Innovation (MICINN).

8.2. COMUNICACIONS EN CONGRESSOS.

Comunicació 1: Pòster

III International Conference of Polyphenols and Health, 2007, Kyoto, Japan.

Urinary Excretion of Polyphenols and Antioxidant Capacity as Biomarkers of Fruits and Vegetables Intake.

Alexander Medina-Remón, Ana Barrionuevo-González, Rita Engel, Raúl Zamora-Ros, Cristina Andrés-Lacueva and Rosa M. Lamuela-Raventós

Urinary Excretion of Polyphenols and Antioxidant Capacity as Biomarkers of Fruits and Vegetables Intake.

Alexander Medina-Remón¹, Ana Barrionuevo-González¹, Rita Engel²,
Raúl Zamora-Ros¹, Cristina Andrés-Lacueva¹ and Rosa M. Lamuela-Raventós¹

¹ Nutrition and Food Science Department, XARTA, INSA, Pharmacy School, University of Barcelona, Av Joan XXIII s/n Barcelona, Spain. * e-mail: lamuela@ub.edu

² Department of Applied Chemistry, Faculty of Food Science, Corvinus University of Budapest, H-1118 Budapest Villányi út 29-43, Budapest, Hungary.



1. INTRODUCTION

The total polyphenols dietary intake is about 1 g/d. It is much higher than all other known dietary antioxidants (1). The health effects of polyphenols depend on their respective intakes, their synergism and their bioavailability, which can vary greatly (2). Epidemiological studies have repeatedly shown an inverse association between the risk of myocardial infarction and the consumption of tea, wine fruit and vegetable and a clear associations have been found between cancer risk and polyphenols consumption (3).

The Folin-Ciocalteu (F-C) assay is affected by several interfering substances typically present in urine. Oasis® MAX 96-well extraction plates allow a reliable Solid Phase Extraction (SPE) in biological fluids. The well plate makes the method particularly well-adapted to the analysis of large batches of samples for clinical and epidemiological studies.

A nutritional biomarker can be any biological specimen that is an indicator of nutritional status with respect to intake or metabolism of dietary constituents. It can be a biochemical, functional or clinical index of status of an essential nutrient or other dietary constituent (4).

AIMS:

1. Evaluate the total polyphenols (TP) and the antioxidant capacity in urine to correlate its with TP intake, to be considered as biomarker of the ingestion, bioavailability and accumulation of these compounds.
2. Improved and optimized the F-C assay described by Roura et al (5) using Oasis® MAX 96-well extraction plates (Mixed-Mode Anion-exChange and Reversed-Phase Solvent).

2. Material and Methods

STUDY DESIGN AND SAMPLES

12 healthy adult volunteers, (8 woman and 4 men; age range 24-54 years).

Prospective randomized, crossover trial



Food groups: cereals, vegetables, legumes, nuts, chocolate, fruits, oils and phenolic beverages.

Total polyphenols content (mg/g fresh matter) were quantified according to Saura-Calixto F. et al (6) taking into count the frequency questionnaire of the volunteers.

Solid Phase Extraction: extraction of total polyphenols

96 well plate cartridges from Waters Oasis® MAX (Milford, MA, USA).

1st Condition: 1 mL of methanol 98-100%.

2nd Condition: 1 mL of sodium acetate 50mM pH 7.

Load: 1mL samples + 1mL Milli-Q water + 34 µL HCl.

Wash: 1 mL sodium acetate 50mM pH 7 / 5% methanol.

Elution: 1.8 mL methanol at 2% formic acid.

ANALYSIS OF TOTAL POLYPHENOLS IN URINES: thermo microtiter 96-well plate

15 µL of each methanolic fraction eluted after SPE, 170 µL of Milli-Q water, 12 µL of F-C reagent 30 µL of sodium carbonate (200 g/L). Incubation 60 min. in the darkroom. After the reaction period: 73 µL of Milli-Q. Measure absorbance at 765 nm

ANALYSIS OF CREATININE IN URINES: thermo microtiter 96-well plate

3 µL of urine, 5 µL of sodium hydroxide (10 %) 60 µL of aqueous picric acid solution at 1% 15 min of reaction in the dark 232 µL of Milli-Q water. Measure absorbance at 500 nm

ABTS RADICAL CATION DECOLORIZATION ASSAY (7)

The antioxidant activity of the compounds is determined by the decolorization of the ABTS^{•+}, through measuring the reduction of the radical cation as the percentage inhibition of absorbance at 734 nm.

calibration

Trolox	Trolox	ABTS ^{•+}
µM	µL	µL
0	5	0
5	5	250
8	5	400
10	5	500
15	5	750

sample

Dilution	Sample	PB
µL	µL	µL
0	0	200
40	5	195
20	10	190
10	20	180

Calculation

$$\text{INH}\% = \frac{(\text{Solvent} - \text{A compound})}{\text{Solvent}} \times 100$$

$$\text{TEAC} = \frac{\text{INH}\% \text{ of sample}}{\text{INH}\% \text{ of trolox}}$$

INSTRUMENTAL: Spectrophotometer UV/VIS Thermo Multiskan Spectrum

Total polyphenols were expressed as mg gallic acid equivalent (GAE)/ g of creatinine and mg catechin equivalent (CE)/ g of creatinine.

The total antioxidant capacity was expressed as Trolox Equivalent Antioxidant Capacity (TEAC).

3. Results

Precision and accuracy

Calibrator Concentration n, mg/L	Means measured concentration, mg/L (n=5)		Precision (RSD), %		Recovery (error), %	
	Gallic acid	Catechin	Gallic acid	Catechin	Gallic acid	Catechin
1	0.9	0.9	0.6	1.1	82.5	85.2
2	2.1	2.0	0.8	0.8	100.7	104.6
4	4.1	4.2	3.5	3.6	105.7	103.0
6	6.0	5.9	5.1	5.3	101.8	99.8
8	7.9	8.0	7.2	6.7	97.7	99.2

Linearity: between 1 and 8 mg/L

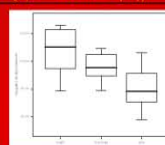
gallic acid calibration curve:
 $y = 0.082x + 0.030$; $r^2 = 0.997$

catechin calibration curve:
 $y = 0.0778x + 0.065$; $r^2 = 0.995$

Limit of detection = 0.07 mg/L

Limit of quantification = 0.11 mg/L

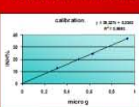
"mg gallic acid equivalent/ g creatinine" excreted in morning urine after the ingestion of the high, normal and low polyphenol diet.



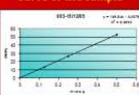
Correlation between consumption of different food groups and spot urinary polyphenols.

Food Group	r	P
Fruits	0,332	0,048
Vegetables	0,504	0,002
Legumes	-0,106	0,539
Cereals	-0,644	0,001
P. beverages	0,293	0,082
Oils	-0,094	0,586
Nuts	0,221	0,195
Chocolate	0,103	0,551
Total phenols	0,482	0,003

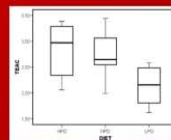
Trolox Calibration curve



Curve of the sample



trolox equivalent antioxidant capacity in different diet.



The antioxidant-rich diet involved high vegetable and fruit intake was positively correlated to the total polyphenol excretion and the antioxidant activity. Cereal consumption was negatively correlated to the total polyphenol excretion. No significant correlations were observed with the rest of the groups.

6. CONCLUSIONS

1. Total polyphenols excretion could be used as biomarkers to evaluate the consumption of total fruit and vegetable.
2. Total polyphenols intake was correlated with total polyphenols measured by F-C assay and antioxidant capacity (TEAC) in spot urine samples, to be considered as biomarker of the ingestion, bioavailability and accumulation of these compounds.
3. A Folin-Ciocalteu assay using Oasis® MAX 96-well extraction plates was improved and optimized to assess total polyphenol in human urine samples.

7. REFERENCES

1. Scalbert A, Williamson G. Dietary intake and bioavailability of polyphenols. J Nutr 2000;130:2073S-85S.
2. Fehér J, Lengyel G. [Nutrition and cardiovascular mortality]. Orv. Hetil. 2006;147:1491-6.
3. Scalbert A, Manach C, Morand C, Remesy C, Jimenez L. Dietary polyphenols and the prevention of diseases. Crit Rev Food Sci Nutr 2005;45:287-306.
4. Pottschman N, Freudenheim JL. Biomarkers of nutritional exposure and nutritional status: an overview. J Nutr 2003;133 Suppl 3:873S-4S.
5. Roura E, Andres-Lacueva C, Estruch R, Lamuela-Raventós RM. Total polyphenol intake estimated by a modified Folin-Ciocalteu assay of urine. Clin Chem 2006;52:749-52.
6. Saura-Calixto F, Goni I. Antioxidant capacity of the Spanish Mediterranean diet. Food Chem 2006;94:442-7.
7. Re R, Pellegrini N, Proteggente A, Pannala A, Min Yang, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine 1999; 26: 1231-1237.

8. SUPPORTED BY.

CICYT s (AGL2005-002823) and Red de Grupo G03/140 and RETICS RD06/0045/0003 from the Instituto de Salud Carlos III. CONSOLIDER CSD2007-063 from Education and Science Ministry (MEC). R. Z.-R. and A. M.-R. were supported by Department d'Universitat, Recerca for the formation of personal investigator. R. E. was supported by the Hungarian Scholarship Board.

Comunicació 2: Presentació oral.

10th European Nutrition Conference, Paris, July 2007

Publicat a: Annals of Nutrition and Metabolism 2007; 51(suppl 1): 70

Normal distribution of urinary polyphenol excretion among male adolescents and changes associated with nutritional intervention with tomato juice (*Lycopersicon esculentum*)

Laila Hussein, Alexander Medina, Anna Barrionuevo, Rosa Maria Lamuela-Raventos, Cristina Andres-Lacueva.

Annals of
**Nutrition &
Metabolism**

10th European Nutrition Conference

July 10–13, 2007, Paris, France

Abstracts

Guest Editors

Paul Guilloteau, Rennes

Daniel Lemonnier, Rouen

Denis Lairon, Marseille

KARGER

Basel • Freiburg • Paris • London • New York •
Bangalore • Bangkok • Singapore • Tokyo • Sydney

OC5-7 Oral Communications**Normal distribution of urinary polyphenol excretion among male adolescents and changes associated with nutritional intervention with tomato juice (*Lycopersicon esculentum*)**

Laila Hussein; A. Medina; A. Barrionuevo;
R.M. Lmuela-Ravestos; Cristina Andres-Lacueva

Departments of Nutrition and Food Science,
National Research Center, Egypt and Pharmacy School,
University of Barcelona, Spain

Introduction: The urinary [P P] was validated as a reliable biomarker for the intake of [P P]- rich fruits and vegetables. Objectives: To assess the normal distribution of urinary [P P] excretion among healthy male adolescents on typical Egyptian diet. To follow – up the impact of nutritional intervention with tomato juice on the [P P] excretion patterns.

Subjects: Forty nine adolescent males collected a 24h urine and completed a dietary record during 7 day period. A nutritional intervention program included daily serving of 240g fresh tomato juice was carried out on a subsample group and terminated for three weeks. Total urinary [P P] were measured before and after the termination of the intervention program.

The analysis of the urinary [P P] was completed after a clean up step using the solid phase extraction. The [P P] was then measured by the modified Folin Ciocalteu colorimetric reaction in 96 well microplates. The results were expressed as mg gallic acid equivalents per 24h urine [GAE/ 24h] or as GAE per gram urinary creatinine [GAE/Cr].

Results: The urinary [P P] excretion averaged 48.6 GAE/24h or 89.5 GAE/Cr. The three week intervention with tomato juice increased the urinary excretion of [P P] by more than 2.5 fold compared to the respective baseline figure. The intervention increased also potential biochemical indicators of the antioxidative status .

Conclusion: The patterns of urinary [P P] excretion suggested that tomato [P P] is highly bioavailable among the male adolescents. The present findings are in good agreement with earlier reports that urinary [P P] excretions is a potential biomarker for the dietary [P P] intake.

OC5-8 Oral Communications**Relative effects of black rice fraction and wood sterols on cholesterol levels and plasma lipid parameters in rats**

Aneta Kopeck; Jerzy Zawistowski; Charles Hu;
David D. Kitts

Food, Nutrition and Health Program, University of British
Columbia, Vancouver, B.C. and Forbes Medi-Tech. Inc.
Vancouver, B.C., Canada

This study reports the individual relative effects, and combinations of black rice fraction (BRF) and wood sterols (WS) in hypercholesterolemic rats exposed to mild hypercholesterolemia (e.g.

absence of bile acids: Exp. I), or extreme hypercholesterolemia (e.g. presence of bile acids: Exp. II), on plasma lipid profile and total hepatic cholesterol accumulation in male rats. Exp I control diet contained 0.5% cholesterol, 1, or 3% BRF and a combination of BRF treatments with WS (e.g. 2%). This experiment was repeated in Exp. II, but with the addition of cholic acid along with 0.5% cholesterol to diets. After 10 weeks feeding experimental diets, animals were sacrificed by exsanguination under halothane anesthesia, and blood was drawn from a heart puncture and collected in heparinized tubes. Plasma and liver total cholesterol (TC), HDL-cholesterol, triacylglycerol (TAG), and antioxidant activity (ORAC method) were measured at the end of experiment. In both experiments, the combination of BRF and WS significantly decreased the level of TC, and LDL. Rats fed control diet with WS showed higher HDL cholesterol levels ($p < 0.05$) than rats fed diets with BRF, or diets with BRF and WS. The LDL cholesterol level was significantly lower in rats fed WS in both experimental groups ($p < 0.01$). Concentration of plasma TAG was significantly lower in severely hypercholesterolemic rats fed a combination of BRF and WS, in presence of bile salts. Liver crude lipids and TC levels were significantly decreased in all experimental groups. Combination of BRF and WS did not alter plasma total antioxidant activity. We conclude that BRF and wood sterols are useful ingredients for formulating functional foods that can modulate cholesterol levels in vivo.

OC6-1 Oral Communications**Influence of gut microbiota on immunological maturation in infancy**

Rikke Brandt Sørensen; Susanne Brix Pedersen;
Hanne Frøkiær

Nutritional Immunology Group, Center for Biological
Sequence Analysis, Technical University of Denmark,
Kgs. Lyngby, Denmark

Maturation and function of the immune system is highly influenced by the establishment of the microbiota in the gut, which in turn, particularly in infancy, is influenced by factors such as maternal microbiota and the environment, including diet. Studies have shown that although lymph nodes are able to elicit mixed Th1/Th2 responses, Th2 responses dominate in the spleen in the neonatal mouse.

In this study, we compared phenotypic markers present on mesenteric lymph nodes (mLNs) and spleens from 3 weeks old mice, to levels found in adult mice. We found that mLNs displayed levels of CD4+ and CD8+ T-cells as well as NK-cells similar to those found in adult mice, while splenocytes expressed severely reduced levels of these markers and were impaired in their ability to proliferate in response to anti-CD3/anti-CD28.

To further characterize the development of immunological maturation in spleens from young mice, female mice were administered different probiotics during pregnancy and lactation and their offspring sacrificed at the age of 3 weeks. Interestingly, intake of *Bb. longum* Q46 or *E. coli* Nissle 1917 resulted in reduced levels of CD4, CD8 and CD49b on the cell surface of splenocytes as well as impaired ex vivo proliferative abilities of T lymphocytes as measured by 3H-TdR incorporation. Furthermore, *Bb. longum* Q46 and *E. coli* Nissle 1917

Comunicació 3: Pòster

XXIV th Conference on Polyphenols Salamanca (ESPAÑA) 2008

Determination of total poliphenols and resveratrol in beer.

Chiva-Blanch, G.; Zamora-Ros, R.; Urpí-Sarda, M.; Medina-Remón, A.;
Estruch, R. and Andres-Lacueva, C.

Determination of total polyphenols and piceid in Beer



Gemma Chiva-Blanch^{1,2}, Raul Zamora-Ros¹, Mireia Urpi-Sardà¹, Alexander Medina-Remón¹, Rosa Lamuela-Raventós¹, Ramon Estruch², Cristina Andres-Lacueva^{1*}.

¹Nutrition and Food Science Department-XaRTA, Faculty of Pharmacy, University of Barcelona, 08028, Barcelona, Spain; ²Laboratory 413, Department of Internal Medicine, Hospital Clínic, Institut d'Investigació Biomèdica August Pi i Sunyer (IDIBAPS), University of Barcelona, 08036, Barcelona, Spain. *Corresponding author: candres@ub.edu



INTRODUCTION



trans- and *cis*-Piceid and *trans*-resveratrol have been found in different hop cultivars in concentrations ranging 0.5-11.7 ppm (Jerkovic, V. and Collin, S. 2007, *J. Agric. Food Chem.* 55:8754-8758). Although about 20-30% of beer polyphenols originate from hop, whereas 70-80% are malt derived, hop is added 100 fold less than malt. So, low concentrations of *trans*- and *cis*-piceid could be expected in final beer.

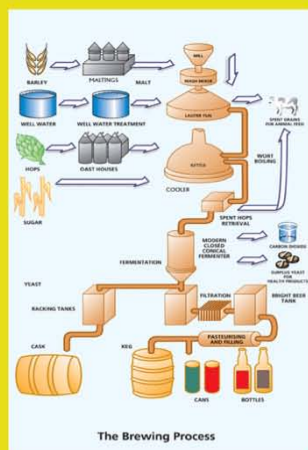
For this reason, the aim of this study is to determine the resveratrol and piceid and total polyphenols content in a wide variety of beers (alcohol free, lager, pilsner, trappist, dark, weissbier).

CONCLUSIONS

1. This is the first time that piceid, the resveratrol glucoside is described in beer. However, no free resveratrol was found.
2. From the 87 commercial beers analyzed, only the 20% had very low amounts of stilbenes.
3. To achieve the same amount of stilbenes from a glass of wine, it is needed 3350 liters of those beers.



1.06 mg stilbenes
(*trans*- and *cis*-resveratrol
and *trans*- and *cis*-piceid)
(125ml of red wine)



MATERIALS AND METHODS

Total polyphenols in beer were quantified by Folin-Ciocalteu method (Roura, E et al. 2006, *Clin. Chem* 52: 749-752), expressed as mg/L of Gallic Acid Equivalents (mg GAE/L).

Resveratrol analyses were carried out by LC-MS/MS after a solid phase extraction of sonicated samples (Urpi-Sardà, M. et al. 2007, *Clin. Chem.* 53:292-299).

RESULTS

Special and trappist beers contained significant higher levels of polyphenols (640.81 ± 142.22 meq AG/L) than pilsener/lager beer (467.98 ± 141.92 meq AG/L, $P < 0.05$), being a source of phenols to consider. Pilsener/lager beer had more polyphenols than alcohol free beer (280.29 ± 87.25 meq AG/L, $P < 0.05$).

The most flavored beers contained more polyphenols than the rest of beers, suggesting that the brewing process is a determinant of the total polyphenol concentration.

17 of 87 of analyzed beers contained piceid in a concentration range between 0.28 and 3.39 ppb, mainly in the *cis* form.

Only 5 of the 17 beers contained *trans*-piceid.

No piceid was found in any alcohol free beer.

TABLE 1. SPECIAL AND TRAPPIST BEER

SAMPLE	Total piceid (µg/L)
Hommel beer	0.63±0.09
Gulden 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

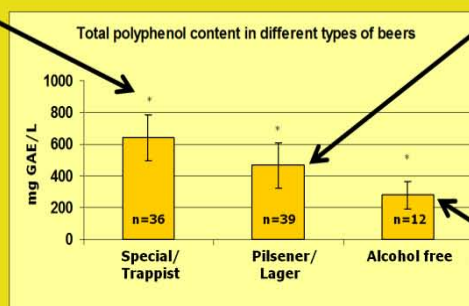


TABLE 2. PILSENER AND LAGER BEER

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)	1.63 ±0.21
Monitz	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

Figure 1. mg GAE/L in Special and trappist beer (n=36), pilsener/lager beer (n=39) and alcohol free beer (n=12). * $p < 0.05$

Table 1 and 2. Concentrations of total piceid (*trans*- and *cis*-piceid) (µg/L) of the different beers analyzed. Values are expressed as mean±sd.

ACKNOWLEDGEMENTS

We are grateful for the financial support of the Ministry of Science and Technology (AGL2006-14228-C03-02/ALI), Ingenio-CONSOLIDER program, FUN-C-Food CSD2007-063 and the CIBER 30/06 Fisiopatología de la Obesidad y la Nutrición, Instituto de Salud Carlos III. Mireia Urpi-Sardà and Alexander Medina-Remon thanks FPI fellowship program from the MEC and FI fellowship program from the Generalitat de Catalunya respectively.

XXIVth International Conference on Polyphenols, July 8-11, 2008, Salamanca, Spain

Comunicació 4: Pòster

XXIVth Conference on Polyphenols Salamanca, 8-11 July 2008

Total Polyphenols in Human Urinary as Biomarkers of Fruits and Vegetables Intake in the PREDIMED Study.

Alexander Medina-Remón, Raúl Zamora-Ros, Cristina Andrés-Lacueva, Ramón Estruch, Javier Díez-Espino, Miguel A. Martínez-González and Rosa M. Lamuela-Raventós



Total Polyphenols in Human Urinary as Biomarkers of Fruits and Vegetables Intake in the PREDIMED Study.

Alexander Medina-Remón¹, Raul Zamora-Ros¹, Cristina Andrés-Lacueva¹, Ramón Estruch², Javier Díez-Espino³, Miguel-Angel Martínez González⁴ and Rosa M. Lamuela-Raventós¹

¹Nutrition and Food Science Department, XaRTA, Pharmacy School, University of Barcelona, Av. Joan XXIII s/n Barcelona, Spain. ²Institut d'Investigacions Biomèdiques August Pi Sunyer, Municipal Institut for Medical Research, University of Barcelona, and Catalan Institute of Health, Barcelona, Spain. ³Tafalla Health Center, Osasunbidea, Tafalla, Spain. ⁴Department of Preventive Medicine and Public Health, Clínica Universitaria, University of Navarra, Pamplona. corresponding author: lamuela@ub.edu

1. INTRODUCTION

Total dietary polyphenol (TP) levels are approximately 1 g/d [1], higher than all other known dietary antioxidants, around 10 times higher than dietary vitamin C and 100 times higher than vitamin E and carotenoid levels. There are few studies that correlated the intake of polyphenols-rich food with the urinary excretion of total phenolic compounds. The health effects of polyphenols depend on their respective intakes, synergism and bioavailability, which can vary greatly. Epidemiological studies have shown an inverse association between the risk of myocardial infarction and the consumption of food rich in phenolic compounds: tea, wine, fruit and vegetables (F&V) and a clear associations have been found between cancer risk and polyphenol consumption. Nutritional markers have several advantages for epidemiologic and clinical assays, over dietary data obtained by food frequency questionnaires (FFQ) [2]. Biomarkers of the intake of some nutrients are more precise than dietary assessment and provide a more accurate measure of specific nutrient intake than FFQ; because it often underestimates intake. The development of biological biomarkers, measured in both blood and urine, are essential for making estimates of polyphenol intake. However, the relationship between dietary intake and nutritional biomarkers is often highly complex [3].

AIMS:

Evaluate the association between TP excretion in spot urine samples and total F&V intake in a free living population with cardiovascular risk to be considered as biomarker of the ingestion, bioavailability and accumulation of total polyphenols intake from total F&V.

2. Subjects and Methods

589 admitted trial participants from PREDIMED study (PREvention with MEDiterranean Diet). This 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 (www.predimed.org) [4].



Urinary polyphenols were analysed by the Folin-Ciocalteu (FC) assay after a solid phase extraction (SPE) to remove the possible interferences with FC reagent. TP consumption from plant food (mg/g fresh matter) were quantified according to Saura-Calixto F. et al [5] taking into account the food frequency questionnaire of the volunteers at the baseline.

SPE: 96 well plate cartridges from Waters Oasis® MAX (Mixed-Mode Anion-exchange and Reversed-Phase Solvent).

1° Condition: 1 mL of methanol 98-100%.

2° Condition: 1 mL of sodium acetate 50mM pH 7.

Load: 1 mL samples + 1 mL Milli-Q water + 34 µL HCl.

Wash: 1 mL sodium acetate 50mM pH 7/ 5% methanol.

Elution: 1.8 mL methanol at 2% formic acid.



ANALYSIS OF TOTAL POLYPHENOLS IN URINES: thermo microtiter 96-well plate.



15 µL of each methanolic fraction eluted after SPE
170 µL of Milli-Q water, 12 µL of F-C reagent
30 µL of sodium carbonate (200 g/L).
Incubation 60 min. in the darkroom.
After the reaction period: 73 µL of Milli-Q
Measure absorbance at 765 nm

ANALYSIS OF CREATININE IN URINES: thermo microtiter 96-well plate.



60 µL of aqueous picric acid solution at 1%
3 µL of urine and 5 µL of sodium hydroxide (10%)
15 min of reaction in the dark
232 µL of Milli-Q water, vortex during 1 min.
Measure absorbance at 500 nm

Equipe: Spectrophotometers UV/VIS Thermo Multiskan Spectrum

Total polyphenols in urine samples were expressed as mg gallic acid equivalent (GAE) g of creatinine.

Statistical analysis

Analyses were performed using SPSS software version 14.0. Descriptive statistical tests were expressed as percentage or means and SD. Because of the TP content in morning urine was not normally distributed it was necessary a boxcox transformation.

The Rho Spearman correlation model was used to examine the simple correlation between TP excreted in urine samples and fruit, vegetable, F&V and TP intake. P-values <0.05 (two-tailed) were considered to be significant.

Multivariate linear regression were performed to assess the relation between TP excreted in urine as the dependent variable and F&V (serving of 100g) as exposure variable with adjustment for potential confounders.



XXIVth Conference on Polyphenols Salamanca, 8-11 July 2008

3. Results

Table 1: Baseline Characteristics of the study participants by quartile of total polyphenols excretion expressed as mg GAE/g creatinine.

	Urine mg GAE/g creatinine concentration quartile				P for trend ¹
	Q1 (<88.99)	Q2 (89-119.46)	Q3 (119.47-160.22)	Q4 (>160.23)	
No of subjects	147	148	147	147	0.742
Women, n (%)	51 (34.7)	83 (56.1)	85 (57.8)	107 (72.8)	<0.001
Mean (SD) age, y	66.0 (5.7)	68.3 (6.1)	68.0 (5.8)	69.3 (6.0)	<0.001
Mean (SD) BMI, (kg/m ²)	29.6 (2.9)	29.6 (3.7)	29.2 (3.7)	29.1 (3.4)	0.171
Overweight or obese (BMI ≥25 Kg/m ²), n (%)	138 (93.9)	137 (92.6)	128 (87.1)	135 (91.8)	0.263
Systolic blood pressure (mm Hg), mean (SD)	152.0 (18.2)	152.4 (18.2)	155.5 (21.8)	155.9 (32.4)	0.125
Diastolic blood pressure, mean (SD)	87.6 (10.9)	86.3 (9.8)	85.8 (11.9)	84.5 (9.1)	0.013
Cardiovascular Frequency, mean (SD)	69.2 (10.4)	70.0 (11.9)	68.3 (10.1)	71.7 (10.1)	0.148
Energy expenditure in physical activity (kcal/d), mean (SD)	256.0 (215.5)	245.9 (220.7)	293.1 (231.0)	255.3 (205.3)	0.576
Current smoker, n (%)	39 (26.5)	20 (13.5)	22 (15.0)	14 (9.5)	<0.001
Vegetables (g)	253.1 (80.2)	261.1 (81.1)	262.6 (84.0)	272.7 (92.1)	0.053
Fruits (g)	304.6 (143.6)	319.3 (141.3)	315.1 (132.9)	360.8 (151.2)	0.002
Total fruits and vegetable (g)	557.7 (176.8)	580.4 (173.3)	577.7 (162.1)	633.5 (190.1)	0.001
Total Polyphenols intake (mg GAE)	1075.6 (354.9)	1057.5 (320.2)	1086.2 (322.3)	1222.5 (436.8)	0.001
Total Energy, Kcal/d	2380.1 (586.8)	2238.0 (472.0)	2205.1 (547.4)	2138.5 (476.8)	<0.001
Urine Total Polyphenol (mg GAE/g creatinine)	72.8 (11.6)	103.1 (8.2)	138.2 (11.1)	226.1 (69.8)	<0.001

¹A general linear model was used for continuous variables, and chi-square test was used for categorical variables; GAE: gallic acid equivalent; BMI: body mass index

Table 2: Result of simple correlation analyses between total polyphenol excreted expressed as mg GAE/g creatinine and the different food groups as serving of 100 g.

	Total population (n=589)		Model	β	SE	Beta	P	95 % CI	R ²
	r	P							
Vegetables	0.088	0.032	Model 1	0.131	0.035	0.155	<0.001	0.064 to 0.198	0.024
Fruits	0.118	0.004	Model 2	0.112	0.033	0.132	0.001	0.047 to 0.177	0.111
Fruit and vegetable (F&V)	0.137	0.001	Model 3	0.107	0.034	0.126	0.002	0.040 to 0.174	0.122
Total polyphenols intake	0.109	0.008	Model 4	0.127	0.036	0.150	<0.001	0.056 to 0.198	0.171



β: Non-standardized coefficient (regression line coefficient); SE: Standard error; Beta: Standardized coefficient; CI: Confidence interval; P: two-sided test of significance; R²: determination coefficient; Model 1, unadjusted; Model 2 was adjusted by sex, age and weight; Model 3 as in Model 2 and smoking status, physical activity, educational level and energy expenditure in physical activity; Model 4 was adjusted as in Model 3 and total polyphenol intake, olive oil, beer, chocolate, natural orange juice, tea, cereals, milk products, meat or meat product, nuts, pastries-cakes-sweets, fruit and vegetables, wine and coffee intake; GAE: Gallic acid equivalent.

The result of the simple correlation analysis (Table 2) shows that the TP excreted in spot urine expressed as mg GAE/g creatinine was positively correlated with fruit, vegetables, F&V and TP intake.

In linear regression analyses (Table 3) were observed a significantly positively association between the total polyphenols excreted in spot urine samples and daily intake of 100 g (one serving) of F&V in unadjusted model. In multivariate regression, after adjusting for potential confounding factors (model 2, 3 and 4) the association remained positively statistically significant.



4. CONCLUSIONS

1. Total polyphenols excretion could be used as biomarkers to evaluate the consumption of total fruit and vegetable intake in epidemiologic studies.
2. Total polyphenols intake was correlated with total polyphenols measured by F-C assay in spot urine samples, to be considered as biomarker of the ingestion, bioavailability and accumulation of these compounds.
3. A Folin-Ciocalteu method to assess total polyphenols in spot human urine samples with Oasis® MAX 96-well extraction plates is fast, simple and allows the analysis of a large number of samples, as many as 96 samples. The methodology could potentially be used to detect a wide range of polyphenols in urine samples, with the high throughput of 96-well microtiter plates and reader

5. REFERENCES

- [1] Scalbert A, et al. (2000) J Nutr. 130:2073S-95S.
- [2] Marshall JR. (2003) J Nutr. 133 Suppl 3:881S-7S.
- [3] Spencer JP, et al. (2008) J C. Br J Nutr 99:12-22.
- [4] Estruch R, et al. (2006) Ann. Intern. Med. 145:1-11.
- [5] Saura-Calixto F, et al. (2006) Food Chemistry 94:442-7.

6. SUPPORTED BY.

CICYT's (AGL2005-002823) and Red de Grupo G03/140 and RETICS RD06/0045/0003 from the Instituto de Salud Carlos III (PI 052443, PI070240). R. Z-R. was supported by Departament d'Universitats, Recerca and A. M-R. was supported by the Generalitat de Catalunya for the formation of personal investigator (2006FI 01399).

Comunicació 5: Pòster

12^{as} Jornades d'anàlisi instrumental. Congrés Internacional: Barcelona (ESPAÑA) 2008

High sensitive determination of resveratrol in beer by liquid chromatography tandem mass spectrometry

Chivam Blanch, G.; Zamora Ros, R.; Urpí Sardà, M.; Medina Remón, A.; Estruch, R. and Andres Lacueva, C.

HIGH SENSITIVE DETERMINATION OF RESVERATROL IN BEER BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY



Gemma Chiva-Blanch^{1,2}, Raul Zamora-Ros¹, Mireia Urpi-Sardà¹, Alexander Medina-Remón¹, Rosa Lamuela-Raventós¹, Ramon Estruch², Cristina Andres-Lacueva^{1*}.

¹Nutrition and Food Science Department-XaRTA, INSA affiliated to INGENIO-CONSOLIDER program, Fun-c-food CSD2007-063, Faculty of Pharmacy, University of Barcelona, 08028, Barcelona, Spain; ²Laboratory 413, Department of Internal Medicine, Hospital Clínic, Institut d'Investigació Biomèdica August Pi i Sunyer (IDIBAPS), University of Barcelona, 08036, Barcelona, Spain affiliated to CIBER de Fisiopatología de la Obesidad y la Nutrición (CIBEROBN), and RETICS RD06/0045, Instituto de Salud Carlos III, Spain.

*Corresponding author: candres@ub.edu

INTRODUCTION

trans- and *cis*-Piceid and *trans*-resveratrol have been found in different hop cultivars in concentrations ranging 0.5-11.7 ppm (Jerkovic, V. and Collin, S., 2007, *J. Agric. Food Chem.* 55:8754-8758). Although about 20-30% of beer polyphenols originate from hop, whereas 70-80% are malt derived, hop is added 100 fold less than malt. So, low concentrations of *trans*- and *cis*-piceid could be expected in final beer.

For this reason, the aim of this study is to set up a high sensitive methodology able to determine the potential low content of resveratrol and piceid extracted from hop. For this purpose we have analyzed a wide variety of different kinds of beers (alcohol free, lager, pilsner, trappist, dark, weissbier).

RESULTS AND DISCUSSION

17 of 87 of analyzed beers contained piceid in a concentration range between 0.28 and 3.39 ppb, mainly in the *cis* form.

Only 5 of the 17 beers contained *trans*-piceid.

No piceid was found in any alcohol free beer.

TABLE 1. 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)	1.63 ±0.21
Montz	1.97±0.32
El Corte Inglés (german)	2.3 ±0.39
Samuel Adams	3.39±0.16

TABLE 2. SPECIAL AND TRAPPIST BEER

SAMPLE	Total piceid (µg/L)
Hommel bier	0.63±0.09
Gulden 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 1 and 2. Concentrations of total piceid (*trans*- and *cis*-piceid) (µg/L) of the different beers analyzed. Values are expressed as mean±sd.

TABLE 3. ALCOHOL FREE BEER

SAMPLE	Total piceid (µg/L)
All samples	Non detected

CONCLUSIONS

The use of Multiple Reaction Monitoring (MRM) allowed the identification and quantification of resveratrol and piceid in beers in very low levels (ng/mL).

This is the first time that piceid, the resveratrol glucoside is quantified in beer. However, no free resveratrol was found.

From the 87 commercial beers analyzed, only the 20% had very low amounts of stilbenes.

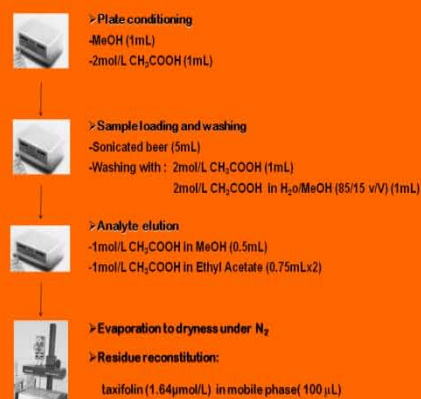
To achieve the same amount of stilbenes from a glass of wine, it is needed 3350 liters of beer containing resveratrol.

ACKNOWLEDGEMENTS

We are grateful for the financial support of the Ministry of Science and Technology (AGL2006-14228-C03-02/ALI), Ingenio-CONSOLIDER program, FUN-C-Food CSD2007-063 and the CIBER 30/06 Fisiopatología de la Obesidad y la Nutrición, Instituto de Salud Carlos III, Mireia Urpi-Sardà and Alexander Medina-Remon thanks FPI fellowship program from the MEC and FI fellowship program from the Generalitat de Catalunya respectively. We also thank Isidre Ferrer, Olga Jauregui and the Scientific and Technical Unit from UB for their collaboration and technical assistance.

MATERIALS AND METHODS

Resveratrol analyses were carried out by LC-MS/MS after a solid phase extraction of sonicated samples based on the method described by Urpi-Sardà, M. et al. 2007 (*Clin. Chem.* 53:292-299) adapted to beer samples.



LC conditions:
LC Instrument: Agilent 1100 system (Waldbronn, Germany)
Column: Phenomenex Luna C18[50 x 2.0 mm i.d., 5µm] (Torrance, CA)
Solvents: phase A (0.5 mL/L acetic acid) and phase B (700 mL/L CH₃(CO)CH₃, 300 mL/L ACN with 0.4 mL/L CH₃COOH)
Linear gradient (v/v) of phase B (t(min), % B): (0, 10), (0.5, 15), (5, 100), (5.6, 100).
Flow rate: 500 µl/min
Injection volume: 15 µl

MS/MS conditions:
Instrument: Triple Quadrupole mass spectrometer API 3000 (Applied Biosystems, PE Sciex, Concord, Ontario, Canada)
Turbo IonSpray
Ionization mode: negative

MRM experiments were performed for quantification purposes.

Comunicació 6: Pòster

II International Symposium CIBERobn, November 2008 “La Toja” Spain.

Publicado en: Obesity and Metabolism Vol.5, Suppl. to No.1, 2009 March p15

EVALUATION OF POLYPHENOLS INTAKE IN THE PREDIMED STUDY

Alexander Medina-Remón, Raúl Zamora-Ros, Cristina Andrés-Lacueva, Maria Rotchés-Ribalta, Ramón Estruch, Javier Díez-Espino, Miguel-Angel Martínez González and Rosa M. Lamuela-Raventós.



EVALUATION OF POLYPHENOLS INTAKE IN THE PREDIMED STUDY.

Alexander Medina-Remón¹, Raúl Zamora-Ros¹, Cristina Andrés-Lacueva¹, Maria Rotchés-Ribalta¹, Ramón Estruch², Javier Díez-Espino³, Miguel-Angel Martínez González⁴ and Rosa M. Lamuela-Raventós^{1*}

¹Nutrition and Food Science Department, XaRTA, INSA, Pharmacy School, University of Barcelona, Av. Joan XXIII s/n Barcelona, Spain; ²Institut d'Investigacions Biomèdiques August Pi Sunyer, Municipal Institut for Medical Research, University of Barcelona, and Catalan Institute of Health, Barcelona, Spain; ³Tatalla Health Center, Osasunbidea, Tafalla, Spain; ⁴Department of Preventive Medicine and Public Health, Clínica Universitaria, University of Navarra, Pamplona *corresponding author: lamuela@ub.edu

1. INTRODUCTION

Total dietary polyphenol (TP) levels are approximately 1 g/d [1], higher than all other known dietary antioxidants, around 10 times higher than dietary vitamin C and 100 times higher than vitamin E and carotenoid levels. There are few studies that correlated the intake of polyphenols-rich food with the urinary excretion of total phenolic compounds. The health effects of polyphenols depend on their respective intakes, synergism and bioavailability, which can vary greatly. Epidemiological studies have shown an inverse association between the risk of myocardial infarction and the consumption of food rich in phenolic compounds: tea, wine, fruit and vegetables (F&V) and a clear associations have been found between cancer risk and polyphenol consumption. Nutritional markers have several advantages for epidemiologic and clinical assays, over dietary data obtained by food frequency questionnaires (FFQ) [2]. Biomarkers of the intake of some nutrients are more precise than dietary assessment and provide a more accurate measure of specific nutrient intake than FFQ; because it often underestimates intake. The development of biological biomarkers, measured in both blood and urine, are essential for making estimates of polyphenol intake. However, the relationship between dietary intake and nutritional biomarkers is often highly complex [3].

Objectives:

The aim of the present study was to evaluate the association between TP excretion in spot urine samples and total F&V intake in a free living population with cardiovascular risk to be considered as biomarker of the ingestion, bioavailability and accumulation of total polyphenols intake from total F&V.

2. Subjects and Methods

589 admitted trial participants from PREDIMED study (PREvention with MEDiterranean Diet). This 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 (www.predimed.org) [4].



Urinary polyphenols were analysed by the Folin-Ciocalteu (FC) assay after a solid phase extraction (SPE) to remove the possible interferences with FC reagent. TP consumption (from plant food (mg/g fresh matter) were quantified according to Saura-Calixto F. et al [5] taking into account the food frequency questionnaire of the volunteers at the baseline.

SPE: 96 well plate cartridges from Waters Oasis® MAX (Mixed-Mode Anion-exchange and Reversed-Phase Solvent).

1st Condition: 1 mL of methanol 98-100%.

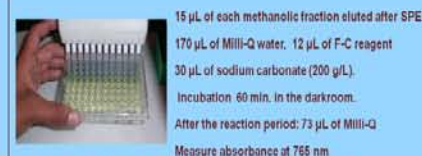
2nd Condition: 1 mL of sodium acetate 50mM pH 7.

Load: 1mL samples + 1mL Milli-Q water + 34 µL HCl.

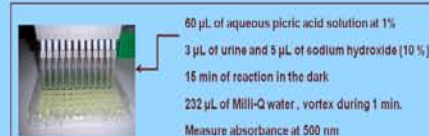
Wash: 1 mL sodium acetate 50mM pH 7/ 5% methanol.

Elution: 1.8 mL methanol at 2% formic acid.

ANALYSIS OF TOTAL POLYPHENOLS IN URINES: thermo microtiter 96-well plate.



ANALYSIS OF CREATININE IN URINES: thermo microtiter 96-well plate.



Equipos: Spectrophotometers UV/VIS Thermo Multiskan Spectrum

Total polyphenols in urine samples were expressed as mg gallic acid equivalent (GAE)/g of creatinine.

Statistical analysis

Analyses were performed using SPSS software version 14.0. Descriptive statistical tests were expressed as percentage or means and SD. Because of the TP content in morning urine was not normally distributed it was necessary a boxcox transformation.

The Rho Spearman correlation model was used to examine the simple correlation between TP excreted in urine samples and fruit, vegetable, F&V and TP intake. P-values <0.05 (two-tailed) were considered to be significant.

Multivariate linear regression were performed to assess the relation between TP excreted in urine as the dependent variable and F&V (serving of 100g) as exposure variable with adjustment for potential confounders.



Food Chemistry (2009-1000)



3. Results

Table 1: Baseline Characteristics of the study participants by quartile of total polyphenols excretion expressed as mg GAE/g creatinine.

	Urine mg GAE/g creatinine concentration quartile				P for trend ^a
	Q1 (<58.99)	Q2 (59-119.48)	Q3 (119.47-169.22)	Q4 (>169.23)	
No of subjects	147	148	147	147	0.742
Women, n (%)	51 (34.7)	83 (56.1)	85 (57.8)	107 (72.8)	<0.001
Mean (SD) age, y	66.0 (5.7)	68.3 (6.1)	68.0 (5.8)	69.3 (6.0)	<0.001
Mean (SD) BMI, (kg/m ²)	29.6 (2.9)	29.6 (3.7)	29.7 (3.7)	29.1 (3.4)	0.171
Overweight or obese (BMI ≥25 Kg/m ²), n (%)	138 (93.9)	137 (92.6)	128 (87.1)	135 (91.8)	0.263
Systolic blood pressure (mm Hg), mean (SD)	152.0 (18.2)	152.4 (18.2)	155.5 (21.8)	155.9 (23.4)	0.125
Diastolic blood pressure, mean (SD)	87.6 (10.9)	86.3 (9.8)	85.8 (11.9)	84.5 (9.1)	0.013
Cardiovascular Frequency, mean (SD)	69.2 (10.4)	70.0 (11.9)	68.3 (10.1)	71.7 (10.1)	0.148
Energy expenditure in physical activity (kcal/d), mean (SD)	256.0 (215.5)	245.9 (220.7)	293.1 (231.0)	255.3 (205.3)	0.578
Current smoker, n (%)	39 (26.5)	20 (13.5)	22 (15.0)	14 (9.5)	<0.001
Vegetables (g)	253.1 (80.2)	261.1 (81.1)	262.6 (84.0)	272.7 (92.1)	0.053
Fruits (g)	304.6 (143.8)	310.3 (141.3)	315.1 (152.9)	360.8 (151.2)	0.002
Total fruits and vegetable (g)	557.7 (178.8)	580.4 (173.3)	577.7 (162.1)	633.5 (160.1)	0.001
Total Polyphenols intake (mg GAE)	1075.6 (354.9)	1097.5 (320.2)	1086.2 (322.3)	1222.5 (439.8)	0.001
Total Energy, kcal/d	2380.1 (566.8)	2236.0 (472.0)	2205.1 (547.4)	2156.5 (476.8)	<0.001
Urine Total Polyphenol (mg GAE/g creatinine)	72.8 (11.6)	103.1 (8.2)	138.2 (11.1)	226.1 (89.8)	<0.001

^a A general linear model was used for continuous variables, and chi-square test was used for categorical variables. GAE: gallic acid equivalent; BMI: body mass index.

Table 2: Result of simple correlation analyses between total polyphenol excreted expressed as mg GAE/g creatinine and the different food groups as serving of 100 g.

	Total population (n=589)	
	r	P
Vegetables	0.089	0.032
Fruits	0.116	0.004
Fruit and vegetable (F&V)	0.137	0.001
Total polyphenols intake	0.109	0.008



Total urine polyphenols, mean (SD) was 135.28 (87.91) expressed as mg GAE/g creatinine with a total polyphenols intake of 1110.00 (366.09) mg Gallic Acid Equivalent/d. Total fruit, mean (SD) intake was 325.65 (144.02) g/d and total vegetable, means (SD) was 262.45 (84.84) g/d in this population. In Table 1 are shown the baseline characteristics of the study participants by quartile of total polyphenols excretion expressed as mg GAE/g creatinine. The result of the simple correlation analysis (Table 2) shows that the TP excreted in spot urine expressed as mg GAE/g creatinine was positively correlated with fruit, vegetables, F&V and TP intake.

In linear regression analyses (table 3) were observed a significantly positively association between the total polyphenols excreted in spot urine samples and daily intake of 100 g (one serving) of F&V in unadjusted model. In multivariate regression, after adjusting for potential confounding factors (model 2, 3 and 4) the association remained positively statistically significant.

4. CONCLUSIONS

1. Total polyphenols excretion could be used as biomarkers to evaluate the consumption of total fruit and vegetable intake in epidemiologic studies.
2. Total polyphenols intake was correlated with total polyphenols measured by F-C assay in spot urine samples, to be considered as biomarker of the ingestion, bioavailability and accumulation of these compounds.
3. A Folin-Ciocalteu method to assess total polyphenols in spot human urine samples with Oasis® MAX 96-well extraction plates is fast, simple and allows the analysis of a large number of samples, as many as 96 samples. The methodology could potentially be used to detect a wide range of polyphenols in urine samples, with the high throughput of 96-well microtiter plates and reader.

5. REFERENCES

- [1] Scalbert A, et al. (2000) J Nutr. 130:2073S-85S.
- [2] Marshall JR, (2003) J Nutr. 133 Suppl 3:881S-7S.
- [3] Spencer JP, et al. (2008) J. Br J Nutr 99:12-22.
- [4] Estruch R, et al. (2008) Ann. Intern. Med. 145:1-11.
- [5] Saura-Calixto F, et al. (2006) Food Chemistry 94:442-7.

6. SUPPORTED BY.

El CIBERobn es una iniciativa del ISCIII. This work has been supported by CICYT's (AGL2005-002823) and "Red de Grupo" G03/140, Ministries of Education and Science and Health (PI 052443, PI070240) and RETICS RD06/0045/0003 from the "Instituto de Salud Carlos III" and program INGENIO-CONSOLIDER FUN-C-FOOD (CSD2007-063) from Education and Science Ministry.

II International Symposium CIBERobn "La Toja", Spain 26-29 of November 2008

Comunicació 7: Pòster

In Vino Analytica Scientia 2009, 2-4 July, Angers France

Total polyphenols excretion is associated with wine polyphenols in a mediterranean diet: PREDIMED Study.

Alexander Medina-Remón, Raul Zamora-Ros, Maria Rotchés-Ribalta, Cristina Andrés-Lacueva, Ramón Estruch, Miguel-Ángel Martínez-González and Rosa M. Lamuela-Raventós.



Total polyphenols excretion is associated with wine polyphenols in a mediterranean diet: PREDIMED Study.

Alexander Medina-Remón^{1,2}, Raul Zamora-Ros^{1,3}, Maria Rotchés-Ribalta^{1,2}, Cristina Andrés-Lacueva^{1,3}, Ramón Estruch^{2,4}, Miguel-Ángel Martínez-González^{2,5,6} and Rosa M. Lamuela-Raventós^{1,2*}

¹Nutrition and Food Science Department, XaRTA, INSA, Pharmacy School, University of Barcelona, Av. Joan XXIII s/n Barcelona, Spain; ²CIBER 06/003 Physiopathology of Obesity and Nutrition, (CIBEROBN), and RETICS RD06/0045/0003, Instituto de Salud Carlos III, Spain; ³Ingenio-CONSOLIDER program, FUN-C-FOOD, Instituto de Salud Carlos III, Spain; ⁴Department of Internal Medicine, Hospital Clinic, Institut d'Investigació Biomèdica August Pi i Sunyer (IDIBAPS), University of Barcelona, Spain; ⁵Department of Preventive Medicine and Public Health, Clínica Universitaria, University of Navarra, Pamplona; ⁶Nutrition and Food Science Department, University of Barcelona. Telephone +34-934034843. Fax +34-93-4035931; e-mail lamuela@ub.edu

1. INTRODUCTION

Polyphenols are the most abundant antioxidants in human diets. They represent a wide variety of diverse structures from different subclasses¹. Total dietary polyphenol levels are approximately 1 g/d², higher than all other known dietary antioxidants, around 10 times higher than dietary vitamin C and 100 times higher than vitamin E and carotenoid levels. Epidemiologic data have shown an inverse association between the risk of overall mortality or cardiovascular disease and the consumption of polyphenols-rich foods such as fruit and vegetables, tea, olive oil and wine³⁻⁵. There are few studies that correlated the intake of polyphenols-rich food with the urinary excretion of total phenolic (TP) compounds.

AIMS:

The present study was designed to evaluate the association between the polyphenols rich foods in a normal Mediterranean diet with the excretion of TP in spot urine samples at the beginning of the recruitment period in a free living population with cardiovascular risk. Explain to what extent wine influences on the excretion of TP.

2. Subjects and Methods

589 admitted trial participants from PREDIMED study (PREVENCIÓN con Dieta MEDiterránea) study is a large, parallel-group, multicenter, randomized, controlled clinical trial of 5-year duration aimed to assess the effects of the Med-Diet on the primary prevention of cardiovascular disease (www.predimed.org; ISRCTN35739639).

263 men (55 to 80 years old)

326 women (60 to 80 years)

Urinary polyphenols were analysed by the Folin-Ciocalteu (FC) assay, after a solid phase extraction (SPE) with Oasis® MAX 96-well plate to remove the possible interferences with FC reagent. TP consumption from plant food and beverages (mg/g fresh matter) were quantified according to Saura-Calixto F. et al⁶ taking into account the frequency questionnaire of the volunteers.

SPE: 96 well plate cartridges from Waters Oasis® MAX (Mixed-Mode Anion-exchange and Reversed-Phase Solvent).

1° Condition: 1 mL of methanol 98-100%.

2° Condition: 1mL of sodium acetate 50mM pH 7.

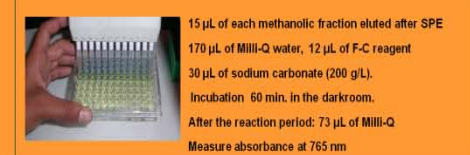
Load: 1mL samples + 1mL Milli-Q water + 34 µL HCl.

Wash: 1 mL sodium acetate 50mM pH 7/ 5% methanol.

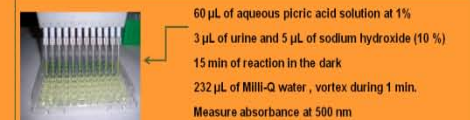
Elution: 1.8 mL methanol at 2% formic acid.



Analysis of total polyphenols in urines: thermo microtiter 96-well plate.



ANALYSIS OF CREATININE IN URINES: thermo microtiter 96-well plate.



Equipel: Spectrophotometers UV/VIS Thermo Multiskan Spectrum

Total polyphenols in urine samples were expressed as mg gallic acid equivalent (GAE)/g of creatinine.

Statistical analysis

Analyses were performed using SPSS software version 14.0. Baseline characteristics of the participants were expressed as means or percentages and standard deviations (SD). Parametric χ^2 -tests were used for analysis of categorical variables and one-factor ANOVA for means of comparison of continuous variables. Multivariate linear regression was performed to assess the relation between the total phenols excreted (TPE) normalized in urine as the dependent variable, and total F&V (100 g), and wine (100 mL) as the exposure variables with adjustment for potential confounders. The relationship between the urinary TPE quartile and systolic or diastolic BP was assessed by linear regression model adjusted for potential confounders

3. Results

Table 1: Consumption of daily food and urinary total polyphenol excreted, expressed as mg GAE/g creatinine.

	Urine mg GAE/g creatinine concentration quartile				P for trend ¹
	Q1 (<89.0)	Q2 (89.1-119.5)	Q3 (119.6-160.2)	Q4 (>160.3)	
No. of subjects	147	148	147	147	0.742
Olive oil (g)	48.7 (14.3)	47.3 (13.7)	45.1 (14.6)	45.0 (15.7)	0.014
Total nuts (g)	8.6 (10.7)	9.2 (11.3)	9.6 (12.2)	9.1 (10.9)	0.680
Legumes (g)	17.9 (7.0)	17.9 (7.5)	17.7 (7.0)	16.5 (7.6)	0.107
Total fruits and vegetables (g)	557.7 (176.8)	580.4 (173.3)	577.7 (162.1)	633.5 (190.1)	0.001
Fish or seafood intake(g)	86.5 (34.0)	82.2 (35.8)	88.7 (36.9)	94.1 (39.0)	0.030
Meat or meat products (g)	132.0 (49.6)	129.7 (43.7)	134.3 (48.5)	134.2 (47.5)	0.531
Pastries, cakes or sweets (g)	34.8 (31.6)	27.9 (24.8)	25.3 (25.9)	23.7 (28.7)	<0.001
Cereals (g)	243.7 (106.7)	235.8 (91.6)	228.7 (98.3)	211.3 (83.3)	0.003
Milk products (mL)	352.1 (214.8)	378.8 (216.5)	396.5 (222.1)	403.1 (215.1)	0.033
Wine (mL)	121.1 (147.0)	81.5 (137.9)	101.1 (161.5)	81.3 (149.3)	0.071
Coffee (mL)	63.3 (47.7)	69.6 (52.9)	67.5 (48.3)	74.5 (56.0)	0.097
Tea (mL)	3.92 (16.4)	3.25 (11.4)	4.16 (12.6)	5.47 (20.4)	0.332
Chocolate (g)	3.1 (6.2)	2.4 (5.2)	2.3 (5.5)	1.9 (4.8)	0.063
Total polyphenols intake (mg GAE)	1075.6 (354.9)	1057.5 (320.2)	1086.2 (322.3)	1222.5 (439.8)	0.001
Sodium intake (mg/d)	3347.7 (959.2)	3088.0 (905.4)	3123.2 (1006.5)	3145.4 (877.2)	0.100
Potassium intake (mg/d)	3926.7 (722.3)	3929.4 (700.9)	3994.8 (805.5)	4029.7 (659.7)	0.161
Total energy, Kcal/d	2380.1 (586.8)	2238.0 (472.0)	2205.1 (547.4)	2138.5 (476.8)	<0.001
Urine total polyphenol (mg GAE/g creatinine)	72.8 (11.6)	103.1 (8.2)	138.2 (11.1)	226.1 (69.8)	<0.001
Age, (y) mean (SD)	66.0 (5.7)	68.3 (6.1)	68.0 (5.8)	69.3 (6.0)	<0.001
Weight (Kg), mean (SD)	80.0 (11.1)	75.3 (11.2)	74.1 (10.3)	71.7 (11.2)	<0.001
Diastolic BP (mmHg), mean (SD)	88.9 (10.2)	86.2 (9.8)	85.5 (11.8)	83.4 (8.5)	<0.001
Systolic BP (mmHg), mean (SD)	156.0 (15.6)	152.2 (18.3)	155.0 (21.6)	149.1 (16.0)	0.015

Table 2: Multivariate linear regression analysis with total polyphenol excreted in spot urine samples (mg GAE/g creatinine) as the dependent variable, and total fruit and vegetable intake (100g/d), wine (100mL/d) and coffee (100mL/d) as exposure variable, adjusted for potential confounders.

	Model	β	SE	Beta	P	95% CI
Fruits and Vegetables (100g)	Model 1	0.131	0.035	0.155	<0.001	0.064 to 0.199
	Model 2	0.127	0.036	0.150	<0.001	0.056 to 0.198
Wine (100 mL)	Model 1	-0.090	0.041	-0.090	0.029	-0.171 to -0.009
	Model 2	0.121	0.051	0.120	0.019	0.020 to 0.222

β : Non-standardized coefficient (regression line coefficient); SE: Standard error; Beta: Standardized coefficient; CI: Confidence interval; P: two-sided test of significance. Model 1: unadjusted; Model 2 was adjusted by sex, age, weight, smoking status, physical activity, educational level, energy expenditure in physical activity, total fish/seafood, olive oil, beer, chocolate, natural orange juice, tea, cereals, milk products, meat or meat products, nuts, pastries/cakes/sweets, fruits and vegetables, wine and coffee intake. GAE: Gallic acid equivalent.

Table 3: Multivariate linear regression analyses with systolic blood pressure and diastolic blood pressure as the dependent variables, and quartile of total polyphenol excreted in spot urine samples (mg GAE/g creatinine) as exposure variable, adjusted for potential confounders.

	Model	β	SE	Beta	P	95% CI
Systolic blood pressure	Model 1	-1.743	0.712	-0.104	0.015	-3.141 to -0.345
	Model 2	-1.708	0.758	-0.102	0.025	-3.197 to -0.219
Diastolic blood pressure	Model 1	-1.705	0.397	-0.180	<0.001	-2.485 to -0.925
	Model 2	-1.313	0.418	-0.138	0.002	-2.134 to -0.491

Model 1: unadjusted; Model 2: adjusted by sex, age, weight, smoking status, physical activity, educational level, drug consumed in the last month, and sodium and potassium intake.

4. CONCLUSIONS

In multivariate regression we observed a significantly positively association between the TP excreted in spot urine samples and daily intake of 100 mL of wine ($\beta=0.121$, $P<0.05$) and daily intake of 100 g of F&V ($\beta=0.127$; $P<0.001$) after adjusting for potential confounding factors. The standardized coefficients shows that 100g of F&V intake (Beta=0.150) have a higher contribution on TP excreted than 100 mL of wine (Beta=0.120).

Systolic and diastolic BP were negatively associated with the quartile of TPE in spot urine samples. The non-standardized coefficients, $\beta=-1.708$ ($P=0.025$) and $\beta=-1.313$ ($P=0.002$) represent the expected change of systolic and diastolic BP, respectively, when the TPE quartile changes to the next step. The systolic BP decreases 1.708 mm Hg (CI, -3.197 to -0.219) and diastolic BP -1.313 mm Hg (CI, -2.134 to -0.491) when the participants change, from one smaller quartile of urine TPE to the next one.

According to our results wine contributes to total polyphenols excretion in a Mediterranean diet in a population with high cardiovascular risk.

Wine consumption may contribute to the decrease in blood pressure observed among individuals who followed a Mediterranean diet rich in polyphenols

5. REFERENCES

- 1) Birt F, George S, Bellamy A et al. Daily polyphenol intake in France from fruit and vegetables. *J Nutr* 2006 September;136(9):2368-73.
- 2) Suredut A, Williamson G. Dietary intake and bioavailability of polyphenols. *J Nutr* 2006 August;136(8 Suppl):2073S-489S.
- 3) Casas M, Fito M, Marrugat J et al. [Coronary disease protective factors: antioxidant effect of olive oil]. *Thromb* 2001 September;36(9):607-11.
- 4) Casas M, Nyyssönen K, Poulsen HE et al. The effect of polyphenols in olive oil on heart disease risk factors: a randomized trial. *Ann Intern Med* 2006 September 5;145(5):323-31.
- 5) Manson C, Mozar A, Scallert A. Polyphenols and prevention of cardiovascular diseases. *Curr Opin Lipidol* 2006 February;16(1):77-84.
- 6) Medina-Remón A, Barrio-Neuvel-González A, Zamora-Ros R et al. Rapid Folin-Ciocalteu method using microtiter 96-well plate cartridges for solid phase extraction to assess urinary total phenolic compounds, as a biomarker of total polyphenols intake. *Analytica Chimica Acta* 2009 February 16;641(1):54-60.
- 7) Saura-Calixto F, Goni I. Antioxidant capacity of the Spanish Mediterranean diet. *Food Chemistry* 2006 February;94(2):442-7.

6. SUPPORTED BY

We would like to thank all of the volunteers involved in the PREDIMED study for their valuable cooperation. The authors express their gratitude for financial support from CICYT's (HLS2005-00557), FIS2006-10228-CO2-02 and FIS2007-60426-CO2-01) and "Red de Grupos" CO2-140, the Spanish Ministry of Science and Innovation (MCINN), RETICS RD06/0045/0003 and CIBER CB06/03/004, from the "Instituto de Salud Carlos III". This work has been funded by the CONSOLIDER INGENIO 2010 Programme: FUN-C-FOOD CSO2007-602 also from MCINN. A. S&R received support from the Generalitat of Catalonia for the training of researchers

Comunicació 8: Presentació oral

**5th Indo-Italian Workshop on Chemistry and Biology of Antioxidants,
Roma, July 2009**

Total antioxidant capacity assay, Folin Ciocalteu, as biomarker of total polyphenols intake

Medina-Remón A, Barrionuevo-González A, Zamora-Ros R, Andres-Lacueva C, Estruch R, Martínez-González MA, Díez-Espino J and Lamuela-Raventós RM.

**5th Indo-Italian Workshop on
Chemistry and Biology of Antioxidants**

Organized by

**Department of Physiology and
Pharmacology
“Vittorio Erspamer”
Sapienza University of Rome**



**Institute for
Biomolecular
Chemistry of
CNR**



July 5-9, 2009

Location

*Rooms A and C
Department of Chemistry
Sapienza University
P.le Aldo Moro 5, Rome*

TOTAL ANTIOXIDANT CAPACITY ASSAY, FOLIN CIOCALTEU, AS BIOMARKER OF TOTAL POLYPHENOLS INTAKE.

Alexander Medina-Remón^{1,3}, Ana Barrionuevo-González^{1,3}, Raúl Zamora-Ros^{1,4}, Cristina Andres-Lacueva^{1,4}, Ramón Estruch^{2,3}, Miguel-Ángel Martínez-González^{3,4}, Javier Díez-Espino⁵ and Rosa M. Lamuela-Raventós^{1,3*}

¹ Nutrition and Food Science Department, XaRTA, INSA. Pharmacy School, University of Barcelona, Av. Joan XXIII s/n Barcelona, Spain; ² Department of Internal Medicine, Hospital Clinic, Institut d'Investigació Biomèdica August Pi i Sunyer (IDIBAPS), University of Barcelona, Barcelona, Spain; ³ CIBER 06/003 Fisiopatología de la Obesidad y Nutrición, (CIBEROBN), and RETICS RD06/0045/0003. Instituto de Salud Carlos III, Spain; ⁴ Ingenio-CONSOLIDER program, FUN-C-FOOD. Instituto de Salud Carlos III, Barcelona, Spain; ⁵ Department of Preventive Medicine and Public Health, Clínica Universitaria, University of Navarra, Pamplona; ⁶ Tafalla Health Center, Osasunbidea, Tafalla, Spain. *Address correspondence to this author at: Nutrition and Food Science Department, XaRTA, INSA Pharmacy School, University of Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain. e-mail lamuela@ub.edu

Nutritional markers have several advantages for epidemiologic and clinical assays, over dietary data obtained by food frequency questionnaires. Few studies have assessed whether total polyphenols (TP) compounds is a valid biomarker for TP intake. Total dietary polyphenol levels are approximately 1 g/ day (1), higher than all other known dietary antioxidants, around 10 times higher than dietary vitamin C and 100 times higher than vitamin E and carotenoid levels. The health effects of polyphenols depend on their respective intakes, synergism and bioavailability, which can vary greatly (2). For most polyphenols, urinary excretion values have reported to be consistent with plasma kinetics data. The Folin-Ciocalteu (F-C) assay for total phenols (TP) is affected by several interfering substances such as sulfur dioxide, ascorbic acid, sugar, aromatic amines, organic acid, Fe(II) and nonphenolic organic substances that react with the F-C reagent (3). Roura et al in their research on cocoa (4) studied these interferences after a single Solid Phase Extraction (SPE) cleanup procedure and these substances were not in the eluate, so cannot react with F-C reagent. We report a antioxidant assay, Folin-Ciocalteu method, to determine TP in urine samples using Oasis® MAX 96-well plate cartridges for solid phase extraction. These plates allows to analyze a high number of samples at the same time. It was performed a prospective randomized crossover trial and one cross-sectional study with 60 volunteers from the PREDIMED trial, these attempts to evaluate if the TP in urine are correlated with polyphenol intake, to be considered as a marker of intake of these compounds. The methodology could potentially be used to detect a TP in urine samples, with the high throughput of 96-well microtiter plates and reader. The method is fast, simple and allows the analysis of a large number of samples at the same time.

Selected Publications

- 1) A. Scalbert and G. Williamson, Dietary intake and bioavailability of polyphenols, *Nutr.* 130 (2000) 2073S-2085S.
- 2) J. Feher and G. Lengyel, [Nutrition and cardiovascular mortality], *Orv. Hetil.* 147 (2006) 1491-1496.
- 3) V.L. Singleton, R. Orthofer, and R.M. Lamuela-Raventós, Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent, *Oxidants and Antioxidants*, Pt A 299 (1999) 152-178.
- 4) E. Roura, C. Andres-Lacueva, R. Estruch, and R.M. Lamuela-Raventós, Total polyphenol intake estimated by a modified Folin-Ciocalteu assay of urine, *Clin. Chem* 52 (2006) 749-752.
- 5) Medina-Remón, A. Barrionuevo-González, Zamora-Ros R, C, Andres-Lacueva A, R. Estruch MA, Martínez-González, J, Díez-Espino RM, and Lamuela-Raventós. Rapid Folin-Ciocalteu method using microtiter 96-well plate cartridges for solid phase extraction to assess urinary total phenolic compounds, as a biomarker of total polyphenols intake. *Anal. Chim. Acta.* 2009 Feb 16; 634(1): 54-60.

Comunicació 9: Pòster

Educació Mèdica versió ISSN 1575-1813 Educ . méd vol.12 suppl.2 Barcelona 2009

Adaptació dels crèdits de pràctiques de l'assignatura de Nutrició i Bromatologia de Farmàcia a l'EEES

Riu-Aumatell, M.; Alemany, M.; Esteve, M.; Fernandez, J.A.; Gallardo, J.; Grasa, M.; Izquierdo, M.; Latorre, M.L.; López Tamames, E.; Medina-Remón, A.; Rafecas, M.; Remesar, X.; Rotchés-Ribalta, M.; Serra, A.; Tres, A.; Urpí-Sardà, M.; Veciana, T.; Vichi, S.; Vidal M.C.; Zamora-Ros, R.

ADAPTACIÓ DELS CRÈDITS PRÀCTICS DE L'ASSIGNATURA DE NUTRICIÓ I BROMATOLOGIA DE FARMÀCIA A L'EEES

Riu-Aumatell, M.; Alemany, M.; Esteve, M.; Fernandez, J.A.; Gallardo, J.; Grasa, M.; Izquierdo, M.; Latorre, M.L.; López Tamames, E.; Medina-Remón, A.; Rafecas, M.; Remesar, X.; Rotchés-Ribalta, M.; Serra, A.; Tres, A.; Urpí-Sardà, M.; Veciana, T.; Vichi, S.; Vidal M.C.; Zamora-Ros, R.
Dept. Nutrició i Bromatologia, Facultat de Farmàcia, Avda. Joan XXIII, s/n. 08028 Barcelona.

INTRODUCCIÓ

Nutrició i Bromatologia és una assignatura troncal de segon cicle de l'ensenyament de Farmàcia corresponent al tercer curs. Dels 8.5 crèdits de l'assignatura 6 són teòrics i 2.5 pràctics. Per tal de donar a les pràctiques de l'ensenyament un paper més significatiu en l'aprenentatge de l'assignatura i adaptar-les a l'EEES es va proposar un canvi metodològic que significà també un canvi en els criteris d'avaluació. Ens vam plantejar els següents **objectius**:

- ✓ Aplicar noves eines d'ensenyament més d'acord a l'EEES
- ✓ Incrementar la versatilitat fent una organització modular
- ✓ Reduir el cost econòmic

Per això s'ha proposat i posat en pràctica un canvi en l'estructuració i avaluació de les pràctiques (Taula 1).

	Cursos anteriors	Curs 2008-09
Teoria	80%	70% (obligatorietat de treure una nota mínima de 4 a l'examen)
Treball dirigit	10%	30% (5 activitats corresponents cada una al 6% de la nota)
Pràctiques	10% (90% nota de l'examen test + 10% aptitud)	

- 1- Anàlisi reflexiu de l'etiquetatge nutricional
- 2- Additius alimentaris i la informació del consumidor
- 3- Biodisponibilitat de minerals
- 4- Avaluació de l'estat nutricional individual
- 5- Avaluació de la idoneïtat de la dieta

Les activitats 1, 2 i 3 s'han realitzat a un laboratori químic. L'activitat 4 a una sala habilitada per realitzar mesures antropomètriques i la 5 a l'aula d'informàtica. Per l'avaluació, els alumnes han efectuat un lliurament, per cadascuna de les activitats, a través del campus virtual.

Taula 1: Comparativa i ponderació dels factors que intervien en l'avaluació de l'assignatura de Nutrició i Bromatologia del curs 2008-09 i els anteriors.

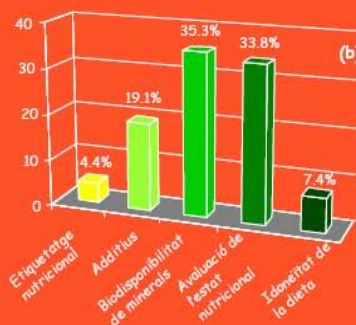
RESULTATS

Amb el canvi, cada professor implicat en l'assignatura s'ocupa d'un o dos dels mòduls per tal d'afavorir l'especialització del professor. Aquest model permet també facilitar la incorporació del professorat novell, ja que a més del guió necessari per l'alumne, s'ha creat paral·lelament un guió disponible per tot el professorat. Igualment aquests mòduls poden utilitzar-se en altres assignatures de diversos ensenyaments, on els continguts puguin ser similars.

La gestió de les pràctiques (grups, professorat, tutories, lliuraments, etc.) s'ha realitzat mitjançant el Campus Virtual (Plataforma Moodle). Això suposa certs avantatges, com ara els sistemes de comunicació alumne-alumne, professor-professor i alumne-professor; la qualificació automàtica; el registre de dades; el control de les activitats realitzades; l'estalvi en el paper i saturacions del correu electrònic personal amb l'enviament massiu de les activitats i la possibilitat de realitzar consultes i estadístiques. La satisfacció de l'alumne s'ha avaluat mitjançant una consulta voluntària en el campus virtual (Figura 1a i 1b):



Figura 1: Histogrames representatius de les respostes dels alumnes a la consulta sobre la satisfacció de les pràctiques (a) i la pràctica que els ha agradat més (b).



La satisfacció i implicació per part dels professors i alumnes ha quedat reflectida en les qualificacions corresponents a la part de pràctiques. Respecte l'any anterior en el curs actual, han disminuït notablement els suspensos i aprovats i per contra, ha augmentat significativament el percentatge de notables (Figura 2).

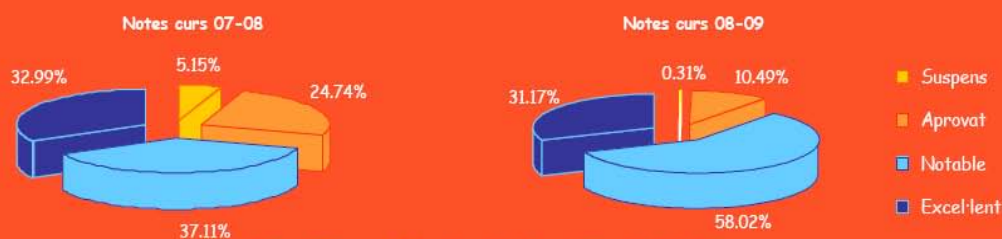


Figura 2: Comparació de les notes de pràctiques obtingudes els dos últims cursos.

Comunicación 10: Presentació oral

1er Congrés tecnològic del tomàquet per a indústria, Badajoz, España 2009

Folin-Ciocalteu assay for total polyphenols analysis in tomato samples needs a previous clean up procedure

Anna Vallverdú-Queralt¹, Alexander Medina-Remón^{1,2}, Cristina Andrés-Lacueva^{1,3} and Rosa M. Lamuela-Raventós^{1,2}

¹Nutrition and Food Science Department, XaRTA, INSA. Pharmacy School, University of Barcelona, Av. Joan XXIII s/n Barcelona, Spain. ²CIBER 06/003 Physiopathology of Obesity and Nutrition, (CIBEROBN), and RETICS RD06/0045/0003. Instituto de Salud Carlos III, Spain. ³Ingenio-CONSOLIDER program, FUN-C-FOOD. Instituto de Salud Carlos III, Spain

Tomatoes are one of the most widely consumed vegetables worldwide, not only in fresh form, but also in a wide range of processed products. The Folin-Ciocalteu(F-C) assay has been used for many years to determine the total polyphenol (TPs) content in tomatoes(1,2). However, it is affected by several interfering substances(3). The aim of this study was to improve the determination of TPs in tomato samples using solid phase extraction(SPE) with Oasis[®] MAX 96-well plate in order to eliminate these substances before the F-C assay.

The F-C assay was optimised, to eliminate interferences by reducing substances, and validated. The sensitivity, linearity, precision and accuracy were evaluated by spiking the tomato matrix with known levels of gallic acid standard at six different concentrations. The stability after successive freeze and thaw cycles, and short- and long-term temperature stability were also evaluated. We used high-performance liquid chromatography to calculate the recovery of nine representative polyphenols with different polarity, after spiking the tomato samples with four different concentrations of each standard. Seven tomato samples were analysed and TPs were expressed as mg of gallic acid equivalents (GAE)/100g dry material (DM).

The results of the F-C assay were linear over the working range 1 to 75 mgL⁻¹. Assay precision and accuracy were between the accepted values according to AOAC INTERNATIONAL(4). The samples and standards were stable under the storage and sample handling condition used and after successive freeze and thaw cycles. The recoveries for nine polyphenol standards after SPE were between 79.12% and 108.75%. The concentrations of TPs for tomato samples after SPE were between 149.99 and 247.12 mg of GAE/100g DM.

We have optimized and validated a method for analysis of TPs in tomato samples, eliminating the interferences with the F-C assay using 96-well plate cartridges to carry out SPE. This methodology is fast and simple, and it allows the analysis of a large number of samples at the same time.

- (1) Gautier H. et al. J.Agric. Food Chem. 2008;56(4):1241-50.
- (2) Hussein L. et al. Int.J. Food Sci. Nutr 2008 Jul 4;1-10.
- (3) Medina-Remón A et al. Analytica Chimica Acta 2009 Feb 16;634(1):54-60.
- (4) Horwitz W. Official Method of Analysis of AOAC INTERNATIONAL (Appendix D and E). 17 th Ed. Revision 2, Vol. 2 ed. 2003.

Comunicació 11: Pòster

1er Congrés tecnològic del tomàquet per a indústria, Badajoz, España 2009

The extraction of the solid phase of tomato samples before the Folin-Ciocalteu method to reduce interferences.

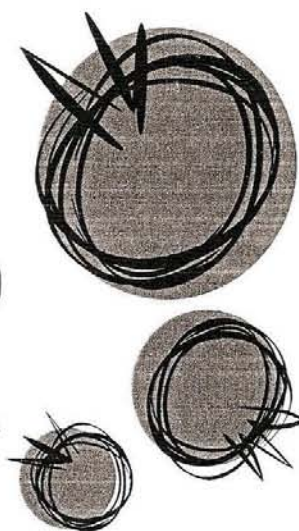
Anna Vallverdú-Queralt, Vaila Mallace, Alexander Medina-Remón, Cristina Andrés-Lacueva and Rosa M. Lamuela-Raventos

Colección
Monografías

**I^{er} CONGRESO
TECNOLOGICO
DEL TOMATE PARA
INDUSTRIA**

12-13-NOVIEMBRE

**Palacio de Congresos
de Badajoz "Manuel Rojas"**





Folin-Ciocalteu assay for total polyphenols analysis in tomato samples needs a previous clean up procedure

Anna Vallverdú-Queralt¹, Alexander Medina-Remón^{1,2}, Cristina Andrés-Lacueva^{1,3} and Rosa M. Lamuela-Raventós^{1,2*}

¹Nutrition and Food Science Department, XaRTA, INSA, Pharmacy School, University of Barcelona, Av. Joan XXIII s/n Barcelona, Spain; ²CIBER 06/03 Physiopathology of Obesity and Nutrition, (CIBEROBN), and RETICS RD06/0045/0003, Instituto de Salud Carlos III, Spain; ³Ingenio-CONSOLIDER program, FUN-C-FOOD, Instituto de Salud Carlos III, Spain ^{*}Nutrition and Food Science Department, University of Barcelona. Telephone +34-934034843. Fax +34-93-4035931; e-mail ramuela@ub.edu

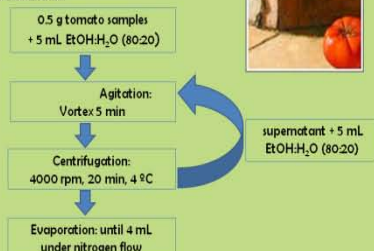
1. Introduction

Tomatoes are one of the most widely consumed vegetables worldwide, not only in fresh form, but also in a wide range of processed products. Foods rich in polyphenols such as tomatoes may have an important effect in preventing diseases, specifically they have been related with a lower risk of cancer and cardiovascular diseases. The Folin-Ciocalteu (F-C) assay has been used for many years to determine the total polyphenol (TPs) content in tomatoes. However, TPs content is sometimes overestimated for some reducing substances such as ascorbic acid, sugars and aminoacids which interfere in the assay.

Aim: The aim of this study was to eliminate non-phenolics interfering substances before the F-C assay using solid phase extraction (SPE) in order to improve the determination of TPs in tomato samples. This is the first study that has assessed TPs content in tomato samples after SPE, allowing the analysis of a large number of samples at the same time, using Oasis® MAX 96-well so as to eliminate these interfering substances.

2. Method

Sample preparation



Sample cleaning-up: Oasis MAX® 96 well plate

- 1st Condition: 1 mL of methanol 98-100%.
- 2nd Condition: 1 mL of sodium acetate 50mM pH 7.
- Load: 1 mL samples + 1 mL Milli-Q water + 34 µL HCl.
- Cleaning: 1 mL sodium acetate 50mM pH 7 / 5% methanol
- Elution: 1.8 mL methanol at 2% formic acid.

Analysis of total polyphenols in tomatoes, in thermo microtiter 96 well plate.

- 20 µL of each methanolic fraction eluted after SPE.
- 188 µL of Milli-Q water.
- 12 µL of F-C reagent.
- 30 µL of sodium carbonate (200 g/L).
- Incubation 60 min. in the darkroom.
- After the reaction period: 50 µL of Milli-Q
- Measure absorbance at 765 nm at the Spectrophotometers UV/VIS Thermo Multiskan Spectrum.

Validation:

-Linearity:

0.25-10 mg/L (gallic acid)

-Sensitivity:

LoD= 3*SD

LoQ= 10*SD

(10 blanks at 765 nm)

-Accuracy: % recoveries

-Precision: RSD=SD/X*100

-Stability

-Short-term

-Long-term

-After successive freeze-thaw cycles

-Recoveries of gallic, caffeic, p-coumaric, protocatechuic, p-hydroxybenzoic and chlorogenic acids, naringenin, rutin and quercetin, at 5, 10, 25 and 50 mg/L.

-Seven samples were analysed and results were expressed as mg of gallic acid equivalents/100g dry material.

3. Results

Linearity:

0.25-10mg/L $\rightarrow$ 0.1 x - 0.003

Sensitivity:

LOD: 0.006 mg/L

LOQ: 0.019 mg/L



The intra and inter-day precision and accuracy are between the accepted values for the AOAC International (Table 1).

Table 1: Intra- and inter-day accuracy and precision of the assay

Calibrator concentration (mg/L)	Means measured concentration (mg/L)		Precision, R.S.D. (%)		Recovery, error (%)	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
1.12	1.07	1.01	0.98	0.10	90.57	91.97
2.24	2.14	2.24	2.06	0.10	100.08	96.25
4.48	4.27	4.38	4.25	0.09	97.77	99.60
6.73	6.41	6.69	6.40	0.09	99.38	99.87
8.97	8.55	8.93	8.21	0.06	99.58	96.00
11.21	10.69	11.62	11.03	0.05	103.67	103.21
13.40	13.81	13.89	14.51	0.04	103.66	105.10

RSD: relative standard deviation

Stability

Table 2: Freeze-thaw cycle, short and long - term stability

Temperature	Freeze-thaw cycles stability	Short-term stability	Long-term stability
-20 °C	94.9-106.2 %	90.1-104.0 %	95 %
-80 °C	91.7-106.5 %	85.2-102.6 %	95 %

Absolute recoveries

Table 3: Absolute recoveries of the nine polyphenols

Polyphenols	Mean recoveries %	Standard deviation (S.D.)
Gallic acid	92.47	4.45
Naringenin	98.20	3.39
Caffeic acid	96.74	10.72
Rutin	87.32	4.81
Quercetin	77.52	5.43
p-Coumaric acid	96.14	3.36
Protocatechuic acid	108.75	3.15
p-Hydroxybenzoic acid	99.53	2.00
Chlorogenic acid	101.11	5.17

The concentrations of TPs for tomato samples vary between 260.80 and 472.23 mg of GAE/100 g DM when SPE was not used and between 113.32 and 174.85 mg of GAE/100g DM after the SPE. So this means that the results have been overestimated until now.

4. Conclusions

- It is the first time that SPE has been used to eliminate interfering nonphenolics reductants before the F-C assay.
- The method described may be useful as a routine control assay for total polyphenols determination in food, especially food such as fruit and vegetables that are rich in non phenolic reductant substances that react with FC reagent and interfere in the analysis.
- Samples require minimal sample preparation before TPs analysis. It is ideal to analyse large batches of samples, as the Oasis® MAX 96-well plate accommodates much larger sample volumes.

5. References

- (1) Capanoglu E. et al., J Agric Food Chem 2008, 56(3), 964-73.
- (2) Long M. et al., Phytochemistry 2006, 67(16), 1750-7.
- (3) Hussein L. et al., Int J Food Sci Nutr., 2008, 4, 1-10.
- (4) Medina-Remón A. et al., Analytica Chimica Acta, 2009, 634(1), 54-60.
- (5) Horwitz W. Official Method of Analysis of AOAC INTERNATIONAL (Appendix D and E), 17th Ed. Revision 2, Vol. 2 ed. 2003.
- (6) Singleton VL, Orthofer R, and Lamuela-Raventós RM (1999) Methods Enzymol.
- (7) Rosa E, Andrés-Lacueva C, Estruch R, and Lamuela-Raventós RM (2006) Clin. Chem 52:749-752.

6. Supported by

The authors express their gratitude to **CONEIA** for the samples and to CICYT (AGL2006-14228-C03-02 and AGL2007-66638-C02-01), RETICS RD06/0045/0003 from the Spanish Ministry of Science and Innovation (MICINN) and grant PI070240 from Instituto de Salud Carlos III, Spain for financial support.

The CIBERobn CB06/03 is an initiative from the Instituto de Salud Carlos III, Spain. This work has been funded in part by the CONSOLIDER INGENIO 2010 Programme, FUN-C-FOOD CSD2007-043 and also from MICINN. A. V-Q received support from MICINN and A. M-R from the Generalitat of Catalonia for training of researchers.

I International conference about industrial tomato, Extremadura España

Comunicació 12: Pòster

III Symposium Ciber Fisiopatología de la Obesidad y Nutrición (CIBERObn), Illa de la Toja Novembre 2009, Espanya.

High polyphenol consumption related to their excretion reduces blood pressure in high cardiovascular risk subjects.

Alexander Medina-Remón; Raul Zamora-Ros; Maria Rotchés-Ribalta; Cristina Andres-Lacueva; Ramón Estruch; Miguel Angel Martínez-González; Maria Isabel Covas; Jordi Salas-Salvadó and Rosa Ma. Lamuela-Raventos.



HIGH POLYPHENOL CONSUMPTION RELATED TO THEIR EXCRETION REDUCES BLOOD PRESSURE IN HIGH CARDIOVASCULAR RISK SUBJECTS.

Alexander Medina-Remón^{1,2,3}, Raul Zamora-Ros^{1,4}, Maria Rotchés-Ribalta^{1,4}, Cristina Andres-Lacueva^{1,4}, Ramón Estruch^{2,3,5}, Miguel Angel Martínez-González^{2,6}, Maria Isabel Covas^{2,7}, Jordi Salas-Salvadó^{2,8} and Rosa Ma. Lamuela-Raventós^{1,2,3}

¹Nutrition and Food Science Department, IRTA, INSA, Pharmacy School, University of Barcelona; ²CIBER Fisiopatología de la Obesidad y Nutrición and ³RETICS RD06/045/0001, Instituto de Salud Carlos III, Spain; ⁴Unipen-CONSOLIDER program, FUND-FOOD (CS2007-463) Barcelona; ⁵Department of Internal Medicine, Hospital Clinic, IDIBAPS, University of Barcelona; ⁶Department of Preventive Medicine and Public Health, School of Medicine-Clinica, University of Navarra, Pamplona; ⁷Cardiovascular Epidemiology Unit, Municipal Institute for Medical Research (IMM), Barcelona; ⁸Human Nutrition Unit, School of Medicine, KSPV, University Rovira i Virgili, Reus. Nutrition and Food Science Department, University of Barcelona. Telephone +34 934034843; e-mail: lamuela@ub.edu

1. INTRODUCTION

Hypertension is a major global public health problem, affecting approximately 1 billion individuals causing 7.6 million premature deaths and 6% of all cases of disability-adjusted life years worldwide(1). It is also one of the main modifiable cardiovascular risk factors in the elderly. A healthy diet and lifestyle modification are the first steps for the management of hypertension(2).

Epidemiologic data have shown an inverse association between the risk of overall mortality or cardiovascular disease and the consumption of polyphenol-rich foods such as fruit and vegetables (F&V), tea, olive oil and wine(3-5).

AIMS: We undertook, therefore, a substudy within a larger clinical trial, the PREDIMED study in order to evaluate whether polyphenol-rich food intake, assessed as the excretion of total polyphenols (TPs) in spot urine samples, was associated with average blood pressure (BP) and/or with the prevalence of hypertension.

3b. Results

Table 2: Multivariate linear regression analysis with total polyphenol excreted normalized, in spot urine samples (mg GAE/g creatinine) as the dependent variable, and total polyphenol (100mg/d), total fruit and vegetable (100g/d), wine (100mL/d) and coffee (100mL/d) intakes, as exposure variable, adjusted for potential confounders.

	Model	β	SE	Beta	P	95% CI
Total polyphenol intake (100mg)	Model 1	0.073	0.017	0.179	<0.001	0.041 to 0.106
	Model 2	0.116	0.020	0.283	<0.001	0.077 to 0.154
Fruit and Vegetables (100g)	Model 1	0.131	0.035	0.155	<0.001	0.064 to 0.199
	Model 2	0.127	0.036	0.150	<0.001	0.056 to 0.198
Wine (100 mL)	Model 1	-0.090	0.041	-0.090	0.029	-0.171 to -0.009
	Model 2	0.121	0.051	0.120	0.019	0.020 to 0.222
Coffee (100 mL)	Model 1	0.261	0.120	0.089	0.030	0.025 to 0.497
	Model 2	0.414	0.119	0.141	0.001	0.181 to 0.647

β : Non-standardized coefficient (regression line coefficient); SE: Standard error; Beta: Standardized coefficient; CI: Confidence interval; P: two-sided test of significance; Model 1: unadjusted; Model 2: adjusted by sex, age, weight, smoking status, physical activity, educational level, energy expenditure in physical activity, total fat, fish, poultry, olive oil, beer, chocolate, natural orange juice, tea, cereals, nuts and dairy products, meat or meat products, nuts, pastries/sweets/breads, F&V, wine and coffee intake; GAE: Gallic acid equivalent.

Table 3: Baseline characteristics of the study participants by quartile of total polyphenol excretion expressed as mg GAE/g creatinine.

	Urine mg GAE/g creatinine concentration quartile				
	Q1 (-88.99)	Q2 (89-119.46)	Q3 (119.47-160.22)	Q4 (>160.23)	P for trend
No. of subjects	147	148	147	147	
Age, (y) mean (SD)	66.0 (5.7)	68.3 (6.1)	68.0 (5.8)	69.3 (6.0)	<0.001
Women, n (%)	51 (34.7)	83 (56.1)	85 (57.8)	107 (72.8)	<0.001
Overweight or obese (BMI ≥ 25 Kg/m ²), n (%)	138 (93.9)	137 (92.6)	128 (87.1)	135 (91.8)	0.263
Systolic BP (mm Hg), mean (SD)	156.0 (15.6)	152.2 (18.3)	155.0 (21.6)	149.1 (16.0)	0.105
Diastolic BP (mmHg), mean (SD)	88.9 (10.2)	86.2 (9.8)	85.5 (11.8)	83.4 (8.5)	<0.001
Hypertension, n (%)	123 (83.7)	126 (85.1)	113 (76.9)	114 (77.6)	0.067
Energy expenditure in physical activity (kcal/d), mean (SD)	256.0 (215.5)	245.9 (220.7)	293.1 (231.0)	255.3 (205.3)	0.576

Table 4: Multivariate linear regression analyses with systolic blood pressure and diastolic blood pressure as the dependent variables, and quartile of total polyphenol excreted in spot urine samples (mg GAE/g creatinine) as exposure variable, adjusted for potential confounders.

	Model	β	SE	Beta	P	95% CI
Systolic blood pressure	Model 1	-1.743	0.712	-0.104	0.015	-3.141 to -0.345
	Model 2	-1.731	0.765	-0.103	0.024	-3.233 to -0.228
Diastolic blood pressure	Model 1	-1.705	0.397	-0.180	<0.001	-2.485 to -0.925
	Model 2	-1.264	0.422	-0.133	0.003	-2.092 to -0.435

β : Non-standardized coefficient (regression line coefficient); SE: Standard error; Beta: Standardized coefficient; CI: Confidence interval; P: two-sided test of significance; Model 1: unadjusted; Model 2: adjusted by sex, age, weight, smoking status, physical activity, educational level, medication use (angiotensin-converting enzyme inhibitors, diuretics, statins or other hypolipemic drugs, insulin, oral hypoglycemic drugs and aspirin or other antiplatelet drugs) consumed in the last month, sodium and potassium intake and glomerular filtration rate (GFR); GAE: Gallic acid equivalent.

Table 5: Multivariate adjusted odds ratios (95% confidence intervals) for cardiovascular risk factors (hypertension) according to quartiles of total polyphenol excretion expressed as mg GAE/g creatinine using the lowest quartile group as reference category.

	Urine mg GAE/g creatinine concentration quartile				
	Q1 (-88.99)	Q2 (89-119.46)	Q3 (119.47-160.22)	Q4 (>160.23)	P for trend
Hypertension, n (%)	123 (83.7)	126 (85.1)	113 (76.9)	114 (77.6)	0.067
Model 1	1.00	1.82 (0.33-10.13)	0.67 (0.29-1.55)	0.71 (0.53-0.95)	0.021
Model 2	1.00	1.39 (0.19-10.34)	0.55 (0.20-1.48)	0.64 (0.45-0.92)	0.047

Model 1: unadjusted; Model 2: adjusted for sex, age, weight, smoking status, physical activity, educational level, energy expenditure in physical activity, medication intake: ACE inhibitors, diuretics, statins (hypolipemic drugs), insulin, oral hypoglycemic drugs, aspirin or other antiplatelet drugs, supplements taken in the last month, sodium and potassium intake and glomerular filtration rate (GFR); GAE: Gallic acid equivalent.

4. CONCLUSIONS

We observed an inverse association between urinary TPE, an objective measurement of polyphenol intake, and the risk of hypertension in a large Spanish cohort of elderly participants at high cardiovascular risk. Compared with the lowest quartile of TPE, in the participants in the highest quartile, the odds of hypertension were reduced by 36% after adjustment for potential confounding factors.

Systolic and diastolic BP measurements correlated negatively with urinary TPE after adjustment for potential confounders. A high intake of polyphenol-rich diet may help to decrease BP in elderly hypertensive populations in order to lower their cardiovascular risk.

Total phenol, F&V, coffee and wine intake in the Mediterranean diet are positively correlated with the excretion of TPs in spot urine samples. The standardized coefficients (Beta) showed that the intake of F&V contributed more to urinary TPE than coffee, and wine consumption.

Medina-Remón A, et al. Total polyphenols excretion and blood pressure in subjects at high cardiovascular risk. *Nutr Metab Cardiovasc Dis*. doi:10.1016/j.numecd.2009.10.019. in press.

5. REFERENCES

- Lawes CM, et al. *Lancet* 2008 May 3;371(9623):1513-8.
- Mancia G, et al. *J Hypertens* 2007 Jun;25(6):1105-47.
- Covas MI, et al. *Thrombosis* 2001 Sep;55(5):507-11.
- Covas MI, et al. *Ann Intern Med* 2006 Sep 5;145(5):332-41.
- Manach C, et al. *Curr Opin Lipidol* 2005 Feb;15(1):77-84.
- Medina-Remón A, et al. *Análisis Químico Acta* 2009 February 16;334(1):54-60.

6. SUPPORTED BY

We would like to thank all of the volunteers involved in the PREDIMED study for their valuable cooperation. The authors express their gratitude for financial support from CICYT (AGL2006-05937, AGL2006-14228-C03-02 and AGL2007-66638-C02-01) and "Red de Grupos" (RD06/045), the Spanish Ministry of Science and Innovation (MICINN), RETICS RD06/045/0001 and CIBER CB06/03/024, from the "Instituto de Salud Carlos III". This work has been funded by the CONSOLIDER INGENIO 2010 Programme, FUND-FOOD (CS2007-063) also from MICINN. A. M.R. received support from the Generalitat de Catalunya for the training of researchers.

2. Subjects and Methods

589 admitted trial participants from the PREDIMED (PREvención con Dieta MEDiterránea) study is a large, parallel-group, multicenter, randomized, controlled 5-year clinical trial aimed to assess the effects of the Med-Diet on the primary prevention of cardiovascular disease (www.predimed.org; ISRCTN35739639).

At baseline:

Validated semiquantitative food frequency questionnaire (FFQ) with 136-items.

The validated Spanish version of the Minnesota Leisure Time Physical Activity Questionnaire.

47-item questionnaire about education, lifestyle, history of illnesses and medication use.

BP thrice with a validated semi-automatic oscillometer (Omron) was measured and morning urine samples were collected.

Energy and nutrient intake was derived from Spanish food composition tables.

TPs consumption from plant food and beverages (mg/g fresh matter) was quantified from the FFQ.

Urinary total polyphenols were analyzed by the Folin-Ciocalteu (FC) assay, after a solid phase extraction (SPE) (6).



SPE: 96 well plate cartridges from Waters Oasis® MAX (Mixed-Mode Anion-Exchange and Reversed-Phase Solvent) to avoid any interference with F-C reagent.

Analysis of TPs and creatinine in urines: In thermo microtiter 96-well plate at 765 nm and 500 nm respectively (6).

TPs were expressed as mg gallic acid equivalent (GAE)/g of creatinine.

Statistical analysis

Analyses were performed using SPSS software version 14.0. Baseline characteristics of the participants were expressed as means or percentages and standard deviations (SD).

Multivariate linear regression models were used to assess the relationship between the total phenols excreted (TPE) as the dependent variable and TPs intake, total F&V, wine and coffee, as the exposure variable with adjustment for potential confounders.

The relationship between the urinary TPE quartile and systolic or diastolic BP was assessed by linear regression models adjusted for potential confounders.

Logistic regression models were fitted to assess the relationship between urinary TPE (categorized in quartiles) and hypertension status. Odds ratios (ORs) and 95% confidence intervals (CI) were calculated using the lowest quartile as the reference category. P-values<0.05 (two-tailed) were considered to be significant.

3a. Results

Table 1: Daily intake of selected foods according to quartiles of excreted total urinary polyphenols, expressed as mg GAE/g creatinine.

	Urine mg GAE/g creatinine concentration quartile				
	Q1 (-88.99)	Q2 (89-119.46)	Q3 (119.47-160.22)	Q4 (>160.23)	P for trend
Urinary total polyphenols (mg GAE/g creatinine)	72.8 (31.6)	103.1 (19.5)	138.2 (31.1)	226.1 (89.6)	<0.001
No. of subjects	147	148	147	147	
Vegetables (g)	253.1 (60.2)	261.1 (31.1)	262.6 (34.0)	272.7 (92.3)	0.033
Fruit (g)	304.6 (143.6)	319.3 (141.3)	315.1 (132.9)	368.1 (151.2)	0.002
Total fruit and vegetables (g)	557.7 (176.8)	580.4 (173.3)	577.7 (142.1)	631.1 (138.1)	0.001
Wine (mL)	121.1 (47.0)	81.5 (37.9)	181.1 (61.5)	81.3 (48.3)	0.073
Coffee (mL)	63.3 (47.7)	69.6 (52.9)	67.5 (41.3)	74.5 (54.8)	0.097
Total polyphenols intake (mg GAE)	1075.4 (534.9)	1057.5 (320.2)	1086.2 (332.3)	1222.5 (636.8)	0.001
Alcohol (g)	15 (7.7)	10 (3.0)	12 (3.5)	9.9 (7.8)	0.018
Fiber (g)	22.1 (8.2)	22.0 (5.5)	21.9 (5.2)	22.5 (6.3)	0.406
Sodium (mg/d)	3347 (959.2)	3688.8 (965.4)	3123.2 (1086.5)	3145.4 (977.2)	0.100
Protein (mg/d)	3326.7 (722.3)	3924.4 (700.9)	3994.8 (805.5)	4829.7 (959.7)	0.181
Total energy: Kcal/d	2386.1 (586.8)	2238.0 (472.0)	2295.1 (547.4)	2138.5 (476.8)	<0.001

Urine total polyphenols were expressed as mg GAE/g creatinine; Mean (standard deviation); GAE: gallic acid equivalent.

Comunicació 13: Presentació oral

“The 4th International Conference on Polyphenols and Health” Harrogate International Centre, Yorkshire, England, 2009.

“Polyphenol consumption and hypertension status: a baseline cross-sectional analysis within the PREDIMED trial”

Alexander Medina-Remón^{1,2}, Raúl Zamora-Ros^{1,3}, Maria Rotchés-Ribalta^{1,2}, Cristina Andres-Lacueva^{1,3}, Ramón Estruch^{2,4}, Miguel-Angel Martínez-González^{2,5}, Maria-Isabel Covas^{2,6} and Rosa M. Lamuela-Raventós^{1,2*}

¹Nutrition and Food Science Department, XaRTA, INSA. Pharmacy School, University of Barcelona, Av. Joan XXIII s/n Barcelona, Spain; ²CIBER 06/003 Physiopathology of Obesity and Nutrition, (CIBEROBN), and RETICS RD06/0045/0003. Instituto de Salud Carlos III, Spain; ³Ingenio-CONSOLIDER program, FUN-C-FOOD (CSD2007-063) Barcelona, Spain; ⁴Department of Internal Medicine, Hospital Clinic, IDIBAPS, University of Barcelona; ⁵Department of Preventive Medicine and Public Health, School of Medicine-Clínica, University of Navarra, Pamplona; ⁶Cardiovascular Epidemiology Unit, Municipal Institute for Medical Research (IMIM), Barcelona.

*Address correspondence to this author at: Nutrition and Food Science Department, XaRTA, INSA Pharmacy School, University of Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain. Telephone +34-934034843. Fax +34-93-4035931; e-mail lamuela@ub.edu

Hypertension is a major global public health problem. A healthy diet and lifestyle modifications are the first steps in the management of hypertension (1). Epidemiologic data has shown an inverse association between the risk of overall mortality or cardiovascular disease, and the consumption of polyphenol-rich food (2). Our aim was to assess the relationship between total polyphenol excretion (TPE) in urine, as a correlate of total polyphenol intake, and blood pressure (BP) or with the prevalence of hypertension in an elderly population at high cardiovascular risk.

A cross-sectional substudy with baseline data from the PREDIMED trial (3) was designed. Participants were 263 men and 326 women, aged 55–80, with a high risk of coronary heart disease. The anthropometric data and blood pressure were measured. The volunteers were divided into quartiles according to their TPE, it was measured in spot urine samples by the Folin-Ciocalteu assay (4).

A significant positive association between TPE in urine and daily intake of fruit and vegetables (F&V), coffee or wine was observed after adjusting for potential confounders. The intake of 100g of F&V (Beta=0.150; $P<0.001$) contributed higher to TPE than 100mL of coffee (Beta=0.141; $P=0.001$), and the latter two foods greater than the consumption of 100mL of wine (Beta=0.120; $P=0.019$). An inverse association between urinary TPE and the prevalence of hypertension was observed; participants in the highest quartile of urinary TPE had a reduced prevalence of hypertension compared to those in the lowest quartile (ORs=0.64; 95% CI 0.45 to 0.92; $P=0.016$). Systolic and diastolic BP were inversely associated with urinary TPE after adjustment for potential confounders ($P=0.025$; $P=0.002$, respectively).

Polyphenol intake, assessed via TPE in urine, was negatively associated with BP levels and prevalence of hypertension in an elderly Mediterranean population at high cardiovascular risk. A dietary intervention directed to increase polyphenol-rich food intake may be useful in the prevention and treatment of hypertension.

- (1)Mancia G et al. J.Hypertens 2007,25(6):1105-87.
- (2)Manach C et al. Curr.Opin.Lipidol 2005,16(1):77-84.
- (3)Estruch R et al. Ann.Intern.Med. 2006,145(1):1-11.
- (4)Medina-Remón A et al. Anal.Chim. Acta 2009,634(1):54-60.

Comunicació 14: Pòster

“The 4th International Conference on Polyphenols and Health” Harrogate International Centre, Yorkshire, England, 2009.

“Green mouthfeel wines have different phenolic profile”

Alexander Medina-Remón, Anna Tresserra-Rimbau, Cristina Andres-Lacueva, NuriaTobella, Mireia Torre and Rosa M. Lamuela-Raventos



Green mouthfeel wines have different phenolic profile

Alexander Medina-Remón^{1,2}, Anna Tresserra-Rimbau^{1,2}, Cristina Andres-Lacueva^{1,3}, Nuria Tobella⁴, Mireia Torres⁴ and Rosa M. Lamuela-Raventós^{1,2}

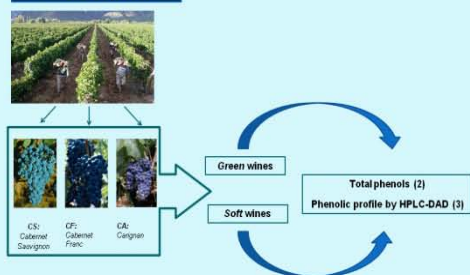
¹Nutrition and Food Science Department, XARTA, INSA, Pharmacy School, University of Barcelona, Av Joan XXIII s/n Barcelona, Spain; ²CIBER 06/03 Physiopathology of Obesity and Nutrition, (CIBEROBN) and RETICS RD06/0045/0003, Instituto de Salud Carlos III, Spain; ³Ingenuo-CONSOLIDER program, FUN-C-FOOD, Barcelona, Spain; ⁴Bodega Miguel Torres, Miquel Torres i Carbó 6, 08720 Vilafranca del Penedès, Barcelona, Spain; Cent DEMETER, Centre for the Development of Industrial Technology (CDTI), Nutrition and Food Science Department, University of Barcelona, Telephone +34-934034843; e-mail: lamuela@ub.edu

1. INTRODUCTION

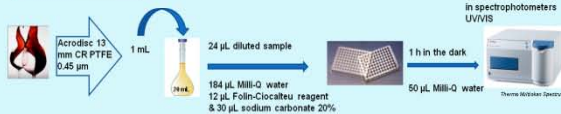
In some climate conditions, minor compounds from grapes, secondary metabolites, do not evolve at the same rate than the major compound of grapes sugars and acids, they are still immature or *green* at grape maturity. Wines made from these *green* have an adequate alcohol concentration and acidity but the minor compounds give to the wine green sensory properties, characterized by excess of acidity and astringency compared to *soft* wines that have a light and finely textured astringency (1).

AIMS: The aim of this work was to determine differences between *green* and *soft* wines across to specific parameters of the wine and characterize some indicator responsible of this green mouthfeel properties.

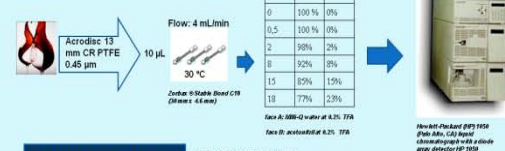
2. Samples



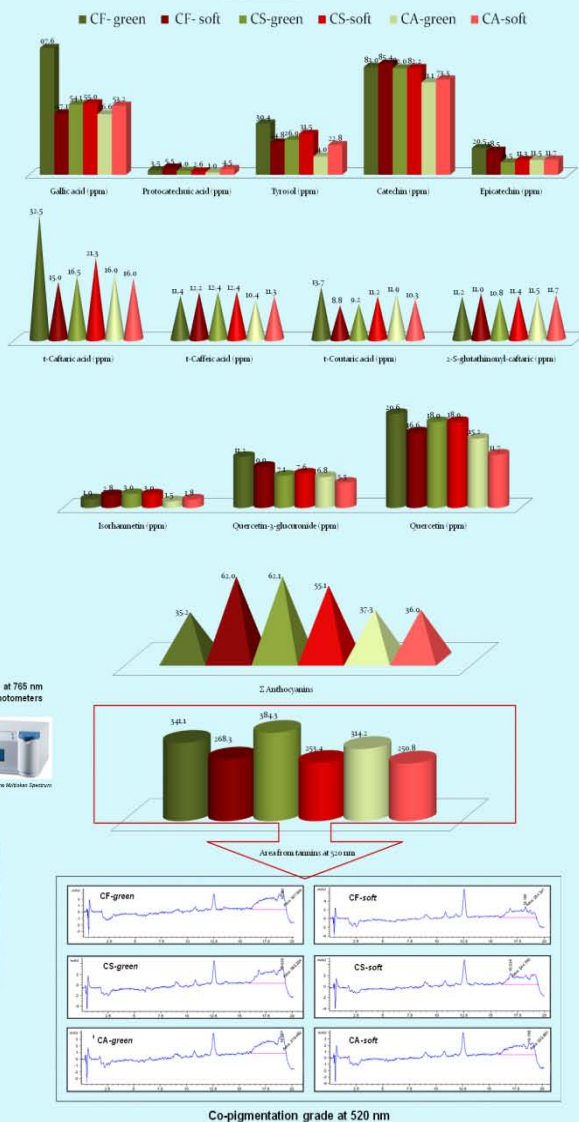
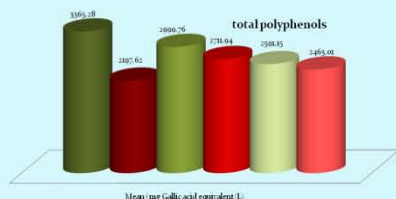
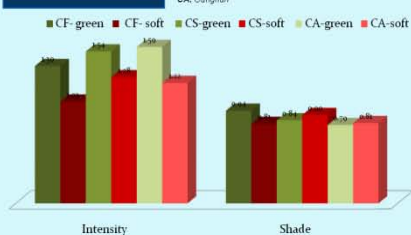
Total polyphenols (2)



Phenolic profile by HPLC-DAD (3)



3. Results



4 CONCLUSIONS

The color intensity of the *green* "green" wines are higher than "soft" wines from the corresponding grapevines.

Differences in total phenolics contents were observed among the "green" and the soft wines.

"Green" wines have higher grade of co-pigmentation than soft wines. Moreover the polymerized anthocyanins at 520 nm may be a good parameter to differentiate green and soft mouthfeel wines, however more analysis with different are need

5.REFERENCES

1. Gawel R. et al. 2000; 6(3):203-207.
2. Medina-Remón A. et al. *Anal. Chim. Acta* 2009; 634:54-60.
3. Ibern-Gómez M. et al. *Am. J. Enol. Vitic.* 2002; 53:3.

6. SUPPORTED BY



The authors express their gratitude for financial support from Cent DEMETER, Centre for the Development of Industrial Technology (CDTI) from the Spanish Ministry of Science and Innovation (MICINN); "Red de Grupo" G03140, RETICS RD06/0045/0003 and CIBER CB06/031024, from the "Instituto de Salud Carlos III". This work has been funded by the CONSOLIDER INGENIO 2010 Programme; FUN-C-FOOD CSD2007-663 also from MICINN.

The 4th International Conference on Polyphenols and Health. 7th – 11th December 2009; Yorkshire, England.

Comunicació 15: Pòster

“The 4th International Conference on Polyphenols and Health” Harrogate International Centre, Yorkshire, England, 2009.

“An improved method to determine total polyphenols in tomato samples eliminating interferences with Folin-Ciocalteu assay”

Anna Vallverdú-Queralt, Alexander Medina-Remón, Vail Mallace, Cristina Andrés-Lacueva and Rosa M. Lamuela-Raventós



An improved method to determine total polyphenols in tomato samples eliminating interferences with Folin-Ciocalteu assay.

Anna Vallverdú-Queralt¹, Alexander Medina-Remón^{1,2}, Vail Mallace³, Cristina Andrés-Lacueva^{1,4} and Rosa M. Lamuela-Raventós^{1,2}

¹Nutrition and Food Science Department, XaRTA, INSA, Pharmacy School, University of Barcelona, Av. Joan XXIII s/n Barcelona, Spain; ²CIBER 06/003 Physiopathology of Obesity and Nutrition, (CIBEROBN), and RETICS RD06/0048/0003, Instituto de Salud Carlos III, Spain; ³Department of Food Science and Nutrition, University of Leeds; ⁴Ingenio-CONSOLIDER program, FUN-C-FOOD, Instituto de Salud Carlos III, Spain; ⁵Nutrition and Food Science Department, University of Barcelona, Telephone +34-934034843, Fax +34-93-4035931; e-mail

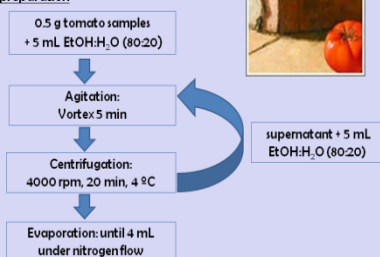
1. Introduction

Tomatoes are one of the most widely consumed vegetables worldwide, not only in fresh form, but also in a wide range of processed products. Foods rich in polyphenols such as tomatoes may have an important effect in preventing diseases, they have specifically been related with a lower risk of cancer and cardiovascular diseases. The Folin-Ciocalteu (F-C) assay has been used for many years to determine the total polyphenol (TP) content in tomatoes. However, TP content is sometimes overestimated for some reducing substances such as ascorbic acid, sugars and aminoacids which interfere in the assay.

Aim: The aim of this study was to eliminate non-phenolic interfering substances before the F-C assay using solid phase extraction (SPE) in order to improve the determination of TP in tomato samples. This is the first study that has assessed TP content in tomato samples after SPE, allowing the analysis of a large number of samples at the same time, using Oasis® MAX 96-well so as to eliminate these interfering substances.

2. Method

Sample preparation



Sample cleaning-up: Oasis MAX® 96 well plate

- 1st Condition: 1 mL of methanol 98-100%.
- 2nd Condition: 1 mL of sodium acetate 50 mM pH 7.
- Load: 1 mL samples + 1 mL Milli-Q water + 34 µL HCl.
- Cleaning: 1 mL sodium acetate 50 mM pH 7/ 5% methanol
- Elution: 1.8 mL methanol at 2 % formic acid.

Analysis of total polyphenols in tomatoes, in thermo microtiter 96 well plate.

- 20 µL of each methanolic fraction eluted after SPE.
- 188 µL of Milli-Q water.
- 12 µL of F-C reagent.
- 30 µL of sodium carbonate (200 g/L).
- Incubation 60 min. in the darkroom.
- After the reaction period: 50 µL of Milli-Q
- Measure absorbance at 765 nm at the Spectrophotometers UV/VIS Thermo Multiskan Spectrum.

Validation:

-Linearity:

0.25-10 mg/L (gallic acid)

-Sensitivity:

LoD= 3*SD

LoQ= 10*SD

(10 blanks at 765 nm)

-Accuracy: % recoveries

-Precision: RSD=SD/X*100 } 0.25-10 mg/L

-Stability

-Short-term

-Long-term

-After successive freeze-thaw cycles } -20 °C and -80 °C

-Recoveries of gallic, caffeic, p-coumaric, protocatechuic, p-hydroxybenzoic and chlorogenic acids, naringenin, rutin and quercetin, at 5, 10, 25 and 50 mg/L.

-Seven samples were analysed and results were expressed as mg of gallic acid equivalents/100g dry material.

3. Results

Linearity:

0.25-10 mg/L $y = 0.1x - 0.003$

Sensitivity:

LOD: 0.006 mg/L

LOQ: 0.019 mg/L



The intra and inter-day precision and accuracy are between the accepted values for AOAC International (Table 1).

Table 1: Intra- and inter-day accuracy and precision of the assay

Calibrator concentration (mg/L)		Means measured concentration (mg/L)		Precision, R.S.D. (%)		Recovery, error (%)	
Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
1.12	1.07	1.01	0.98	0.10	0.12	90.57	91.97
2.24	2.14	2.24	2.06	0.10	0.11	100.08	96.25
4.48	4.27	4.38	4.25	0.09	0.09	97.77	99.60
6.73	6.41	6.69	6.40	0.09	0.10	99.38	99.87
8.97	8.55	8.93	8.21	0.06	0.08	99.58	96.00
11.21	10.69	11.62	11.03	0.05	0.07	103.67	103.21
13.40	13.81	13.89	14.51	0.04	0.05	103.66	105.10

RSD: relative standard deviation

Stability

Table 2: Freeze-thaw cycle, short and long - term stability

Temperature	Freeze-thaw cycles stability	Short-term stability	Long-term stability
-20 °C	94.9-106.2 %	90.1-104.0 %	95 %
-80 °C	91.7-106.5 %	85.2-102.6 %	95 %

Absolute recoveries

Table 3. Absolute recoveries of the nine polyphenols

Polyphenols	Mean recoveries %	Standard deviation (S.D.)
Gallic acid	92.47	4.45
Naringenin	98.20	3.39
Caffeic acid	96.74	10.72
Rutin	87.32	4.81
Quercetin	77.52	5.43
p-Coumaric acid	96.14	3.36
Protocatechuic acid	108.75	3.15
p-Hydroxybenzoic acid	99.53	2.00
Chlorogenic acid	101.11	5.17

The concentrations of TPs for tomato samples vary between 260.80 and 472.23 mg of GAE/100 g DM when SPE was not used and between 113.32 and 174.85 mg of GAE/100g DM after the SPE. So this means that the results have been overestimated until now.

4. Conclusions

- It is the first time that SPE has been used to eliminate interfering non-phenolic reductants before the F-C assay.
- The method described may be useful as a routine control assay for total polyphenol determination in food, especially food such as fruit and vegetables that are rich in non phenolic reductant substances that react with FC reagent and interfere in the analysis.
- Samples require minimal sample preparation before TP analysis. It is ideal to analyse large batches of samples, as the Oasis® MAX 96-well plate accommodates much larger sample volumes.

5. References

- (1)Capanoglu E. et al. J Agric Food Chem. 2008, 56(3), 964-73.
- (2)Long M. et al. Phytochemistry, 2006, 67(6), 1750-1757.
- (3)Hussein L. et al. Int J Food Sci Nutr. 2008, 4, 1-10.
- (4)Medina-Remón A. et al. Analytica Chimica Acta. 2009, 634(1), 54-60.
- (5)Horwitz W. Official Method of Analysis of AOAC INTERNATIONAL (Appendix D and E), 17th Ed. Revision 2, Vol. 2 ed. 2003.
- (6)Singleton VL, Orthofer R., and Lamuela-Raventós RM. Methods Enzymol. 1999
- (7)Roura E., Andrés-Lacueva C., Estruch R., and Lamuela-Raventós RM. Clin. Chem. 2006, 52, 749-752.

6. Supported by

The authors express their gratitude to **CONESA** for the samples and to CICYT's (AGL2006-14228-C03-02 and AGL2007-66638-C02-01), RETICS RD06/0048/0003 from the Spanish Ministry of Science and Innovation (MICINN) and grant PI070240 from Instituto de Salud Carlos III, Spain for financial support.

The CIBERBN CB06/03 is an initiative from the Instituto de Salud Carlos III, Spain. This work has been funded in part by the CONSOLIDER INGENIO 2010 Programme, FUN-C-FOOD CSD2007-063 and also from MICINN. A. V-Q received support from MICINN and A. M-R from the Generalitat de Catalunya for training of researchers.

Comunicació 16: Pòster

“The 4th International Conference on Polyphenols and Health” Harrogate International Centre, Yorkshire, England, 2009.

“METABOLIC PROFILE OF POLYPHENOLS FOLLOWING ADMINISTRATION OF ALMOND (PRUNUS DULCIS (MILL.) D.A. WEBB) EXTRACT IN HUMANS”

Mireia Urpí-Sarda, Ignacio Garrido, María Monagas, Carmen Gómez-Cordovés, Alexander Medina-Remón, Cristina Andres-Lacueva, Begoña Bartolomé



METABOLIC PROFILE OF POLYPHENOLS FOLLOWING ADMINISTRATION OF ALMOND (PRUNUS DULCIS (MILL.) D.A. WEBB) EXTRACT IN HUMANS



Mireia Urpi-Sarda¹, Ignacio Garrido², Maria Monagas², Carmen Gómez-Cordovés², Alexander Medina-Remón¹, Cristina Andres-Lacueva¹, Begoña Bartolomé²

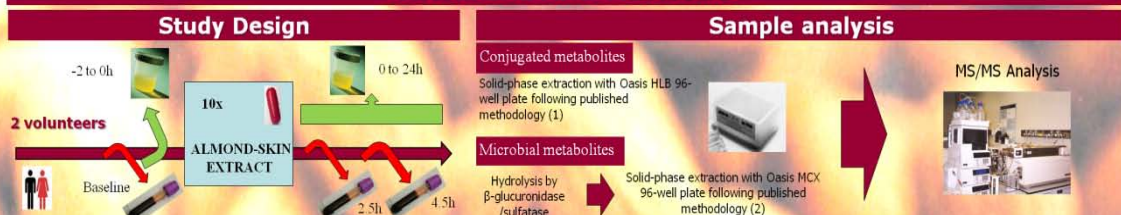
¹Nutrition and Food Science Department, XaRTA INSA, Pharmacy Faculty, University of Barcelona, Spain.

²Instituto de Fermentaciones Industriales (CSIC), Madrid, Spain. *candres@ub.edu

INTRODUCTION

Nut skins are considered a rich source of polyphenols and may be partially responsible for the numerous health effects associated with nut consumption. However, more bioavailability studies of nut skin polyphenols are needed in order to understand the health effects derived from nut consumption. The aim of the present study was to determine, in a pilot study, the profile of both phase II and microbial-derived phenolic metabolites in plasma and urine samples before and after the intake of almond skin polyphenols by healthy human subjects (n=2).

MATERIAL AND METHODS



RESULTS

Table 1. Concentration of individual phenolic compounds in the encapsulated almond skin extract

	Mean ^a	SD
Non-flavonoids		
Protocatechuic acid	134.86 ± 20.77	
Vanillic acid	272.82 ± 41.16	
Ellagic acid	9.00 ± 1.34	
Flavonoids		
Kaempferol-3-O-glucuronide	22.24 ± 1.31	
Kaempferol-3-O-rutinoside	235.63 ± 23.50	
Kaempferol-3-O-glucoside	32.45 ± 1.86	
Isorhamnetin-3-O-glucuronide	289.51 ± 32.34	
Isorhamnetin-3-O-rutinoside	1253.06 ± 130.76	
Isorhamnetin-3-O-glucoside	185.85 ± 20.11	
Kaempferol	4.26 ± 0.34	
Isorhamnetin	24.21 ± 3.53	
Naringenin-7-O-glucuronide	55.19 ± 6.31	
Eriodictyol-7-O-glucuronide	5.17 ± 0.69	
Naringenin	54.82 ± 6.72	
Eriodictyol	7.82 ± 1.67	
Dihydroquercetin	46.99 ± 8.13	
Catechin	682.83 ± 55.46	
Epicatechin	292.48 ± 34.46	
Procyanidin dimer B3-B1	269.45 ± 41.69	
Procyanidin dimer B2	355.34 ± 7.18	
Procyanidin dimer B7	112.63 ± 8.29	
Unknown A-type procyanidin dimer	99.37 ± 7.14	
Unknown A-type procyanidin dimer	33.54 ± 2.57	
Unknown B-type propylgallate dimer	69.03 ± 7.49	
Unknown B-type propylgallate dimer	117.91 ± 15.76	
Unknown A-type propylgallate dimer	50.44 ± 0.44	
Procyanidin trimer C1	173.97 ± 10.27	

Table 2. Concentration of conjugated phenolic metabolites detected in non-hydrolyzed human plasma and urine before and after the consumption of the almond skin extract

Metabolite	MRM transition	Compound	Plasma ^a		Urine ^b	
			Before Mean SD	After Mean SD	Before Mean SD	After Mean SD
Flavan-3-ols						
Catechin-O-glucuronide #1	465→289	1	nd	nd	0.55 ± 0.78	6.95 ± 3.29
Catechin-O-glucuronide #2	465→289	2	nd	nd	2.25 ± 0.05	6.39 ± 0.89
Catechin-O-glucuronide #3	465→289	3	nd	19.51 ± 3.99	3.08 ± 0.17	21.35 ± 3.91
O-methyl catechin-O-glucuronide #1	479→303	4	nd	8.54 ± 3.47	1.93 ± 0.70	25.30 ± 9.74
O-methyl catechin-O-glucuronide #2	479→303	5	nd	7.94 ± 3.98	2.28 ± 0.62	46.92 ± 7.28
Catechin sulfate #1	369→289	6	nd	5.33 ± 1.77	972.70 ± 344.64	
Catechin sulfate #2	369→289	7	nd	14.71 ± 9.02	10.62 ± 5.79	200.40 ± 28.55
O-methyl catechin sulfate #1	383→303	8	nd	41.82 ± 17.17	8.24 ± 4.84	586.31 ± 90.34
O-methyl catechin sulfate #2	383→303	9	nd	17.83 ±	7.14 ± 1.83	537.34 ± 132.70
O-methyl catechin sulfate #3	383→303	10	nd	nd	2.90 ± 0.57	87.52 ± 10.37
Hydroxyphenylvalerolactones						
5- γ -valerolactone glucuronide derivative #1	383→207	11	nd	nd	18.03 ± 3.95	2939.65 ± 501.22
5- γ -valerolactone glucuronide derivative #2	383→207	12	nd	nd	35.28 ± 17.30	7682.78 ± 1942.14
5- γ -valerolactone sulfate derivative	287→207	13	278.81 ± 160.36	368.23 ± 224.37	3969.68 ± 263.23	44950.92 ± 146817.62
5- γ -valerolactone glucuronide derivative #1	397→221	14	7.62 ± 6.11	5.94 ± 4.84	362.61 ± 97.5	832.89 ± 291.5
5- γ -valerolactone glucuronide derivative #2	397→221	15	nd	nd	6.18 ± 1.68	117.89 ± 41.19
5- γ -valerolactone sulfate derivative #1	301→221	16	nd	nd	57.26 ± 50.50	1495.68 ± 828.12
5- γ -valerolactone sulfate derivative #2	301→221	17	nd	nd	14.36 ± 7.15	704.80 ± 266.53
Flavanones						
Naringenin-O-glucuronide #1	447→271	18	0.84 ± 0.15	3.35 ± 0.17	2.81 ± 1.44	14.97 ± 6.41
Naringenin-O-glucuronide #2	447→271	19	0.61 ± 0.05	1.27 ± 0.17	8.15 ± 8.35	15.43 ± 5.61
Flavanols						
Isorhamnetin-O-glucuronide #1	491→315	20	1.68 ± 0.03	2.40 ± 1.46	5.08 ± 2.74	6.71 ± 0.55
Isorhamnetin-O-glucuronide #2	491→315	21	1.14 ± 0.81	1.49 ± 0.54	4.54 ± 1.38	4.89 ± 1.72
Isorhamnetin sulfate	395→315	22	nd	nd	37.39 ± 4.54	62.75 ± 29.13

Table 3. Concentration of microbial-derived phenolic metabolites detected in hydrolyzed human plasma and urine before and after the consumption of the almond skin extract.

Metabolite	MRM transition	Plasma ^a		Urine ^b	
		Before Mean SD	After Mean SD	Before Mean SD	After Mean SD
Hydroxyphenylvalerolactones					
5- γ -valerolactone	207→163	nd	nd	121.09 ± 11.70	23553.03 ± 1230.73
5- γ -valerolactone	191→147	nd	nd	48.81 ± 10.12	229.21 ± 54.93
Hydroxyphenylpropionic acids					
3,4-Dihydroxyphenylpropionic acid	181→137	2880.36 ± 365.83	2787.25 ± 114.53	2805.14 ± 415.97	3618.37 ± 707.73
3-Hydroxyphenylpropionic acid	165→121	2038.17 ± 367.60	1780.23 ± 335.28	81.67	51.61
Hydroxyphenylacetic acids					
3,4-Dihydroxyphenylacetic acid	167→123	406.28 ± 112.62	385.83 ± 30.04	1759.11 ± 559.81	3122.01 ± 161.09
3-Hydroxyphenylacetic acid	151→107	986.95 ± 143.93	939.50 ± 52.28	2421.92 ± 236.71	7012.71 ± 4401.08
4-Hydroxy-3-methoxyphenylacetic acid	181→137	755.81 ± 189.16	571.49 ± 162.27	6327.50 ± 2186.86	25212.61 ± 10960.16
Phenylacetic acid	135→91	861.61 ± 2343.59	873.33 ± 2384.11	5377.11 ± 116.15	6716.26 ± 921.82
Hydroxycinnamic acids					
p-Coumaric acid	163→119	17.94 ± 9.58	22.93 ± 4.98	15.54 ± 8.91	29.35 ± 17.15
p-Coumaric acid	163→119	29.29 ± 0.46	19.14 ± 2.66	14.86 ± 17.72	20.65 ± 12.90
Caffeic acid	179→135	509.99 ± 95.85	484.26 ± 24.04	809.06 ± 319.26	1200.01 ± 43.82
Ferulic acid	193→134	46.50 ± 2.68	25.38 ± 12.68	111.13 ± 17.70	365.45 ± 258.27
3-Hydroxy-4-methoxy-cinnamic acid	193→134	71.52 ± 26.28	66.74 ± 5.61	3034.48 ± 128.10	4541.98 ± 1705.02
Hydroxybenzoic acids					
3-Hydroxybenzoic acid	137→93	nd	nd	206.26 ± 140.96	315.91 ± 10.28
4-Hydroxybenzoic acid	137→93	6477.80 ± 824.05	6527.09 ± 234.47	5529.69 ± 1759.09	7034.03 ± 240.22
Protocatechuic acid	153→109	4553.05 ± 792.78	4222.13 ± 265.67	9012.44 ± 1414.96	9451.31 ± 1943.37
Vanillic acid	167→152	2821.25 ± 478.51	3087.02 ± 273.06	218.39 ± 111.27	1676.85 ± 1183.74
Hydroxyhippuric acids					
4-Hydroxyhippuric acid	191→100	14.56 ± 1.47	11.46 ± 0.11	15475.48 ± 13393.70	17909.39 ± 12733.87

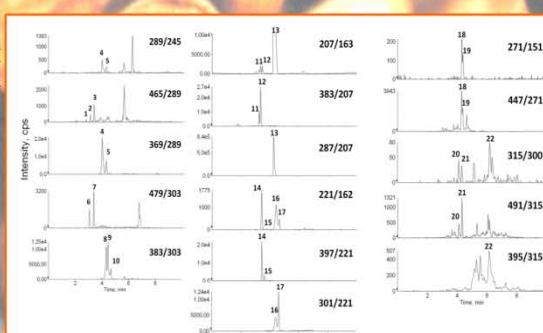


Figure 1. MRM trace chromatograms of (epi)catechin, hydroxyvalerolactones, naringenin and isorhamnetin metabolites described in Table 2.

CONCLUSIONS

In conclusion, to our knowledge this study constitutes the most complete report of the bioavailability of almond skin polyphenols in humans, including both phase II and microbial metabolism. Almond skin polyphenols were absorbed as phase-II metabolites, mainly including: glucuronide, O-methyl glucuronide, sulfate and O-methyl sulfate derivatives of (epi)catechin which arise from the metabolism of monomeric flavanols, as well as glucuronide and sulfate derivatives of isorhamnetin and glucuronide derivatives of naringenin. Of particular importance was the detection of microbial-derived metabolites of flavanols: 5-(dihydroxyphenyl)- γ -valerolactone and 5-(hydroxy-methoxy-phenyl)- γ -valerolactone in their glucuronide and sulfate forms, which confirmed the absorption of both monomeric and polymeric flavanols from almond skins and subsequent conjugation in the liver. Numerous metabolites derived from the further microbial degradation of hydroxyvalerolactones were also detected. The urinary excretion of these metabolites accounted for a larger proportion of the total polyphenol ingested than phase II metabolites of monomeric flavanols, indicating the important role of intestinal bacteria in the metabolism of highly polymerized almond skin polyphenols.

REFERENCES

- Urpi-Sarda et al. *J. Chromatogr. A* **2009**, 1216 (43), 7258-7267.
- Urpi-Sarda et al. *Anal. Bioanal. Chem.* **2009**, 394 (6), 1545-1556
- Urpi-Sarda et al. *J. Agric. Food Chem.* **2009**, 57(21), 10134-42.

ACKNOWLEDGMENTS

I.G. was the recipient of a fellowship from the ISP Program funding by the European Social Fund. A.M-R thanks the FI program for the training of researchers from the Generalitat of Catalonia. M.M thanks the "Juan de la Cierva" post-doctoral program. This work has received financial support from AGL2004-07075-C02-02, AGL2004-08378-C02-01, AGL2006-14228-C03-02 and the CONSOLIDER INGENIO 2010 Programme; RUN-C-FOOD CSD2007-463 from the Spanish Ministry of Science and Innovation (MICINN).

4th International Conference on Polyphenols and Health, Harrogate, December 7th-11th 2009

Comunicació 17: Presentació oral

II Congrés FESNAD, 2010 Barcelona, España.

L'alt consum de polifenols relacionat amb la seva excreció redueix la pressió arterial en subjectes d'alt risc cardiovascular.

Publicat a: Nutrición Hospitalaria. 2010; (supl. 1) vol. 25: p20-21

A. Medina-Remón^{1,2}, R. Zamora-Ros^{1,3}, M. Rotchés-Ribalta^{1,2}, C. Andres-Lacueva^{1,3}, R. Estruch^{2,4}, MA. Martínez-González^{2,5}, MI. Covas^{2,6}, J Salas-Salvadó^{2,7} and RM._Lamuela-Raventos^{1,2*}

Nutrición Hospitalaria

ÓRGANO OFICIAL DE LA SOCIEDAD ESPAÑOLA DE NUTRICIÓN PARENTERAL Y ENTERAL

ÓRGANO OFICIAL DE LA SOCIEDAD ESPAÑOLA DE NUTRICIÓN

ÓRGANO OFICIAL DE LA FEDERACIÓN LATINO AMERICANA DE NUTRICIÓN PARENTERAL Y ENTERAL

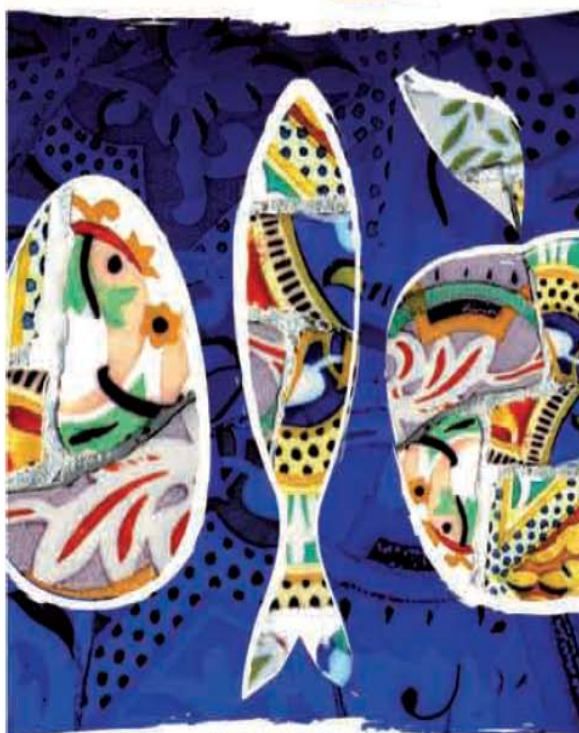
ÓRGANO OFICIAL DE LA FEDERACIÓN ESPAÑOLA DE SOCIEDADES DE NUTRICIÓN, ALIMENTACIÓN Y DIETÉTICA

II CONGRESO FESNAD BARCELONA 2010

Hacia una alimentación responsable

3 A 5 DE MARZO 2010

PALAU DE CONGRESSOS DE CATALUNYA



www.fesnad2010.com



Nutr Hosp. 2010;(Supl. 1):25:1-228 • ISSN (Versión papel): 0212-1611 • ISSN (Versión electrónica): 1699-5198 • CODEN NUHOEQ • S.V.R. 318

Incluida en EMBASE (Excerpta Medica), MEDLINE (Index Medicus), Chemical Abstracts, Cinahl, Cochrane plus, Ebsco, Índice Médico Español, preIBCS, IBECS, MEDES, SENIOR, Scielo, Science Citation Index Expanded (SciSearch), Cancerlit, Toxline, Aidsline y Health Planning Administration

www.nutricionhospitalaria.com

Nutrición Hospitalaria

www.nutriciónhospitalaria.com

ÓRGANO OFICIAL DE LA SOCIEDAD ESPAÑOLA DE NUTRICIÓN
PARENTERAL Y ENTERAL

ÓRGANO OFICIAL DE LA SOCIEDAD ESPAÑOLA DE NUTRICIÓN
ÓRGANO OFICIAL DE LA FEDERACIÓN LATINO AMERICANA
DE NUTRICIÓN PARENTERAL Y ENTERAL

ÓRGANO OFICIAL DE LA FEDERACIÓN ESPAÑOLA
DE SOCIEDADES DE NUTRICIÓN, ALIMENTACIÓN Y DIETÉTICA

Suplemento 1. Vol. 25. Marzo 2010

Edición y Administración
AULA MÉDICA EDICIONES
(Grupo Aula Médica, S.L.)

OFICINA

C/ Isabel Colbrand, 10-12 Nave 78. 2ª planta
Ciudad Industrial Venecia - Edificio Alfa
28050 Madrid
Tel.: 91 358 64 78 - Fax: 91 358 99 79
www.libreriasaulamedica.com

Suscripción y pedidos
AULA MÉDICA EDICIONES
(Grupo Aula Médica, S.L.)

- Por teléfono:
91 358 64 78
- Por fax:
91 358 99 79
- Por e-mail:
suscripciones@grupoaulamedica.com

Dep. Legal: M-34.850-1982
Soporte válido: 19/05-R-CM
ISSN (Versión papel): 0212-1611
ISSN (Versión electrónica): 1699-5198



www.grupoaulamedica.com • www.libreriasaulamedica.com

© AULA MÉDICA EDICIONES (Grupo Aula Médica, S.L.) 2010

Reservados todos los derechos de edición. Se prohíbe la reproducción
o transmisión, total o parcial de los artículos contenidos en este número,
ya sea por medio automático, de fotocopia o sistema de grabación,
sin la autorización expresa de los editores.



Miembro de:



FEDERACIÓN INTERNACIONAL
DE LA PRENSA PERIÓDICA

Métodos: Análisis longitudinal de 12.552 graduados universitarios de la una cohorte prospectiva y dinámica SUN (Seguimiento Universidad de Navarra) (edad media: 38 años, 42% varones). La ingesta de huevos se evaluó con un cuestionario semi-cuantitativo de frecuencia de consumo de alimentos previamente validado en España. La frecuencia de consumo de huevos se categorizó en categorías (desde el consumo de menos de 1 huevo/semana hasta el consumo de 4 o más huevos/semana). Se usaron modelos de regresión de Cox estimando hazard ratios (riesgos relativos [RR]) para ajustar por posibles factores de confusión.

Resultados: Se observaron 103 casos de ECV incidente durante el seguimiento. La frecuencia de consumo de huevos no se asoció con un mayor riesgo de ECV en ningún modelo. Después de ajustar por edad, sexo, ingesta energética total, tabaco, patrón dietético, actividad física e índice de masa corporal, un mayor consumo de huevos (> 4 raciones/semana) no se asoció con la incidencia de eventos cardiovasculares durante el seguimiento (HR cuartil 4 vs cuartil 1: 0,97; [IC 95%: 0,44-2,11]) (p de interacción con el sexo = 0,591). Al categorizar el consumo de huevos en quintiles como análisis de sensibilidad, tampoco se observó ninguna asociación entre la ingesta de huevos y la incidencia de ECV.

Conclusiones: Una mayor frecuencia de consumo de huevos no se relaciona con un mayor riesgo cardiovascular en una cohorte mediterránea de graduados universitarios.

Financiación: Instituto de Salud Carlos III (RD 06/0045 PI030678, PI042241, PI050514, PI050976, PI070240, PI081943) y Dpto. de Salud, Gobierno de Navarra (PI41-2005, PI36/2008).

OR37 Modificaciones en las concentraciones de lípidos en VLDL tras el consumo de dieta mediterránea. El estudio PREDIMED

Sánchez Perona J¹, Covas M^{2,3}, Fitó M², Cabello Moruno R¹, Aros F², Corella D⁴, Ros E⁵, Estruch R⁶, Martínez González MA⁶, Ruiz-Gutiérrez V⁷

¹Instituto de La Grasa (CSIC). ²Institut Municipal d'Investigació Mèdica (IMIM). ³Hospital Txagorritxu. ⁴Universidad de Valencia. ⁵Institut d'Investigacions Biomèdiques August Pi Suñer. ⁶Universidad de Navarra.

Introducción: Los niveles de TG séricos son un factor de riesgo coronario independiente, que están fuertemente determinados por la composición de las VLDL. Los primeros resultados del estudio PREDIMED mostraron que la dieta mediterránea (DM), suplementada con aceite de oliva virgen (AOV) o frutos secos (FS), reducía las concentraciones séricas de colesterol y triglicéridos (TG),

mientras que incrementaba el colesterol asociado a HDL, en comparación con una dieta baja en grasa.

Objetivo: Evaluar la composición triglicéridica de VLDL en participantes del estudio PREDIMED, tras la ingesta de DM suplementada con AOV o frutos secos para evaluar su influencia sobre las concentraciones de TG totales en suero.

Métodos: Se reclutaron 50 sujetos con un leve riesgo cardiovascular según los criterios del estudio PREDIMED. Los voluntarios fueron distribuidos en 3 grupos a los que se administraron una MD suplementada con AOV (DM+AOV) o frutos secos (DM+FS) o una dieta baja en grasa (DBG) durante 3 meses. A partir de muestras de sangre se aislaron las VLDL, que se analizaron mediante técnicas cromatográficas para determinar su composición lipídica y triglicéridica.

Resultados: Solamente la DM+AOV fue capaz de reducir las concentraciones de TG y colesterol en VLDL, así como el ratio TG/apolipoproteína B, que se emplea como indicador del tamaño de dichas partículas. Aunque la composición en ácidos grasos de las VLDL se modificó ligeramente, se encontraron importantes diferencias en las especies moleculares de TG, encontrando una mayor presencia de TG ricos en ácido linoleico (18:2, n-6) en el grupo que recibió DM+FS y de ácido oleico (18:1, n-9) en el que recibió DM+AOV.

Conclusión: La reducción en las concentraciones sistémicas de TG observada tras el consumo de DM puede ser explicada por una reducción en el contenido lipídico de las VLDL y una modificación selectiva de la composición en especies moleculares de TG en la partícula.

OR38 El alto consumo de polifenoles relacionado con su excreción reduce la presión arterial en sujetos de alto riesgo cardiovascular

Medina Remón A^{1,2}, Zamora Ros R^{1,2}, Rotchés-Ribalta M^{3,4,5}, Andres-Lacueva C^{1,2}, Estruch R⁶, Martínez González MA^{2,5}, Covas M^{2,3,6}, Salas Salvadó J^{2,7}, Lamuela Raventos RM^{2,1,2}

¹Departamento de Nutrición y Bromatología. XaRTA. INSA Facultad Farmacia. Universidad de Barcelona. ²CIBER 06/003 Fisiopatología de la Obesidad y Nutrición CIBEROBN, y RETICS RD06/0045/0003. Instituto de Salud Carlos III de Madrid. ³Programa Ingenio CONSOLIDER. FUN-C-FOOD (CSD2007-063) de Barcelona. ⁴Departamento de Medicina Interna Hospital Clínico. IDIBAPS Universidad de Barcelona. ⁵Departamento de Medicina Preventiva y Salud Pública Escuela de Medicina. Clínica de la Universidad de Navarra. Pamplona. ⁶Unidad Cardiovascular de Epidemiología Instituto Municipal de Investigación Médica (IMIM) de Barcelona. ⁷Unidad de Nutrición Humana Facultad de Medicina. IISPV Universidad de Rovira i Virgili. Reus. Tarragona.

Una dieta saludable y modificaciones en el estilo de vida son los primeros pasos en la prevención de la hipertensión, un importante problema mundial a nivel de salud pública.

Objetivo: Evaluar el efecto del consumo de los polifenoles en la hipertensión arterial en una población adulta con alto riesgo cardiovascular.

Métodos: Como parámetro para determinar la ingesta de polifenoles totales se ha utilizado la excreción de polifenoles totales (EPT), biomarcador de la ingesta de polifenoles. Se ha llevado a cabo un sub-estudio transversal con datos de referencia del estudio PREDIMED (www.predimed.org). A los participantes, 263 hombres y 326 mujeres con edades entre 55-80, se les tomaron los datos antropométricos, la presión arterial, y un cuestionario de frecuencia de consumo. Los EPT se determinaron utilizando el método de Folin-Ciocalteu, adaptado por nuestro grupo de investigación a muestras biológica y los resultados fueron divididos en cuartiles según el índice de EPT.

Resultados: Se observa una correlación positiva estadísticamente significativa entre la EPT y la ingesta total de café, vino y frutas-verduras (F&V). La ingesta de una porción de 100 g de F&V (Beta = 0,150; $P < 0,001$) contribuyó más a la EPT que una de 100 mL de café (Beta = 0,141; $P = 0,001$) y que una porción de 100 mL de vino (Beta = 0,120; $P = 0,019$). El análisis de regresión logística mostró una asociación inversa entre EPT (por cuartiles) y la prevalencia de hipertensión; participantes del cuartil más alto de EPT urinaria tuvieron una reducción de la prevalencia de hipertensión en comparación con el cuartil más bajo (OR = 0,64; $P = 0,016$). En el análisis de regresión lineal múltiple la PA sistólica (Beta = 1,731; $P = 0,024$) y diastólica (Beta = 1,264; $P = 0,003$) se asociaron inversamente con la EPT urinaria después de ajustar por posibles factores de confusión.

Conclusión: La ingesta de polifenoles, evaluados a través de EPT en la orina, se asoció negativamente con los niveles de PA y la prevalencia de la hipertensión en una población mediterránea de edad avanzada con alto riesgo cardiovascular. Una intervención dietética dirigida a incrementar la ingesta de alimentos ricos en polifenoles, podría ser eficiente en la prevención y el tratamiento de la hipertensión.

OR39 Asociación transversal de la ingesta de frutos secos y la adiposidad en una población mediterránea

Casas-Agustench P¹, Bulló M¹, Ros E², Basora J³, Salas-Salvadó J⁴

¹Unidad de Nutrición Humana. Hospital Universitario Sant Joan de Reus. ²Unidad de Lípidos. Servicio de Endocrinología y Nutrición Institut d'Investigacions Biomèdiques August Pi Sunyer (IDIBAPS). Hospital Clínic de Barcelona. CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBN). Instituto de Salud Carlos III. ³Direcció d'Atenció Primària. Institut Català de la Salut de Reus. Tarragona.

Introducción: En diversos estudios prospectivos la ingesta de frutos secos (FS) se ha relacionado inversamente con el índice de masa corporal (IMC).

Objetivo: Examinar los determinantes dietéticos de la adiposidad en sujetos ancianos que viven en una región mediterránea con un consumo elevado de FS.

Métodos: Estudio transversal realizado en 847 sujetos (56% mujeres, 67 años, IMC 29,7 kg/m²) con un elevado riesgo cardiovascular reclutados en el estudio PREDIMED. El consumo alimentario se estimó mediante un cuestionario semi-cuantitativo validado, la actividad física a través del cuestionario Minnesota Leisure Time Activity, y las variables antropométricas mediante mediciones estándar.

Resultados: La ingesta de FS disminuyó a medida que aumentaron los quintiles de IMC y circunferencia de la cintura (P -tendencia $< 0,005$; en ambos casos). La ingesta de alcohol se relacionó inversamente con el IMC (P -tendencia = 0,020) y directamente con la circunferencia de la cintura (P -tendencia = 0,011), mientras que la ingesta de carne se asoció directamente con la circunferencia de la cintura (P -tendencia = 0,018). En un modelo multivariable ajustado, la ingesta de FS se asoció inversamente ($P = 0,002$) y la ingesta de carne directamente ($P = 0,042$) con el IMC. La ingesta de FS ($P = 0,002$) y vegetales ($P = 0,040$) se asociaron inversamente con la circunferencia de la cintura, mientras que la ingesta de carne se asoció directamente ($P = 0,009$). A partir de los resultados del análisis de regresión se predijo que por cada ración de 30 g de FS el IMC y la circunferencia de la cintura disminuyeron en 0,78 kg/m² y 2,1 cm, respectivamente. Los resultados fueron similares en hombres y mujeres.

Conclusión: El consumo de FS se asoció negativamente con la adiposidad independientemente de otras variables de estilo de vida. A pesar de que es posible que existan otros factores confusores relacionados con un estilo de vida más saludable entre los consumidores de FS que puedan explicar en parte estos resultados, el presente estudio confirma las recomendaciones del consumo de FS como un componente importante de la dieta saludable.

Comunicació 18: Pòster

II Congrés FESNAD, 2010 Barcelona, España.

Polifenols totals en mostres de tomàquet eliminant substàncies interferents pel mètode de Folin-Ciocalteu

Anna Vallverdú-Queralt, Alexander Medina-Remón, Cristina Andrés-Lacueva and Rosa M. Lamuela-Raventós.

Publicat a: Nutrición Hospitalaria. 2010; (supl. 1) vol. 25: p97

Nutr Hosp Vol. 25. Suplemento 1. Marzo 2010

nivel de significancia del 5% ($p < 0,05$), hay una diferencia estadísticamente significativa para la cantidad total de fósforo, siendo los valores más altos en yogur de cabra, mientras que calcio y magnesio no muestran diferencias estadísticamente significativas entre las distintas especies.

P154 Polifenoles totales en muestras de tomate eliminando sustancias interferentes por el método de Folin-Ciocalteu

Lamuela Raventós RM^{1,2}, Vallverdú Queralt A¹, Medina Remón A^{1,2}, Lacueva CA^{1,3}

¹Departamento de Nutrición y Bromatología. XaRTA. INSA Facultad de Farmacia Universidad de Barcelona. ²CIBER 06/003 Fisiopatología de la obesidad y nutrición (CIBERON) y RETICS RD06/0045/0003 Instituto de Salud Carlos III. ³Ingenio-CONSOLIDER, FUN-C-FOOD. Instituto de Salud Carlos III-Madrid.

Introducción: El método de Folin-Ciocalteu (F-C) se ha utilizado durante años para determinar el contenido de polifenoles totales (PTs) en muestras de tomate; no obstante otras sustancias presentes en el tomate con poder reductor (como ácido ascórbico, dehidráscorbico, azúcares...) interfieren en la cuantificación de los PTs.

Objetivo: El objetivo de nuestro estudio es mejorar la determinación de polifenoles totales en muestras de tomate utilizando la extracción en fase sólida (SPE) con placas Oasis® MAX 96 con el objetivo de eliminar las sustancias interferentes antes de la determinación de PTs con el método de F-C.

Método: El método de F-C ha sido optimizado, para reducir las interferencias causadas por sustancias reductoras, y validado. Se ha determinado la sensibilidad, precisión, exactitud y linealidad del método añadiendo 6 concentraciones de ácido gálico a la matriz de tomate. La estabilidad a corto y largo plazo y después de sucesivos ciclos de congelación-descongelación también se ha evaluado. Mediante cromatografía líquida de alta resolución se han determinado las recuperaciones de nueve polifenoles con diferente polaridad, después de adicionar a la matriz de tomate 4 concentraciones distintas de cada patrón estudiado. Se han analizado siete muestras de tomate y los resultados se expresan en mg equivalentes de ácido gálico/100 g de materia seca.

Resultados: El intervalo de linealidad del método F-C oscila entre 1 y 75 mg/L. Los valores de precisión y exactitud están comprendidos entre los niveles que acepta la AOAC INTERNATIONAL. Las muestras y patrones son estables tanto a corto como a largo plazo, así como después de los sucesivos ciclos de conge-

lación descongelación. Las recuperaciones de los nueve polifenoles después de la SPE están comprendidos entre 79,12% y 108,75%. Las concentraciones de PTs en las muestras de tomate después de SPE oscilan entre 149,99 Y 247,12 mg equivalentes de ácido gálico/100 g materia seca.

Conclusión: Se ha optimizado y validado un método de análisis de PTs en muestras de tomate eliminando las interferencias con el método F-C utilizando SPE con placas Oasis® MAX 96 obteniendo unos resultados entre 149,99 Y 247,12 mg de GAE/100 g materia seca.

P155 Valor nutritivo de tomate maduro en base a su perfil metabólico

Oms Oñu G¹, Hertog TM², Nicolaï BM²

¹Departamento de Tecnología de Alimentos. TPV-XaRTA. Universidad de Lleida. ²BIOSYST-MeBioS División. Katholieke Universiteit Leuven. Bélgica.

Introducción: Las modernas técnicas de análisis global, denominadas 'omics' permiten estudiar la complejidad nutritiva de los alimentos. La Metabolómica se ha aplicado en este trabajo como herramienta para explorar el valor nutritivo de tomate maduro en base a su perfil metabólico.

Objetivo: obtener un mapa global de los metabolitos polares que componen el tomate tales como azúcares, ácidos orgánicos y aminoácidos.

Métodos: La cromatografía de gases-espectrometría de masas (GC/MS) permitió el análisis simultáneo de los metabolitos polares presentes en tomate. No obstante, la inyección de la muestra en un GC requiere una conversión previa de los compuestos a derivados volátiles mediante un proceso de derivatización. La identificación de los metabolitos se realizó a través de la librería "Fiehn GC/MS metabolomic library". Las concentraciones relativas de los metabolitos se calcularon a partir de los factores respuesta de cada uno de los compuestos, relacionando el área de cada pico con la del estándar interno (ribitol) presente en cada cromatograma.

Resultados: En los extractos de tomate maduro se identificaron 41 metabolitos. Los compuestos mayoritarios en tomate (glucosa, fructosa y ácido cítrico) se detectaron en concentraciones relativas de 1.344,72, 1.739,46 y 352,16, respectivamente. No obstante, se detectaron otros azúcares como la galactosa, sacarosa, inositol, ácido galacturónico, manosa, maltosa, glucosa 6-fosfato, rafinosa, manitol, galactitol, sorbitol, y ácidos tales como el isocítrico, gulónico, glucónico, tartárico, sacárico, succínico, maleico, láctico, citramálico, fumárico,



POLIFENOLES TOTALES EN MUESTRAS DE TOMATE ELIMINANDO SUSTANCIAS INTERFERENTES POR EL MÉTODO DE FOLIN-CIOCALTEU.

Anna Vallverdú-Queralt^{1,2}, Alexander Medina-Remón^{1,2}, Cristina Andrés-Lacueva^{1,2} and Rosa M. Lamuela-Raventós^{1,2*}

¹Departamento de Nutrición y Bromatología, XaRTA, INSA. Facultad de Farmacia, Universidad de Barcelona, Av. Joan XXIII s/n Barcelona, Spain., ²CIBER 06/003 Fisiopatología de la obesidad y nutrición, (CIBEROBN), y RETICS RD06/0045/0003. Instituto de Salud Carlos III, España., ³Ingenio-CONSOLIDER, FUN-C-FOOD. Instituto de Salud Carlos III, España. * Departamento de nutrición y Bromatología, Universidad de Barcelona. Teléfono +34-934034843. Fax +34-93-4035931; e-mail lamuela@ub.edu

1. Introducción

Los tomates son una de las hortalizas más consumidas en todo el mundo, no sólo en forma fresca, sino también en una amplia gama de productos transformados. Los alimentos ricos en polifenoles, como los tomates pueden tener un efecto importante en la prevención de enfermedades, en concreto, se han relacionado con un menor riesgo de cáncer y enfermedades cardiovasculares. El método del Folin-Ciocalteu (FC) se ha utilizado durante muchos años para determinar el contenido de polifenoles totales (PT) en los tomates. Sin embargo, el contenido de TPs es a veces sobreestimado debido a la presencia de algunas sustancias reductoras como el ácido ascórbico, azúcares y aminoácidos que interfieren en el ensayo del F-C.

Objetivo: El objetivo de este estudio fue eliminar las sustancias no fenólicas que interfieren en el método del F-C mediante extracción en fase sólida (SPE) a fin de mejorar la determinación de PT en muestras de tomate. Este es el primer estudio que ha evaluado el contenido real de PT en las muestras de tomate después de la SPE, utilizando placas Oasis MAX 96, lo que permite el análisis de un gran número de muestras a la vez.

2. Método

Preparación de la muestra



Limpieza de la muestra: Placas Oasis MAX 96

- 1ª Acondicionamiento: 1 mL de metanol 98-100%.
- 2ª Acondicionamiento: 1 mL de sodium acetate 50mM pH 7.
- Carga: 1 mL muestra + 1 mL agua Milli-Q + 34 µL HCl.
- Limpieza: 1 mL acetato sódico 50mM pH 7 / 5% metanol
- Elución: 1.8 mL metanol al 2% ácido fórmico.



Analysis of total polyphenols in tomatoes, in thermo microtiter 96 well plate.

- 20 µL del extracto eluido en la SPE.
- 188 µL de agua Milli-Q.
- 12 µL del reactivo F-C.
- 30 µL de carbonato sódico (200 g/L).
- Incubación 60 min. en la oscuridad
- Después del periodo de reacción: 50 µL de agua Milli-Q
- Medir absorbancia a 765 nm al espectrofotómetro UV/VIS Thermo Multiskan Spectrum.



Validación:

- Linealidad:
0.25-10 mg/L (ácido gálico)
- Sensibilidad:
LoD= 3°SD
LoQ= 10°SD
(10 blancos a 765 nm)
- Exactitud: % recuperaciones
- Precisión: RSD=SD/X*100 } 0.25-10 mg/L
- Estabilidad
- Corto plazo
- Largo plazo
- Después de ciclos de congelación-descongelación -20 °C and -80 °C



- Recuperaciones de ácido gálico, caféico, p-coumarico, protocatequico, p-hidroxibenzoico y clorogénico y naringenina, rutina y quercetina a 5, 10, 25 and 50 mg/L.

- Siete muestras fueron analizadas y los resultados fueron expresados como mg of gallic acid equivalentes/100g muestra seca.

3. Resultados

Linealidad:

0.25-10mg/L $y = 0.1x - 0.003$

Sensibilidad:

LOD: 0.006 mg/L

LOQ: 0.019 mg/L



Los resultados de precisión y exactitud intradía e interdía están comprendidos entre los valores establecidos por la AOAC International (Tabla 1).

Tabla 1: Exactitud y precisión intra e interdía del método F-C

Patrones (mg/L)		Concentración obtenida (mg/L)		Precisión, R.S.D. (%)		Recuperaciones, error (%)	
Intra-día	Inter-día	Intra-día	Inter-día	Intra-día	Inter-día	Intra-día	Inter-día
1.12	1.07	1.01	0.98	0.10	0.12	90.57	91.97
2.24	2.14	2.24	2.06	0.10	0.11	100.08	96.25
4.48	4.27	4.38	4.25	0.09	0.09	97.77	99.60
6.73	6.41	6.69	6.40	0.09	0.10	99.38	99.87
8.97	8.55	8.93	8.21	0.06	0.08	99.58	96.00
11.21	10.69	11.62	11.03	0.05	0.07	103.67	103.21
13.40	13.81	13.89	14.51	0.04	0.05	103.66	105.10

RSD: desviación estándar relativa

Estabilidad

Tabla 2: Después de ciclos de congelación-descongelación, a corto y largo plazo

Temperatura	Estabilidad cíclica	Estabilidad a corto plazo	Estabilidad a largo plazo
-20 °C	94.9-106.2 %	90.1-104.0 %	95 %
-80 °C	91.7-106.5 %	85.2-102.6 %	95 %

Recuperaciones absolutas

Tabla 3: Absolute recoveries of the nine polyphenols

Polifenoles	% Recuperaciones (promedio)	Desviación estándar (S.D.)
Ácido gálico	92.47	4.45
Naringenina	98.20	3.39
Ácido caféico	96.74	10.72
Rutina	87.32	4.81
Quercetina	77.52	5.43
Ácido p-coumarico	96.14	3.36
Ácido protocatequico	108.75	3.15
Ácido p-hidroxibenzoico	99.53	2.00
Ácido clorogénico	101.11	5.17

Las concentraciones de TPs en muestras de tomate varían entre 260.80 y 472.23 mg of GAE/100g MS cuando la SPE no fue empleado. No obstante, los valores varían entre 113.32 y 174.85 mg of GAE/100g MS después de la SPE. Por lo tanto, los resultados se han sobreestimado hasta ahora.

4. Conclusiones

- Es la primera vez que la SPE ha sido utilizado para eliminar las interferencias no fenólicas antes del método FC en muestras de tomate.
- El método descrito puede ser útil como un ensayo rutinario para la determinación de TPs en alimentos, especialmente en alimentos como las frutas y verduras que son ricas en sustancias fenólicas no reductoras que reaccionan con el reactivo de FC e interfieren en el análisis.
- Las muestras requieren una preparación mínima antes del análisis de TPs. Este método es ideal para analizar grandes cantidades de muestras, ya que la placa OASIS MAX 96 puede acomodar volúmenes de muestra mucho más grandes.

5. Referencias

- (1) Capanoglu E. et al., J Agric Food Chem 2008, 56(3), 964-73.
- (2) Long M. et al., Phytochemistry 2006, 67(16), 1750-7.
- (3) Hussein L. et al., Int J Food Sci Nutr., 2008, 4, 1-10.
- (4) Medina-Remón A. et al., Analytica Chimica Acta, 2009, 634(1), 54-60.
- (5) Horwitz W. Official Method of Analysis of AOAC INTERNATIONAL (Appendix D and E). 17 th Ed. Revision 2, Vol. 2 ed. 2003.

6. Ayudas:

Los autores expresan su gratitud por el apoyo financiero (CICYT de AGL2006-14228-C03-02 y AGL2007-66638-C02-01), RETICS RD06/0045/0003 del Ministerio español de Ciencia e Innovación (MICINN) y la concesión por el Instituto PI070240 de Salud Carlos III, España. B CB06/03 CIBERobn es una iniciativa del Instituto de Salud Carlos III, España. Este trabajo ha sido financiado en parte por la iniciativa INGENIO 2010 del Programa CONSOLIDER, FUN-C-CSD2007 ALIMENTARIA-063 y también del MICINN. A.V.Q. recibió el apoyo del MEC para la formación de investigadores.

Comunicació 19: Pòster

VIII Congrés Internacional de Barcelona sobre la Dieta Mediterrània 24-25 març del 2010 Barcelona, España.

Following a Mediterranean diet pattern increases total polyphenol consumption and produces a significant decrease in blood pressure. The PREDIMED randomized trial after one year.

Medina-Remón A., Estruch R., Andres-Lacueva C., Martínez-González M.A., Covas M.I., Corella D., Salas-Salvadó J., Serra- Majem, L. and Lamuela-Raventos R.M., on behalf of the PREDIMED Study Investigators.



Following a Mediterranean diet pattern increases total polyphenol consumption and produces a significant decrease in blood pressure. The PREDIMED randomized trial after one year.

Medina-Remón A.^{a,b,c,d}, Estruch R.^{a,b,c,d}, Andres-Lacueva C.^{a,b}, Martínez-González M.A.^{e,f}, Covas M.I.^{g,h}, Corella D.^{h,i}, Salas-Salvadó J.^{h,i}, Serra-Majem L.^{j,k} and Lamuela-Raventós R.M.^{a,b,c,d}, on behalf of the PREDIMED Study Investigators.

^aNutrition and Food Science Department, XaRTA, INSA, Pharmacy School, University of Barcelona, Barcelona, Spain; ^bCIBER CB06/03 Fisiopatología de la Obesidad y la Nutrición, (CIBEROBN), and ^cRETICS RD06/0045/0003, Instituto de Salud Carlos III, Spain; ^dDepartment of Internal Medicine, Hospital Clinic, IDIBAPS, University of Barcelona; ^eIngenio-CONSOLIDER program, FUN-C-FOOD, Barcelona; ^fDepartment of Preventive Medicine and Public Health, School of Medicine, University of Navarra, Pamplona; ^gCardiovascular Epidemiology Unit, Municipal Institute for Medical Research (IMM), Barcelona; ^hDepartment of Epidemiology, Preventive Medicine and Public Health, School of Medicine, University of Valencia, Valencia; ⁱHuman Nutrition Unit, School of Medicine, ISPV, University Rovira i Virgili, Reus; ^jDepartment of Clinical Sciences, University of Las Palmas de Gran Canaria, Las Palmas de Gran Canaria; ^kNutrition and Food Science Department, University of Barcelona. Telephone: +34-934034843; e-mail: rmedina@ub.edu

1. INTRODUCTION

The first step in its management of hypertension is to follow a healthy diet such as the traditional Mediterranean diet (TMD) [1, 2, 3] and/or to improve lifestyle, for instance, by reducing body weight and increasing physical activity [4]. Adherence to the TMD in numerous epidemiological studies have demonstrated an inverse association with a reduction in hypertension; this protective effect has been attributed in part, to the richness of this diet in antioxidants. Olive oil is the main natural fat in the Mediterranean diet [6], the virgin olive oil (VOO) has the highest antioxidant phenolic content [7]; nuts are also typical from TMD, they are a rich source of nutrients and antioxidant phytochemicals [8]. Previous studies have demonstrated that polyphenol intake, assessed in urine are correlate with polyphenol consumption [9].

AIMS: We undertook this substudy in the frame of the PREDIMED study, in order to evaluate the effect of a one-year intervention with two traditional Mediterranean diets supplemented with virgin olive oil (VOO) or nuts on blood pressure and their relationship with polyphenol consumption.

2. Subjects and Methods

1139 admitted trial participants from the PREDIMED (PREvención con Dieta MEDiterránea) study is a large, parallel-group, multicenter, randomized, controlled 5-year clinical trial (www.predimed.org; ISRCTN35739639).

- Men: 55-80 years old
- Women: 60-80 years old
- High CV risk without CVD
- type 2 diabetics
- ≥3 risk factors

All free of CVD at baseline

random

1. Smoking
2. Hypertension
3. LDL
4. HDL
5. Overweight/obesity
6. Family history CVD



solid phase extraction (SPE): 96 well plate cartridges from Waters Oasis® MAX (Mixed-Mode Anion-Exchange and Reversed-Phase Solvent) to avoid any interference with Folin-Ciocalteu reagent.



Urinary total polyphenols were analyzed by the Folin-Ciocalteu assay, after a solid phase extraction [9].

3. Results

Table 1: Baseline of the study participants completing 1 year of follow-up.

	DMed+VOO	DMed+Nuts	Control Diet	P for trend ^a
No. of subjects	394	366	379	
Age, (y) mean (SD)	67.2 (6.1)	67.2 (6.0)	68.3 (5.9)	0.025
Women, n (%)	219 (55.6)	181 (49.5)	228 (60.2)	0.013
BMI, (kg/m ²) mean (SD)	29.2 (3.2)	29.3 (3.4)	29.7 (3.5)	0.128
Overweight or obese (BMI ≥ 25 kg/m ²), n (%)	356 (90.4)	332 (90.7)	340 (89.7)	0.896
Systolic BP (mm Hg), mean (SD)	150.4 (17.5)	152.2 (19.2)	152.5 (17.8)	0.230
Diastolic BP (mm Hg), mean (SD)	83.9 (9.7)	85.3 (10.7)	84.0 (8.9)	0.145
Hypertension, n (%)	302 (76.6)	285 (77.9)	314 (82.8)	0.082
Diabetes, n (%)	168 (42.6)	161 (44.0)	176 (46.4)	0.561
Dyslipidemia, n (%)	256 (65.0)	242 (66.1)	242 (63.9)	0.678
Current smoker, n (%)	64 (16.2)	58 (15.8)	64 (16.9)	0.927
Family history of CHD, n (%)	76 (19.3)	64 (17.5)	64 (17.0)	0.875
Energy expenditure in physical activity (kcal/d), mean (SD)	311.9 (241.3)	289.1 (212.3)	240.7 (187.3)	<0.001



Table 3: Baseline level and 1-year changes in adiposity, blood pressure and total polyphenol excreted.^a

	N	DMed+VOO	P Value ^b	DMed+Nuts	P Value ^b	Control Diet	P Value ^b	P Value ^c
Urine total polyphenol (mg GAE/g creatinine)	394	366		379				
Baseline		110.8 (47.1)		127.5 (66.5)		138.2 (81.3)	0.903	<0.027 ^d
1 year		133.5 (74.7)	<0.001	149.3 (85.6)	<0.001	139.7 (87.8)		
Weight (kg)								
Baseline		74.8 (10.7)		75.3 (11.3)		75.2 (11.2)	0.195	0.330 ^e
1 year		74.7 (10.9)	0.392	74.9 (11.3)	0.654	76.8 (49.9)		
BMI								
Baseline		29.2 (3.2)		29.3 (3.4)		29.7 (3.5)	0.208	0.043 ^e
1 year		29.2 (3.3)	0.384	29.1 (3.5)	0.601	31.3 (22.2)		
Waist, cm								
Baseline		97.2 (9.7)		97.5 (10.3)		97.9 (10.4)	0.001	0.435 ^e
1 year		96.5 (9.6)	0.022	96.3 (10.2)	<0.001	97.2 (10.9)		
Systolic BP (mm Hg), mean (SD)								
Baseline		150.4 (17.5)		152.2 (19.2)		152.5 (17.8)	0.071	0.001 ^d
1 year		147.5 (16.9)	0.003	149.2 (18.6)	0.008	151.9 (18.2)		
Diastolic BP (mm Hg), mean (SD)								
Baseline		83.9 (9.7)		85.3 (10.7)		84.0 (9.9)	0.370	0.056 ^e
1 year		82.6 (9.7)	0.002	84.1 (11.0)	0.021	84.0 (9.5)		

^aData are given as means (SD). ^bDifferences from baseline by non-parametric Wilcoxon test, ^cP=0.05 indicates statistical significance. DMed: Mediterranean diet, VOO: virgin olive oil, GAE: gallic acid equivalent, ^dP values for differences among diets, ^eP values for differences between DMed+VOO and Control diet, ^fbetween DMed+Nuts and Control diet, ^gbetween DMed+VOO and DMed+Nuts.

Figure 1: Mean ± SD changes in total polyphenols excreted in spot urine samples, systolic and diastolic blood pressure after one-year with different intervention.

Table 2: Covariate analysis with systolic blood pressure and diastolic blood pressure at one-year as the dependent variables; intervention groups as the fixed factor and baseline measures as covariates.

	Model	B	P	95% CI
Systolic blood pressure	Model adjusted			
	DMed+VOO vs. Control diet	-3.429	0.002	-5.598 to -1.261
Diastolic blood pressure	Model adjusted			
	DMed+Nuts vs. Control diet	-2.266	0.042	-4.450 to -0.086
Systolic blood pressure	Model adjusted			
	DMed+VOO vs. Control diet	-1.382	0.014	-2.479 to -0.285
Diastolic blood pressure	Model adjusted			
	DMed+Nuts vs. Control diet	-0.696	0.217	-1.803 to 0.410

B: Non-standardized coefficient, CI: Confidence Interval, P: two-sided test of significance, Model adjusted by sex, age, weight, smoking status, physical activity, education level at baseline, medication status: ACE inhibitor, diuretics, statins (myocardial drugs), insulin, oral hypoglycemic drugs, aspirin or other antiplatelet drug supplements taken in the last month, sodium and potassium intake and glomerular filtration rate. DMed: Mediterranean diet, VOO: virgin olive oil.

4. CONCLUSIONS

Urinary total polyphenols excreted are statistically significant increased in Mediterranean diets supplemented with virgin olive oil and nuts (figure 1).

The weight and BMI have not differences from baseline in any intervention groups or between groups after one year.

Participants in both Mediterranean diets had decreased systolic and diastolic blood pressure (figure 1).

A one-year intervention with a traditional Med-diet supplemented with either VOO or nuts increases total polyphenols excreted in urine samples from elderly participants, and decreases systolic and diastolic BP. Both Med-diets exhibited beneficial effects on cardiovascular risk factors.

5. REFERENCES

- [1] C.M.Lewes, et al., Lancet 371 (2008) 1513-1518.
- [2] R.Estruch, et al., Ann Intern Med. 145 (2006) 1-11.
- [3] G.Mancini, et al., J Hypertens. 25 (2007) 1105-1107.
- [4] Serra-Majem L, et al., Nutr Rev. 64 (2006) 27-47.
- [5] Medina-Remón A, et al., Nutr Metab Cardiovasc Dis. 2009;19:1018.

- [6] A.Trichopoulos, et al., N Engl J Med. 348 (2003) 2599-2608.
- [7] C.de la Torre-Carbayo, et al., J Agric Food Chem. 53 (2005) 4321-4340.
- [8] P.M.Kris-Etherton, et al., Nutr Rev. 59 (2001) 102-111.
- [9] Medina-Remón A, et al., Analytica Chimica Acta 2009;16:4341-154-60.

6. SUPPORTED BY



We would like to thank all of the volunteers involved in the PREDIMED study for their valuable cooperation. The authors express their gratitude for financial support from CICT's (AGL2005-05597, AGL2006-14228-C03-02 and AGL2007-46638-C02-01) and "Red de Grupo" G03140, the Spanish Ministry of Science and Innovation (MICINN), RETICS RD06/0045/0003 and CIBER CB06/03/024, from the "Instituto de Salud Carlos III". This work has been funded by the CONSOLIDER INGENIO 2010 Programme; FUN-C-FOOD CSD2007-063 also from MICINN. A. M.R. received support from the Generalitat de Catalunya for the training of researchers.

VIII Congreso Internacional de Barcelona sobre la Dieta Mediterránea 24-25 Marzo del 2010

El vi només es disfruta amb moderació

Rosa M. Lamuela^{a,b,*}, A. Medina-Remón^{a,b}, Cristina Andres-Lacueva^{a,c} i

Ramon Estruch^{b,d}

**E-mail:lamuela@ub.edu, Tel :+34 934034843*

^a*Grupo de Investigación Antioxidantes Naturales :Polifenoles*

<http://www.ub.es/depnutricioibromatologia/GrupAntioxidants>

^aDepartment of Nutrition and Food Science, XaRTA, INSA. Pharmacy School, University of Barcelona, Barcelona, Spain; ^bCIBER 06/003 Fisiopatología de la Obesidad y la Nutrición, (CIBEROBN) y RETICS RD06/0045/0003. Instituto de Salud Carlos III, Spain.; ^cIngenio-CONSOLIDER program, FUN-C-FOOD, Barcelona, ^dDepartment of Internal Medicine, Hospital Clinic, IDIBAPS, University of Barcelona.

RESUM

El consum de vi ha estat molt lligat a l'evolució de la civilització a la conca mediterrània. Es té constància que fa més de 2000 anys ja s'atribuïen al vi efectes saludables. Recentment, nombrosos estudis epidemiològics han observat que el consum moderat de vi i begudes alcohòliques té un paper protector davant les malalties cardiovasculars. Els consumidors moderats d'alcohol presenten una menor incidència de malaltia coronària que els abstemis. Tanmateix, el vi, a més d'alcohol, conté altres múltiples components, com els compostos fenòlics o polifenols. Aquests components, que procedeixen majoritàriament del raïm, han demostrat una intensa activitat biològica. Per això, el vi, al contenir tant etanol com una elevada concentració de polifenols, sembla tenir un efecte protector davant les malalties cardiovasculars superior al d'altres begudes alcohòliques.

La cultura del vi ha estat molt lligada a la civilització des del període neolític i, possiblement, des de molt abans, tant a nivell econòmic, religiós, social, mèdic (1). La primera constància històrica d'un vas que havia contingut vi data dels anys 5.400-5.000 a.C., i va ser trobada en una cuina neolítica ubicada entre els rius Tigris i Eufrates, en l'actual Iraq. Durant el període de l'Antic Egipte ja s'elaboraven diferents tipus de vins: blanc, negre i fins i tot un vi amb un tipus d'elaboració més complexa, que seria com un actual vi de Màlaga, el Shedeheh (2,3,4).

Així mateix, es té constància que fa més de 2.000 anys ja s'atribuïen efectes saludables al consum moderat de vi. Tanmateix, cap a l'any 1.800, coincidint amb l'avanç de la medicina científica d'aquella època, es van perdre les nocions referents als possibles efectes beneficiosos del consum de vi, per considerar només els seus efectes tòxics (5). L'interès en aquest tòpic es va reiniciar fa uns 30 anys després de l'anàlisi dels resultats de varis estudis epidemiològics i constatar que el consum moderat d'alcohol podria ser beneficiós per la salut (6-8). Els investigadors d'aquests estudis van trobar una associació negativa entre el risc relatiu d'infart de miocardi i el consum moderat de begudes alcohòliques. Les corbes que relacionen la mortalitat i incidència de malaltia cardiovascular amb el consum d'alcohol segueixen una forma en U o de J. És a dir, els bevedors moderats presentaven un menor risc de mort cardiovascular que els abstemis (braç esquerra de la U o la J), però també inferior als grans bevedors (braç dret de la U o la J).

Aquest efecte antiaterogènic del consum moderat de begudes alcohòliques es va atribuir inicialment a canvis en el perfil lipídic, principalment un increment de l'HDL-colesterol, i a efectes sobre la funció hemostàtica, com una reducció

Annexes

de l'agregació plaquetària i un increment de la fibrinolisi. No obstant, més recentment s'han apuntat altres possibles mecanismes com un efecte antiinflamatori sobre la paret arterial, una funció endotelial més eficient i una menor concentració plasmàtica d'homocisteïna en els bevedors moderats (9). Aquest efecte beneficiós no només s'observa en la prevenció primària de la malaltia cardiovascular, sinó també s'observa en persones que han patit un episodi de cardiopatia isquèmica o un accident vascular cerebral (prevenció secundària) (10).

Però existeixen notables discrepàncies a l'hora d'atribuir aquests efectes a l'etanol que contenen aquestes begudes, als seus components no alcohòlics, principalment polifenols, o a ambdós. En altres paraules, existeixen dubtes sobre si hi ha alguna diferència en consumir l'alcohol en forma de vi, cava, cervesa, o begudes alcohòliques d'alta graduació como conyac o whisky.

De totes maneres, són molts els estudis epidemiològics portats a terme a Europa i Estats Units que assenyalen que el vi tindria un efecte cardioprotector addicional superior a la cervesa, licors o destil·lats (11-13). De fet, s'han publicat varies meta-anàlisi sobre els efectes del consum de vi, cervesa i licors sobre el risc cardiovascular (14). Per exemple, després de l'anàlisi conjunt de 13 estudis que incloïen un total de 209.418 subjectes, es va concloure que el risc relatiu de malaltia vascular associat amb el consum de vi era de 0,68 (interval de confiança 95% [IC] 0,59 a 0,77) comparat amb els no bevedors (15). És a dir, el consum moderat de vi reduiria en un 32% la possibilitat de patir una complicació cardiovascular. Els mateixos autors van analitzar altres 15 estudis en els que s'havien inclòs 208.036 subjectes, i van calcular que el risc relatiu del consum moderat de cervesa era de 0,78 (IC 0,70 a 0,86).

L'efecte protector de la cervesa seria, doncs, d'un 22%. Tanmateix, en altres estudis prospectius s'ha comprovat que el consum moderat de begudes alcohòliques de major graduació (licors) també tindria un cert efecte cardioprotector, per la qual cosa alguns investigadors, sobretot d'origen anglosaxó, consideren que gran part dels efectes beneficiosos de les begudes alcohòliques es deu al propi etanol que contenen, per el qual l'efecte protector és independent del tipus de beguda alcohòlica consumida (16).

En aquest sentit, s'han realitzat estudis clínics d'intervenció que han tractat de diferenciar els efectes de l'etanol dels components no alcohòlics del vi sobre diferents paràmetres relacionats amb l'aparició i progressió de l'arteriosclerosi (17-20). Així, a un grup de 40 homes sans, sense factors de risc vascular, se'ls va administrar 30 g d'alcohol en forma de vi negre o de ginebra durant un mes. Com que la ginebra es pràcticament alcohol pur (no conté polifenols), amb aquest disseny es va pretendre separar els efectes de l'alcohol etanol (ginebra) de l'efecte de la mateixa quantitat d'etanol més polifenols (vi negre). Tant el consum moderat de ginebra com de vi va mostrar efectes positius sobre la pressió arterial, lipoproteïnes plasmàtiques i coagulació, però després del consum de vi es va apreciar una major reducció dels paràmetres d'inflamació vascular, especialment de la concentració de molècules d'adhesió i quemoquines que participen en el pas dels monòcits sanguinis a l'interior de l'endoteli vascular, una de les primeres fases del procés arterioscleròtic. D'altra banda, després del consum de ginebra també es van apreciar alguns efectes negatius com una reducció de la concentració sanguínia i eritrocitària d'àcid fòlic i un increment d'algunes molècules d'adhesió. En canvi, després del consum de vi, no es va apreciar cap efecte negatiu. Altres assaigs clínics

realitzats amb altres tipus de vi i cava en homes i dones (19-20) han obtingut resultats similars. Aquest tipus d'estudi ajuda a comprendre els mecanismes mitjançant els quals el vi podria exercir els seus efectes cardioprotectors i, paral·lelament, aporten l'evidència científica necessària per reafirmar les principals conclusions dels estudis epidemiològics realitzats, és a dir, que el consum moderat de vi, que és un dels principals components de la dieta mediterrània, redueix la mortalitat global i molt especialment la mortalitat cardiovascular i la incidència d'infart de miocardi i d'accidents vasculars cerebrals.

Bibliografia

1. Mc Govern, PE Ancient wine: the search for the origins of viniculture. PRINCETON (NJ).Princeton University Press, 2003
2. Donaldson IM. Bon sante: Is wine good for your health? Intern Med J. 2004; 34: 221-3.
3. Guasch-Jané, R; Ibern-Gómez, M.; Andrés-Lacueva, C. ; Jáuregui, O, M. Lamuela-Raventós, R.M. Liquid chromatography with mass spectrometric in tandem mode applied for the identification of wine markers in residues from ancient Egyptian vessels. Anal. Chem.2004, 76, 1672-1677.
4. Guasch-Jané, MR; Andrés-Lacueva, C.; Jáuregui, O.; Lamuela-Raventós, R.M. The origin of the Ancient Egyptian drink shedeh revealed using LC/MS/MS. Journal of Archaeological Science 2006, 33, 98-101
5. Guasch-Jané, M.R.; Andrés-Lacueva, C.; Jáuregui, O.; Lamuela-Raventós, R.M.. First evidence of white wine in ancient Egypt from Tutankhamun's tomb. Journal of Archaeological Science 2006, 33:1075-1080.

6. Yano K, Rhoads GG, Kagan A. Coffee, alcohol and risk of coronary heart disease among Japanese men living in Hawaii. *N Engl J Med*. 1977; 297: 405-9.
7. Kagan A, Yano K, Rhoads GG, McGee DL. Alcohol and cardiovascular disease: the Hawaiian experience. *Circulation*. 1981; 64: III 27-31
8. Klatsky AL, Friedman GD, Siegelaub AB. Alcohol use and cardiovascular disease: the Kaiser-Permanente experience. *Circulation*. 1981; 64: III 32-41.
9. Estruch R, Sacanella E. Alcohol, ¿Tónico o Tóxico Cardiovascular? *Clin Invest Arterioscl*. 2005; 17: 183-95.
10. Muntwyler J, Hennekens CH, Buring JE, Gaziano JM. Mortality and light to moderate alcohol consumption after myocardial infarction. *Lancet*. 1998; 352: 1882-5.
11. Renaud SC, Gueguen R, Siest G, et al. Wine, beer, and mortality in middle-aged men from eastern France. *Arch Intern Med* 1999; 159: 1865–70.
12. Gronbaek M, Becker U, Johansen D, Gottschau A, Schnohr P, Hein HO, Jensen G, Sorensen TI. Type of alcohol consumed and mortality from all causes, coronary heart disease, and cancer. *Ann Intern Med*. 2000; 133: 411-9.
13. Klatsky AL, Friedman GD, Armstrong MA, Kipp H. Wine, liquor, beer, and mortality. *Am J Epidemiol*. 2003; 158: 585-95.
14. Di Castelnuovo A, Costanzo S, Bagnardi V, Donati MB, Iacoviello L, De Gaetano G. Alcohol drinking and total mortality in men and women. An updated meta-analysis of 34 prospective studies. *Arch Intern Med* 2006; 166: 2437-2445.
15. Di Castelnuovo, Rotondo S, Iacoviello L, Donati MB, De Gaetano G. Meta-analysis of wine and beer consumption in relation to vascular risk. *Circulation* 2002; 105: 2836 - 2844.
16. Mukamal KJ, Conigrave KM, Mittlemen MA, et al. Roles of drinking pattern and type of alcohol consumed in coronary heart disease in men. *N Engl J Med* 2003; 348: 109 – 18.

17. Estruch R, Sacanella E, Badia E, Antunez E, Nicolas JM, Fernandez-Sola J, Rotilio D, de Gaetano G, Rubin E, Urbano-Marquez A. Different effects of red wine and gin consumption on inflammatory biomarkers of atherosclerosis: a prospective randomized crossover trial. Effects of wine on inflammatory markers. *Atherosclerosis*. 2004; 175: 117-23.
18. Lamuela-Raventos R M, Andres-Lacueva C. Wine in Mediterranean diet. *Archivos Latinoamericanos de Nutrición*, 2004; 54: 79-82
19. Vázquez-Agell M, Sacanella E, Tobias E, Monagas M, Antúnez E, Zamora-Ros R, Andrés-Lacueva C, Lamuela-Raventós RM, Fernández-Solá J, Nicolás JM, Estruch R. Inflammatory markers of atherosclerosis are decreased after moderate consumption of cava (sparkling wine) in men with low cardiovascular risk. *J Nutr*. 2007;137:2279-84.
20. Sacanella E, Vázquez-Agell M, Mena MP, Antúnez E, Fernández-Solá J, Nicolás JM, Lamuela-Raventós RM, Ros E, Estruch R. Down-regulation of adhesion molecules and other inflammatory biomarkers after moderate wine consumption in healthy women: a randomized trial. *Am J Clin Nutr*. 2007;86:1463-9.