UNIVERSIDAD DE GRANADA

FACULTAD DE CIENCIAS

Departamento de Química Analítica

Grupo de Investigación FQM-297 “Control Analítico, Ambiental, Bioquímico y Alimentario”

Centro Tecnológico de Investigación y Desarrollo del Alimento Funcional (CIDAF)

AN ANALYTICAL APPROACH: FROM NATURAL SOURCES OF BIOACTIVE COMPOUNDS TO NUTRACEUTICALS AND FUNCTIONAL FOODS. DEVELOPMENT OF EXTRACTION, CHARACTERIZATION AND BIOACTIVITY EVALUATION STRATEGIES

Memoria presentada por

Celia Rodríguez Pérez

Para optar al grado de

Doctor Internacional en Química por la Universidad de Granada

Granada, Junio de 2016

LOS DIRECTORES DE LA TESIS

Dr. Antonio Segura Carretero

Dra. Rosa María Quirantes Piné

Editor: Universidad de Granada. Tesis Doctorales
Autora: Celia Rodríguez Pérez
ISBN: 978-84-9125-991-6
URI: http://hdl.handle.net/10481/44074

Esta Tesis doctoral ha sido realizada gracias a una beca predoctoral de Formación de Profesorado Universitario (FPU) concedida por el Ministerio de Educación (AP2010-1551) y a la financiación con cargo a fondos del Centro Tecnológico de Investigación y Desarrollo del Alimento Funcional (CIDAF) procedentes de diferentes proyectos, contratos y subvenciones de las Administraciones Central y Autonómica, Plan Propio de Investigación de la Universidad de Granada, así como de empresas interesadas en los resultados de investigación.

AN ANALYTICAL APPROACH: FROM NATURAL SOURCES OF BIOACTIVE COMPOUNDS TO NUTRACEUTICALS AND FUNCTIONAL FOODS. DEVELOPMENT OF EXTRACTION, CHARACTERIZATION AND BIOACTIVITY EVALUATION STRATEGIES

POR

Celia Rodríguez Pérez

Granada, Junio, 2016

Fdo. Dr. Antonio Segura Carretero

Fdo. Dra. Rosa María Quirantes Piné

Memoria para optar al grado de Doctor Internacional en Química por la Universidad de Granada.

Fdo. Celia Rodríguez Pérez

El Prof. Dr. Antonio Segura Carretero, Catedrático en el Departamento de Química Analítica de la Universidad de Granada y Coordinador de Investigación en el Centro Tecnológico de Investigación y Desarrollo del Alimento Funcional (CIDAF),

CERTIFICA:

Que el trabajo realizado en la presente Tesis doctoral titulada: "**AN ANALYTICAL APPROACH: FROM NATURAL SOURCES OF BIOACTIVE COMPOUNDS TO NUTRACEUTICALS AND FUNCTIONAL FOODS. DEVELOPMENT OF EXTRACTION, CHARACTERIZATION AND BIOACTIVITY EVALUATION STRATEGIES",** se ha realizado bajo su dirección y la de la Dra. Rosa María Quirantes Piné, en los laboratorios del Departamento de Química Analítica de la Universidad de Granada, del Centro Tecnológico de Investigación y Desarrollo del Alimento Funcional (Parque Tecnológico de la Salud), así como también de manera parcial en las instalaciones del Richardson Centre for Functional Foods and Nutraceuticals (Manitoba, Canadá) y en los laboratorios del Instituto de Investigación en Ciencias de la Alimentación (CIAL) (Madrid, España) perteneciente al Consejo Superior de Investigaciones Científicas (CSIC), reuniendo todos los requisitos legales, académicos y científicos para hacer que la doctoranda Dña. Celia Rodríguez Pérez pueda optar al grado de Doctor Internacional en Química por la Universidad de Granada.

Y para que así conste, expido y firmo el presente certificado en Granada a 25 de Abril de 2016.

La doctoranda Celia Rodríguez Pérez y los directores de la Tesis Dr. Antonio Segura Carretero y Dra. Rosa María Quirantes Piné, garantizamos al firmar esta Tesis doctoral que el trabajo ha sido realizado por la doctoranda bajo nuestra dirección y hasta donde nuestro conocimiento alcanza, durante la realización del trabajo, se han respetado los derechos de otros autores al ser citados cuando se han utilizado sus resultados o publicaciones.

Granada a 25 de Abril de 2016

Directores de la Tesis

Fdo.: Dr. Antonio Segura Carretero Fdo.: Dra. Rosa María Quirantes Piné

Doctoranda

Fdo.: Celia Rodríguez Pérez

Agradecimientos

Siempre pensé que esta parte sería la más fácil de escribir y por ello decidí escribirla una vez terminada la tesis. Y ahora, aquí estoy frente a un folio en blanco, intentando dejar que fluyan todos los sentimientos que como estrellas fugaces se cruzan en mi cabeza. Sin embargo, son tantas las personas que han pasado por mi vida durante estos últimos años y tantas las cosas que tengo que agradecer, que se me hace cuesta arriba encontrar la mejor forma de hacerlo. Vayamos por partes…

Para empezar, me gustaría agradecer a mis directores de Tesis, Antonio Segura Carretero y Rosa Quirantes Piné, el esfuerzo y dedicación que han puesto en mí durante estos años. Antonio, gracias por darme la oportunidad de trabajar en este grupo de investigación, por confiar en mí y en mis capacidades desde el principio, por orientarme y por guiarme en este mundo tan complicado como es el mundo de la investigación. Rosa, gracias por estar a mi lado en todo momento, especialmente agradecerte esos ratitos en el laboratorio en festivos y fines de semana en los que no tenías que haber estado pero estuviste, por enseñarme la mayor parte de las cosas que sé de química analítica, por tu paciencia y tus consejos. Siempre dije que tuve suerte de tenerte como codirectora y lo mantengo. La verdad es que sin vosotros, Antonio y Rosa, esto no habría sido posible.

Agradecerte especialmente a ti, Alberto, el que me abrieras las puertas del grupo de investigación en el que tanto he aprendido.

Agradecer, como no, a mis compañeros y amigos de viaje, a los que se fueron y a los que están, por esos buenos ratitos que hemos pasado juntos y por las risas, los enfados y las lágrimas, pero sobretodo por las risas. Nunca olvidaré los congresos de Sitges, Londres y Serbia, no sólo académicamente hablando sino porque fueron la excusa perfecta para conocer un poquito mejor a algunas de mis compañeras, hoy amigas como son Patri, Ceci y Aranxa (o Arantxa o Arantxzazu o como se escriba. Axa, para mí). Con vosotras he pasado algunos de los mejores momentos de estos años. Gracias por saber escuchar, por estar ahí cuando lo he necesitado, por vuestra ayuda y por las risas, siempre las risas. No se me olvidará lo que pudimos reír el día

que fuimos a comprar los mecheros para desatascar el tapón del matraz o la risa al usar el traductor de google después de estar en el lab más de 10 horas sin parar de escribir aquel artículo…culumbeleeeeee. El lab no es lo mismo sin vosotras…

Aunque con vosotros no he tenido la suerte de estar de congreso, he vivido tantos buenos momentos (barbacoas, desayunos, excursiones, tapillas… y por supuesto muchas horas en el laboratorio, que conste) que difícilmente podré olvidar. Nass, echo de menos tus ocurrencias y espontaneidad, tu sonrisa y tu alegría. Vuelve! Mar, siempre dispuesta a ayudar…gracias por compartir tus conocimientos con los demás sin esperar nada a cambio. Eres una de las mejores científicas que he conocido y mejor persona. Hakim, mi niño, gracias por cederme parte de tu positividad, por preocuparte por mí y ayudarme siempre que lo he necesitado. Siempre tendrás las puertas de mi casa abiertas. Eli, mi vasca favorita, te conocí tarde pero dicen que más vale tarde que nunca, no? Gracias por transmitirme tu energía y carácter, por decir las cosas como son y por estar, simplemente por estar (ya tú sabes). Lidia, gracias por tu disposición para todo, para lo bueno y para lo malo. Hemos compartido muy buenos momentos, la mayoría fuera del lab…y los que nos quedan, eh?. Franxu69, gracias esos ricos desayunos, por tu generosidad y tus sonrisas. El lab está mucho más silencioso desde que marchaste (literalmente, jejeje). Sandra, especialmente agradecerte tu sonrisa cada día. Me encanta la ilusión que le pones a todo y tus ganas de aprender…también tus ganas inagotables de fiesta (ya se te pasarán, ya…que tú también te haces mayor aunque no lo quieras creer…jajaja). Pequeña Ana, admiro tu fuerza y dedicación. Con la peque debe de ser difícil y aún así, ahí estás. Queda pendiente el viaje a Sudáfrica. Álvaro, no paras chiquillo…siempre serás el pequeño del grupo, pero te querremos igual. Gracias por transmitirme tu afán de superación y por tus ánimos. Cristina, me encanta la visión que tienes del mundo. Gracias por ponerme los pies en la tierra y por tus consejos. Luzia, gracias por estar para todo, desde rescatar perritos de la calle hasta para instalarme un programa en el ordenador. Vales mucho y eso nadie lo puede negar. No podré olvidar ese primer verano trabajando hasta las mil contigo, esos días en los que Salva nos tenía esclavizadas pero aún así él, Jesús, Rosa, tú y yo aprovechábamos

la mínima oportunidad para escaparnos a tomar cafés infinitos o irnos de fiesta (ahora que los jefes no nos oyen). Isa, no tendrías que haberte ido de vacaciones! Fueron días muy bonitos… Ana, Vito y David gracias por echar una mano siempre que se os necesita, en especial gracias a vosotros, pareja, por compartir conmigo tantos buenos momentos. Javier, gracias por preocuparte en los momentos difíciles. A las nuevas incorporaciones Rafa, Carlos y Jose Antonio agradeceros vuestra disposición, simpatía y ganas de ayudar.

Por supuesto agradecer a todas las personas que han venido de estancia por la huella que han dejado en mí. Gracias por enriquecerme cultural e ideológicamente, por enseñarme a ver la vida con más perspectiva y por hacer mi mundo más amplio. Gracias Ameni, Cristiano, Carol, Andreia, Ihsan, Dianita, Xiaoqin, Geovanni, Reham, Sonda, Estefanía, Inés, Nataša e Ibrahim, espero no dejarme a nadie, aunque con esta memoria que tengo…no me extrañaría nada…

-- *De todos vosotros he aprendido algo que me ha hecho crecer como persona, así que una vez más, gracias por ser como sois* --

Por supuesto tengo que agradecer a Elena Ibáñez, del grupo de investigación Foodomics del Instituto de Investigación en Ciencias de la Alimentación (CIAL) de Madrid el haberme dado la oportunidad de trabajar con ella, así como agradecer tanto a ella como al grupo "Fiestomics" la gran acogida y todas las facilidades que me proporcionaron durante mi estancia. Especialmente agradecer a Jose y a Bienve que me enseñaran tantas cosas de extracción, su dedicación absoluta y toda la ayuda que me han ofrecido con la simpatía que les caracteriza. A ellos y a mis "losers" favoritos, especialmente Lidia y Andrea por esas cervecitas ricas después de trabajar. Gracias a todos vosotros mi estancia fue inmejorable, incluso teniendo la rodilla pocha. Queda pendiente otro partido de fútbol…

I would like also thank Dr. Jones for giving to me the great opportunity to do a stay in the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN), Manitoba, Canada and Vanu Ramkumar for being my supervisor during that period. This stay gave me the opportunity to know other ways to work, to make contact with

the clinical trials' world and to learn about other culture. I want also thank all my colleagues for their help, especially David, Ali, Katima and Dr. A (or Momo…hehehe). Without you, my work would not been possible. Paquitas (Ale y Sandra), cómo iba a olvidarme de vosotras weeyy? Lo mejor de mi estancia en Canadá. Sin vosotras ese período no habría sido ni la mitad de bueno de lo que fue. Hacer amigas en tres meses es posible y después de dos años, aquí estamos…Gracias por todo. Queda menos para vernos por México. Gracias a ti, Raúl por estar en los buenos y malos momentos, gracias por tu amistad que también durará para siempre.

Gracias a Jimmy y a Herminia del departamento de Nutrición por ser quienes me ayudaron a dar mis primeros pasos en el mundo de la investigación. Jimmy, no olvidaré toda la ayuda que me brindaste cuando entré allí con mi beca de colaboración. Gracias a los dos y al resto de profesores que lo hicieron posible.

No quiero dejar pasar la oportunidad de agradecer también a mis niñas, mis amigas de siempre Ana, María y Rocío por estar siempre a las duras y a las maduras, por ser capaces de sacarme una sonrisa incluso el día más gris. Ramón, hemos pasado por esto juntos y a la vez, has sido un gran apoyo para mí. Suerte tuve de conocerte en Italia. Bonitas casualidades de la vida…

No podría terminar de otra manera los agradecimientos, sino mencionando a mi maravillosa familia, el pilar de mi vida. Gracias a todos, especialmente gracias papá y mamá por educarme en vuestros valores, por transmitirme parte de vuestra fuerza de voluntad, vuestro coraje y vuestro carácter. Sin eso, sin vosotros, no habría llegado a donde hoy me encuentro. Y por supuesto, gracias a ti, Jose Ángel, gracias por complementarme como lo haces, por las (des)horas de skype, por tu paciencia, tus risas y tu ayuda infinita, sobretodo en esta última etapa. Estos últimos meses han sido una locura pero…nadie dijo que fuera fácil. Aunque, no lo he llevado tan mal, ¿no? jajaja.

-- ***Dicen que la familia no se escoge, te toca. Pero si tuviera que elegir, elegiría la mía una y mil veces*** --

Defiende tu derecho a pensar, porque
incluso pensar de manera errónea es mejor
que no pensar en absoluto.

-Hipatia de Alejandría-

TABLE OF CONTENTS

LIST OF ABBREVIATIONS **23**

LIST OF FIGURES **27**

OBJECTIVES/OBJETIVOS **29**

- **Objectives** **31**
- **Objetivos** **33**

SUMMARY/RESUMEN **35**

- **Summary** **37**
- **Resumen** **41**

INTRODUCTION **47**

- **Nutraceutical and functional food development** **49**
  - **1.1. Current status of functional foods and nutraceuticals** **50**
  - **1.2. Workflow in nutraceutical and functional food development** **55**
- **Bioactive compounds from natural sources and health** **59**
  - **2.1. Phenolic compounds** **61**
  - **2.2. Phytosterols** **68**
  - **2.3. Fatty acids** **71**
- **Extraction of bioactive compounds** **78**
  - **3.1. Extraction of bioactive compounds from plant sources** **78**
    - 3.1.1. Conventional extraction 80
    - 3.1.2. Ultrasound-assisted extraction (UAE) 83
    - 3.1.3. Supercritical fluid extraction (SFE) 85
    - 3.1.4. Pressurized liquid extraction (PLE) 89
    - 3.1.5. Microwave-assisted extraction (MAE) 92
  - **3.2. Extraction of bioactive compounds from biological matrices** **94**
- **Analytical tools for characterizing and quantifying bioactive compounds** **95**
  - 4.1. Chromatographic techniques **95**
    - 4.1.1. High-performance liquid chromatography 96
      - a) HPLC coupled to UV-Vis detector 99
      - b) HPLC coupled to mass spectrometry 100
        - Quadrupole-time-of-flight (QTOF) mass analyzer 102
    - 4.1.2. Gas chromatography 103
      - a) GC coupled to flame-ionization detector (GC-FID) 106
      - b) GC coupled to a quadrupole mass analyzer 107
  - **4.2. Spectrophotometric techniques** **109**
    - 4.2.1. Total phenolic content (TPC) 109

4.2.2. Total flavonoid content (TFC) ... 110
4.2.3. Dimethylaminocinnamaldehyde (DMAC) assay ... 111
4.2.4. Enzyme-linked immunosorbent assay (ELISA) ... 112

❖ **Fractionation and purification of bioactive compounds** 114

❖ **Methodologies for determining the bioactivity** 115

6.1. *In vitro* evaluation of antioxidant activity ... 115

6.1.1. 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH•) assay ... 115
6.1.2. Trolox equivalent antioxidant capacity (TEAC) ... 117

6.2. *In vitro* evaluation of antibacterial activity ... 118

❖ **Plant-based matrices under study** 120

7.1. Natural sources of bioactive compounds ... 121

7.1.1. *Pistacia lentiscus* ... 121
7.1.2. *Cucumis melo* ... 122
7.1.3. *Moringa oleifera* ... 124

7.2. Nutraceuticals and functional foods ... 125

7.2.1. *Vaccinium macrocarpon* ... 125
7.2.2. Canola oil ... 127

EXPERIMENTAL PART. RESULTS AND DISCUSSION ... 129

Section I. "The search for new sources of bioactive compounds" 131

Chapter 1 ... **133**

Optimization of extraction method to obtain a phenolic compounds-rich extract from *Moringa oleifera* Lam leaves.

Chapter 2 ... **165**

Optimization of microwave assisted extraction and pressurized liquid extraction of phenolic compounds from *Moringa oleifera* leaves by multi-response surface methodology.

Chapter 3 ... **197**

Green downstream processing using supercritical fluid, CO_2-expanded ethanol and pressurized hot water extractions for recovering bioactive compounds from *Moringa oleifera* leaves.

Chapter 4 ... **237**

A metabolite-profiling approach allows the identification of new compounds from *Pistacia lentiscus* leaves.

Chapter 5 ... **267**

Comparative characterization of phenolic and other polar compounds in Spanish melon cultivars by using high-performance liquid chromatography coupled to electrospray ionization quadrupole-time of flight mass spectrometry.

Section II. "Stability, in vitro and in vivo bioactivity evaluation of nutraceuticals and functional foods" 299

Chapter 6 .... **301**

Assessment of the stability of proanthocyanidins and other phenolic compounds in cranberry syrup after gamma-irradiation treatment and during storage.

Chapter 7 .... **329**

Antibacterial activity of isolated phenolic compounds from cranberry (*Vaccinium macrocarpon*) against *Escherichia coli.*

Chapter 8 .... **359**

Docosahexaenoic acid attenuates cardiovascular risk factors via a decline in proprotein convertase subtilisin/kexin type 9 (PCSK9) plasma levels.

CONCLUSIONS/CONCLUSIONES 385

- ❖ **Conclusions 387**
- ❖ **Conclusiones 390**

LIST OF ABBREVIATIONS

ABTS	2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
AFSSA	Agence Française de Securité Sanitaire des Aliments
ALA	α-Linolenic acid
ANOVA	One-way analysis of variance
AOH	Antioxidant compound
Apo A	Apolipoprotein A
Apo B	Apolipoprotein B
ASE	Accelerated solvent extraction
BPC	Base peak chromatogram
CE	Capillary electrophoresis
CE	Conventional extraction
CH	Carbohydrates
COMIT	Canola Oil Multicenter Intervention Trial
CV	Coefficient of variation
CVD	Cardiovascular disease
DAD	Diode array detector
DHA	Docosahexaenoic acid
DMAC	Dimethylaminocinnamaldehyde
DPPH	2,2'-diphenyl-1-picrylhydrazyl radical
EFSA	European Food Safety Authority
EI	Electron impact
ELISA	Enzyme-linked immunosorbent assay
EMEA	Committee for Human Medicinal Products
EPA	Eicosapentaenoic acid
ESI	Electrospray ionization
FAO	Food & Agriculture Organization of the United Nations
FID	Flame ionization detector
FINUT	Fundación Iberoamericana de Nutrición
FOSHU	Foods for Specified Health Use
FUFOSE	European Commission Concerted Action on Functional Food Science in Europe
GC	Gas chromatography
GRAS	Generally recognized as safe
GXL	Gas-expanded liquid
HDL	High density lipoprotein
HPLC	High-performance liquid chromatography
HSCCC	High-speed countercurrent chromatography

ILSI	International Life Science Institute
I.S.	Internal standard
IT	Ion trap
LA	Linoleic acid
LDL	Low density lipoprotein
LDLR	LDL-receptor
LLE	Liquid-liquid extraction
LOD	Limits of detection
LOQ	Limits of quantification
m/z	Mass-to-charge ratio
MAE	Microwave-assisted extraction
MAGRAMA	Ministerio de Agricultura, Alimentación y Medio Ambiente
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MUFAs	Monounsaturated fatty acids
NGO	Non-Governmental Organization
NMR	Nuclear magnetic resonance
OA	Oleic acid
OD	Optical density
PACs	Proanthocyanidins
PBS	Phosphate buffer saline
PCA	Principal-component analysis
PCSK9	Proprotein convertase subtilisin/kexin type 9
PHWE	Pressurized hot water extraction
PLE	Pressurized liquid extraction
PPAR	Peroxisome proliferator-activated receptor
Prep-HPLC	Preparative high performance liquid chromatography
PS	Plant sterols
PT	Proteins
PUFAs	Polyunsaturated fatty acids
Q	Quadrupole
QTOF	Quadrupole-time-of-flight
RH	Relative humidity
ROS	Reactive oxygen species
RP	Reversed-phase
RSD	Relative standard deviation
RSM	Response surface methodology
RT	Retention time
SAS	Statistical analysis software

SAT	Salt aggregation test
SD	Standard deviation
SE	Soxhlet extraction
Semiprep-HPLC	Semipreparative high performance liquid chromatography
SFE	Supercritical fluid extraction
SLE	Solid-liquid extraction
S/N	Signal-to-noise ratio
SOD	Superoxide dismutase
SPE	Solid-phase extraction
SPSS	Statistical package for the social sciences
SREBP	Response element-binding proteins
TAG	Triglycerides
TC	Total cholesterol
TEAC	Trolox Equivalent Antioxidant Capacity
TF	Total flavonoids content
TM	Tube method
TOF	Time-of-flight
TPC	Total phenolic content
TSB	Tryptic soy broth
UAE	Ultrasound assisted extraction
UPEC	Urophatogenic strains of *Escherichia coli*
USDA	United States Department of Agriculture
UTIs	Urinary tract infections
UV-Vis	Ultraviolet-Visible
VLDL	Very low-density lipoprotein
WHO	World Health Organization

LIST OF FIGURES

Figure 1. Functional food products on the European market in 2004 51
Figure 2. The difference between a drug and nutraceutical. 53
Figure 3. The market forecast from 2015 to 2025. 54
Figure 4. Workflow in nutraceutical and functional foods development 56
Figure 5. Ingredients with polyphenols associated to different claims....................... 57
Figure 6. Some bioactive compounds from foods, classified according to their chemical nature 60
Figure 7. General pathways for production of secondary metabolites. 62
Figure 8. Classification of phenolic compounds.. 63
Figure 9. Mechanism of action of natural antioxidants against oxidative stress. 65
Figure 10. Biosynthesis of phytosterols. 69
Figure 11. Cholesterol, plant sterol and plant stanol structures. 69
Figure 12. Simplified mechanism of action of phytosterols. 70
Figure 13. The general biochemical pathway of synthesis of PUFAs in plants 71
Figure 14. Some of the most representative fatty acids in the diet. 73
Figure 15. Mechanism of action of oleic acid... 75
Figure 16. Nuclear mechanism of DHA 77
Figure 17. LDL-C metabolism in the presence of PCSK9. 78
Figure 18. Types of extraction techniques used for the extraction of bioactive compounds. 80
Figure 19. Schematic diagram of the equipment used to perform supercritical fluid extractions. 86
Figure 20. Schematic equipment used to perform pressurized liquid extractions. 90
Figure 21. Main types of compounds extracted by PLE from plant sources, and an example of each.. 90
Figure 22. Schematic representation of yield *vs.* time in extraction processes. 93
Figure 23. HPLC instrumentation. 96
Figure 24. The most widely used detection systems in liquid chromatography. 99
Figure 25. Schematic representation of a mass spectrometer 101

Figure 26. Schematic diagram of an ESI source. 101
Figure 27. Schematic representation of a QTOF mass spectrometer. 103
Figure 28. Scheme of a gas chromatograph. 104
Figure 29. Scheme of a flame-ionization detector. 106
Figure 30. Scheme of a quadrupole mass analyzer. 108
Figure 31. Scheme of a GC-MS with EI ionization and quadrupole mass analyzer. 108
Figure 32. Product of the DMAC reaction with the flavan-3-ol terminal unit of proanthocyanidins. 112
Figure 33. ELISA performance. 113
Figure 34. Reaction of the stable radical DPPH with an antiradical compound (RH). 116
Figure 35. Oxidation reaction of ABTS by potassium persulfate to generate $ABTS^{\bullet+}$ and its reaction with an antioxidant compound (AOH). 117
Figure 36. Scheme of bacterial pathogenesis. 119

Objectives/Objetivos

❖ OBJECTIVES

The scientific and cultural development of our society has caused notable changes in dietary habits. In this regard, consumers are aware of the diet-health relationship which is reflected in the higher demand of products capable of preventing or alleviating different diseases. Therefore, the search for bioactive compounds from natural sources and the evaluation of their bioactivity are being increasingly undertaken with the aim of developing nutraceuticals and functional foods to help consumers maintain or improve their quality of life. For this goal, it is necessary to optimize extraction techniques and analytical methods which enable the production and characterization of extracts rich in bioactive compounds, but it is also necessary to use different assays to evaluate their bioactivity.

Therefore, the **overall goal** of this Thesis is:

- To explore different steps of the nutraceutical and functional food development such as the use of different extraction techniques that enable the production of extracts rich in bioactive compounds, their qualitative and quantitative characterization by advanced analytical techniques, and also the assessment of the stability and the *in vitro* bioactivity of nutraceuticals and the *in vivo* bioactivity of functional foods.

For this objective, the following **specific objectives** were established:

✓ To optimize and to compare the conventional and ultrasound-assisted extraction systems to produce an extract rich in phenolic compounds from *Moringa oleifera* Lam leaves.

✓ To optimize, by means of multi-response surface methodology, the extraction of phenolic compounds from *Moringa oleifera* leaves using the microwave-assisted and pressurized liquid extraction systems, as well as to characterize by high-performance liquid chromatography coupled to quadrupole-time-of-flight

mass spectrometry (HPLC-ESI-QTOF-MS) and to evaluate the antioxidant activity of the extracts produced under optimal conditions.

- ✓ To develop and to optimize a green platform based on a three-step downstream processing using pressurized fluid extraction, gas-expanded liquid and pressurized hot-water extraction to produce different fractions rich is bioactive compounds from an extract of *Moringa oleifera* leaves for their subsequent characterization by HPLC-ESI-QTOF-MS and GC-MS.
- ✓ To optimize and validate analytical methods through HPLC-DAD-QTOF-MS to study the metabolite-profiling from *Pistacia lentiscus* leaves to ascertain in depth its qualitative and quantitative composition.
- ✓ To compare different melon varieties (Galia, Cantaloupe, and Piel de Sapo) by multivariate statistical analysis methods such as principal component analysis (PCA) using the chromatographic profile.
- ✓ To evaluate the stability of phenolic compounds from a cranberry syrup during the storage and after applying a preservative method.
- ✓ To study the *in vitro* antibacterial activity of fractions isolated from commercial capsules of cranberry against *Escherichia coli*.
- ✓ To test the *in vivo* impact of the intake of different fatty-acid-enriched canola oils to the sterol and PCSK9 plasma concentration in volunteers with metabolic syndrome.

❖ OBJETIVOS

El desarrollo científico y cultural de nuestra sociedad ha dado lugar a cambios importantes en los hábitos alimentarios. En este sentido, los consumidores cada vez son más conscientes de la relación dieta-salud, la cual queda reflejada en el aumento de la demanda de productos que contribuyan a la prevención o mejora de enfermedades. Es por ello que tanto la búsqueda de compuestos bioactivos procedentes de matrices vegetales naturales como la evaluación de su bioactividad están siendo cada vez más estudiadas a fin de desarrollar nutracéuticos y alimentos funcionales que ayuden al consumidor a mantener o mejorar su calidad de vida. Para conseguirlo, es necesario llevar a cabo la optimización tanto de sistemas de extracción como de métodos analíticos que permitan obtener extractos ricos en compuestos bioactivos y caracterizarlos, como el empleo de ensayos para evaluar la bioactividad de los mismos.

Por ello, el **objetivo principal** que se pretenden conseguir con la presente Tesis Doctoral es:

- Explorar diferentes etapas del proceso de desarrollo de nutracéuticos y alimentos funcionales como son: el uso de diferentes técnicas de extracción que permitan conseguir extractos ricos en compuestos bioactivos, la caracterización cualitativa y cuantitativa de los extractos obtenidos mediante técnicas analíticas avanzadas así como el estudio de la estabilidad y bioactividad *in vitro* de nutracéuticos y la bioactividad *in vivo* de alimentos funcionales.

Para la consecución del objetivo principal, se establecieron una serie de **objetivos específicos** que se presentan a continuación:

- ✓ Optimizar y comparar los sistemas de extracción convencional y asistida por ultrasonidos a fin de conseguir un extracto rico en compuestos fenólicos de hojas de *Moringa oleifera* Lam.

- ✓ Optimizar, mediante el método de superficie multirespuesta, la extracción de compuestos fenólicos de la hoja de *Moringa oleifera* usando la extracción

asistida por microondas y la extracción mediante líquidos presurizados, así como caracterizar mediante cromatografía líquida de alta resolución acoplada a espectrometría de masas mediante analizador tiempo de vuelo (HPLC-ESI-QTOF-MS) y evaluar la capacidad antioxidante de los extractos obtenidos bajo las condiciones óptimas.

- ✓ Desarrollar y optimizar un proceso de extracciones consecutivas limpias hasta agotar la muestra mediante el uso de fluidos supercríticos, seguido de etanol expandido y extracción con agua presurizada que permita obtener diferentes fracciones ricas en compuestos bioactivos de un extracto de hojas de *Moringa oleifera* para su posterior caracterización mediante HPLC-ESI-QTOF-MS y GC-MS.
- ✓ Optimizar y validar métodos analíticos mediante el uso de HPLC-DAD-QTOF-MS para la obtención del perfil metabólico de las hojas de *Pistacia lentiscus* a fin de conocer en profundidad su composición tanto cualitativa como cuantitativa.
- ✓ Comparar la composición de distintas variedades de melón (Galia, Cantaluz y Piel de Sapo) mediante métodos de análisis estadísticos multivariantes como es el análisis de componentes principales (PCA) usando el perfil cromatográfico.
- ✓ Evaluar la estabilidad durante el almacenamiento y tras aplicar un método de conservación, de los compuestos fenólicos en un nutracéutico a base de arándano rojo americano.
- ✓ Estudiar la actividad antibacteriana *in vitro* frente a *Escherichia coli* de fracciones aisladas de cápsulas comerciales a base de arándano rojo americano.
- ✓ Comprobar el impacto *in vivo* del consumo de diferentes aceites de canola enriquecidos con diversos ácidos grasos sobre el metabolismo de los esteroles y la concentración en plasma de PCSK9 en voluntarios con síndrome metabólico.

Summary/Resumen

❖ SUMMARY

The current report encompasses all the results found during work carried out for the PhD Thesis entitled: **"An analytical approach: from natural sources of bioactive compounds to nutraceuticals and functional foods. Development of extraction, characterization, and bioactivity evaluation strategies"**. This Thesis provides data on the extraction and characterization of different plant matrices such as *Moringa oleifera* and *Pistacia lentiscus* leaves, and various melon varieties. In addition, the *in vitro* bioactivity of different nutraceuticals made from cranberry (*Vaccinium macrocarpon*) is evaluated, and the *in vivo* bioactivity of functional foods such as docosahexaenoic-acid-enriched canola oil is assessed.

This work is divided into two main sections: **Introduction** and **Experimental part, results and discussion**.

The first part of the **Introduction** section presents the state of the art of nutraceuticals and functional foods and the workflow for their development, from the search for new sources of bioactive compounds to their marketing. Firstly, bioactive compounds are classified and their health implications are reviewed, focusing mainly on the three target families of bioactive compounds studied in this Thesis: phenolic compounds, plant sterols, and unsaturated fatty acids. Next, conventional (LLE, SLE, SPE) and alternative extraction techniques (UAE, SFE, PLE, and MAE) used for plant matrices and extraction techniques for biological matrices are described. Afterwards, the chromatographic analytical techniques for the separation (HPLC and GC) and detection (UV-Vis, QTOF-MS for HPLC and FID as well as Q-MS for GC) of bioactive compounds and the spectrophotometric methods (TPC, TFC, DMAC, and ELISA assays) for the quantification of bioactive compounds are detailed. After a section dedicated to the fractionation and purification of bioactive compounds, a section is included to describe the methods used to evaluate bioactivity. At the same time, this section is divided into methods for determining antioxidant activity and

methods for evaluating antimicrobial activity. Finally, the plant matrices, nutraceuticals, and functional foods under study are briefly presented.

The **Experimental part, results, and discussion** section is split into two subsections. The first focuses on searching for new sources of bioactive compounds while the second evaluates the *in vitro* and *in vivo* bioactivity of nutraceuticals and functional foods. Specifically, **Subsection I** examines the optimization of extraction systems for producing extracts rich in bioactive compounds from different matrices such as *Moringa oleifera*, *Pistacia lentiscus,* and three *Cucumis melo* varieties (melon) for a subsequent characterization and evaluation of their antioxidant activity. This subsection is divided in 5 chapters. In **Chapter 1**, two extraction systems, namely ultrasound-assisted extraction and conventional solid-liquid extraction, are optimized using different solvents and water-solvent mixtures for producing an extract with the highest total phenolic content from *Moringa oleifera* leaves. The optimal extract was characterized by HPLC–ESI–QTOF–MS, a powerful analytical tool that enabled the tentative characterization of 59 compounds, 30 of which, mainly flavonoids, were tentatively characterized for the first time in this matrix. **Chapter 2** and **Chapter 3** were prepared in collaboration with the Institute of Food Science Research (CIAL-CSIC), Madrid, after a 3-month-long predoctoral mobility fellowship called "Estancias breves FPU". Due to the great potential of moringa leaves, in **Chapter 2,** a comparative analysis was made between two green extraction techniques, i.e. microwave-assisted extraction and pressurized liquid extraction. This time, both extraction methods were optimized through multi-response surface methodology. The parameters studied were temperature, solvent percentage, and extraction time while the extraction yield, total phenolic content, total flavonoid content, and the antioxidant activity measured by TEAC and DPPH assays were considered as response variables. Both extraction systems proved efficient for the extraction of phenolic compounds. However, pressurized liquid extraction allowed the recovery of more polar and temperature-sensitive compounds compared to the microwave-assisted extraction, which was better for the extraction of flavonoids and their glycosylated derivatives.

Continuing with the same matrix, **Chapter 3** approaches the composition of bioactive compounds from different fractions of moringa leaves after developing a green platform based on a three-step downstream processing that includes the use of supercritical fluid extraction, following by gas-expanded liquid (GXL), and ending with pressurized hot-water extraction (PHWE), all previously optimized. The extract by SFE was analyzed by GC–MS while the extract gained by PHWE was analyzed by HPLC–ESI–QTOF–MS. Meanwhile, the extract by GXLs was characterized employing both analytical techniques. The chromatographic analysis revealed that the volatile fraction from moringa leaves was a good source of alcohols, ketones, fatty acids, esters, and alkanes while the mid-polarity fraction and the most polar fraction were rich in glycosylated flavonoids and phenolic acids, respectively. Moreover, the content of total phenolics and total flavonoids was calculated and the antioxidant activity was also evaluated by TEAC assay.

In **Chapter 4**, an analytical method was optimized in order to characterize the leaves of *Pistacia lentiscus*, commonly known as mastic tree, by means of a metabolite-profiling strategy. This chapter was prepared in collaboration with the Mira University, Bejaia, Algeria. The main parameters optimized were the mobile-phase composition, elution gradient, flow rate, and volume of injection to achieve the best resolution and sensitivity by HPLC–ESI–QTOF–MS. The optimized method enabled the tentative identification of 46 compounds, 20 of which were identified for the first time in this matrix. In addition, the quantification revealed flavonoids and phenolic acids as the most abundant in mastic leaves.

Chapter 5 is dedicated to comparing the polar fraction of 14 extracts from 3 varieties of melon (Galia, Cantaloupe, and Piel de Sapo) most consumed in Spain. The HPLC–ESI–QTOF–MS analysis allowed the tentative characterization of amino acids and derivatives, nucleosides, organic acids, phenolic acids, esters, lignans, flavonoids, and other polar compounds. Furthermore, a principal component analysis (PCA) was applied to explore the distribution of the 3 varieties according to their composition, revealing a good differentiation between them.

The **Subsection II** evaluates the stability as well as the *in vitro* and *in vivo* bioactivity of nutraceuticals and functional foods. This subsection is made up of 3 chapters. **Chapters 6** and **7** were prepared in collaboration with the Pediatric Unit of San Cecilio Hospital from Granada and Chapter 7 was also included collaboration with the Microbiology Department from the aforementioned hospital. **Chapter 6** was dedicated to the study of the stability of commercial cranberry syrup irradiated with gamma radiation stored for 6 months at 25°C and 60% relative humidity and under accelerated stability conditions. Compounds were characterized and quantified through HPLC–ESI–QTOF–MS and the proanthocyanidin quantification was also made by DMAC assay, both at the beginning and after 1, 3, and 6 months of storage. The majority of the quantified compounds were stable until 3 months of storage at 25ºC. In **Chapter 7**, 25 fractions from commercial cranberry capsules were isolated by semi-preparative chromatography. All the fractions and the complete extract were characterized by HPLC–ESI–MS and 13 fractions where chosen (the purest one) to assess their *in vitro* effect against a mix of 14 uropathogenic strains of *Escherichia coli* adherence and biofilm formation. The results suggest that apart from proanthocyanidins, other compounds, mainly flavonoids, could have antibacterial effects against uropathogenic *E. coli* by altering the surface hydrophobicity and inhibiting the biofilm formation *in vitro*. Lastly, **Chapter 8** was prepared in collaboration with the "Richardson Centre for Functional Foods and Nutraceuticals" from the University of Manitoba (Canada) with a long-term predoctoral CEI-Biotic mobility fellowship of 3 months. In this chapter, the impact of different canola oils — conventional canola, high oleic canola oil, and high oleic canola oil enriched with docosahexaenoic acid (DHA)— was studied with respect to the sterol metabolism and PCSK9 concentration (a novel proprotein that regulates the cholesterol metabolism discovered in 2003) in plasma from 54 volunteers with at least one symptom of metabolic syndrome. The results demonstrated that the intake of omega-3 could reduce the risk factors associated to cardiovascular disease by diminishing the plasma PCSK9 concentration.

❖ RESUMEN

La presente memoria recoge los resultados obtenidos durante el período de realización de la Tesis doctoral titulada: **"Un enfoque analítico: de fuentes naturales de compuestos bioactivos a nutracéuticos y alimentos funcionales. Desarrollo de estrategias de extracción, caracterización y evaluación de la bioactividad"** en la que se recogen datos de extracción y caracterización de diversas matrices vegetales tales como las hojas de *Moringa oleifera, Pistacia lentiscus* y de diferentes variedades de melón, y la evaluación de la bioactividad *in vitro* de nutracéuticos a base de arándano rojo americano (*Vaccinium macrocarpon*) en diversos formatos de presentación y la bioactividad *in vivo* de alimentos funcionales como es el aceite de canola enriquecido en ácido docosahexaenoico. La memoria está dividida en dos secciones principales: **Introducción** y **Parte experimental, resultados y discusión**.

La primera parte de la **Introducción** presenta el estado del arte de los nutracéuticos y los alimentos funcionales, así como el proceso de trabajo para el desarrollo de los mismos, desde la búsqueda de compuestos bioactivos, hasta su puesta en el mercado. Seguidamente se hace una clasificación de los compuestos bioactivos, haciendo énfasis en las tres familias de compuestos bioactivos estudiadas en esta Tesis, como son los compuestos fenólicos, esteroles vegetales y ácidos grasos insaturados, así como sus implicaciones en la salud. A continuación, se describen por un lado las técnicas de extracción tanto convencionales (LLE, SLE o SPE) como alternativas (UAE, SFE, PLE y MAE) para matrices vegetales y por otro lado los sistemas de extracción para muestras biológicas. Posteriormente se detallan las técnicas analíticas cromatográficas empleadas para la separación (HPLC y GC) y detección (UV-Vis, QTOF-MS para HPLC y FID y Q-MS para GC) de compuestos bioactivos así como los métodos espectrofotométricos (determinación de TPC y TFC, así como ensayos DMAC y ELISA) para la cuantificación de los mismos. Tras un apartado dedicado al fraccionamiento y purificación de compuestos bioactivos, se incluye una sección en la que se describen brevemente los métodos empleados para la evaluación de la bioactividad que, a su vez, están divididos en métodos para

determinar la capacidad antioxidante y métodos para determinar la capacidad antibacteriana. Por último, se presentan brevemente las matrices vegetales, nutracéuticos y alimentos funcionales objeto de estudio en esta memoria.

La **Parte experimental, resultados y discusión** consta de dos bloques temáticos. El primer bloque se centra en la búsqueda de nuevas fuentes de compuestos bioactivos, mientras que el segundo bloque se centra en la evaluación de la estabilidad y la bioactividad *in vitro* e *in vivo* de nutracéuticos y alimentos funcionales. Concretamente, el **Bloque I** se ha enfocado a la optimización de sistemas de extracción que permitan la obtención de extractos ricos en compuestos bioactivos para su posterior caracterización y evaluación de su actividad antioxidante a partir de diferentes matrices vegetales, como son las hojas de *Moringa oleifera*, *Pistacia lentiscus* y tres variedades de *Cucumis melo* (melón). A su vez, este primer bloque se subdivide en cinco capítulos. En el **Capítulo 1** se probaron dos sistemas de extracción como son la extracción convencional sólido-líquido y la extracción asistida por ultrasonidos utilizando diferentes disolventes y diversas mezclas de estos disolventes con agua con el fin de obtener un extracto con la mayor concentración en compuestos fenólicos totales de hojas de *Moringa oleifera*. El extracto óptimo se caracterizó mediante el uso de HPLC–ESI–QTOF–MS, una potente técnica analítica que permitió la identificación tentativa de 59 compuestos de los cuales 30, mayoritariamente flavonoides, nunca antes se habían identificado en esta matriz. El segundo y tercer capítulo (**Capítulo 2** y **Capítulo 3**) se desarrollaron en colaboración con el Centro de Investigación en Ciencias de la Alimentación del Consejo Superior de Investigaciones Científicas de Madrid (CIAL-CSIC) gracias a una beca predoctoral de “Estancias breves FPU” de tres meses de duración. Dado el gran potencial que presentaron las hojas de moringa, en el **Capítulo 2** se realizó una comparativa de técnicas de extracción limpias como son la extracción asistida por microondas y la extracción con fluidos presurizados. En esta ocasión, ambos métodos fueron optimizados mediante superficie multirespuesta en los que los parámetros estudiados fueron la temperatura, el porcentaje de disolvente y el tiempo de extracción, siendo las respuestas el

rendimiento, el contenido total en compuestos fenólicos, el contenido total en flavonoides y la capacidad antioxidante medida mediante los ensayos TEAC y DPPH. Ambos sistemas de extracción demostraron ser eficientes a la hora de extraer compuestos fenólicos. Sin embargo, la extracción con fluidos presurizados permitió una mayor recuperación de los compuestos más polares y sensibles a la temperatura comparada con la extracción asistida por microondas que resultó ser más adecuada para la extracción de flavonoides y sus derivados glicosados. Por su parte y siguiendo con la misma matriz, el **Capítulo 3** recoge la composición en compuestos bioactivos de distintas fracciones de la hoja de moringa tras realizar extracciones sucesivas mediante sistemas de extracción limpios hasta agotar la muestra empezando por el uso de extracción con fluidos supercríticos (SFE), seguida de una extracción mediante líquidos expandidos (GXLs) y terminando con una extracción con agua subcrítica (PHWE), todas ellas previamente optimizadas. El extracto obtenido mediante SFE fue analizado mediante GC–MS, mientras que el obtenido mediante PHWE se analizó mediante HPLC–ESI–QTOF–MS. Por su parte, el extracto generado por GXLs se caracterizó empleando ambas técnicas. Los análisis cromatográficos revelaron que la fracción volátil de las hojas de moringa es fuente de alcoholes, cetonas, ácidos grasos, ésteres y alcanos, mientras que la fracción de polaridad media y la más polar es rica en compuestos tales como flavonoides glicosados y ácidos fenólicos, respectivamente. Además se calculó el contenido en compuestos fenólicos y flavonoides totales y se evaluó la capacidad antioxidante de los extractos mediante el ensayo TEAC.

En el **Capítulo 4** se optimizó un método analítico que permitió la caracterización de las hojas de *Pistacia lentiscus,* comúnmente conocida como lentisco, mediante una estrategia de perfilado metabólico. Este capítulo se llevó a cabo en colaboración con la Universidad de Mira, Bejaia, Argelia. Los principales parámetros optimizados fueron la composición de las fases móviles, el gradiente de separación, el flujo y el volumen de inyección con el fin de conseguir la máxima resolución y sensibilidad mediante HPLC–ESI–QTOF–MS. El método optimizado permitió la identificación tentativa de 46 compuestos, 20 de los cuales fueron

identificados por primera vez en esta matriz. Además se llevó a cabo la cuantificación de los compuestos mayoritarios, siendo los más abundantes los flavonoides y los ácidos fenólicos.

El **Capítulo 5** se dedicó a la comparación de la fracción polar de 14 extractos de las 3 variedades de melón más consumidas en España como son Galia, Cantaloupe o Cantaluz y Piel de Sapo. El análisis mediante HPLC–ESI–QTOF–MS permitió identificar tentativamente aminoácidos y derivados, nucleósidos, ácidos orgánicos, ácidos fenólicos, ésteres, lignanos, flavonoides y otros compuestos polares. Además, se aplicó un análisis de componentes principales (PCA) para explorar la distribución de las 3 variedades según su composición lo que permitió una buena diferenciación entre variedades.

El **Bloque II** está dedicado a la evaluación de la estabilidad y la bioactividad *in vitro* e *in vivo* de nutracéuticos y alimentos funcionales. Este bloque consta de 3 capítulos, los cuales se resumen a continuación. Los **Capítulos 6** y **7** se llevaron a cabo en colaboración con la Unidad de Pediatría del Hospital Clínico San Cecilio de Granada y concretamente el Capítulo 7 también con el Departamento de Microbiología del mismo hospital. El **Capítulo 6** se dedicó al estudio de la estabilidad durante 6 meses de almacenamiento de un jarabe de arándano rojo americano rico en proantocianidinas con capacidad antibacteriana tras irradiación gamma. Se investigó la estabilidad a 25ºC con un valor de humedad relativa del 60% y bajo condiciones aceleradas. Los compuestos fueron caracterizados y cuantificados mediante HPLC–ESI–QTOF–MS y también se llevó a cabo la cuantificación de las proantocianidinas mediante el ensayo DMAC, ambos al inicio y tras 1, 3 y 6 meses de almacenamiento, permitiendo concluir que la mayor parte de los compuestos cuantificados se mantuvieron estables hasta los 3 meses de almacenamiento a 25ºC. Por su parte, en el **Capítulo 7** se aislaron 25 fracciones de cápsulas comerciales de arándano rojo americano mediante cromatografía semipreparativa. Las fracciones y el extracto completo se caracterizaron mediante HPLC–ESI–MS y se eligieron 13 de ellas (por presentar mayor pureza) para probar su efecto *in vitro* sobre la adherencia y la

formación de biofilm de una mezcla de 14 cepas de *Escherichia coli* uropatógena. Los resultados sugirieron que aparte de las proantocianidinas, otros compuestos, principalmente flavonoides, podrían tener efecto antibacteriano frente a *E. coli* uropatógena a través de la modificación de la hidrofobicidad de superficie e inhibiendo la formación de biofilm.

Por último, el **Capítulo 8** fue desarrollado en colaboración con el “Richardson Centre for Functional Foods and Nutraceuticals” de la Universidad de Manitoba (Canadá) gracias a una beca predoctoral del CEI-Biotic a través de una estancia de 3 meses de duración. En este capítulo se estudió el impacto de diferentes aceites de canola: aceite de canola convencional, aceite de canola rico en ácido oleico y aceite de canola alto oleico rico en DHA sobre el metabolismo de los esteroles vegetales y sobre las concentraciones de PCSK9 (una novedosa proproteína descubierta en 2003 que regula el metabolismo del colesterol) en plasma de 54 voluntarios con al menos un síntoma de síndrome metabólico. Los resultados demostraron que el consumo de omega 3 podría disminuir los factores de riesgo de enfermedad cardiovascular a través de la disminución de la concentración de PCSK9 en plasma.

Introduction

❖ NUTRACEUTICAL AND FUNCTIONAL FOOD DEVELOPMENT

Throughout history, humans have relied upon nature for survival. The plant kingdom offers a variety of species still used as remedies for several diseases in many parts of the world[1]. Much research has pointed out that the Neanderthal diet included plants even before the appearance of agriculture and it has also been suggested they had knowledge about nutritional and medicinal properties of some plants[2,3]. In addition, in the fifth century B.C., Hippocrates mentioned over 400 medicinal plants. The Bible also describes 30 healing plants while Arab and Asian cultures have a full pharmacopeia, as reported by Cowan in a notable review[4].

Currently, the interest in nature as a source of potential chemotherapeutic agents is increasing. In fact, some authors have reported that natural products and their derivatives represent more than 50% of all the drugs in clinical use in the world. Higher plants contribute over 25% of the total[5]. Plant-based diets play an important role in health care of many cultures. In fact, the World Health Organization (WHO) estimated that over 65% of world's population relies mainly on plant and food-derived traditional medicines for their primary health care[6]. In addition, diet is the preferred

[1] Brusotti, G., Cesari, I., Dentamaro, A., Caccialanza, G., & Massolini, G. (2014). Isolation and characterization of bioactive compounds from plant resources: The role of analysis in the ethnopharmacological approach. *Journal of Pharmaceutical and Biomedical Analysis*, 87, 218-228.

[2] Sistiaga, A., Mallol, C., Galván, B., & Summons, R.E. (2014). The Neanderthal meal: A new perspective using faecal biomarkers. *PLoS ONE*, 9(6), 1-6.

[3] Buckley, S., Usai, D., Jakob, T., Radini, A., & Hardy, K. (2014). Dental calculus reveals unique insights into food items, cooking and plant processing in prehistoric central Sudan. *PLoS ONE*, 9(7), 1-10.

[4] Cowan, M.M. (1999). Plant products as antimicrobial agents. *Clinical Microbiology Reviews*, 12, 564-582.

[5] Gurib-Fakim, A. (2006). Medicinal plants: Traditions of yesterday and drugs of tomorrow. *Molecular Aspects of Medicine*, 27(1), 1-93.

[6] Gragg, G.M., Grothaus, P.G., & Newman, D.J. (2012). Wiley series in drug discovery and development, 17. In: Plant bioactives and drug discovery: Principles, practice and perspectives, 1-42. John Wiley & Sons, Inc.

treatment option for some conditions, including early type-2 diabetes and hypercholesterolemia[7].

The concept of a healthy diet has been changing over recent years. In the industrialized world, due to new challenges from increased costs of health care, longer life expectancy, new scientific knowledge, and the development of new technologies, consumers' interest in healthy eating is moving food, nutrition and biochemical sciences to design products that promote optimal health and reduce the risk of disease, allowing a higher quality of life. In this context, the development of nutraceuticals and functional foods has emerged as a promising tool for preventing nutrition-related diseases and improve physical and mental well-being of consumers.

1.1. Current status of functional foods and nutraceuticals

Much has changed since the Japanese Minister of health and Welfare established Labeling Regulations for Foods for Specified Health Use (FOSHU) in the 1980s[8]. Today, different definitions of functional foods are being used depending on the country. In fact, Eastern and Western cultures diverge considerably concerning the nature of functional foods[9]. In accordance with the European Commission Concerted Action on Functional Food Science in Europe (FUFOSE) which is coordinated by the International Life Science Institute (ILSI), the working definition of functional foods is "*food which has satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and wellbeing and/or reduction of risk of disease*"[10]. In addition, the European definition emphasizes that functional foods must be consumed as a component of the normal diet, rather than pills or capsules. On this point it differs from the Japanese definition, which since 2001 has included dietary

---

[7] Hooper, L., & Cassidy, A. (2006). A review of the health care potential of bioactive compounds. *Journal of the Science of Food and Agriculture*, 86(12), 1805-1813.
[8] Arai S. (2000). Functional food science in Japan. *Biofactors*, 12: 13-6.
[9] Siró, I., Kápolna, E., Kápolna, B., & Lugasi, A. (2008). Functional food. Product development, marketing and consumer acceptance-A review. *Appetite*, 51(3), 456-467.
[10] Howlett, J. (2008). Functional foods from science to health and claims. ILSI Europe concise monograph series. (http://www.ilsi.org/Europe/Publications/C2008Func_FoodEng.pdf).

supplements in the aforementioned forms to the original definition of FOSHU. The classification of functional foods has been widely described. Briefly, in general terms, it is possible to differentiate natural and processed functional foods, which the latest include: foods to which a component has been added, foods from which a component has been removed, foods where the nature of one or more components has been modified, foods in which the bioavailability of one or more components has been modified or any combination of the aforementioned possibilities[11].

The market of functional foods started growing since the emergence of that term. Currently the United States has the largest market segment, followed by Europe and Japan. **Figure 1** shows the main European market in 2004. Specifically, in Spain it is expected to become about 40% in 2040[9].

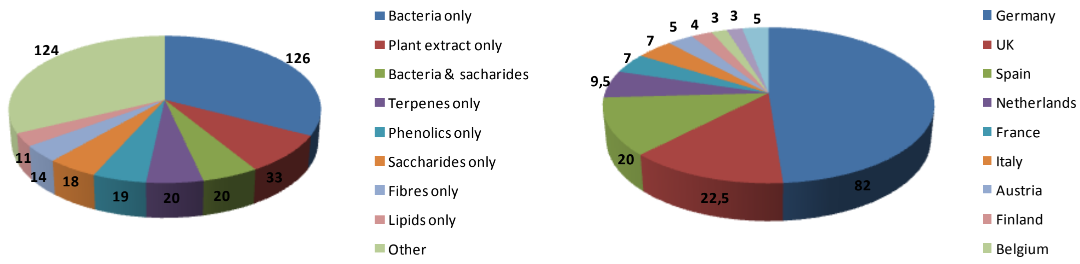


Figure 1. Functional food products on the European market in 2004, by ingredients (left); Number and location of companies on the European market for functional food in 2004 (right). Adapted from Stein *et al.*[12]

On one hand, many foods, mainly plant-based foods, could be considered natural functional foods due to their composition in bioactive compounds. However, some discrepancies exist about the real functionality of many of them and about the target compound responsible for their bioactivity. Despite that a consensus does not exist, many lists of natural functional foods have been proposed[13]. Some examples of

[11] Roberfroid, M.B. (2002). Global view on functional foods: European perspectives. *British Journal of Nutrition*, 88(2), 133-138.
[12] Stein, A.J. & Rodríguez-Cerezo, E. (2010). Functional Food in the European Union IPTS – Institute for Prospective Technological Studies, JRC, Seville, Spain. ISBN. 978-92-79-09071-4.
[13] Silveira Rodríguez, M.B., Monereo Megías, S., & Molina Baena, B. (2003). Functional foods and optimum nutrition: A way or away? *Revista Española de Salud Pública*, 77(3), 317-331.

natural functional foods could be: cranberry, soya, tea, grape, olive oil or cocoa as sources of phenolic compounds; some vegetable oils as sources of phytosterols and olive oil; and blue fish and nuts as a source of unsaturated fatty acids. On the other hand, the commercialized processed functional foods offer more specific information on their functionality. This is true of milk enriched with isoflavones[14]; fat spreads, yogurt, and the so-called tall oil (a by-product of manufacturing wood pulp), all of these being enriched with phytosterols[15-17]; eggs; milk; and infant formulas or oils enriched with n-3 fatty acids[18-20].

Over the last few years, functional foods have been wrongly called nutraceuticals. However, despite that the term nutraceutical has been traditionally defined as "*a food or parts of food that provide medical or health benefits, including the prevention and/or treatment of disease*"[21], in the European Union the main difference with functional foods is that nutraceuticals are presented in pharmaceutical form (pills, tables or capsules) and not as a food *per se* (**Figure 2**).

[14] García-Martín, A., Quesada Charneco, M., Álvarez Guisado, A., Jiménez Moleón, J.J., Fonollá Joya, J., & Muñoz-Torres, M. (2012). Effect of milk product with soy isoflavones on quality of life and bone metabolism in postmenopausal Spanish women: Randomized trial. *Medicina Clinica*, 138(2), 47-51.

[15] Palou Oliver, A., Picó Segura, C., Bonet Piña, M.L., Oliver Vara, P., Serra Vich, F., Rodríguez Guerrero, A.M., & Ribot Riutort, J. (2005), El Libro Blanco de los Esteroles Vegetales en Alimentación, 2 Edición, Unilever Foods S.A, Barcelona, 173 p.

[16] De Los Luis, M.G.J., & Montaña, C.H. (2010). Novel foods: Sterols margarines enriched. Case study: Flora proActiv margarine. *Nutrición Clínica y Dietética Hospitalaria*, 30(1), 35-43.

[17] De Graaf, J., de Sauvage Nolting, P.R.W., Marjel van Dam, E.M.B., Kastelein, J.J.P., Pritchard, P.H., & Stalenhoe, A.F.H. (2002). Consumption of tall oil-derived phytosterols in a chocolate matrix significantly decreases plasma total and low-density lipoprotein-cholesterol levels. *British Journal of Nutrition*, 88, 479-488.

[18] Rahmawaty, S., Lyons-Wall, P., Charlton, K., Batterham, M., & Meyer, B.J. (2014). Effect of replacing bread, egg, milk, and yogurt with equivalent ω-3 enriched foods on ω-3 LCPUFA intake of Australian children. *Nutrition*, 30(11-12), 1337-1343.

[19] López-Huertas, E. (2010). Health effects of oleic acid and long chain omega-3 fatty acids (EPA and DHA) enriched milks. A review of intervention studies. *Pharmacological Research*, 61(3), 200-207.

[20] Lane, K.E., & Derbyshire, E. (2014). Systematic review of omega-3 enriched foods and health. *British Food Journal*, 116(1), 165-179.

[21] DeFelice, S.L. (1995). The nutraceutical revolution: its impact on food industry R&D. *Trends in Food Science and Technology*, 6(2), 59-61.

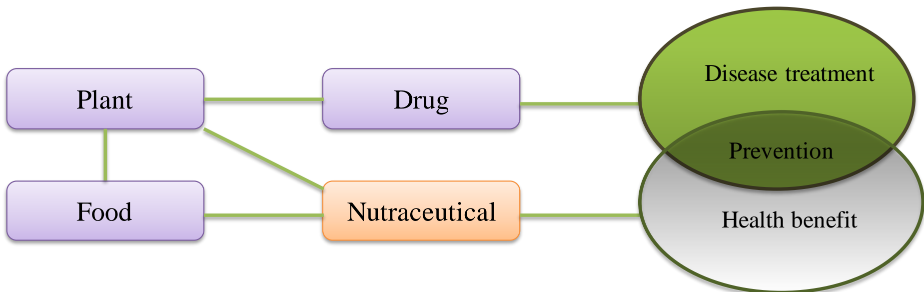


Figure 2. The difference between a drug and nutraceutical. Adapted from Gulati, *et al.*[22]

Since the term nutraceuticals emerged from fusing the words "nutrition" and "pharmaceutical", it is considered as a mid-term between foods and drugs. Nutraceuticals may range from substances with established nutritional functions (such as vitamins, minerals, amino acids, and fatty acids, herbs or botanical products as concentrated or herbal extracts) to chemical agents that are derived from other sources and that provide specific functions[23]. The term nutraceutical also includes dietary supplements.

In 2011, the global nutraceutical market was dominated by the USA (36%), EU (25%), and Japan (22%). In 2010, the best-selling ingredients in the European market were vitamins and minerals, omega-3 fatty acids, amino acids and probiotics[24]. The market forecast from 2015 to 2025 is displayed in **Figure 3**.

[22] Gulati, O.P., & Berry Ottaway, P. (2006). Legislation relating to nutraceuticals in the European Union with a particular focus on botanical-sourced products. *Toxicology*, 221(1), 75-87.

[23] Chauhan, B., Kumar, G., Kalam, N., & Ansari, S.H. (2013). Current concepts and prospects of herbal nutraceutical: A review. *Journal of Advanced Pharmaceutical Technology and Research*, 4(1), 4-8.

[24] Frost & Sullivan. (2011). Global nutraceuticals industry: Investing in healthy living. Frost & Sullivan India Pvt Ltd, 1-50. (www.frost.com/prod/servlet/cio/236145272)

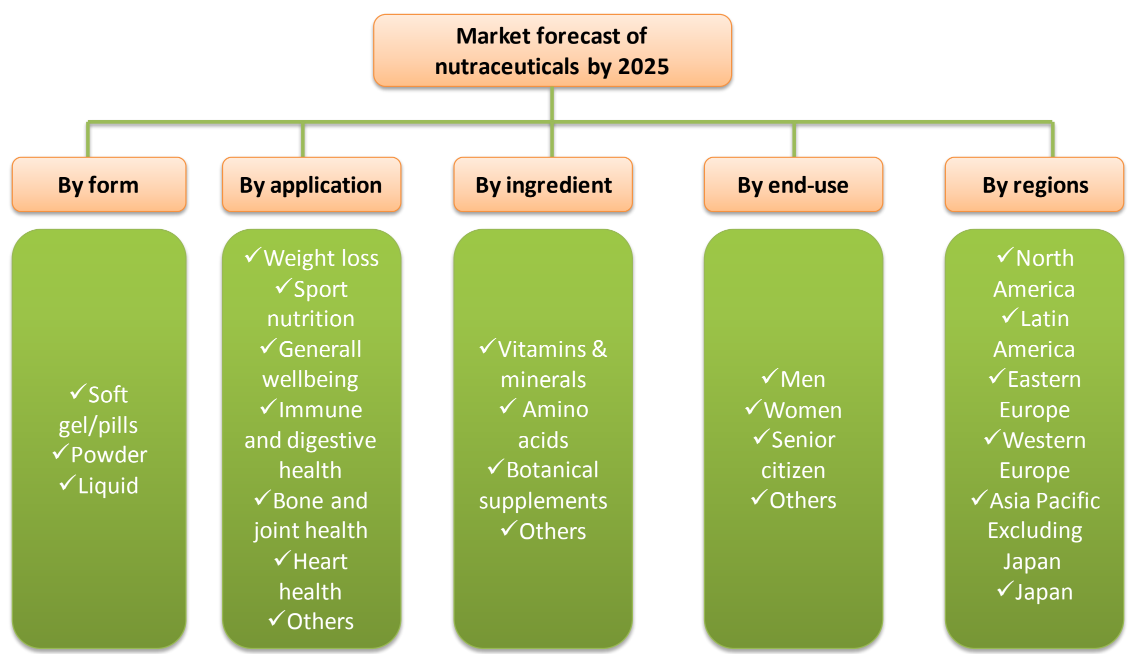


Figure 3. The market forecast from 2015 to 2025. Adapted from Future Market Insights[25]

Many nutraceuticals have been marketed and a large number of publications have focused on the study of the effects of different types of these products. The vast majority of these publications have centered their attention to cardiometabolic risk factors such as hypercholesterolemia, hypertriglyceridemia, metabolic syndrome, diabetes, arterial hypertension, and obesity through nutraceuticals such as omega-3 polyunsaturated fatty acids (PUFAs), cocoa flavonoids, aged garlic extract or resveratrol, among others[26-28]. In addition, there is a good number of *in vitro* studies based on the antibacterial effects of several herbal extracts[29,30], whereas the *in vivo*

[25] Dietary Supplements Market – Driven by increasing demand for healthp: Global industry analysis and Opportunity Assessment 2015-2025. http://www.futuremarketinsights.com/reports/dietary-supplements-market.

[26] Choudhary, M., & Grover, K. (2012). Development of functional food products in relation to obesity. *Functional Foods in Health and Disease*. 2(6), 188- 197.

[27] Volpe, R. & Sotis, G. (2015). Nutraceuticals: Definition and Epidemiological Rationale for Their Use in Clinical Practice. *High Blood Pressure & Cardiovascular Prevention*, 22, 199-201.

[28] Cicero, A.F.G., & Colletti, A. (2015). Nutraceuticals and blood pressure control: Results from clinical trials and meta-analyses. *High Blood Pressure & Cardiovascular Prevention*, 22(3), 203-213.

[29] Nascimento, G.G.F., Locatelli, J., Freitas, P.C., & Silva, G.L. (2000). Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria. *Brazilian Journal of Microbiology*, 31(4), 247-256.

effects of nutraceuticals such cranberry and other plants have been less thoroughly described[31,32].

1.2. Workflow in nutraceutical and functional food development

Food and pharmaceutical companies are aware of the increasing cost of healthcare, the desire of the population to improve their quality of life and the increase in life expectancy and they take advantage of this situation by creating products that help in this sense. **Figure 4** shows an overview of the steps for developing nutraceuticals and functional foods. The initial step for developing nutraceuticals and functional foods is to identify a diet-disease interaction, which means hypothesize how one or more component from food could interact with a function of the body, thus promoting healthy effects. Afterwards, it will be necessary look for the most appropriate sources of compounds of interest (plants, plant-based foods, foods, etc.) and undertake the extraction, characterization, and the study of their stability (these processes will be detailed in the following sections). These first steps usually involve a prior search of the scientific literature.

[30] Su, X., Howell, A.B., & D'Souza, D.H. (2012). Antibacterial effects of plant-derived extracts on methicillin-resistant *Staphylococcus aureus*. *Foodborne Pathogens and Disease*, 9(6), 573-578.

[31] Uberos, J., Rodríguez-Belmonte, R., Rodríguez-Pérez, C., Molina-Oya, M., Blanca-Jover, E., Narbona-López, E., & Muñoz-Hoyos, A. (2015). Phenolic acid content and antiadherence activity in the urine of patients treated with cranberry syrup (*Vaccinium macrocarpon*) vs. trimethoprim for recurrent urinary tract infection. *Journal of Functional Foods*, 18, 608-616.

[32] Ocheng, F., Bwanga, F., Joloba, M., Borg-Karlson, A., Gustafsson, A., & Obua, C. (2014). Antibacterial activities of extracts from Ugandan medicinal plants used for oral care. *Journal of Ethnopharmacology*, 155(1), 852-855.

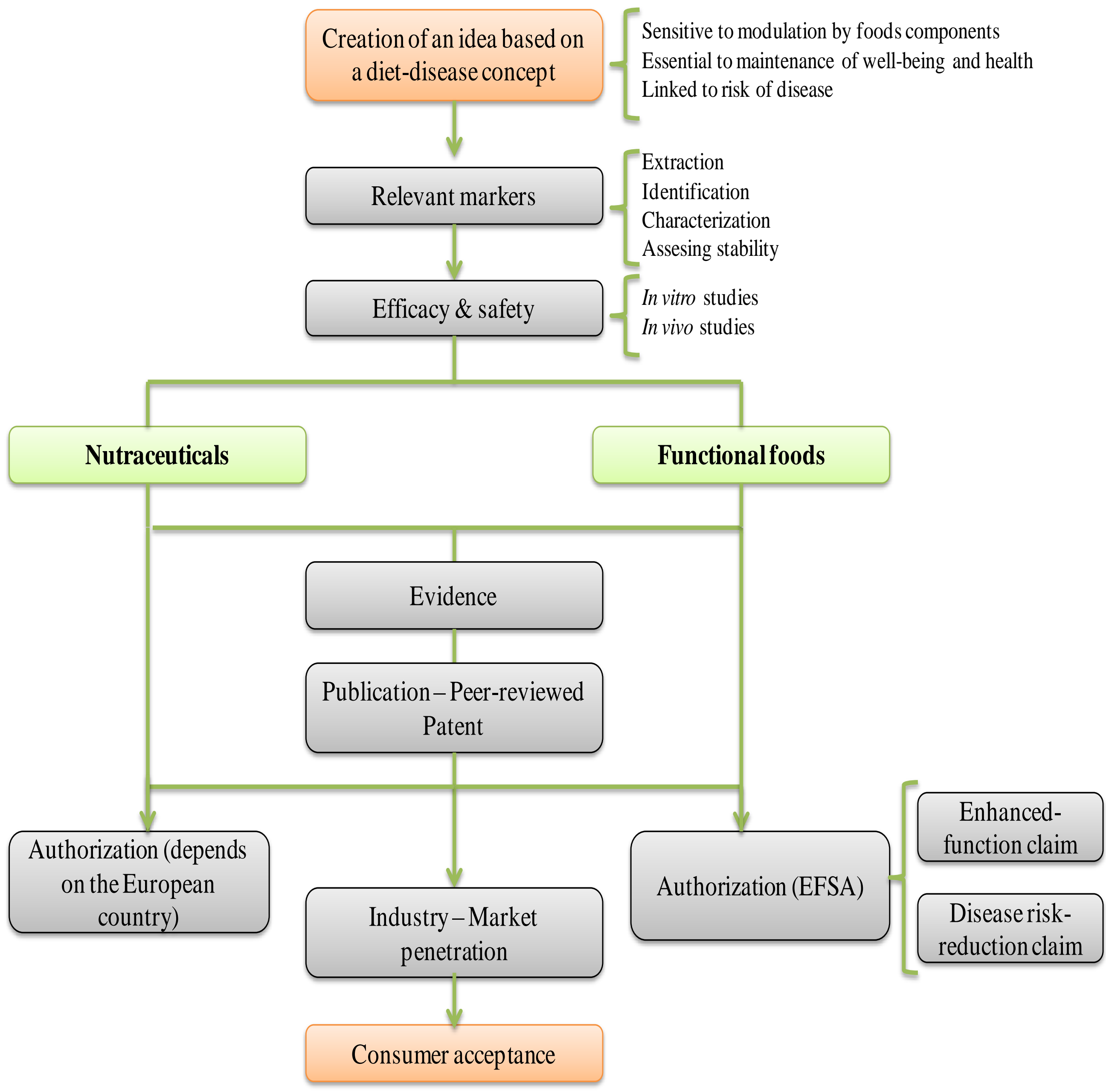


Figure 4. Workflow in nutraceutical and functional foods development. Adapted from Roberfroid, *et al.* and Jones *et al.*[11,33]

[33] Jones, P.J., & Jew, S. (2007). Functional food development: concept to reality. *Trends in Food Science & Technology*, 18(7), 387-390.

To determine the bioactivity of foods or individual target compounds, *in vitro* assays followed by *in vivo* assays and clinical trials need to be performed. Once their bioactivity is demonstrated, and before marketing, their approval is required by the respective authority, which in the European Union is the European Food Safety Authority (EFSA).

Functional foods are currently regulated by COMMISSION REGULATION (EU) No 1924/2006 on nutrition and health claims made on foods. This regulation is designed to obligate companies to provide strong substantiation of the evidence of the effect of health claims for their products. This strict regulation has brought about a drop in the number of health claims allowed. The most affected areas have been related to natural-plant extracts or probiotics[34]. This is exemplified by the sectors associated with phenolic compounds (**Figure 5**).

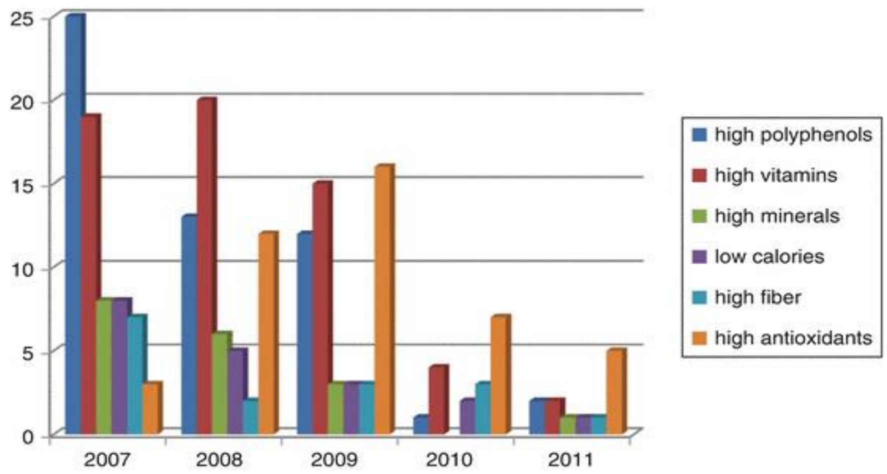


Figure 5. Ingredients with polyphenols associated to different claims[34].

In addition, COMMISSION REGULATION (EU) No 432/2012 of 16 May 2012 established a list of permitted health claims made on foods, other than those referring to the reduction of disease risk and to child development and health. Some examples of compounds with approved health claims are oleic acid (EFSA journal

[34] Valls, J., Pasamontes, N., Pantaleón, A., Vinaixa, S., Vaqué, M., Soler, A., Millán, S., & Gómez, X. (2013). Prospects of functional foods/ Nutraceuticals and markets. In: K.G. Ramawat, J.M. Mérillon (Eds.), *Natural Products*, Springer, Germany, 2491-2522.

number 2011;9(4):2043), PUFAs (EFSA journal numbers 2011;9(4):2069 and 2011;9(6):2203), polyphenols from olive oil (EFSA journal numbers 2011;9(4):2033), plant sterols and plant stanols (2010;8(10):1813 and 2011;9(6):2203).

The regulatory status of nutraceuticals in the EU remains confusing due to the differences in traditions, in historical and cultural backgrounds, and in the legislation of each member state[22]. Nevertheless, in the Paragraph 8 from the DIRECTIVE 2002/46/EC on the approximation of the laws of the Member States relating to food supplements, it is specified that "*specific rules concerning nutrients, other than vitamins and minerals, or other substances with a nutritional or physiological effect used as ingredients of food supplements should be laid down at a later stage, provided that adequate and appropriate scientific data about them become available. Until such specific Community rules are adopted and without prejudice to the provisions of the Treaty, national rules concerning nutrients or other substances with nutritional or physiological effect used as ingredients of food supplements, for which no Community specific rules have been adopted, may be applicable*". In addition, the lists of vitamins and minerals and their forms that can be added to foods, including food supplements, are regulated by the COMMISSION REGULATION (EC) No 1170/2009.

The last step is the market launching and consumer acceptance. The demands of functional foods and nutraceuticals vary significantly within EU countries due to food tradition and cultural heritage[35]. A representative example is the fact that the interest of consumers is higher in Central and Northern European countries than in Mediterranean countries, where people consider natural and fresh food the healthiest option[9]. However, in general, consumers are not properly informed about what functional foods and nutraceuticals actually are and for this reason carrying out educational campaigns to consumers by public authorities seems to be necessary[35,36].

[35] Annunziata, A., & Vecchio, R. (2011). Functional foods development in the European market: A consumer perspective. *Journal of Functional Foods*, 3(3), 223-228.

[36] Verbeke, W. (2005). Consumer acceptance of functional foods: socio-demographic, cognitive and attitudinal determinants. *Food Quality and Preference*, 16(1), 45-57.

Thus, for successful nutraceutical and functional foods development, it is necessary to undertake effective scientific research, including the search for new sources of bioactive compounds related to the diet-disease concept. This should include the extraction, identification, characterization, stability assessment, and a bioactivity evaluation of these compounds. Therefore, the following sections will focus on exploring in detail all the aforementioned steps.

❖ BIOACTIVE COMPOUNDS FROM NATURAL SOURCES AND HEALTH

The role of the human diet has been traditionally associated with the contribution of macro- (carbohydrates, lipids, and proteins) and micronutrients (vitamins and minerals) for the proper functioning of the body. However, numerous scientific works support the contention that the healthy effect of the consumption of nutraceuticals and functional food is closely related to the presence of other non-nutrient compounds, known as **bioactive compounds**. In this regard, bioactive compounds can be defined as natural substances, usually found in small amounts in plants (phytochemicals) and certain foods that provide health benefits beyond the basic nutritional value of the products. It should be noted that although edible plants are capable of producing bioactive compounds, they could also be found in animal-based food, bacteria, and fungi.

Plant foods contain an array of bioactive compounds with different structures and bioactivity and therefore different classifications have been proposed according to their clinical function, botanical categorization, biochemical classes, and pathways[37]. The primary metabolites are found in all plants and perform metabolic roles that are essential. They include: phytosterols, fatty acids, nucleotides, amino acids, and organic acids. In addition, plants produce secondary metabolites which do not participate

[37] Vermerris, W. & Nicholson, R. (2006). Families of phenolic compounds and means of classification. Phenolic compound biochemistry. In: Phenolic compound biochemistry. Springer, Netherlands, 1-34.

directly in growth and development but they play a major role in the adaptation of plants to their environment.

The most accepted classification is made on the basis of their chemical nature. In this regard, bioactive compounds can be classified into **inorganic compounds** which include minerals and **organic compounds** such as phenolic compounds, alkaloids, nitrogen-containing compounds, organosulfur compounds, phytosterols, carotenoids, unsaturated fatty acids, oligosaccharides (prebiotics), and polysaccharides (starch). A more detailed classification is displayed in **Figure 6**.

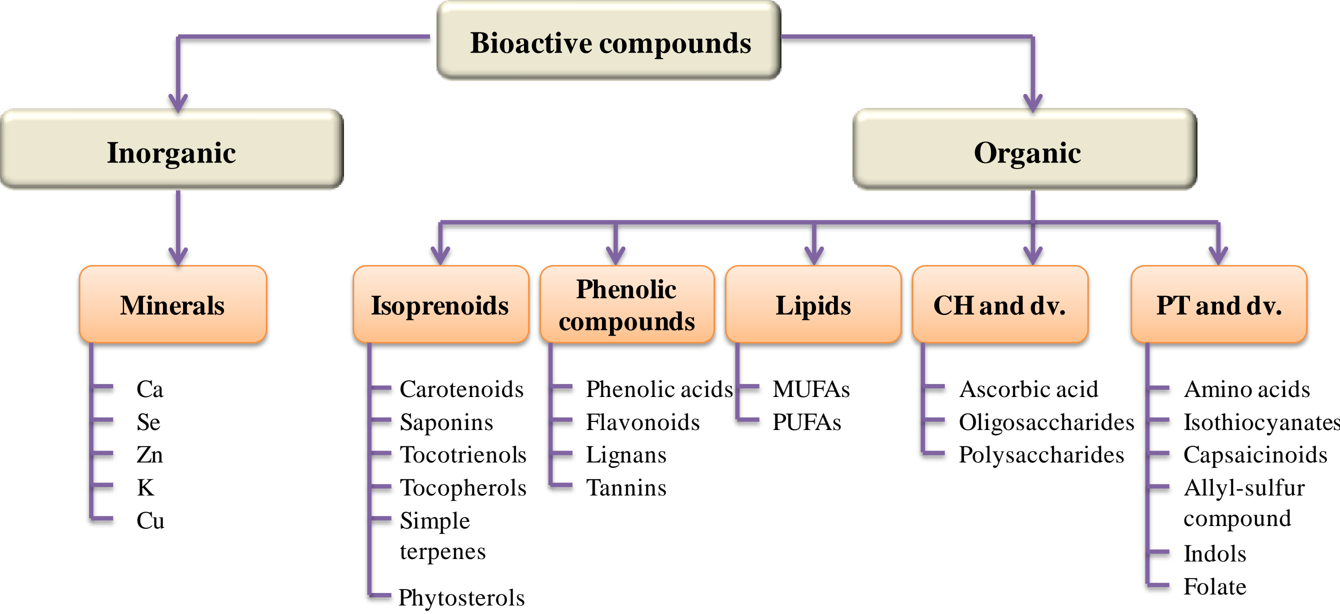


Figure 6. Some bioactive compounds from foods, classified according to their chemical nature. CH, carbohydrates; PT, protein; dv., derivatives. Adapted from Martí del Moral *et al.*[38]

Among all classes of bioactive compounds, the current Thesis has been focused on the study of phenolics, phytosterols, and unsaturated fatty acids and therefore these will be described in more detail in the following sections.

[38] Martí del Moral, A.A., & Martínez-Hernández, J.A. (2005). ¿Sabemos realmente lo que comemos?: alimentos transgenicos, ecológicos y funcionales. ENUSA, Barañain, Navarra. 160 pages. ISBN: 9788431323257.

2.1. Phenolic compounds

Phenolics constitute a large group of compounds produced by plants as secondary metabolites under normal and stress conditions. They arise biogenetically from the shikimic and malonic pathways as shown in **Figure 7**. Their structure is characterized by possessing one or more aromatic rings with one or more hydroxyl groups. So far, more than 8000 phenolics with very diverse chemical structures have been identified[39]. Phenolic compounds are commonly bound to other molecules such as sugars (glycosyl residue) or proteins but can also be linked to lipids, carboxylic acids or other phenolic compounds[40].

Among the main families of phenolic compounds, we can distinguish between a) **flavonoids** which are polyphenolic compounds made up fifteen carbons with two aromatic rings connected by a three-carbon bridge, and b) **non-flavonoids**, being phenolic acids such as gallic acid and stilbenes the most representative of the diet[41] (**Figure 8**). These latter are the most common and widely distributed phenolic compounds in all plants and plant-based foods. Indeed, more than 5000 plant-derived flavonoids have been isolated from different plants[42]. They offer antibiotic, antifungal, antiviral plant protection and anti-feeding properties for insects. In addition, they constitute important UV-absorbing compounds, thus preventing leaf damage from the

[39] Velderrain-Rodríguez, G.R., Palafox-Carlos, H., Wall-Medrano, A., Ayala-Zavala, J.F., Chen, C. O., Robles-Sánchez, M., Astiazarán-García, H., Álvarez-Parrilla, E., & González-Aguilar, G.A. (2014). Phenolic compounds: Their journey after intake. *Food and Function*, 5(2), 189-197.

[40] Bravo, L. (1998). Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition Reviews*, 56(11), 317-333.

[41] Crozier, A., Jaganath, I.B., & Clifford, M.N. (2008). Phenols, polyphenols and tannins: An overview. In: Plant secondary metabolites. Occurrence, structures and role in the human diet. Blackwell Publishing Ltd. 2, 1-22.

[42] Yao, L.H., Jiang, Y.M., Shi, J., Tomás-Barberán, F.A., Datta, N., Singanusong, R., & Chen, S.S. (2004). Flavonoids in food and their health benefits. *Plant Foods for Human Nutrition*, 59(3), 113-122.

light[43]. Phenolics are related to sensory characteristics such as bitterness, astringency, color, flavor, and odor and contribute to oxidative stability in plant-derived foods[44].

There are differences in the distribution of phenolic compounds throughout the plant. For example, roots show lower concentrations than the surrounding cortex or the peel, while leaves have higher phenolic concentrations. In addition, the phenolic content can vary depending on factors such as: climate, agronomic conditions, ripeness, harvest time, storage conditions and cultivar[45]. It should be noted that some of these compounds are widespread while others are characteristic of specific plants families or found only in certain plant organs or at precise development stages[46].

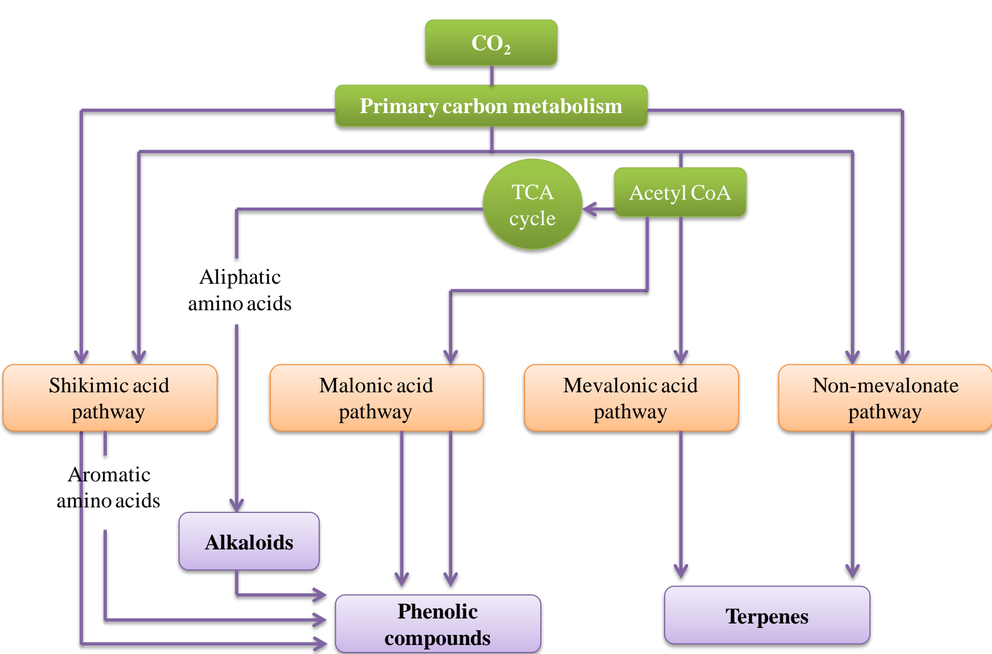


Figure 7. General pathways for production of secondary metabolites.

[43] Bourgaud, F., Gravot, A., Milesi, S., & Gontier, E. (2001). Production of plant secondary metabolites: a historical perspective. *Plant Science*, 161(5), 839-851.
[44] Naczk, M., & Shahidi, F. (2006). Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. *Journal of Pharmaceutical and Biomedical Analysis*, 41(5), 1523-1542.
[45] Klepacka, J., Gujska, E., & Michalak, J. (2011). Phenolic compounds as cultivar and variety distinguishing factors in some plant products. *Plant Foods for Human Nutrition*, 66(1), 64-69.
[46] Cheynier, V. (2012). Phenolic compounds: From plants to foods. *Phytochemistry Reviews*, 11(2-3), 153-177.

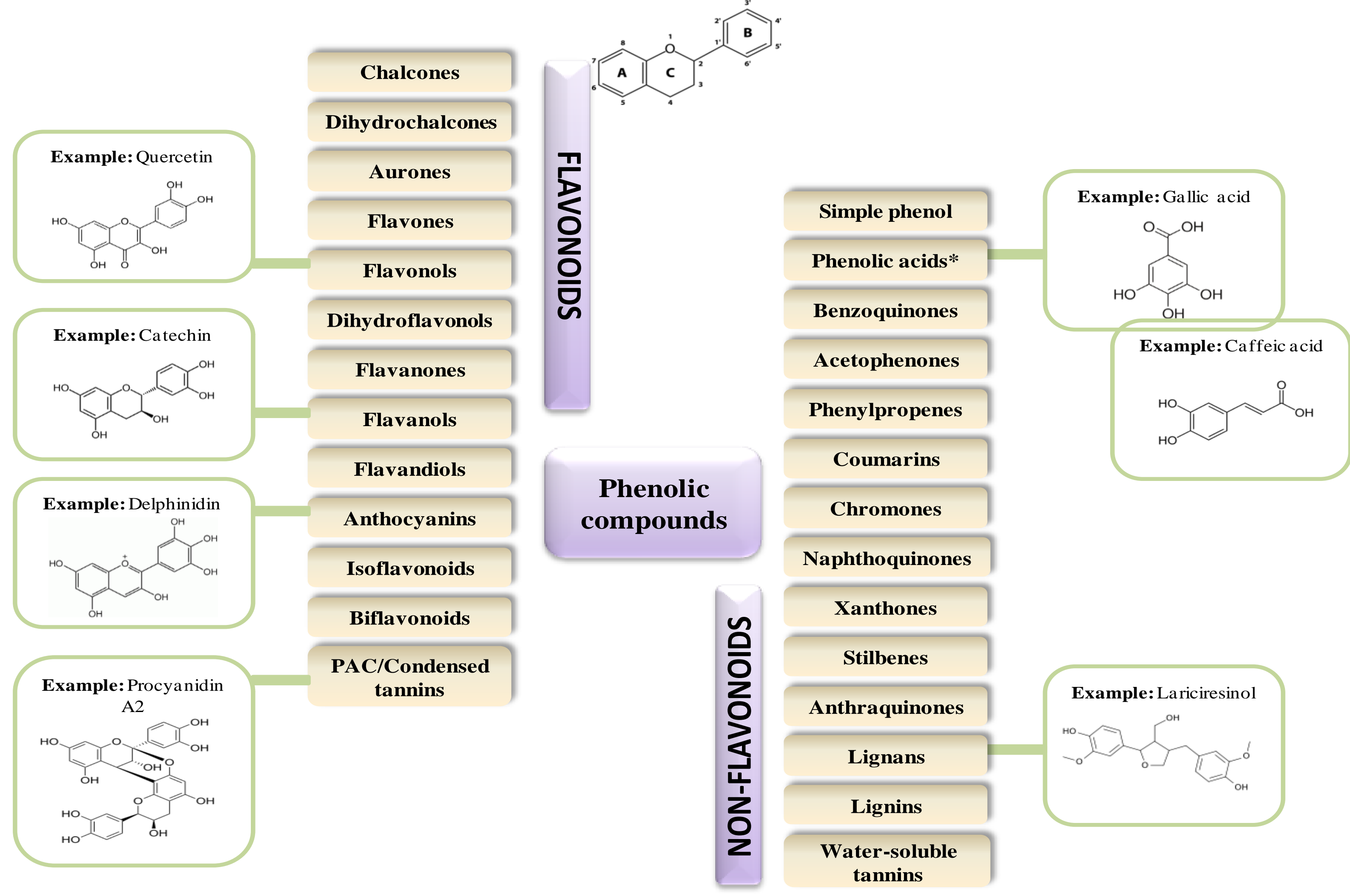


Figure 8. Classification of phenolic compounds. *Phenolic acids can be subdivided into cinnamic acids, benzoic acids, and phenylacetic acids; lignins are complex aromatic polymers; PAC, proanthocyanidin.

Phenolic compounds have become a popular research topic since the 1990s due to epidemiological studies indicating an inverse association between the intake of food rich in phenolics and the incidence of some diseases such as cardiovascular diseases or cancer[47]. Today, the number of publications which conclude that phenolic compounds may provide significant protection against the development and progression of several chronic pathological conditions including cancer, diabetes, cardiovascular diseases, and aging, among other pathologies, are increasing[48]. The beneficial effects derived from phenolic compounds have been attributed to their antioxidant activity[49]. Oxidative stress has been defined as a disturbance in the balance between the production of reactive oxygen species (ROS) and antioxidant defenses[50]. There is growing evidence identifying ROS as one of the primary determinants of human pathologies[51]. In the human body, ROS are metabolized primarily through antioxidant-defense mechanisms using endogenously synthesized compounds made up of enzymes such as superoxide dismutase (SOD) and catalase[52]. However, an additional barrier for the defense of oxidative stress damage is necessary in situations involving an overproduction of ROS. This additional protection is achieved by the dietary intake of antioxidants[51] such as vitamins, minerals, unsaturated fatty acids or phenolic compounds, among other natural bioactive compounds[53].

---

[47] Visioli, F., de la Lastra, C.A., Andres-Lacueva, C., Aviram, M., Calhau, C., Cassano, A., D'Archivio, M., Faria, A., Favé, G., Fogliano, V., Llorach, R., Vitaglione, P., Zoratti, M., & Edeas, M. (2011). Polyphenols and human health: A prospectus. *Critical Reviews in Food Science and Nutrition*, 51(6), 524-546.

[48] Contreras-Gámez, M.M., Rodríguez-Pérez, C., García-Salas, P., & Segura-Carretero, C. (2014). Polyphenols from the mediterranean diet: Structure, analysis and health evidence. In: Occurrences, structure, biosynthesis, and health benefits based on their evidences of medicinal phytochemicals in vegetables and fruits, *Nova Science Publishers, Inc*. 2, 141-209.

[49] Balasundram, N., Sundram, K., & Samman, S. (2006). Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chemistry*, 99(1), 191-203.

[50] Betteridge, D.J. (2000). What is oxidative stress? *Metabolism,* 49(2), 3-8.

[51] De Roos, B., & Duthie, G.G. (2015). Role of dietary pro-oxidants in the maintenance of health and resilience to oxidative stress. *Molecular Nutrition and Food Research*, 59(7), 1229-1248.

[52] Rahal, A., Kumar, A., Singh, V., Yadav, B., Tiwari, R., Chakraborty, S., & Dhama, K. (2014). Oxidative stress, prooxidants, and antioxidants: The interplay. *BioMed Research International*, 1-19.

[53] Lemieux, H., Bulteau, A.L., Friguet, B., Tardif, J., & Blier, P.U. (2011). Dietary fatty acids and oxidative stress in the heart mitochondria. *Mitochondrion*, 11(1), 97-103.

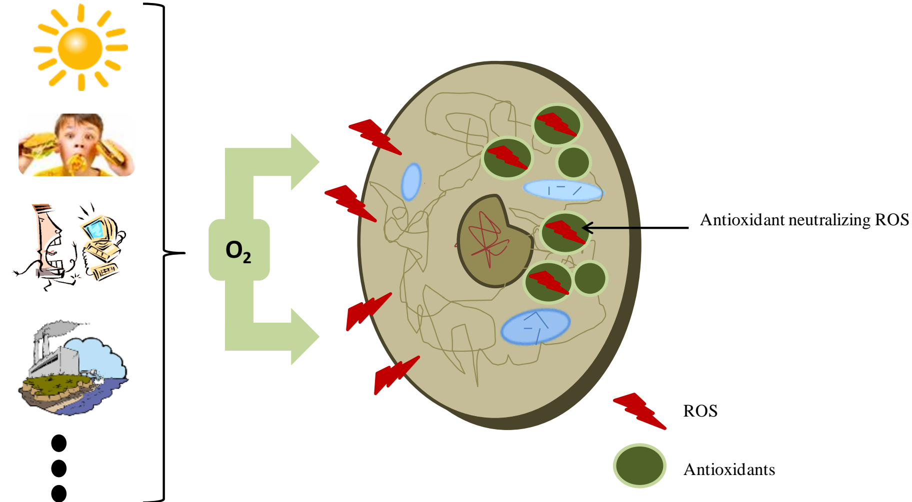


Figure 9. Mechanism of action of natural antioxidants against oxidative stress.

Many *in vitro* assays have been made in order to determine the antioxidant activity of numerous plants and plant-based foods[54-56]. However, antioxidant activity depends largely on the chemical structure of phenolic compounds and most plants and plant-based foods are complex matrices which make it difficult to have accurate results on their antioxidant activity. If we consider the aforementioned relationship between structure and activity, in general terms we can say that flavonoids activity is governed by the number and position of their aromatic –OH groups while for phenolic acids, it has been reported that free hydroxyl groups in the ring provide higher antioxidant activity[57]. Specifically, the antioxidant activity of phenolics lies in their capability to donate hydrogen or an electron and their ability to delocalize the unpaired electron within the aromatic structure[57].

[54] Morales-Soto, A., García-Salas, P., Rodríguez-Pérez, C., Jiménez-Sánchez, C., Cádiz-Gurrea, M.L., Segura-Carretero, A., & Fernández-Gutiérrez, A. (2014). Antioxidant capacity of 44 cultivars of fruits and vegetables grown in Andalusia (Spain). *Food Research International*, 58, 35-46.

[55] Fu, L., Xu, B., Xu, X., Gan, R., Zhang, Y., Xia, E., & Li, H. (2011). Antioxidant capacities and total phenolic contents of 62 fruits. *Food Chemistry*, 129(2), 345-350.

[56] Tiveron, A.P., Melo, P.S., Bergamaschi, K.B., Vieira, T.M.F.S., Regitano-d'Arce, M.A.B., & Alencar, S.M. (2012). Antioxidant activity of Brazilian vegetables and its relation with phenolic composition. *International Journal of Molecular Sciences*, 13(7), 8943-8957.

[57] Fernández-Panchón, M.S., Villano, D., Troncoso, A.M., & García-Parrilla, M.C. (2008). Antioxidant activity of phenolic compounds: From *in vitro* results to *in vivo* evidence. *Critical Reviews in Food Science and Nutrition*, 48(7), 649-671.

Regarding individual phenolic compound, some *in vivo* studies have shown the antioxidant mechanism of action of some flavonoids, e.g. quercetin, naringin or resveratrol. Quercetin has proved to increase antioxidant enzyme activity and apoptotic proteins in cancer-induced rats, preventing prostate cancer[58]. Naringin has also been shown to stimulate some antioxidant enzymes such as SOD in induced-type diabetic rats, concluding that this bioflavonoid ameliorates dyslipidemia, hepatic steatosis, and kidney damage in type 2 diabetic rats by partly regulating oxidative stress[59].

Apart from antioxidant activity, phenolics have demonstrated their potential as antimicrobial agents. The interest of food, pharmaceutical, and nutraceutical companies in finding new antimicrobial agents resides in the increasing number of micro-organisms resistant to the available antibiotics, implying higher health care expenditures, according to the WHO[60]. In this context, the search for natural sources of bioactive compounds is a promising alternative. An antimicrobial is any substance that kills or inhibits the growth of microorganisms causing little or no damage to the host. The action mechanism of phenolics is generally based on the disturbance of the function of bacterial-cell membranes which depress bacterial growth or multiplication[61].

[58] Sharmila, G., Bhat, F.A., Arunkumar, R., Elumalai, P., Raja Singh, P., Senthilkumar, K., & Arunakaran, J. (2014). Chemopreventive effect of quercetin, a natural dietary flavonoid on prostate cancer in *in vivo* model. *Clinical Nutrition*, 33(4), 718-726.

[59] Sharma, A.K., Bharti, S., Ojha, S., Bhatia, J., Kumar, N., Ray, R., Kumari, S., & Arya, D.S. (2011). Up-regulation of PPARγ, heat shock protein-27 and-72 by naringin attenuates insulin resistance, β-cell dysfunction, hepatic steatosis and kidney damage in a rat model of type 2 diabetes. *British Journal of Nutrition*, 106(11), 1713-1723.

[60] WHO. (2015). http://www.who.int/mediacentre/factsheets/fs194/en/

[61] Borrás-Linares, I., Fernández-Arroyo, S., Arráez-Roman, D., Palmeros-Suárez, P.A., Del Val-Díaz, R., Andrade-Gonzáles, I., Fernández-Gutiérrez, A., Gómez-Leyva, J.F., & Segura-Carretero, A. (2015). Characterization of phenolic compounds, anthocyanidin, antioxidant and antimicrobial activity of 25 varieties of Mexican Roselle (*Hibiscus sabdariffa*). *Industrial Crops and Products*, 69, 385-394.

The literature contains a vast number of works on demonstrating the *in vitro* and *in vivo* antibacterial effect of many plants and plant-based foods such as aromatic plants[62,63]. Examples include pomegranate peel and pomegranate juice[64,65]; garlic cloves and ginger[66]; different varieties of roselle[61]; and cranberry[67] among other plant-food-based products. However, only some clinical trials have also demonstrated the antibacterial effect of plant-based products. In this way the activity of cranberry and cranberry-based products[31,68], black tea leaf extract[69] or garlic[70] has been attributed primarily to their composition in phenolic compounds. Despite that many studies have suggested that the antibacterial activity in plant and plant-based foods is owing to a synergistic action of bioactive compounds, mainly phenolics, recent research has shown the antibacterial activity of isolated phenolic compounds.

[62] Proestos, C., Boziaris, I. S., Nychas, G.E., & Komaitis, M. (2006). Analysis of flavonoids and phenolic acids in Greek aromatic plants: Investigation of their antioxidant capacity and antimicrobial activity. *Food Chemistry*, 95(4), 664-671.

[63] Pereira, A.P., Ferreira, I.C.F.R., Marcelino, F., Valentão, P., Andrade, P. B., Seabra, R., Estevinho, L., Bento, A., & Pereira, J.A. (2007). Phenolic compounds and antimicrobial activity of olive (*Olea europaea* L. Cv. Cobrançosa) leaves. *Molecules*, 12(5), 1153-1162.

[64] Kwon, D., Choi, J., Kang, O., Lee, Y., Chae, H., Oh, Y., Brice, O., Kim, M., Sohn, D., Kim, H., Park, H., Shin, D., & Rho, J. (2011). *In vitro* and *in vivo* antibacterial activity of *Punica granatum* peel ethanol extract against *Salmonella*. *Evidence-based Complementary and Alternative Medicine*, 2011.

[65] Betanzos-Cabrera, G., Montes-Rubio, P.Y., Fabela-Illescas, H.E., Belefant-Miller, H., & Cancino-Diaz, J.C. (2015). Antibacterial activity of fresh pomegranate juice against clinical strains of *Staphylococcus epidermidis*. *Food and Nutrition Research*, 59, 1-9.

[66] Karuppiah, P., & Rajaram, S. (2012). Antibacterial effect of *Allium sativum* cloves and *Zingiber officinale* rhizomes against multiple-drug resistant clinical pathogens. *Asian Pacific Journal of Tropical Biomedicine*, 2(8), 597-601.

[67] Iswaldi, I., Arráez-Román, D., Gómez-Caravaca, A.M., Contreras, M.M., Uberos, J., Segura-Carretero, A., & Fernández-Gutiérrez, A. (2013). Identification of polyphenols and their metabolites in human urine after cranberry-syrup consumption. *Food and Chemical Toxicology*, 55, 484-492.

[68] Kaspar, K.L., Howell, A.B., & Khoo, C. (2015). A randomized, double-blind, placebo-controlled trial to assess the bacterial anti-adhesion effects of cranberry extract beverages. *Food and Function*, 6(4), 1212-1217.

[69] Sharquie, K.E., Al-Turfi, I.A., & Al-Salloum, S.M. (2000). The antibacterial activity of tea *in vitro* and *in vivo* (in patients with impetigo contagiosa). *Journal of Dermatology*, 27(11), 706-710.

[70] Ghalambor, A., & Pipelzadeh, M.H. (2009). Clinical study on the efficacy of orally administered crushed fresh garlic in controlling *Pseudomonas aeruginosa* infection in burn patients with varying burn degrees. *Jundishapur Journal of Microbiology*, 2(1), 7-13.

By way of a few examples, different concentrations of 2,4-dihydroxybenzoic, protocatechuic, vanillic and *p*-coumaric acids have shown antibacterial activity against some Gram-negative bacteria such as *Escherichia coli* (*E. coli*), *Pasteurella multocida*, and *Neisseria gonorrhoeae*[71]. Gallic acid, ferulic acid and quercetin also exhibited activity against the latter two species[71]. Other studies were consistent with these results showing an antibacterial activity of phenolic acids such as cinnamic, caffeic, ferulic and chlorogenic acids against Gram-negative bacteria[72]. Proanthocyanidins isolated from berries, have also demonstrated the inhibition of some *E. coli* strains, thus contributing to the maintenance of the urinary tract health[72,73].

2.2. Phytosterols

Phytosterols, also called plant sterols, are a group of lipophilic steroid alcohols that have structure similar to that of cholesterol, but unlike cholesterol, they are not synthesized in the human body. Phytosterols are products of the isoprenoid biosynthetic pathway, as shown in **Figure 10**. Specifically, it consists of a sequence of more than 30 enzyme-catalyzed reactions, all of which are found in the plant membranes.

[71] Alves, M.J., Ferreira, I.C.F.R, Froufe, H.J.C., Abreu, R.M.V., Martins, A., Pintado, M. (2013). Antimicrobial activity of phenolic compounds identified in wild mushrooms, SAR analysis and docking studies. *Journal of Applied Microbiology*, 115(2), 346-357.

[72] Puupponen-Pimiä, R., Nohynek, L., Meier, C., Kähkönen, M., Heinonen, M., Hopia, A., & Oksman-Caldentey, K. (2001). Antimicrobial properties of phenolic compounds from berries. *Journal of Applied Microbiology*, 90(4), 494-507.

[73] Krueger, C.G., Reed, J.D., Feliciano, R.P., & Howell, A.B. (2013). Quantifying and characterizing proanthocyanidins in cranberries in relation to urinary tract health. *Analytical and Bioanalytical Chemistry*, 405(13), 4385-4395.

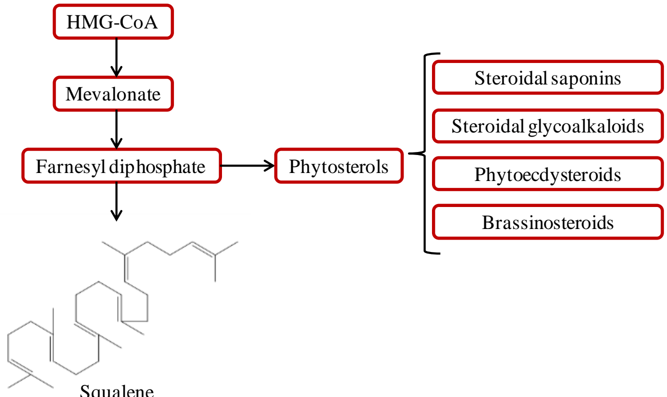


Figure 10. Biosynthesis of phytosterols. Adapted from Moreau *et al.*[74]

Two main classes of phytosterol are found in nature: plant sterols and plant stanols (saturated forms of sterols)[75] (**Figure 11**). The first one can be found at higher concentrations in plants and plant-derived foods. The term phytosterol includes more than 200 compounds. Over 250 naturally occurring sterols have been found in the higher plants. However, the most abundant plant sterols are α-sitosterol (C29), campesterol (C28), and stigmasterol (C28) that represent 95-98% of plant sterols identified in plant extracts and the major plant stanols include sitostanol and campestanol[76]. In addition, they can appear conjugated with fatty acids or ferulic acid or glycosylated[74].

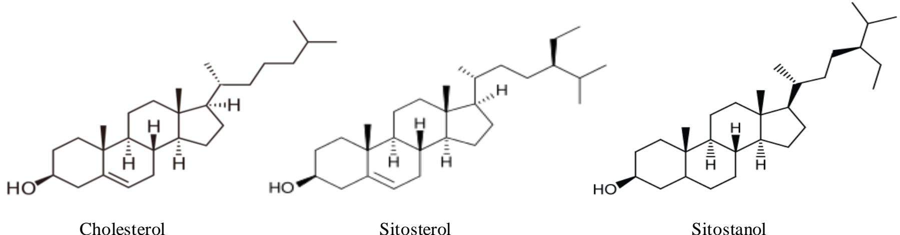


Figure 11. Cholesterol, plant sterol (sitosterol) and plant stanol (sitostanol) structures.

[74] Moreau, R.A., Whitaker, B.D., & Hicks, K.B. (2002). Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. *Progress in Lipid Research*, 41(6), 457-500.

[75] MacKay, D.S., & Jones, P.J.H. (2011). Phytosterols in human nutrition: Type, formulation, delivery, and physiological function. *European Journal of Lipid Science and Technology*, 113(12), 1427-1432.

[76] Trautwein, E.A., & Demonty, I. (2007). Phytosterols: Natural compounds with established and emerging health benefits. *OCL - Oleagineux Corps Gras Lipides*, 14(5), 259-266.

These sterols play an important role in regulating the fluidity and permeability of plant-cell membranes. Their function in plants could be comparable with cholesterol function in animals[77]. Phytosterols are naturally found in plant sources, mainly in oils, legumes or nuts[77,78] and, similarly to phenolic compounds, the composition in plants and plant-based food is influenced by agronomic parameters, the oil-extraction process, storage conditions, and climate.

Within bioactive compounds, plant sterols have been extensively studied by means of large clinical trials mainly for their ability to lower total and LDL-cholesterol[79,80]. Their absorption is low compared to cholesterol. While cholesterol absorption is around 50%, absorption of sitostanol is 1%, sitosterol 4%, campesterol 10% and campestanol 13%[7]. Their main action mechanism is based on the competition of absorption with cholesterol in the intestine which means that their presence affects the cholesterol solubilization into micelles, increasing its fecal excretion[78] (**Figure 12**).

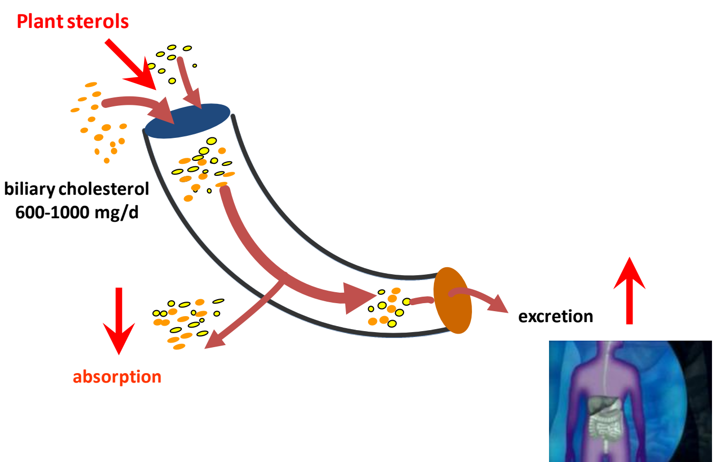


Figure 12. Simplified mechanism of action of phytosterols.

---

[77] Quílez, J., García-Lorda, P., & Salas-Salvadó, J. (2003). Potential uses and benefits of phytosterols in diet: present situation and future directions. *Clinical Nutrition*, 22(4), 343-351.

[78] Gupta, R., Mathur, M., Bajaj, V.K., Katariya, P., Yadav, S., Kamal, R., & Gupta, R.S. (2012). Evaluation of antidiabetic and antioxidant activity of *Moringa oleifera* in experimental diabetes. *Journal of Diabetes*, 4(2), 164-171

[79] Derdemezis, C.S., Filippatos, T.D., Mikhailidis, D.P., & Elisaf, M.S. (2010). Effects of plant sterols and stanols beyond low-density lipoprotein cholesterol lowering. *Journal of Cardiovascular Pharmacology and Therapeutics*, 15(2), 120-134.

[80] Scholz, B., Guth, S., Engel, K., & Steinberg, P. (2015). Phytosterol oxidation products in enriched foods: Occurrence, exposure, and biological effects. *Molecular Nutrition and Food Research*, 59(7), 1339-1352.

Despite existing controversies about the most effective dosage, most studies have consistently shown that the intake of 2-3 g/day of phytosterols is associated with significant lowering (5-15% range) of low-density lipoprotein (LDL-Cholesterol)[75,81]. Nevertheless, a few authors affirm that higher consumption of phytosterol (more than 3 g/day) could produce adverse effects such as the reduction of β-carotene[79,82]. Even though the efficacy in cholesterol-lowering depending on the type of phytosterol (plant sterols or plant stanols) continues to be debated; many clinical trials have shown that both types have similar responses[75]. Although the effectiveness of plant sterols can be influenced by many factors[78], due to their aforementioned properties, phytosterols and their fatty acids esters were one of the first bioactive compounds used to enrich foods[80].

2.3. Fatty acids

Fatty acids are defined as carboxylic acids with a hydrocarbon chain of an even number of carbon atoms ranging from 16-26. The formation of the major fatty acids in plants starts by *the novo* synthesis of saturated long-chain fatty acids through the combined activity of acetyl-CoA carboxylase and fatty acid synthase[83] (**Figure 13**).

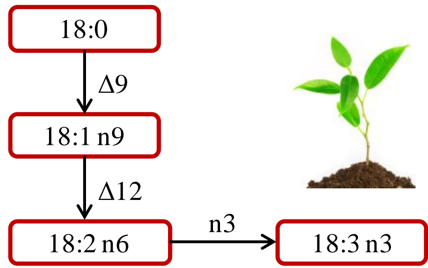


Figure 13. The general biochemical pathway of synthesis of PUFAs in plants. Δ9, Δ^9-desaturase ; Δ12, Δ^{12}-desaturase ; n3, n3-desaturase. Adapted from Wallis, *et al.*[83].

[81] Maki, K.C., & Rains, T.M. (2011). Hypocholesterolemic effects of plant sterols and stanols: Do the dose–response curves diverge? *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 85(1), 5-6.

[82] Katan, M.B., Grundy, S.M., Jones, P., Law, M., Miettinen, T., & Paoletti, R. (2003). Efficacy and safety of plant stanols and sterols in the management of blood cholesterol levels. *Mayo Clinic Proceedings*, 78(8), 965-978.

[83] Wallis, J.G., Watts, J.L., Browse, J. (2002). Polyunsaturated fatty acid synthesis: what will they think of next? *Trends in Biochemical Sciences*, 27(9), 467-473.

While fatty acids with only single bonds between adjacent carbon atoms are defined as saturated fatty acids, those with at least one C=C double bond, are called unsaturated fatty acids.

Among unsaturated fatty acids, we find fatty acids that have only one double bond called monounsaturated fatty acids (MUFAs) and those which have two of more double bonds and which are commonly known as PUFAs (**Figure 14**). At the same time, PUFAs can be classified into short-chain fatty acids (up to 19 carbon atoms), long-chain fatty acids (between 20 to 24 carbon atoms) or very long-chain fatty acids (more than 25 carbon atoms). This nomenclature is based on the position of those bonds and the total chain length. The double bonds from the unsaturated fatty acids are counted from the methyl group determining the metabolic family, noted by ω-x or n-x, where x indicates the position of the first double bond relative to the methyl end.

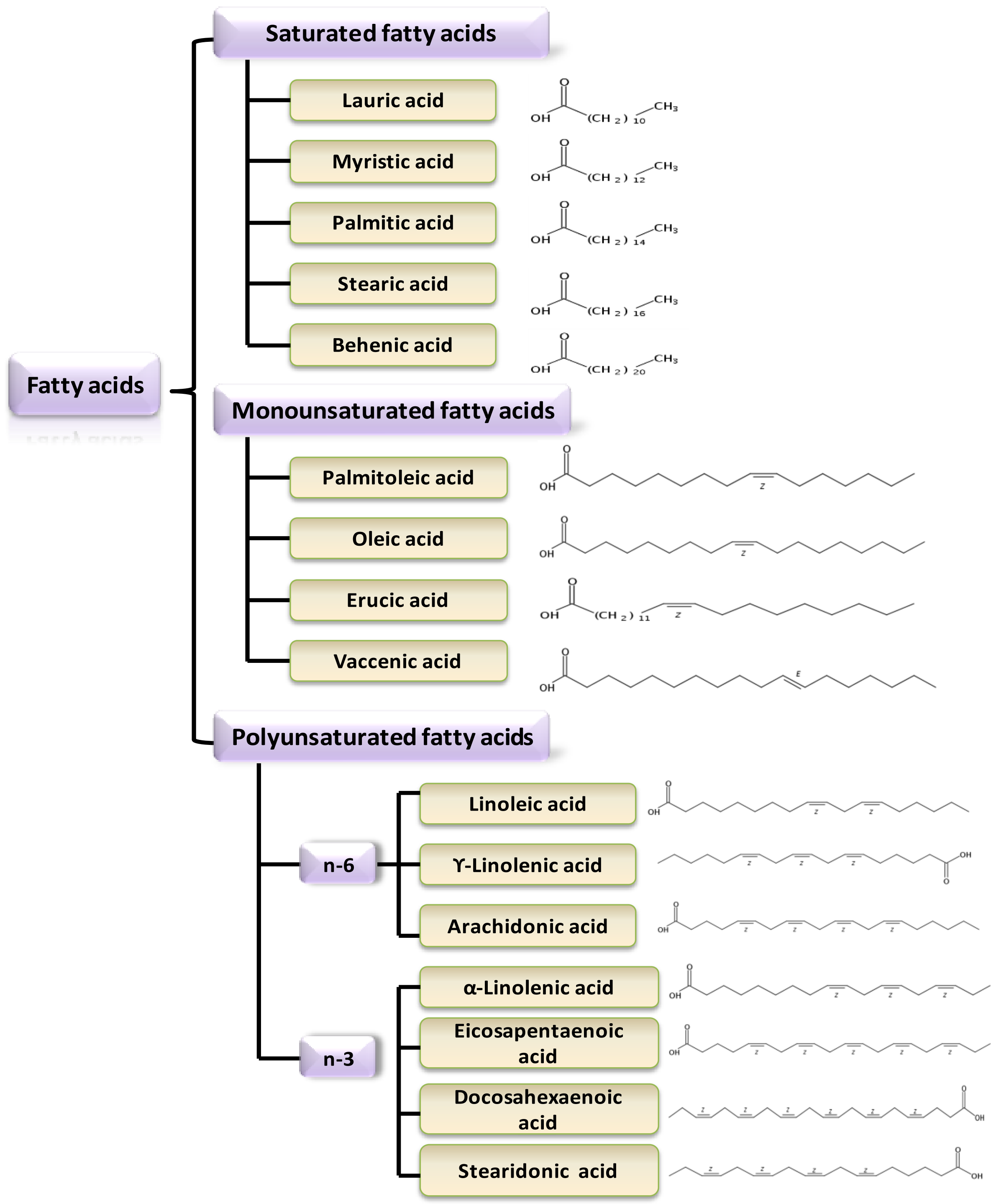


Figure 14. Some of the most representative fatty acids in the diet according to Food & Agriculture Organization of the United Nations (FAO) and Fundación Iberoamericana de Nutrición (FINUT)[84].

[84] Agriculture Organisation of the United Nations (FAO) and Fundación Iberoamericana de Nutrición (FINUT). Terminología de las grasas y los ácidos grasos. Métodos de análisis, digestión y metabolismo lipídico. En: Grasa y ácidos grasos en nutrición humana. Consulta de expertos. ISSN 1014-2916. ISBN 978-92-5-3067336. 2008.

On the topic of fatty acids, the current doctoral Thesis has been mainly focused on unsaturated fatty acids and for this reason; the following sections will be concentrated on these compounds. The main plant sources of MUFAs such as oleic acids or palmitoleic acid are nuts, all vegetable oils, especially olive, canola, sunflower, and avocado oils, among others. Oils, cereal-based products, cereals, vegetables, nuts, and seeds are an important source of n-6 fatty acids[85]. α-Linolenic acid, belonging to the n-3 series, has been found in walnuts, some green leafy vegetables, flaxseed, and rapeseed[86].

The quality of dietary fats plays a significant role in the development and progression of chronic diseases such as cardiovascular disease (CVD) and related pathologies. Actually, the types of fatty acid are more important in influencing the risk of CVD than the total amount of fat in the diet. For that reason the American Heart Association recommends the intake of unsaturated fatty acids instead of saturated fatty acids (http://www.heart.org/). Regarding MUFAs, oleic acid (C18:1) is one of the most studied. The seven-country study was the first major study that investigated the role of the diet and lifestyle in cardiovascular disease. This study revealed that the Mediterranean diet, in which olive oil (rich in oleic acid) is the main source of fat, was associated with a low incidence of CVD[87]. Subsequently, some clinical trials affirmed that when saturated fatty acids are replaced with monounsaturated fats from vegetable oils such as sunflower oil, favorable outcomes in blood lipids occur, mainly by decreasing LDL-cholesterol[88]. Another clinical trial carried out with men with mild

---

[85] Meyer, B.J., Mann, N.J., Lewis, J.L., Milligan, G.C., Sinclair, A.J., & Howe, P.R.C. (2003). Dietary intakes and food sources of omega-6 and omega-3 polyunsaturated fatty acids. *Lipids*, 38(4), 391-398.

[86] Simopoulos, A.P. (1999). Essential fatty acids in health and chronic disease. *American Journal of Clinical Nutrition*, 70(3), 560S-569S.

[87] Keys A., Menotti A., Aravanis C., Blackburn H., Djordevic B.S., Buzina R., Dontas A.S., Fidanza F., Karvonen M.J., Kimura N., et al. (1984). The seven countries study: 2,289 deaths in 15 years. *Preventive Medicine*, 13(2), 141-54.

[88] Alman-Farinelli, M.A., Gomes, K., Favaloro, E.J., & Petocz, P. (2005). A diet rich in high-oleic-acid sunflower oil favorably alters low-density lipoprotein cholesterol, triglycerides, and factor vii coagulant activity. *Journal of the American Dietetic Association*, 105(7), 1071-1079.

hypercholesterolemia and focusing on comparing different dietetic treatments consisting of palm oil, high oleic acid sunflower oil and medium-chain triacylglycerols oil, found that high oleic sunflower oil significantly reduced plasmatic total cholesterol (TC) and LDL-cholesterol (LDL-C) comparing with the other two treatments[89].

Despite that molecular mechanisms underlying this effect remain to be fully elucidated, some research has demonstrated that oleic acid inhibits fatty acids and cholesterol biosynthesis via a downregulation of two enzymes implicated in lipogenesis and cholesterogenesis (acetyl-coenzyme A carboxylase and 3-hydroxy-3-methylglutaryl coenzyme A reductase, respectively)[90] (**Figure 15**). Another theory also pointed out the decrease of apolipoprotein B (apo B) concentrations by means of oleic acid[91].

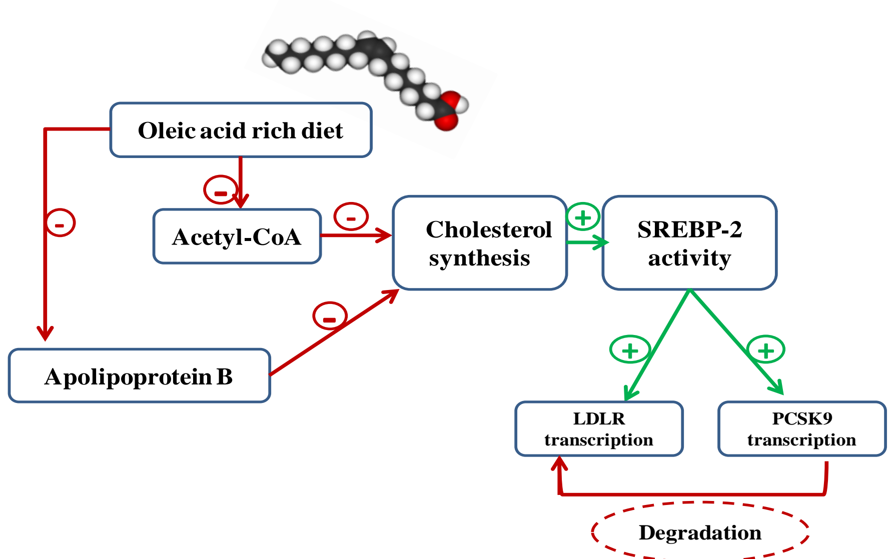


Figure 15. Mechanism of action of oleic acid.

---

[89] Cater, N.B., Heller, H.J., & Denke, M.A. (1997). Comparison of the effects of medium-chain triacylglycerols, palm oil, and high oleic acid sunflower oil on plasma triacylglycerol fatty acids and lipid and lipoprotein concentrations in humans. *American Journal of Clinical Nutrition*, 65(1), 41-45.

[90] Natali, F., Sicuella, L., Salvati, S., & Gnomi, G.V. (2007). Oleic acid is a potent inhibitor of fatty acid and cholesterol synthesis in C6 glioma cells. *Journal of Lipids Research*, 48, 1966-1975.

[91] Chan J.K., Bruce, V.M., & McDonald, B.E. (1991). Dietary alpha-linolenic acid is as effective as oleic acid and linoleic acid in lowering blood cholesterol in normolipidemic men. *The American Society for Clinical Nutrition*, 53(5), 1230-1234.

Within n-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been extensively studied due to their ability to reduce the risk of heart disease[92]. The endogenous synthesis of EPA (C20:5) and DHA (C22:6) from α-linoleic acids is moderately inefficient, and thus dietary intake is necessary for the tissue accumulation. Recently, some studies have also correlated n-3 PUFAs with positive outcomes of inflammation[93], Alzheimer's disease[94] or cancer[95], among others.

Regarding DHA, its benefitial effects are focused on the increase of HDL and the reduction of TAG (**Figure 16**). However, some clinical trials have shown that a diet based on oil enriched in DHA increases TC by 2%[96]. By contrast, in another clinical trial carried out with subjects who consumed 4 g of purified DHA daily for 6 weeks, no correlation was found between DHA and total cholesterol levels, but LDL-C concentrations were found to be significantly increased[97]. The rise in LDL-C levels with DHA intake has been associated with a larger LDL particle size, this in turn being negatively correlated with CV-risk factors[98].

[92] Grundt, H., & Nilsen, D.W.T. (2008). N-3 Fatty Acids and cardiovascular disease. *Haematologica*, 93, 807-812.

[93] Miles, E.A., & Calder, P.C. (2012). Influence of marine n-3 polyunsaturated fatty acids on immune function and a systematic review of their effects on clinical outcomes in rheumatoid arthritis. *British Journal of Nutrition*, 107(2), S171-S184.

[94] De Souza Fernandes, D.P., Canaan Rezende, F.A., Rocha, G.P., Filgueiras, M.D.S., Silva Moreira, P.R., & Goncalves Alfenas, R.C. (2015). Effect of eicosapentaenoic acid and docosahexaenoic acid supplementations to control cognitive decline in dementia and Alzheimer's disease: a systematic review. *Nutrición Hospitalaria*, 32(2), 528-533.

[95] Seo T., Blaner W.S., & Deckelbaum R.J. (2005). N-3 fatty acids: molecular approaches to optimal biological outcomes. *Current Opinion in Lipidology*, 16:11–8.).

[96] Jones, P.J.H., Senanayake, V.K., Pu, S., Jenkins, D.J.A., Connelly, P.W., Lamarche, B., Couture, P., Charest, A., Baril-Gravel, L., West, S. G., Liu, X., Fleming, J.A., McCrea, C.E., & Kris-Etherton, P.M. (2014). DHA-enriched high-oleic acid canola oil improves lipid profile and lowers predicted cardiovascular disease risk in the canola oil multicenter randomized controlled trial. *American Journal of Clinical Nutrition*, 100(1), 88-97.

[97] Mori T.A., Burke V., Puddey I.B., Watts G.F., O'Neal D.N., Best J.D., & Beilin L.J. (2000). Purified eicosapentaenoic and docosahexaenoic acids have differential effects on serum lipids and lipoproteins, LDL particle size, glucose, and insulin in mildly hypedipidemic men. *American Journal of Clinical Nutrition*,71, 1085-94.

[98] Kitessa, S.M., Abeywardena, M., Wijesundera, C., & Nichols, P.D. (2014). DHA-containing oilseed: a timely solution for the sustainability issues surrounding fish oil sources of the health-benefitting long-chain omega-3 oils. *Nutrients*, *6*(5), 2035-2058.

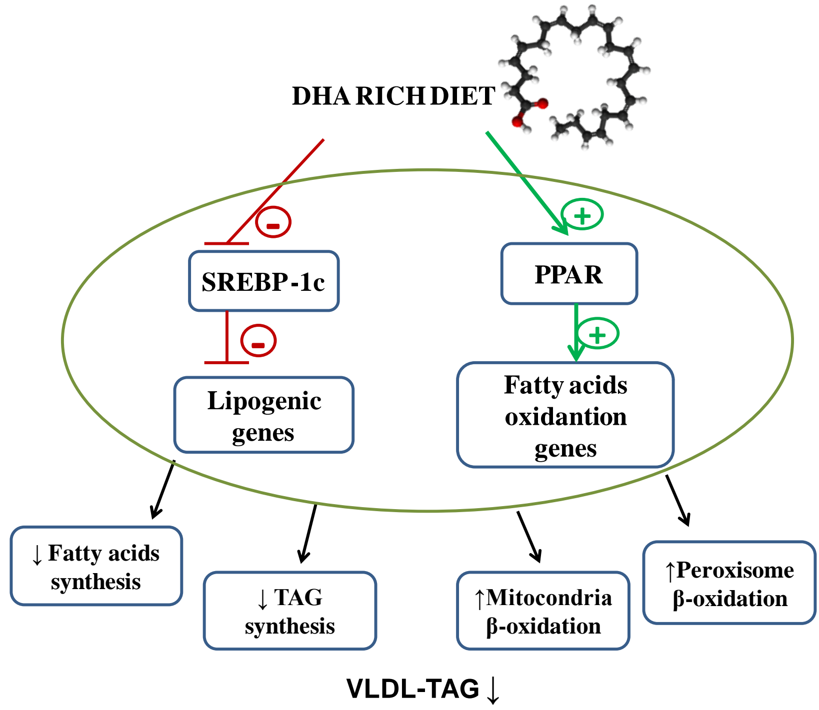


Figure 16. Nuclear mechanism of DHA. SREBP-1c, Sterol regulatory element binding protein-1c; PPAR, peroxisome-proliferator-activated receptor; TAG, triglycerides; VLDL, very low-density lipoprotein. Adapted from Clarke[99].

Recent theories about lowering cholesterol included the suppression of a novel proprotein called proprotein convertase subtilisin/kexin type 9 (PCSK9), which was discovered in 2003[100], thus, allowing recycling the LDL receptor from endosomes to the cell surface (**Figure 17**). Despite that the FDA approved a new drug based on PCSK9 suppression in 2015, only a few studies have focused on finding relationship between diet and decreased PCSK9[101,102].

---

[99] Clarke, S.D. (2001). Molecular mechanism for polyunsaturated fatty acid regulation of gene transcription. *American Journal of Physiology Gastrointestinal Liver Physiology*, 281: 865–869, 2001.

[100] Abifadel, M., Varret, M., Rabès, J., Allard, D., Ouguerram, K., Devillers, M., Cruaud, C., Benjannet, S., Wickham, L., Erlich, D., Derré, A., Villéger, L., Farnier, M., Beucler, I., Bruckert, E., Chambaz, J., Chanu, B., Lecerf, J., Luc, G., Moulin, P., Weissenbach, J., Prat, A., Krempf, M., Junien, C., Seidah, N. G., & Boileau, C. (2003). Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. *Nature Genetics*, 34(2), 154-156.

[101] Richard, C., Couture, P., Desroches, S., Charest, A., & Lamarche, B. (2011). Effect of the Mediterranean diet with and without weight loss on cardiovascular risk factors in men with the metabolic syndrome. *Nutrition, Metabolism and Cardiovascular Diseases*, 21(9), 628-635.

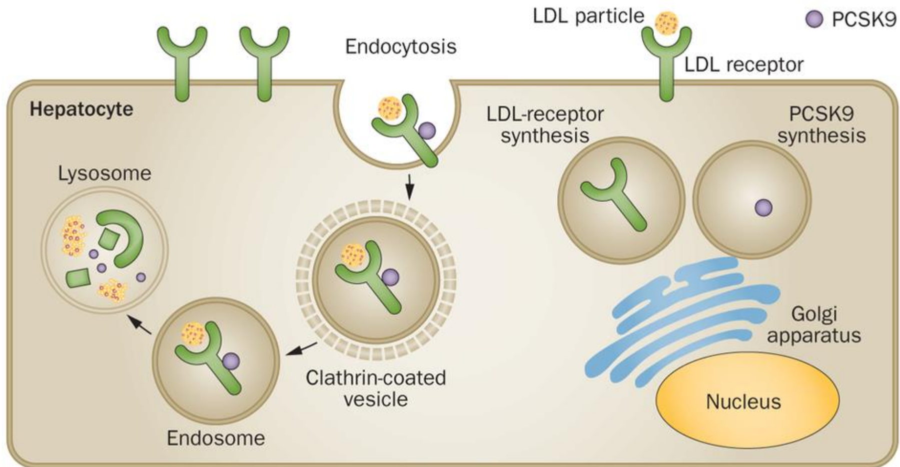


Figure 17. LDL-C metabolism in the presence of PCSK9. Adapted from Dadu & Ballantyne[103].

After the choice of the target family of bioactive compounds to be studied, based on a diet-disease interaction, the selection of the appropriate extraction technique is a critical step that will determine the accuracy of the results. For this reason, following the workflow for nutraceutical and functional foods development, the next section focuses on the most widely used techniques for extracting bioactive compounds from plant and biological matrices.

❖ EXTRACTION OF BIOACTIVE COMPOUNDS

3.1. Extraction of bioactive compounds from plant sources

In recent years, the interest in nutraceutical, functional foods, pharmaceutical, and cosmeceutical companies in developing biologically active compounds from natural sources is increasing. In addition, the use of herbal products has increased

[102] Bjermo, H., Iggman, D., Kullberg, J., Dahlman, I., Johansson, L., Persson, L., Berglund, J., Pulkki, K., Basu, S., Uusitupa, M., Rudling, M., Arner, P., Cederholm, T., Ahlström, H., & Risérus, U. (2012). Effects of n-6 PUFAs compared with SFAs on liver fat, lipoproteins, and inflammation in abdominal obesity: A randomized controlled trial. *American Journal of Clinical Nutrition*, 95(5), 1003-1012.

[103] Dadu, R.T. & . Ballantyne, C.M. (2014). Lipid lowering with PCSK9 inhibitors. *Nature Reviews Cardiology,* 11, 563–575.

dramatically during the last decade[104]. It should be highlighted that extraction is one of the first steps of any analytical process and plays a significant role in the final result. Given the industrial and nutritional value of natural sources, green extraction techniques that enhance yields while minimizing the impact of the quality of the end-product are necessary.

Plant and plant-based products are generally complex matrices made up of a wide range of compounds with different structures and polarities. The solubility of bioactive compounds is governed by the chemical nature of the compound, its possible interaction with other matrix components, as well as the polarity of the solvents used. As an example, phenolics may be associated with other plant components such as carbohydrates and proteins. This makes it difficult to establish a universal extraction procedure suitable for extraction of all plant phenolics. In addition, it should be borne in mind that in the plant kingdom different groups of bioactive compounds are commonly found in the same sample. In this case, additional steps may be necessary to remove the unwanted components which may interfere in the subsequent analysis, causing erroneous results. Different techniques are commonly employed to extract bioactive compounds from plant and plant-based products, depending on the target compound, namely conventional extraction (CE) and alternative extraction techniques such as ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE), pressurized liquid extraction (PLE) and microwave-assisted extraction (MAE). **Figure 18** summarizes these extraction techniques. Nevertheless, the extraction yield and the quality of the final extract depends on the type of solvent used, sample-to-solvent ratio, extraction time, temperature, chemical composition, and physical characteristics of the sample[105]. These parameters affect in different ways depending on the employed

[104] Kelly, J.P., Kaufman, D.W., Kelley, K., Rosenberg, L., Anderson, T.E., & Mitchell, A.A. (2005). Recent trends in use of herbal and other natural products. *Archives of Internal Medicine*, 165(3), 281-286.

[105] Rodríguez-Pérez, C., Jiménez-Sánchez, C., Lozano-Sánchez, J., Quirantes-Piné, R., & Segura-Carretero, A. (2016). Emerging green technologies for the extraction of phenolic compounds from medicinal plants. Recent Progress in Medicinal Plants. 41 – Analytical and processing techniques, 81-104. Studium Press LLC, USA. ISBN 1-62699-078-6.

extraction technique, and therefore they will be discussed in the following sub-sections.

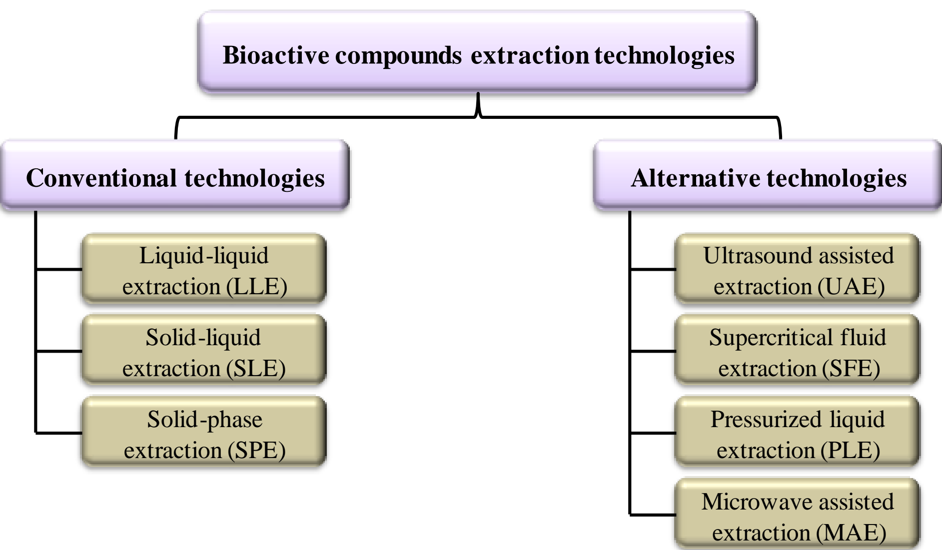


Figure 18. Types of extraction techniques used for the extraction of bioactive compounds.

3.1.1. Conventional extraction

Conventional extraction (CE) methods have been repeatedly used for the extraction of bioactive compounds from plants and plant-based foods. CE is based on the selective transfer of the target compound from a solid or liquid matrix to a solvent. The efficiency of the technique will depend on the difference in solubility between the target compounds and the other compounds of the sample in the solvent employed for the extraction. Classical techniques to obtain bioactive compounds from plant and plant-based products include: liquid-liquid extraction (LLE), solid-liquid extraction (SLE), soxhlet extraction (SE), solid-phase extraction (SPE), hydrodistillation and maceration. The **solvent** employed may be chosen according to the chemical structure of target compounds. In this regard, polar solvents such as methanol, ethanol, acetone and their aqueous mixtures have been the most commonly employed solvents for extracting antioxidant compounds from plants and plant-based foods[106,107] while

[106] Sultana, B., Anwar, F., & Ashraf, M. (2009). Effect of extraction solvent/technique on the antioxidant activity of selected medicinal plant extracts. *Molecules*, 14(6), 2167-2180.

hexane, chloroform, dichloromethane, ethyl acetate, petroleum ether, and mixtures of hexane/isopropanol or hexane/acetone have been used for the extraction of non-polar compounds such as fatty acids or plant sterols from different plant and animal samples[108-110]. Comparing SPE and LLE, we find that the former does not require large quantities of organic solvents, can improve the recovery of some types of bioactive compounds, it is easy to automate, and its configuration into on-line systems is possible[111]. Among the main applications, both maceration and SE in different proportions of ethanol-water mixtures have been used for extracting phenolics and flavonoids from *M. oleifera* leaves[107]. Mixtures of methanol-water have also been used for recovering phenolic compounds from bitter melon[112] by SE. Nevertheless, Murugan *et al.* showed that maceration with methanol can extract tannins more efficiently than SE does, while the latter was more effective in extracting phenolic constituents[113].

In SPE, different cartridges with a large variety of **packing** can be used[114]. In the case of extraction of phenolic compounds, different sorbents including silica

[107] Vongsak, B., Sithisarn, P., & Gritsanapan, W. (2013). Simultaneous determination of crypto-chlorogenic acid, isoquercetin, and astragalin contents in *Moringa oleifera* leaf extracts by TLC-densitometric method. *Evidence-based Complementary and Alternative Medicine*, 2013.

[108] Abidi, S.L. (2001). Chromatographic analysis of plant sterols in foods and vegetable oils. *Journal of Chromatography A*, 935(1–2), 173-201.

[109] Abdolshahi, A., Majd, M.H., Rad, J.S., Taheri, M., Shabani, A., & Teixeira da Silva, J.A. (2015). Choice of solvent extraction technique affects fatty acid composition of pistachio (*Pistacia vera* L.) oil. *Journal of Food Science and Technology*, 52(4), 2422-2427.

[110] Caprioli, G., Giusti, F., Ballini, R., Sagratini, G., Vila-Donat, P., Vittori, S., & Fiorini, D. (2016). Lipid nutritional value of legumes: Evaluation of different extraction methods and determination of fatty acid composition. *Food Chemistry*, 192, 965-971.

[111] García-Salas, P., Morales-Soto, A., Segura-Carretero, A., & Fernández-Gutiérrez, A. (2010). Phenolic-Compound-Extraction Systems for Fruit and Vegetable Samples. *Molecules*, *15*(12), 8813-8826.

[112] Budrat, P., & Shotipruk, A. (2009). Enhanced recovery of phenolic compounds from bitter melon (*Momordica charantia*) by subcritical water extraction. *Separation and Purification Technology*, 66(1), 125-129.

[113] Murugan, R., & Parimelazhagan, T. (2014). Comparative evaluation of different extraction methods for antioxidant and anti-inflammatory properties from *Osbeckia parvifolia* Arn. – An *in vitro* approach. *Journal of King Saud University - Science*, 26(4), 267-275.

[114] Pérez-Magariño, S., Ortega-Heras, M., & Cano-Mozo, E. (2008). Optimization of a solid-phase extraction method using copolymer sorbents for isolation of phenolic compounds in red wines and quantification by HPLC. *Journal of Agricultural and Food Chemistry*, 56(24), 11560-11570.

sorbents such as C_{18}, polymeric materials, functionalized sorbents, carbon or a mixture of the aforementioned are commonly employed for separating phenolic compounds. However, silica-based cartridges have been the most widely used for this purpose[114,115]. Traditionally, the extraction of terpenes (terpenic alcohols and phytosterols) and fatty acids is performed by soxhlet, maceration or hydrodistillation. Among these, SE is probably the most commonly used technique for extracting fats and oils from food matrices[116,117]. In this regard, SE with ethyl acetate and maceration with ethanol were applied for extracting unsaturated fatty acids from pistachio (*Pistacia vera* L.) oil[109] and SE extraction with chloroform:methanol gave the highest oil yield from *Pistacia lentiscus* seeds[118]. It should be mentioned that mechanical extraction systems are also frequently employed for the extraction of oils from plant matrices[117].

Despite that CE methods have been shown to be effective for extracting bioactive compounds of different types, they are usually time- and solvent-consuming with the associated risk for human and environmental health. For this reason, the development of "modern" extraction techniques such as UAE, SFE, PLE, and MAE, with significant advantages over conventional methods is emerging as an alternative. In general, some of these advantages include a reduction in organic-solvent consumption, less degradation, elimination of additional sample clean-up, and concentration steps before chromatographic analysis, improvement in extraction efficiency, selectivity, and/or kinetics, or ease of automation[119].

---

[115] Prior, R.L., Fan, E., Ji, H., Howell, A., Nio, C., Paynef, M.J., & Reed, J. (2010). Multi-laboratory validation of a standard method for quantifying proanthocyanidins in cranberry powders. *Journal of the Science of Food and Agriculture*, 90(9), 1473-1478.

[116] Ruíz-Rodríguez, A., Reglero, G., & Ibáñez, E. (2010). Recent trends in the advanced analysis of bioactive fatty acids. *Journal of Pharmaceutical and Biomedical Analysis*, 51(2), 305-326.

[117] Anwar, P., Bendini, A., Gulfraz, M., Qureshi, R., Valli, E., Di Lecce, G., Naqvi, S.M.S., & Toschi, T.G. (2013). Characterization of olive oils obtained from wild olive trees (*Olea ferruginea* Royle) in Pakistan. *Food Research International*, 54(2), 1965-1971.

[118] Bhutada, P.R., Jadhav, A.J., Pinjari, D.V., Nemade, P.R., & Jain, R.D. (2016). Solvent assisted extraction of oil from Moringa oleifera Lam. seeds. *Industrial Crops and Products*, 82, 74-80.

[119] Huie, C.W. (2002). A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants. *Analytical and Bioanalytical Chemistry*, 373(1-2), 23-30.

3.1.2. Ultrasound-assisted extraction (UAE)

Ultrasound-assisted extraction has been widely used during the last two decades for extracting bioactive compounds by food and pharmaceutical companies[120]. Ultrasound has been defined as a mechanical wave that necessitates an elastic medium to propagate. It differs from the audible sound by the wave frequency. UAE is based on the alteration between physical and chemical properties of the sample after its interaction with ultrasound waves. The cavitational effect facilitates the extraction process, increasing the mass transfer between the solvent and the sample by disrupting the plant-cell walls. When the bubbles collapse onto the solid sample, the high pressure and temperature released generate microjets and shock waves conducted to the solid phase[121]. Briefly, some of the parameters that affect the quality of the UAE are operating temperature, frequencies, ultrasound power and solvents. Most authors agree that elevated **temperatures** improve the efficiency of extraction because of enhanced diffusion rates and solubility of analytes in solvents[122]. However, it should not be forgotten that high temperatures can degrade thermolabile compounds and also solubilize impurities from the sample. Some authors have noticed that when high **frequency ultrasound** is used, the extraction yield does not significantly increase, but the degradation of the plant constituents is diminished[123]. In this sense, the common frequencies described to extract phenolic compounds are in the range from 20 and 45 kHz[124]. Regarding ultrasound power, its increase can promote the formation and collapse of more cavitation bubbles.

---

[120] Esclapez, M.D., García-Pérez, J.V., Mulet, A., & Cárcel, J.A. (2011). Ultrasound-assisted extraction of natural products. *Food Engineering Reviews*, 3(2), 108-120.

[121] Tao, Y., & Sun, D. (2015). Enhancement of food processes by ultrasound: A review. *Critical Reviews in Food Science and Nutrition*, 55(4), 570-594.

[122] Santos, D.T., Veggi, P.C., & Meireles, M.A.A. (2012). Optimization and economic evaluation of pressurized liquid extraction of phenolic compounds from jabuticaba skins. *Journal of Food Engineering*, 108 (3), 444-452.

[123] Vinatoru, M. (2001). An overview of the ultrasonically assisted extraction of bioactive principles from herbs. *Ultrasonics Sonochemistry*, 8(3), 303-313.

[124] Ruíz-Montañez, G., Ragazzo-Sánchez, J.A., Calderón-Santoyo, M., Velázquez-De La Cruz, G., Ramírez De León, J.A., & Navarro-Ocaña, A. (2014). Evaluation of extraction methods for preparative scale obtention of mangiferin and lupeol from mango peels (*Mangifera indica* L.). *Food Chemistry*, 159, 267-272.

The extraction of phenolic compounds through UAE has been especially addressed. Ma *et al.* showed that the extraction yields and the recovery of different phenolic acids were significantly higher than those by maceration[125]. This extraction technique has been applied to recover flavanones and anthocyanins from different plant matrices with promising results[126].

Despite that some authors have found UAE more effective than CE, both extraction techniques have enabled similar recoveries of TPC and total flavonoid content[127]. In addition, UAE has also been used for extracting bioactive compounds such as the triterpenoid charantin from *Momordica charantia* fruit and it was almost 3-fold more efficient than SE[128]. To a lesser extent, UAE has been used for the recovery of oils from different samples. In this regard, UAE allowed the use of considerably smaller extraction time than soxhlet for the extraction of triacylglycerols and fatty acids from papaya seeds[129]. Another study showed that UAE was the best option for recovering fatty acids such as linoleic and palmitic acids from *Piper gaudichaudianum* Kunth leaves[130] compared with SE.

In summary, UAE allows shorter processing time and at a low cost than with conventional extraction techniques. However, its efficiency depends on the nature of the target compounds. In addition, it should be borne in mind that the distribution of

[125] Ma, Y., Chen, J., Liu, D., & Ye, X. (2009). Simultaneous extraction of phenolic compounds of citrus peel extracts: Effect of ultrasound. *Ultrasonics Sonochemistry*, 16(1), 57-62.

[126] Zhu, Z., Guan, Q., Guo, Y., He, J., Liu, G., Li, S., Barba, F. J., & Jaffrin, M.Y. (2016). Green ultrasound-assisted extraction of anthocyanin and phenolic compounds from purple sweet potato using response surface methodology. *International Agrophysics*, 30(1), 113-122.

[127] Dahmoune, F., Spigno, G., Moussi, K., Remini, H., Cherbal, A., & Madani, K. (2014). *Pistacia lentiscus* leaves as a source of phenolic compounds: Microwave-assisted extraction optimized and compared with ultrasound-assisted and conventional solvent extraction. *Industrial Crops and Products*, 61, 31-40.

[128] Ahamad, J., Amin, S., & Mir, S.R. (2015). Optimization of ultrasound-assisted extraction of charantin from *Momordica charantia* fruits using response surface methodology. *Journal of Pharmacy and Bioallied Sciences*, 7(4), 304-307.

[129] Samaram, S., Mirhosseini, H., Tan, C.P., & Ghazali, H.M. (2013). Ultrasound-assisted extraction (UAE) and solvent extraction of papaya seed oil: Yield, fatty acid composition and triacylglycerol profile. *Molecules*, 18(10), 12474-12487.

[130] Péres, V.F., Saffi, J., Melecchi, M.I.S., Abad, F.C., De Assis Jacques, R., Martinez, M.M., Oliveira, E.C., & Caramão, E.B. (2006). Comparison of soxhlet, ultrasound-assisted and pressurized liquid extraction of terpenes, fatty acids and Vitamin E from *Piper gaudichaudianum* Kunth. *Journal of Chromatography A*, 1105(1-2 SPEC. ISS.), 115-118.

the ultrasonic wave is non-uniform and is limited to the vicinity of the ultrasound probe, making its implementation on a large scale an arduous task[105].

3.1.3. Supercritical fluid extraction (SFE)

Supercritical fluid extraction (SFE) is a technique with promising applications in the food industry. SFE is a fast, efficient, and clean technique for extracting natural products from plant and plant-based matrices. Its importance resides in the fact that SFE allows the fabrication of products without toxic residues, with no degradation of active principles, and with high purity[131]. Due to their low viscosity and relatively high diffusivity, supercritical fluids have better transport properties than do liquids. For this reason they can diffuse easily through solid materials, thus, giving faster extraction rates[132]. One of their main characteristics is the possibility of modifying the density of the fluid by changing its pressure and/or temperature. Although many supercritical fluids can be used, for the extraction of non-polar bioactive compounds, carbon dioxide (CO_2) is most commonly used to replace organic solvents for extraction, because it is inert, nonflammable, noncorrosive, inexpensive, easily available, odorless, tasteless, environmentally friendly, and it has GRAS status[133]. SFE consists principally of two different steps: extraction of the soluble substances from the solid substratum by the supercritical fluid solvent and separation of these compounds from the supercritical solvent after the expansion[131]. **Figure 19** shows a general flow diagram of the equipment used to perform supercritical fluid extractions, consisting of a container for the solvent used for the extraction, CO_2 in this case (A), a pump that allows the desired CO_2 flow (B), an oven containing the extraction vessel (C), a back

[131] Pereira, C.G., & Meireles, M.A.A. (2010). Supercritical fluid extraction of bioactive compounds: Fundamentals, applications and economic perspectives. *Food and Bioprocess Technology*, 3(3), 340-372.

[132] Da Silva, R.P.F.F., Rocha-Santos, T.A.P., & Duarte, A.C. (2016). Supercritical fluid extraction of bioactive compounds. *TrAC- Trends in Analytical Chemistry*, 76, 40-51.

[133] Mendiola, J.A., Herrero, M., Cifuentes, A., & Ibáñez, E. (2007). Use of compressed fluids for sample preparation: Food applications. *Journal of Chromatography A*, 1152(1–2), 234-246.

pressure regulator that allows the pressurization of the system (BPR) (D), a trapping vessel (E) and optionally a modifier pump (F)[134].

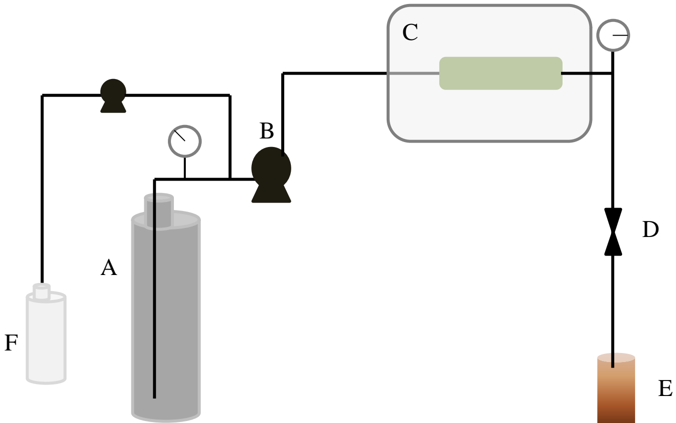


Figure 19. Schematic diagram of the equipment used to perform supercritical fluid extractions. (A) Solvent reservoir, (B) pump, (C) heated extraction cell, (D) BPR, (E) collection vessel and (F) modifier. Adapted from Herrero *et al.*[134]

The main parameters to optimize in SFE are **temperature**, pressure, co-solvent percentage and extraction time. Temperatures ranging from 32 to 86ºC were tested in order to recover the highest amount of lycopene from tomato by-products; the conclusion was that the percentage of lycopene extracted increased at higher temperatures[135]. Santos *et al.* also found that the highest temperature tested (70ºC) was the optimal, allowing the recovery of higher TPC from *Eucalyptus globulus* bark[136]. Additionally, operating **pressure** affects the solvating power of the solvent, and therefore the extraction. It is commonly known that the higher the pressure, the larger the solvation power and the smaller the extraction selectivity. SFE pressure values of

[134] Herrero, M., Castro-Puyana, M., Mendiola, J.A., & Ibáñez, E. (2013). Compressed fluids for the extraction of bioactive compounds. *TrAC - Trends in Analytical Chemistry*, 43, 67-83.

[135] Rozzi, N. L., Singh, R.K., Vierling, R.A., & Watkins, B.A. (2002). Supercritical fluid extraction of lycopene from tomato processing byproducts. *Journal of Agricultural and Food Chemistry*, 50(9), 2638-2643.

[136] Santos, S.A.O., Villaverde, J.J., Silva, C.M., Neto, C P., & Silvestre, A.J.D. (2012). Supercritical fluid extraction of phenolic compounds from *Eucalyptus globulus* Labill bark. *The Journal of Supercritical Fluids*, 71, 71-79.

150–300 bars and temperature of 30-60°C have commonly been used for the extraction of bioactive compounds depending on different plant materials[137].

Also, CO_2 pressures above 200 bars have been applied to extract oil from seeds[138]. The low polarity of CO_2 makes it more effective for the extraction of apolar compounds from natural matrices. To cite some applications, SFE has been used for extracting oils, unsaturated fatty acids, tocopherol-enriched oils, and phytosterols from different plant matrices[139,140]. Nevertheless, when the target compounds are more polar, the use of polar **cosolvents** (modifiers) such as ethanol in small quantities (<10%) together with CO_2 varies the polarity of the solvent significantly, enhancing the extraction of most polar compounds. Borrás-Linares *et al.* extracted bioactive phenolics from rosemary leaves by SFE using 6.6% of ethanol as a modifier[141]. The same methodology was used by Taamali *et al.* for the extraction of phenolic compounds from olive leaves[142]. In addition, as occur in other extraction techniques, the extraction time also influences the overall process. Some authors have reported that up to 120 min the extraction curves of TPC extraction start to decrease[143]. When

[137] Salgin, U. (2007). Extraction of jojoba seed oil using supercritical CO_2 + ethanol mixture in green and high-tech separation process. *Journal of Supercritical Fluids*, 39(3), 330-337.

[138] Passos, C.P., Silva, R.M., Da Silva, F.A., Coimbra, M.A., & Silva, C.M. (2010). Supercritical fluid extraction of grape seed (*Vitis vinifera* L.) oil. Effect of the operating conditions upon oil composition and antioxidant capacity. *Chemical Engineering Journal*, 160(2), 634-640.

[139] Zhao, S., & Zhang, D. (2013). Supercritical fluid extraction and characterisation of *Moringa oleifera* leaves oil. *Separation and Purification Technology*, 118, 497-502.

[140] Uddin, M.S., Sarker, M.Z.I., Ferdosh, S., Akanda, M.J.H., Easmin, M.S., Bt Shamsudin, S.H., & Yunus, K.B. (2014). Phytosterols and their extraction from various plant matrices using supercritical carbon dioxide: A review. *Journal of the Science of Food and Agricultura*, 95, 1385-1394.

[141] Borrás Linares, I., Arráez-Román, D., Herrero, M., Ibáñez, E., Segura-Carretero, A., & Fernández-Gutiérrez, A. (2011). Comparison of different extraction procedures for the comprehensive characterization of bioactive phenolic compounds in *Rosmarinus officinalis* by reversed-phase high-performance liquid chromatography with diode array detection coupled to electrospray time-of-flight mass spectrometry. *Journal of Chromatography A*, 1218(42), 7682-7690.

[142] Taamalli, A., Arráez-Román, D., Barrajón-Catalán, E., Ruíz-Torres, V., Pérez-Sánchez, A., Herrero, M., Ibáñez, E., Micol, V., Zarrouk, M., Segura-Carretero, A., & Fernández-Gutiérrez, A. (2012). Use of advanced techniques for the extraction of phenolic compounds from Tunisian olive leaves: Phenolic composition and cytotoxicity against human breast cancer cells. *Food and Chemical Toxicology*, 50(6), 1817-1825.

[143] Da Porto, C., Decorti, D., & Natolino, A. (2014). Water and ethanol as co-solvent in supercritical fluid extraction of proanthocyanidins from grape marc: A comparison and a proposal. *Journal of Supercritical Fluids*, 87, 1-8.

the objective is the extraction of the highest yield, the best way to establish the extraction time is by performing a kinetic until the sample is exhausted. In this sense, Balvardi *et al.* established 160 min for the recovery of oils from wild almond[144].

In relation to SFE, gas-expanded liquid (GXL) is a novel extraction system that is emerging as a promising technique for extracting bioactive compounds with different polarities. GXL current working definition is "*a liquid whose volume is increased when pressurized with a condensable gas such as* CO_2"[145]. Thus, the expanded liquid phases resulting from the addition of miscible dense gases such as compressed CO_2 to organic solvents such as ethanol offer enhanced properties that may be easily tuned by adjusting the pressure of the system[146]. In addition, GXLs allow working at lower pressures compared with SFE, which makes industrial applications of this technique more attractive[146]. Despite the great potential that GXLs can offer, little research has taken advantage of this technique for the extraction of bioactive compounds from plant matrices[147-149].

[144] Balvardi, M., Mendiola, J.A., Castro-Gómez, P., Fontecha, J., Rezaei, K., & Ibáñez, E. (2015). Development of pressurized extraction processes for oil recovery from wild almond (*Amygdalus scoparia*). *Journal of the American Oil Chemists' Society*, 92, 1503-1511.

[145] Akien, G.R., & Poliakoff, M. (2009). A critical look at reactions in class I and II gas-expanded liquids using CO_2 and other gases. *Green Chemistry*, 11(8), 1083-1100.

[146] Sih, R., Armenti, M., Mammucari, R., Dehghani, F., & Foster, N.R. (2008). Viscosity measurements on saturated gas-expanded liquid systems: Ethanol and carbon dioxide. *Journal of Supercritical Fluids*, 43(3), 460-468.

[147] Gilbert-López, B., Mendiola, J.A., Fontecha, J., Van Den Broek, L.A.M., Sijtsma, L., Cifuentes, A., Herrero, M., & Ibáñez, E. (2015). Downstream processing of *Isochrysis galbana*: a step towards microalgal biorefinery. *Green Chemistry*, 17(9), 4599-4609.

[148] Golmakani, M., Mendiola, J.A., Rezaei, K., & Ibáñez, E. (2012). Expanded ethanol with CO_2 and pressurized ethyl lactate to obtain fractions enriched in γ-Linolenic Acid from *Arthrospira platensis* (Spirulina). *The Journal of Supercritical Fluids*, 62, 109-115.

[149] Reyes, F.A., Mendiola, J.A., Ibáñez, E., & Del Valle, J.M. (2014). Astaxanthin extraction from *Haematococcus pluvialis* using CO_2-expanded ethanol. *Journal of Supercritical Fluids*, 92, 75-83.

In a comparison with CE, Ara *et al.* concluded that SFE was more selective than the steam-distillation method[150]. However, Carvalho *et al.* reported that SE extraction was the most effective, allowing 58.93% of sesame oil vs. 26.47% obtained by SFE[151]. In spite of the large number of studies on SFE, the relatively high cost of investment should be taken into account.

3.1.4. Pressurized liquid extraction (PLE)

Pressurized liquid extraction (PLE) also known as accelerated solvent extraction (ASE) or pressurized hot-water extraction when the solvent employed is water (PHWE), is a technique that involves the extraction of target compounds using solvents at high pressures and temperatures. PLE enables a short extraction time (less than 30 min) under high-pressure conditions (up to 200 bars) and temperature (up to 200ºC). The PLE system works in a similar way as the SE system. However, the most interesting advantage of PLE is that pressurized solvents remain in a liquid state above their boiling point, allowing, thus, higher extraction temperatures without degradation of bioactive compounds compared with CE. The scheme of the equipment required for performing PLE is displayed in **Figure 20**.

In this case, the container for the solvent employed for the extraction is filled with water for PHWE or water and other organic solvents for PLE. Different types of solvents can be used in PLE, which allows the extraction of a wide range of bioactive compounds, as shown in **Figure 21**.

[150] Ara, K.M., Jowkarderis, M., & Raofie, F. (2015). Optimization of supercritical fluid extraction of essential oils and fatty acids from flixweed (*Descurainia sophia* L.) seed using response surface methodology and central composite design. *Journal of Food Science and Technology*, 52(7), 4450-4458.

[151] Carvalho, R.H.R., Galvão, E.L., Barros, J.A.C., Conceição, M.M., & Sousa, E.M.B.D. (2012). Extraction, fatty acid profile and antioxidant activity of sesame extract: (*Sesamum indicum* L.). *Brazilian Journal of Chemical Engineering*, 29(2), 409-420.

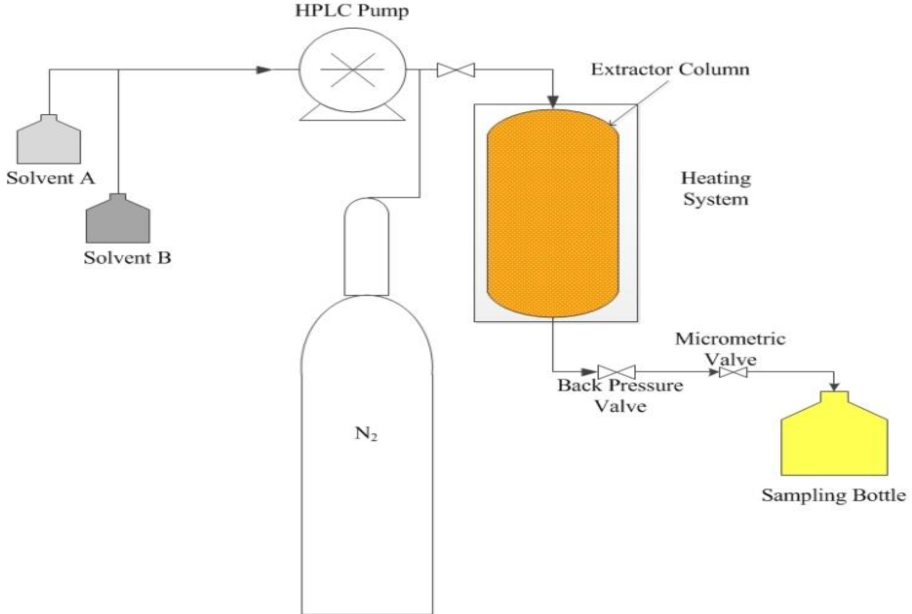


Figure 20. Schematic equipment used to perform pressurized liquid extractions.

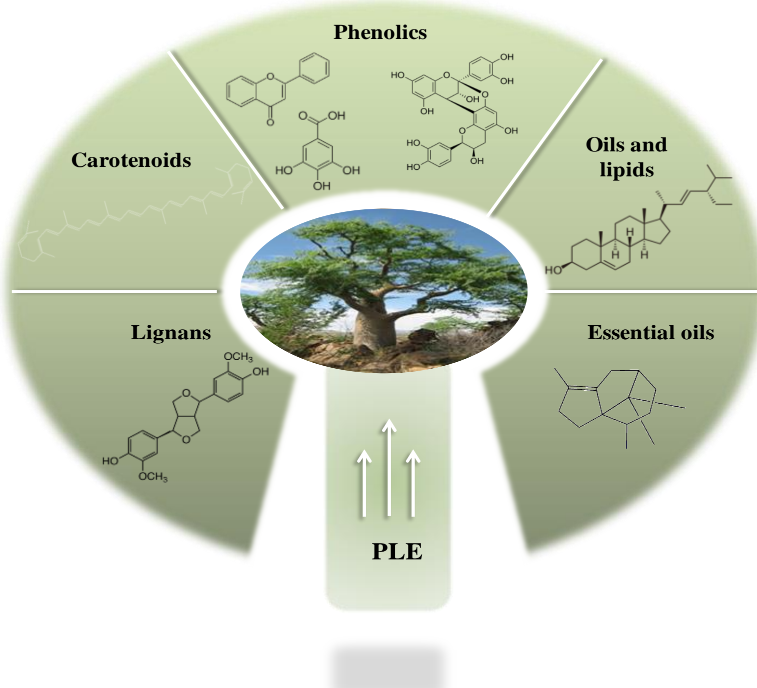


Figure 21. Main types of compounds extracted by PLE from plant sources, and an example of each.

As stated above, for dealing with bioactive compounds, **temperature** is a critical parameter to be examined and optimized. High temperatures lower the viscosity of the solvent, thereby enhancing its penetration into the matrix and resulting in an improved extraction process[152]. Matshediso *et al.* studied the effect of temperature (50, 100, 150, and 200ºC) in TPC, myricetin, quercetin, and kaempferol extraction from plant leaves[153]. These researchers reported that optimum temperature for the extraction of flavonols was 100ºC and total phenols, kaempferol, and myricetin contents remained constant to 150ºC. Another recent study found that TPC recovery from aboveground parts of *Phyllanthus amarus* was significantly higher by PLE at 192.4ºC than CE at 80-90ºC and UAE at 25ºC[154]. In accordance, temperatures between 150 and 200ºC proved to be more suitable for the extraction of phenolic compounds than CE with methanol and SE water extraction[112].

Despite that **pressure** has been pointed out as an important parameter affecting PLE extraction, some authors have failed to find any significant influence of the extraction pressure[152]. Regarding **extraction time**, its influence is closely related to the operation mode, whether dynamic or static[134]. However, there is no commercial or high-throughput system for PLE in the dynamic mode.

Unquestionably, PLE allows higher recoveries of bioactive compounds than CE methods. Authors concluded that PLE significantly decreased the total extraction time, the amount of solvent, and the manipulation of sample and solvents in comparison with SE and UAE. In addition, PLE was more effective for the extraction of terpenic alcohols and phytosterols, fatty acids and vitamin E[130] than CE. However, PLE

---

[152] Mustafa, A., & Turner, C. (2011). Pressurized liquid extraction as a green approach in food and herbal plants extraction: A review. *Analytica Chimica Acta*, 703, 8–18.
[153] Matshediso, P.G., Cukrowska, E., & Chimuka, L. (2015). Development of pressurised hot water extraction (PHWE) for essential compounds from *Moringa oleifera* leaf extracts. *Food Chemistry*, 172, 423-427.
[154] Sousa, A.D., Maia, A.I.V., Rodrígues, T.H.S., Canuto, K.M., Ribeiro, P.R.V., de Cassia Alves Pereira, R., Vieira, R.F., & de Brito, E.S. (2016). Ultrasound-assisted and pressurized liquid extraction of phenolic compounds from *Phyllanthus amarus* and its composition evaluation by UPLC-QTOF. *Industrial Crops and Products*, 79, 91-103.

involves high cost for the high-pressure equipment[155]. Compared with SFE, PLE allows the extraction of more polar compounds and it is able to provide higher extraction yields, although SFE might be more selective and environmentally friendly[141] .

3.1.5. Microwave-assisted extraction (MAE)

Microwave-assisted extraction (MAE) is another technique that is gaining ground in the field of bioactive compounds extraction. Microwave consists of electric and magnetic fields that oscillate perpendicularly to each other in a frequency range from 0.3 to 300 GHz. Thus, microwave is considered an electromagnetic wave that can penetrate some materials, interact with polar compounds, and consequently generate heat. The combination of that heat and mass gradients working in the same direction accelerate the process and raise the extraction yield[156]. As occurs with the other extraction techniques, the **solvent**, **extraction time**, and **temperature** are the main factors to optimize. The extraction yield in MAE is commonly directly proportional to the extraction time but the increase in yield was found to be very small with further increase in time, as displayed in **Figure 22**.

It should be taken into account that ethanol and water mixtures increase the heating capacity of the solvent combination, which could degrade target compounds if the extraction time is prolonged[157].

[155] Heng, M.Y., Tan, S.N., Yong, J.W.H., & Ong, E.S. (2013). Emerging green technologies for the chemical standardization of botanicals and herbal preparations. *TrAC- Trends in Analytical Chemistry*, 50, 1-10.

[156] Veggi, P.C., Martinez, J., & Meireles, M.A.A. (2012). Fundamentals of microwave extraction. In: *Microwave-assisted Extraction for Bioactive Compounds.* Chemat**,** Farid, Cravotto**,** Giancarlo (Eds.),15-52. ISBN 978-1-4614-4830-3.

[157] Routray, W., & Orsat, V. (2012). Microwave-Assisted Extraction of Flavonoids: A Review. *Food and Bioprocess Technology*, 5(2), 409-424.

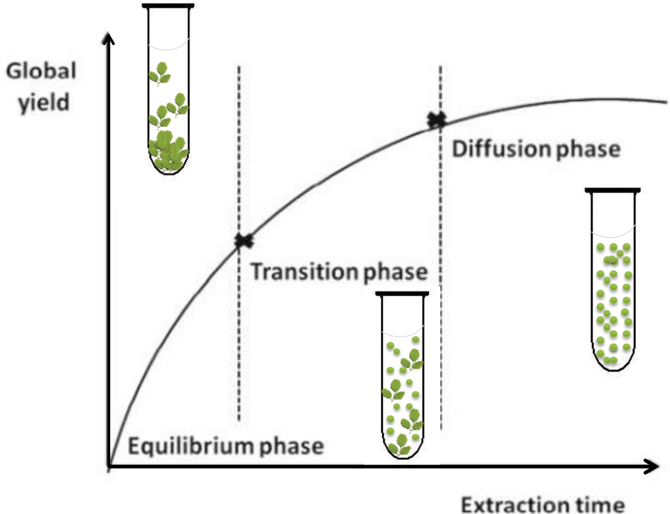


Figure 22. Schematic representation of yield vs. time in extraction processes. Adapted from Veggi *et al.*[156]

Pan *et al.* found that the extraction yield of phenolic compounds from tea leaves did not increase from 4-min extraction time. Also, they found that the extraction of phenolic compounds by MAE was higher than using maceration, UAE, or heat reflux[158]. In addition, optimized MAE at 46% ethanol was the best option for extracting TPC compared with conventional solvent extraction and UAE mixture[127]. MAE was also better than SE for extracting diterpenes[159], despite that oil yields were higher using SE. Likewise, significantly more trans-lycopene was extracted via MAE than via CE[160]. In addition, Qu *et al.* showed that chemical compositions of fatty acids extracted with SE and MAE were mostly similar, whereas relative contents of the

[158] Pan, X., Niu, G., & Liu, H. (2003). Microwave-assisted extraction of tea polyphenols and tea caffeine from green tea leaves. *Chemical Engineering and Processing: Process Intensification*, 42(2), 129-133.

[159] Tsukui, A., Santos Júnior, H.M., Oigman, S.S., De Souza, R.O.M.A., Bizzo, H.R., & Rezende, C.M. (2014). Microwave-assisted extraction of green coffee oil and quantification of diterpenes by HPLC. *Food Chemistry*, 164, 266-271.

[160] Ho, K.K.H.Y., Ferruzzi, M.G., Liceaga, A.M., & San Martín-González, M.F. (2015). Microwave-assisted extraction of lycopene in tomato peels: Effect of extraction conditions on all-*trans* and *cis*-isomer yields. *LWT - Food Science and Technology*, 62, 160–168.

same fatty acids had a few differences, MAE offering the advantage of much higher fatty acid yield[161].

The main advantages of MAE include its suitability for recovering a vast array of compounds. In addition, MAE involves moderate economic cost while allowing shorter extraction times compared with CE and other advanced extraction techniques[162,163].

3.2. Extraction of bioactive compounds from biological matrices

Biological samples are extremely complex matrices containing many components which can interfere with the analyte analysis or be incompatible with subsequent separation techniques. The most common biological matrices in which bioactive compounds are measured include serum, plasma, and urine; however, other samples that can also be used are saliva, tissue, or feces.

The presence of proteins or other organic compounds with structures similar to those of the compounds of interest can interfere in the extraction procedure. Therefore, in most cases it becomes necessary to use solid-phase extraction (SPE), liquid-liquid extraction (LLE) and/or protein precipitation[164]. In this regard, different extraction methods have been used for extracting plant sterols from serum samples. Mackay *et al.* proposed carrying out an alkaline hydrolysis following to two consecutive LLEs using water and cyclohexane as solvents[165]. A sterol hydrolyzation with ethanolic potassium

[161] Qu, W., Mou, Z., Cui, H., & Zhang, Z. (2011). Analysis of fatty acids in *A. szechenyianum* Gay. by microwave-assisted extraction and gas chromatography-mass spectrometry. *Phytochemical Analysis*, 22 (3), 199-204.

[162] Chan, C.H., Yusoff, R., Ngoh, G., & Kung, F.W.L. (2011). Microwave-assisted extractions of active ingredients from plants. *Journal of Chromatography A*, 1218 (37), 6213-6225.

[163] Taamalli, A., Arráez-Román, D., Ibáñez, E., Zarrouk, M., Segura-Carretero, A., & Fernández-Gutiérrez, A. (2012). Optimization of microwave-assisted extraction for the characterization of olive leaf phenolic compounds by using HPLC-ESI-TOF-MS/IT-MS^2. *Journal of Agricultural and Food Chemistry*, 60(3), 791-798.

[164] Ashri, N.Y., & Abdel-Rehim, M. (2011). Sample treatment based on extraction techniques in biological matrices. *Bioanalysis*, 3(17), 2003-2018.

[165] Mackay, D.S., Jones, P.J.H., Myrie, S.B., Plat, J., & Lütjohann, D. (2014). Methodological considerations for the harmonization of non-cholesterol sterol bio-analysis. *Journal of Chromatography B*, 957, 116-122.

hydroxide solution and extraction by LLE with n-hexane was recommended by Matysik *et al.* for extracting plant sterols from plasma samples[166].

❖ ANALYTICAL TOOLS FOR CHARACTERIZING AND QUANTIFYING BIOACTIVE COMPOUNDS

Analytical techniques are key in developing nutraceuticals and functional foods, providing knowledge of the physical and chemical stability of these products, aiding in the selection and design of the dosage form, and allowing the quantification of bioactive compounds and their metabolites. Analytical tools can be divided into two main groups: **separation techniques,** which are used to resolve the compounds from a mixture; and **spectrometric techniques,** which offer precise information on the structural characteristic of each compound analyzed. Roughly speaking, the most suitable techniques of characterizing polar and semi-polar compounds are high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) whereas gas chromatography (GC) is used mainly to characterize non-polar compounds. Nuclear magnetic resonance (NMR) has also been used to elucidate the structure of some compounds from different matrices.

Chromatography and **spectrophotometry** are the two most commonly applied techniques to qualitative and quantitatively characterize bioactive compounds.

4.1. Chromatographic techniques

The separation of analytes is an important step of the analysis of an unknown mixture. In this sense, chromatography, which is a combination of separation techniques based on differential distribution between two phases, appeared as the best option. In chromatography, compounds from a sample are transported by a fluid which moves in a definite direction (mobile phase) into a stationary phase that can be solid or liquid. Solutes from the sample usually have differential partitioning or interactions

[166] Matysik, S., Klunemann, H.H., & Schmitz, G. (2012). Gas chromatography-tandem mass spectrometry method for the simultaneous determination of oxysterols, plant sterols, and cholesterol precursors. *Clinical Chemistry*, 58(11), 1557-1564.

with the mobile and stationary phases. Consequently, each analyte has a specific equilibrium constant and, thus, different elution rate. As a result, analytes that strongly interact with the stationary phase will be retained more (longer retention times) than compounds having lower or no interactions with the stationary phase.

Among the main chromatographic techniques employed for analyzing bioactive compounds such as phenolic compounds, fatty acids, and phytosterols, it is noteworthy to emphasize high-performance liquid chromatography (HPLC) and gas chromatography (GC), which have been used in the current doctoral Thesis.

4.1.1. High-performance liquid chromatography

HPLC is the most widely used separation technique for complex naturally occurring molecules in plants. HPLC instrumentation is generally made up a pump, injector, column, detector, and data acquisition system as showed in **Figure 23**.

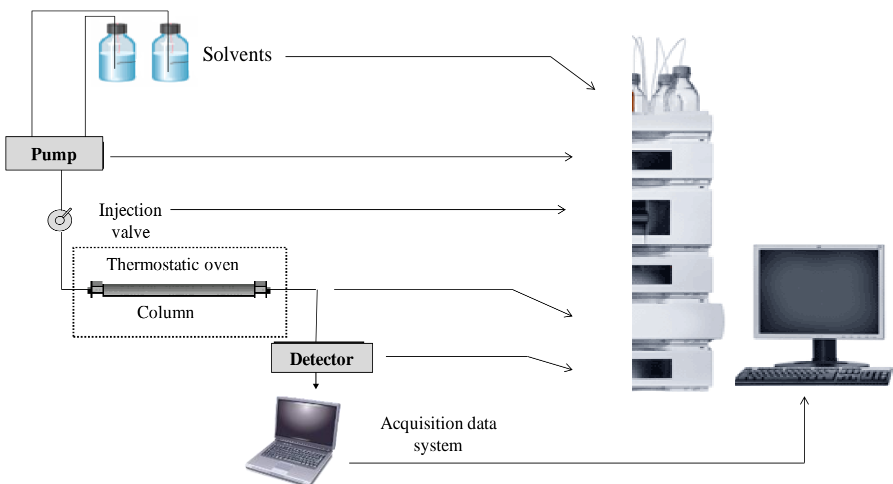


Figure 23. HPLC instrumentation.

In HPLC, the **mobile phase** consists of a solvent or a mixture of solvents in which the pH is often altered by using acids, bases or regulatory solutions depending on the target compound to be separated. The mobile phase is pumped into the chromatographic **column** which contains the stationary phase necessary for the separation of the compounds. The most widely used HPLC mode is reversed-phase (RP) in which the stationary phase is non-polar, while the mobile phase is a polar

mixture of one or more organic solvents and water or a buffer[167]. The choice of a mobile phase depends on the column employed, the analytes of interest, the detection system coupled, while simultaneously the selection of an appropriate column is directly related to the nature of compounds to be separated. When all analytes elute from the column, they are detected, generating an analytical signal. The signal intensity and duration will depend on the quantity and nature of the analyte. Therefore, each analyte will be represented as a peak which has a specific retention time (RT) and area.

The column represents the most determinant component in HPLC separation. Currently, a trend towards the use of still smaller particles (sub-2 µm, > 400 bar) is emerging in order to enhance the efficiency and speed of the analysis[168]. In the case of phenolic compounds, the most commonly used column is C_{18}, which is filled with a packing of octadecylsilyl groups. It can range from 10 to 30 cm long, with an internal diameter from 3.9 to 4.6 mm, and the particle size from 1.8 to 10 µm. The use of C_{18} columns often involves the use of binary systems that consist of acidic aqueous solutions and organic solvents such as methanol or acetonitrile as mobile phases. The separation of more polar phenolic compounds depends heavily on the pH of the mobile phase[169]. The presence of the acid in the aqueous mobile phase avoids the ionization of these compounds, thereby enhancing their separation in the column[170]. However, flavonoids can be separated only by organic solvents because these compounds have

[167] Jandera, P. (2011). Stationary and mobile phases in hydrophilic interaction chromatography: a review. *Analytica Chimica Acta*, 692(1–2), 1-25.

[168] Nguyen, D.T., Guillarme, D., Rudaz, S., & Veuthey, J. (2006). Fast analysis in liquid chromatography using small particle size and high pressure. *Journal of Separation Science*, 29(12), 1836-1848.

[169] Rehová, L., Škeríkova, V., & Jandera, P. (2004). Optimisation of gradient HPLC analysis of phenolic compounds and flavonoids in beer using a CoulArray detector. *Journal of Separation Science*, 27(15-16), 1345-1359.

[170] Schieber, A., Keller, P., & Carle, R. (2001). Determination of phenolic acids and flavonoids of apple and pear by high-performance liquid chromatography. *Journal of Chromatography A*, 910(2), 265-273.

less ionization capacity[171]. Despite that acidified water and acetonitrile has proved to be a good combination for separating flavonoids[172], some researchers affirm that using acidified water and methanol as organic solvent constitutes the best choice for separating phenolic compounds from different plant matrices[173,174].

The **time of analysis** is another parameter that influences the separation of bioactive compounds such as phenolics. This time should range from 10 to 150 min at a flow speed of between 0.2 to 1.5 mL/min. In addition, HPLC has been used to separate lipids and individual plant sterols from plant-based products using different types of columns containing methanol and acetonitrile with water mixtures as mobile phases. The most frequently used columns for this purpose are made of octadecylsilica and range from 3 to 5 µm particle size[108]. As can be seen from the above, HPLC offers a high degree of versatility not found in other chromatographic systems due to its ability to easily separate a large variety of chemical mixtures from plant and plant-based products as well as from biological samples.

After the separation, the following step includes the **detection** of the mixture being eluted from the chromatographic column. A good detector has specific characteristics, including sensitivity, specificity, detectability, linearity, repeatability and dependability. However, no single HPLC detector has all the characteristics of a good detector. **Figure 24** sums up the different detection systems.

[171] Mehta, J.P., Pandya, C.V., Parmar, P.H., Vadia, S.H., & Golakiya, B.A. (2014). Determination of flavonoids, phenolic acid and polyalcohol in *Butea monosperma* and *Hedychium coronarium* by semi-preparative HPLC Photo Diode Array (PDA) Detector. *Arabian Journal of Chemistry*, 7(6), 1110-1115.

[172] Dmitrienko, S.G., Stepanova, A.V., Kudrinskaya, V.A., & Apyari, V.V. (2012). Specifics of separation of flavonoids by reverse phase high performance liquid chromatography on the Luna 5u C18(2) column. *Moscow University Chemistry Bulletin*, 67(6), 254-258.

[173] Talhaoui, N., Gómez-Caravaca, A.M., León, L., De la Rosa, R., Segura-Carretero, A., & Fernández-Gutiérrez, A. (2014). Determination of phenolic compounds of 'Sikitita' olive leaves by HPLC-DAD-TOF-MS. Comparison with its parents 'Arbequina' and 'Picual' olive leaves. *LWT - Food Science and Technology*, 58(1), 28-34.

[174] Nouman, W., Anwar, F., Gull, T., Newton, A., Rosa, E., & Domínguez-Perles, R. (2016). Profiling of polyphenolics, nutrients and antioxidant potential of germplasm's leaves from seven cultivars of *Moringa oleifera* Lam. *Industrial Crops and Products*, 83, 166-176.

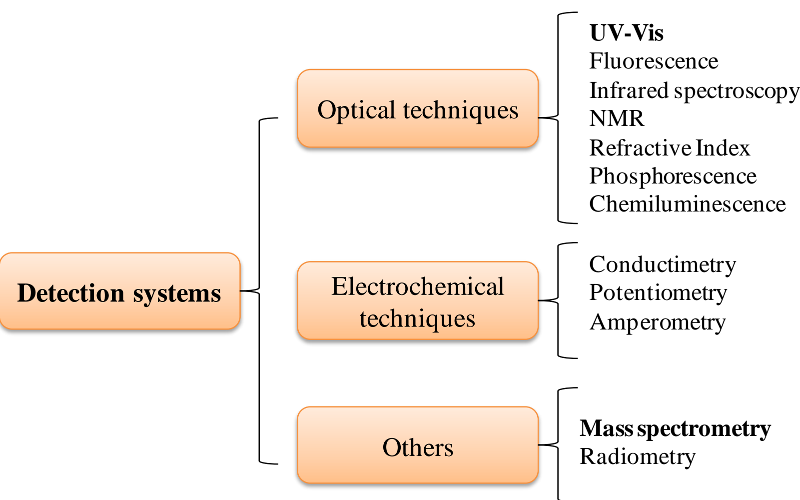


Figure 24. The most widely used detection systems in liquid chromatography.

In the current doctoral Thesis, UV-Visible (UV-Vis) and mass spectrometry (MS) have been used. Both will be described in depth in the following sub-sections.

a) HPLC coupled to UV-Vis detector

HPLC methods based on the UV-Vis detection are the most widely employed because they enable the characterization of a wide range of compounds and functional groups, and have good sensitivity, stability against temperature and flow changes, strong linearity, and high accuracy. Furthermore, UV-Vis detection is more rugged than many other detection systems[175]. Many molecules can absorb radiation UV-Vis and therefore this detector can be considered universal. Three different types of UV-Vis detectors are available: fixed-wavelength, variable-wavelength, and **diode array detectors (DAD)**. Among them, DAD is the most common for the detection of bioactive compounds because it provides the complete UV-Vis absorption spectra of analytes that pass quickly through a measuring cell. The use of DAD coupled to HPLC allows unknown phenolic compounds to be assigned to a specific phenolic class, taking into account its UV-Vis absorption maxima, since each family has specific absorption bands. In addition, HPLC-DAD has been used to characterize and quantify

[175] Lendi, B.E., & Meyer, V.R. (2005). The UV detector for HPLC - An ongoing success story. *LC-GC Europe*, 18(3), 156-163.

16 different anthocyanidins from grape juice[176] and from Mashua (*Tropaeolum tuberosum*)[177]. Despite this detection system can be useful for resolving analytical problems, it presents the disadvantage of low selectivity and it does not provide structural information, so that accurate identification of compounds is not possible without the use of commercial standards.

b) HPLC coupled to mass spectrometry

Mass spectrometry has played a key role in many phases of identifying bioactive compounds due to its sensitivity and speed, and thus has become a standard tool of many laboratories[178]. MS relies on the formation of gas-phase ions (anions or cations) that can be separated electrically or magnetically according to their mass-to-charge ratio (m/z). Mass spectrometric analysis can provide important information about the analytes, including their structure and purity[179]. Despite that different mass spectrometers are available, all consist of an ionization source (interface), mass analyzer (ion-separation system according to their m/z) and a detector together with a signal processor (**Figure 25**).

[176] Oh, Y.S., Lee, J.H., Yoon, S.H., Oh, C.H., Choi, D., Choe, E., & Jung, M.Y. (2008). Characterization and quantification of anthocyanins in grape juices obtained from the grapes cultivated in Korea by HPLC/DAD, HPLC/MS, and HPLC/MS/MS. *Journal of Food Science*, 73(5), 378-389.

[177] Chirinos, R., Campos, D., Betalleluz, I., Giusti, M.M., Schwartz, S.J., Tian, Q., Pedreschi, R., & Larondelle, Y. (2006). High-performance liquid chromatography with photodiode array detection (HPLC-DAD)/HPLC-Mass Spectrometry (MS) profiling of anthocyanins from Andean mashua tubers (*Tropaeolum tuberosum* Ruíz and Pavón) and their contribution to the overall antioxidant activity. *Journal of Agricultural and Food Chemistry*, 54(19), 7089-7097.

[178] Glish, G.L., & Vachet, R.W. (2003). The basics of mass spectrometry in the twenty-first century. *Nature Reviews Drug Discovery*, 2(2), 140-150.

[179] El-Aneed, A., Cohen, A., & Banoub, J. (2009). Mass spectrometry, review of the basics: Electrospray, MALDI, and commonly used mass analyzers. *Applied Spectroscopy Reviews*, 44(3), 210-230.

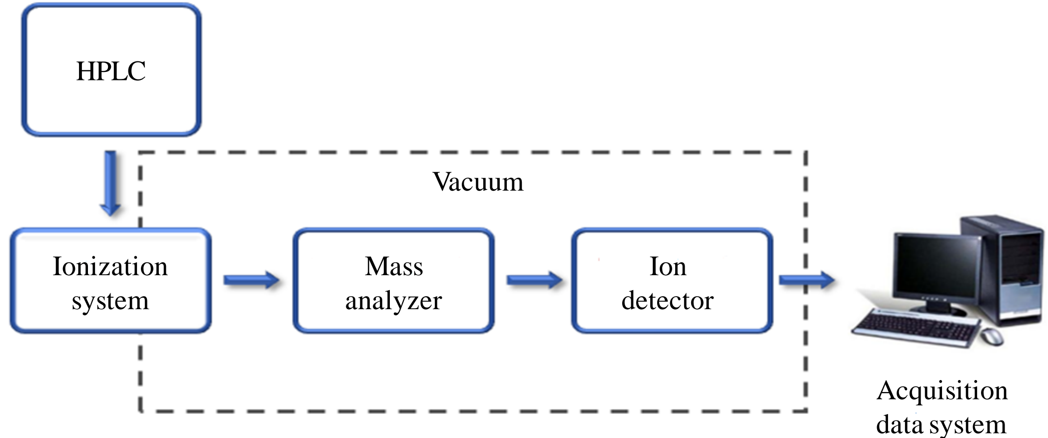


Figure 25. Schematic representation of a mass spectrometer

The combination of HPLC with mass spectrometry (MS) requires to use an interface, also known as the ionization source, where the eluent from the column is evaporated and ionized, allowing the sample molecules to be converted in gas-phase ionic species prior to their separation on the mass spectrometer. In the case of phenolic compounds, the most commonly used interface is **electrospray ionization (ESI)** which is useful mainly for analyzing polar, labile, and high-molecular-weight compounds. Ions in ESI are generated at atmospheric pressure by passing a solution-based sample through a small capillary (internal diameter < 250 µm) that is at a potential difference relative to a counter electrode at voltages between ± 500 and ± 4500 V[178] (**Figure 26**).

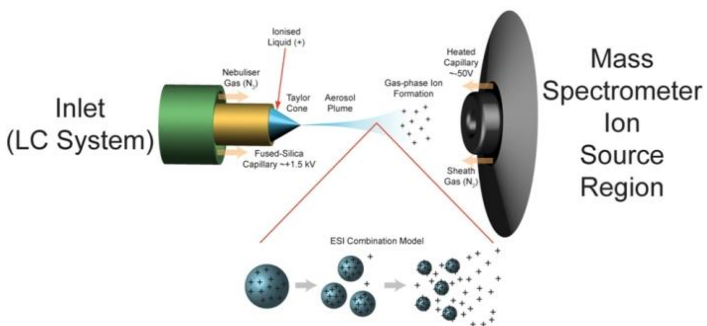


Figure 26. Schematic diagram of an ESI source[180].

[180] Awad, H., Khamis, M.M., & El-Aneed, A. (2015). Mass spectrometry, review of the basics: Ionization. *Applied Spectroscopy Reviews*, 50(2), 158-175.

Once the gas-phase ions have been produced, the next step consists of separating each ion according to its m/z prior to detection. The different types of mass analyzers separate the ions in different ways. Currently, four main mass analyzers are widely used: quadrupole (Q), ion-trap (IT), time-of-flight (TOF) and quadrupole-time-of-flight (QTOF), which differ in terms of size, price, resolution, mass range, and the ability to perform tandem mass-spectrometry experiments (MS/MS)[179]. The current Thesis examines the use of **quadrupole-time-of-flight (QTOF) mass analyzer.**

Quadrupole-time-of-flight (QTOF) mass analyzer

In the quadrupole-time-of-flight (TOF) analyzer, ions previously formed are extracted and accelerated to a high velocity by an electric field into an analyzer consisting on a long, straight drift-tube. Thus, QTOF spectrometer separates ions based on their velocity. The velocity reached by an ion will be inversely proportional to its m/z value. This means that ions with higher m/z value take more time to cross the analyzer whereas, contrarily, smaller ions will reach the detector more quickly[181].

QTOF analyzer has high sensitivity due to the funnels from the ion transmission area which avoid the loss of ions during the ionic transmission and it includes a quadrupole and a collision cell previous to the time-of-flight-tube. As the name suggests, a quadrupole (Q) consists of four cylindrical rods set parallel to each other, enabling the ions to be selected for their fragmentation in a collision cell aided by a collision gas (commonly nitrogen). The quadrupole acts somewhat as a mass filter whereby the fragmented ions are separated into the TOF according to their m/z ratio in the same way as abovementioned. These pairing results are the best combination of some performance characteristic such as accurate mass measurement, the ability to carry out fragmentation.

[181] Verentchikov, A.N., Ens, W., & Standing, K.G. (1994). Reflecting time-of-flight mass spectrometer with an electrospray ion source and orthogonal extraction. *Analytical Chemistry*, 66(1), 126-133.

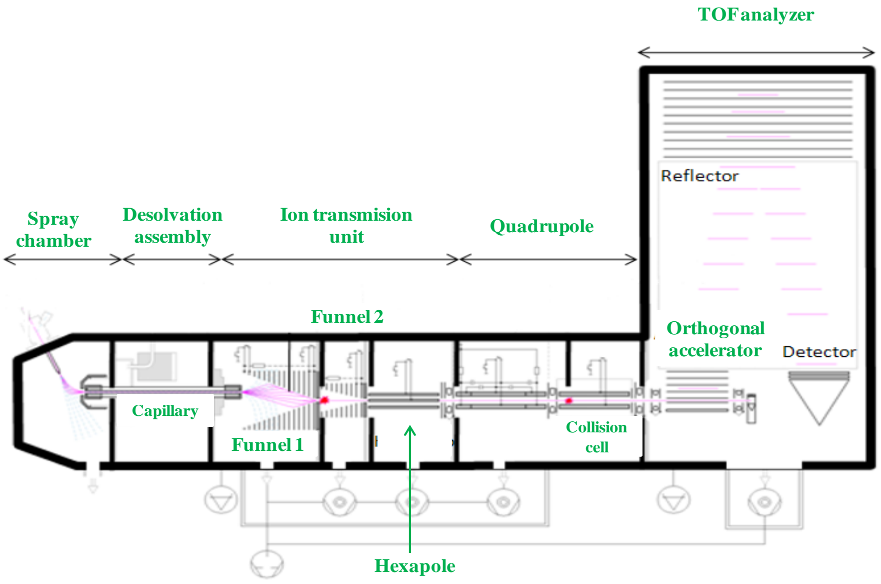


Figure 27. Schematic representation of a QTOF mass spectrometer.

QTOF offers the possibility of analyzing masses ranging from 50-20000 m/z with high resolution and precision of 3 ppm for internal calibration and 5 ppm with external calibration for precursor and fragment ions. QTOF is a powerful tool which can be used for metabolomic, bioavailability and pharmacokinetic studies[67]. QTOF has also been an efficient tool for the tentative characterization of bioactive compounds from plants for the first time in different plant matrices[182,183].

4.1.2. Gas chromatography

Gas chromatography (GC) provides separation for volatile, thermally stable compounds in a broad variety of mixtures, from the simplest to the most complex

[182] García-Salas, P., Gómez-Caravaca, A.M., Morales-Soto, A., Segura-Carretero, A., & Fernández-Gutiérrez, A. (2015). Identification and quantification of phenolic and other polar compounds in the edible part of *Annona cherimola* and its by-products by HPLC-DAD-ESI-QTOF-MS. *Food Research International*, 78, 246-257.

[183] Mekky, R.H., Contreras, M.M., El-Gindi, M.R., Abdel-Monem, A.R., Abdel-Sattar, E., & Segura-Carretero, A. (2015). Profiling of phenolic and other compounds from Egyptian cultivars of chickpea (*Cicer arietinum* L.) and antioxidant activity: a comparative study. *RSC Advances*, 5, 17751-17767.

ones[184]. In the case of GC, the mobile phase is a low-density carrier gas that does not take part in the analyte-distribution process between the two phases. The stationary phase is a liquid retained in a solid support in the column with specific physical and chemical characteristics. Therefore, when the stationary phase is fixed, the only parameter that can alter the equilibrium in the GC separation is temperature. **Temperature** limits commonly range from the lowest temperature at which the stationary phase remains fluid, to the upper limit where thermal degradation of the phase starts[185]. Currently, it is common to use temperature gradients in which the column is progressively heated. This way of operating allows better separation and resolution peaks in reasonable times of analysis. **Figure 28** shows a scheme of a gas chromatograph consisting primarily of a system with a carrier gas (the most common being nitrogen and helium) and flow and pressure regulators to control these parameters in the carrier gas. The amount of sample before injection is regulated by a splitter and the column is inside an oven because the analyses are carried out at high temperatures to ensure that all the analytes are in the gas phase.

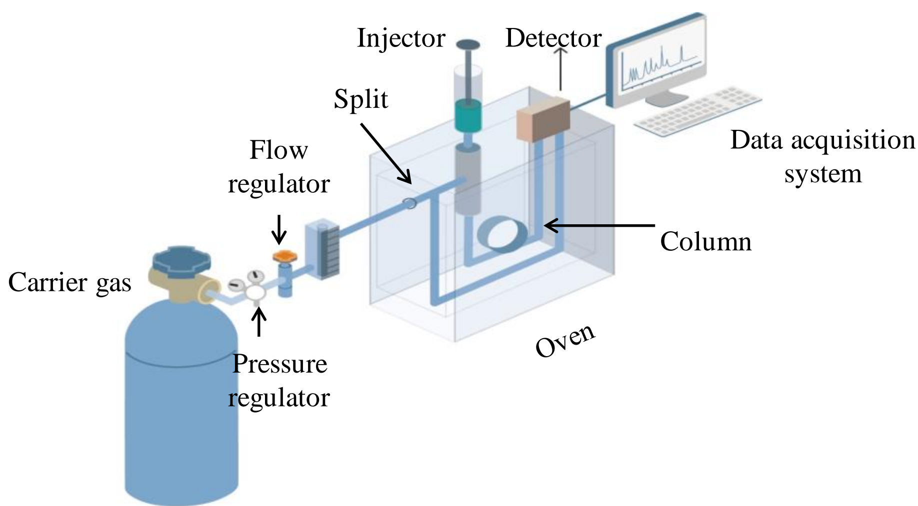


Figure 28. Scheme of a gas chromatograph.

[184] Marriott, P.J. (2004). Chapter 8 Gas chromatography. *Journal of Chromatography Library*, 69, 319-368.
[185] Wang, Z., & Jocelyn Paré, J.R. (1997). Gas chromatography (GC): Principles and applications. *Techniques and Instrumentation in Analytical Chemistry*, 18(C), 61-91.

Currently, this separation technique may be considered to be the main contribution for most of the knowledge of the fatty acid (FA) composition in oilseed plants, biosynthesis, and human metabolism.

In this case, the most common capillary columns used in GC are those made of silica with different stationary phases such as nonpolar dimethylpolysiloxane (DB-1, DB-5 or CPSil-5) and more nonpolar polymers of polyethylene glycol including Carbowax®, DB-Wax, and HP-20M columns. In this regard, a 007-CW Carbowax has been used to separate the components of oils from plant matrices[144] and a DB-5 and HP-5 have been employed to characterize volatile compounds from different plant extracts[186,187]. Temperature needs to be compatible with the stationary phase used because the liquid stationary phases can volatilize or decompose at high temperatures. Some authors have pointed out that in the case of sterols, high-temperature capillary GC **columns** provide better degrees of detection sensitivity and component resolution[108].

One of the main limitations of GC is that the analytes must be volatile and thermally stable, making it a derivatization step essential prior to GC analysis of many compounds. Due to their low volatility and presence of polar functional groups on their structure, this derivatization step is required for example to analyze triterpene pentacyclic acids in plants and plant-based foods[188]. Similarly, the polarity of OH

[186] Sánchez-Camargo, A.d.P., Valdés, A., Sullini, G., García-Cañas, V., Cifuentes, A., Ibáñez, E., & Herrero, M. (2014). Two-step sequential supercritical fluid extracts from rosemary with enhanced anti-proliferative activity. *Journal of Functional Foods*, 11, 293-303.

[187] Morales-Soto, A., Oruna-Concha, M.J., Elmore, J.S., Barrajón-Catalán, E., Micol, V., Roldán, C., & Segura-Carretero, A. (2015). Volatile profile of Spanish Cistus plants as sources of antimicrobials for industrial applications. *Industrial Crops and Products*, 74, 425-433.

[188] Caligiani, A., Malavasi, G., Palla, G., Marseglia, A., Tognolini, M., & Bruni, R. (2013). A simple GC-MS method for the screening of betulinic, corosolic, maslinic, oleanolic and ursolic acid contents in commercial botanicals used as food supplement ingredients. *Food Chemistry*, 136(2), 735-741.

groups of phenolic compounds, together their thermal lability, makes their derivatization necessary prior to GC analysis[189].

As with HPLC, several detectors can be used in GC. The GC detection systems employed in the current Thesis include a **flame-ionization detector (FID)** and a **quadrupole mass analyzer**.

a) GC coupled to flame-ionization detector (GC-FID)

FID is extensively used in GC for signal detection. It works basically because the burning of carbon compounds produces ions. When a carbon compound is eluted from the GC column into the hydrogen flame of the detector, the ions generated produce an electrical current which passes between electrodes located near the flame and held at a suitable potential, producing a recordable signal (**Figure 29**)[190].

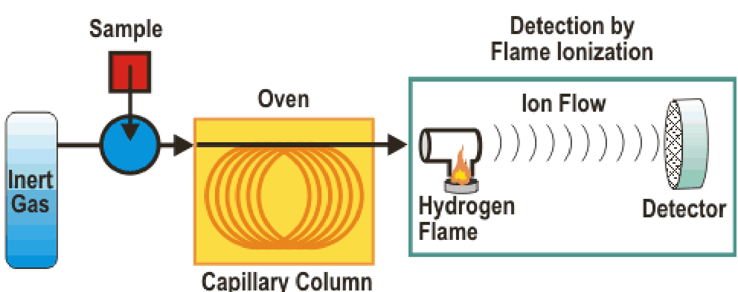


Figure 29. Scheme of a flame-ionization detector.

Notable among the main characteristics of FID are its ease of operation, wide linearity range, rapid response, and low detection limit[191]. FID is considered a

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

[190] Holm, T. (1999). Aspects of the mechanism of the flame ionization detector. *Journal of Chromatography A*, 842(1–2), 221-227.

[191] Visentainer, J.V., Claus, T., Santos Jr, O.O., Chiavelli, L.U.R., & Maruyama, S.A. (2014). Analytical aspects of the flame ionization detection in comparison with mass spectrometry with emphasis on fatty acids and their esters. In: Advanced in gas chromatography. Chapter 2. http://cdn.intechopen.com/pdfs-wm/46047.pdf

universal detector and is the most common choice in fatty acid composition analysis because of the aforementioned properties[191]. GC coupled with FID has also been effectively used to characterize essential oils from a good number of sources[184]. In addition, 15 phytosterols from virgin olive oil were characterized and quantified by GC-FID[192]. Nevertheless, GC-FID has been more frequently used for analyzing and quantifying bioactive compounds such as fatty acids and plant sterols from biological samples[193]. However, at the same that UV-Vis, FID does not provide structural information, so, the accurate identification of compounds is not possible without the use of commercial standards, and consequently this detector is not suitable to identify unknown compounds.

b) GC coupled to a quadrupole mass analyzer

Quadrupole is probably the most widely used mass analyzer in routine laboratories due to its high sensitivity and reproducibility, and because it provides spectrometric information on separated compounds[194]. As mentioned above, Q consists of four cylindrical rods, set parallel to each other, and each opposing rod pair is oppositely charged to the other nearby rod pair, as shown in **Figure 30**. Its resolution depends on the number of ion oscillations on crossing the quadrupole. The higher the oscillation numbers, the higher will be the resolution achieved.

[192] Sivakumar, G., Bati, C.B., Perri, E., & Uccella, N. (2006). Gas chromatography screening of bioactive phytosterols from mono-cultivar olive oils. *Food Chemistry*, 95(3), 525-528.

[193] Mackay, D.S., Gebauer, S.K., Eck, P.K., Baer, D.J., & Jones, P.J.H. (2015). Lathosterol-to-cholesterol ratio in serum predicts cholesterol-lowering response to plant sterol consumption in a dual-center, randomized, single-blind placebo-controlled trial. *American Journal of Clinical Nutrition*, 101(3), 432-439.

[194] Dodds, E.D., McCoy, M.R., Rea, L.D., & Kennish, J.M. (2005). Gas chromatographic quantification of fatty acid methyl esters: Flame ionization detection vs. electron impact mass spectrometry. *Lipids*, 40(4), 419-428.

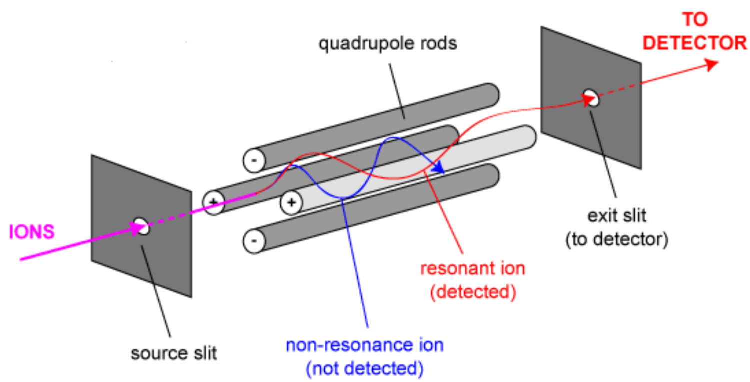


Figure 30. Scheme of a quadrupole mass analyzer.

In the case of GC-MS, the most accepted type of the ionization is **electron impact (EI) ionization** (**Figure 31**). During EI ionization, the vaporized molecules enter the MS ion source, where they are bombarded with free electrons emitted from a heated filament, and then the kinetically activated electrons (70 eV) collide with the molecules, causing their ionization and fragmentation in a characteristic and reproducible way[195].

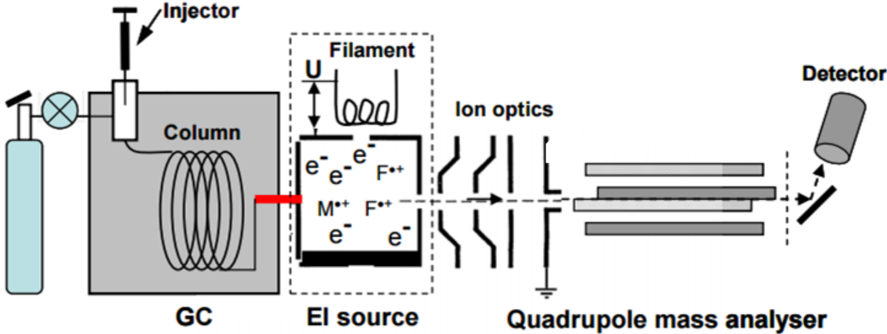


Figure 31. Scheme of a GC-MS with EI ionization and quadrupole mass analyzer.

In contrast to GC-FID, GC–MS is widely used for the characterization of bioactive compounds from plants and plant-based products. In fact, GC-MS is the most important technique for the characterization of essential oils. Mass spectrometry was also chosen for the analysis of pentacyclic triterpenes and oils from various plant and plant-based food extracts[139] as well as for characterizing flavonoids from *Ginkgo biloba* L.[189].

[195] Guo, X., & Lankmayr, E. (2012). *Hyphenated techniques in gas chromatography*: INTECH Open Access Publisher.

4.2. Spectrophotometric techniques

Spectrophotometry is defined as the quantitative measurement of the reflection or transmission properties of a material as a function of wavelength. Despite that chromatographic techniques are the best choices for quantifying individual bioactive compounds, other methodologies based on spectrophotometry are very useful approaches for screening purposes and for acquiring a general estimate of some groups of bioactive compounds in different matrices. These methods are based on natural UV absorption and chemical reactions, and their main advantages include their low time and labor consumption. In this regard, the total phenolic content (TPC), total flavonoid content (TFC) or proanthocyanidin quantification can be estimated by colorimetric methods. Likewise, enzyme-linked immunosorbent assay enables the quantification of antigens by spectrophotometry.

4.2.1. Total phenolic content (TPC)

The Folin-Ciocalteu assay is based on the chemical reduction of Folin-Ciocalteu reagent (made up molybdenum and tungsten) in the presence of certain reducing agents such as phenolic compounds in alkaline medium, leading a blue complex. The intensity of the color, which is measured at 760 nm, will be directly correlated with the presence of reducing substances. For many years the Folin-Ciocalteu assay has been the most widely specrophotometric assay used to measure total phenolics in food, beverages, and plant materials.

For example, the TPC assay has been used on *P. lentiscus* aboveground parts[196], *M. oleifera* leaves[107], 23 commercially available vegetable juices[197], different berries[198], and even in plasma samples[199].

[196] Gardeli, C., Vassiliki, P., Athanasios, M., Kibouris, T., & Komaitis, M. (2008). Essential oil composition of *Pistacia lentiscus* L. and *Myrtus communis* L.: Evaluation of antioxidant capacity of methanolic extracts. *Food Chemistry*, 107(3), 1120-1130.

[197] Wootton-Beard, P.C., Moran, A., & Ryan, L. (2011). Stability of the total antioxidant capacity and total polyphenol content of 23 commercially available vegetable juices before and after *in vitro* digestion measured by FRAP, DPPH, ABTS and Folin–Ciocalteu methods. *Food Research International*, 44(1), 217-224.

Although some authors have affirmed that this method could overestimate the content of phenolic compounds because some other compounds such as sugars, some organic acids, and amino acids can interfere[200], Folin-Ciocalteu assay could be considered the standardized specrophotometric assay for determining TPC in analytical and routine laboratories due to its ease and rapidity, serving for the screening of numerous samples in a relatively short period of time and at low cost[201].

4.2.2. Total flavonoid content (TF)

The total flavonoid content (TF) assay, based on aluminum chloride complex formation, is the most commonly accepted analytical procedure applied to determine the flavonoid content. The principle of the analysis involves the formation of stable complexes with either the C-3 and C-5 hydroxyl groups of flavones and flavanols and acid labile complexes with the orthodihydroxyl groups in the A- or B-ring of flavonoids with aluminum chloride ($AlCl_3$) producing a yellow color[202]. The absorbance of these complexes are subsequently measured at 425 nm, using quercetin as standard. As the same that occurred with Folin-Ciocalteu assay, some researchers pointed out that flavones/flavonols did not react uniformly with $AlCl_3$ in comparison with different flavonoids, indicating that this method is inadequate for estimating the total flavonoid content in unknown samples[203]. Nevertheless, a large number of

---

[198] Zheng, W., & Wang, S.Y. (2003). Oxygen radical absorbing capacity of phenolics in blueberries, cranberries, chokeberries, and lingonberries. *Journal of Agricultural and Food Chemistry*, 51(2), 502-509.

[199] Torabian, S., Haddad, E., Rajaram, S., Banta, J., & Sabaté, J. (2009). Acute effect of nut consumption on plasma total polyphenols, antioxidant capacity and lipid peroxidation. *Journal of Human Nutrition and Dietetics*, 22(1), 64-71.

[200] Prior, R.L., Wu, X., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry*, 53(10), 4290-4302.

[201] Tan, J.B.L., & Lim, Y.Y. (2015). Critical analysis of current methods for assessing the *in vitro* antioxidant and antibacterial activity of plant extracts. *Food Chemistry*, 172, 814-822.

[202] Bag, G.C., Grihanjali Devi, P., & Bhaigyaba, T. (2015). Assessment of total flavonoid content and antioxidant activity of methanolic rhizome extract of three Hedychium species of Manipur valley. *International Journal of Pharmaceutical Sciences Review and Research*, 30(1), 154-159.

[203] Mammen, D., & Daniel, M. (2012). A critical evaluation on the reliability of two aluminum chloride chelation methods for quantification of flavonoids. *Food Chemistry*, 135(3), 1365-1368.

research articles following publication of this method have described the TFC in fruits, vegetables, cereals, spices, and medicinal plant samples[107-205].

4.2.3. Dimethylaminocinnamaldehyde (DMAC) assay

Proanthocyanidins constitute the other main class of phenolic compounds measured by spectrophotometry. Different methods such as vanillin and DMAC assays have been used to determine proanthocyanidins from plants and plant-based products. However, DMAC has shown up to 5-fold higher sensitivity than in the vanillin assay, and some authors have concluded that DMAC is fairly specific for PACs while the vanillin assay tends to overestimate the PAC content[206,207]. DMAC is an aromatic aldehyde that reacts with flavan-3-ols and PAC to form a green chromophore with maximum absorbance at approximately 640 nm. The condensation of DMAC with PACs (**Figure 32**) is measured spectrophotometrically and quantified with a PAC standard curve using procyanidin A2. This method served mainly for PAC quantification in cranberry and cranberry-based products[208].

[204] Sulaiman, C.T., & Balachandran, I. (2012). Total phenolics and total flavonoids in selected Indian medicinal plants. *Indian Journal of Pharmaceutical Sciences*, 74(3), 258-260.

[205] Al-Owaisi, M., Al-Hadiwi, N., & Khan, S.A. (2014). GC-MS analysis, determination of total phenolics, flavonoid content and free radical scavenging activities of various crude extracts of *Moringa peregrina* (Forssk.) fiori leaves. *Asian Pacific Journal of Tropical Biomedicine*, 4(12), 964-970

[206] Hümmer, W., & Schreier, P. (2008). Analysis of proanthocyanidins. *Molecular Nutrition and Food Research*, 52(12), 1381-1398.

[207] Grace, M.H., Massey, A.R., Mbeunkui, F., Yousef, G.G., & Lila, M.A. (2012). Comparison of health-relevant flavonoids in commonly consumed cranberry products. *Journal of Food Science*, 77(8), 176-183.

[208] Howell, A.B., Botto, H., Combescure, C., Blanc-Potard, A., Gausa, L., Matsumoto, T., Tenke, P., Sotto, A., & Lavigne, J. (2010). Dosage effect on uropathogenic *Escherichia coli* anti-adhesion activity in urine following consumption of cranberry powder standardized for proanthocyanidin content: a multicentric randomized double blind study. *BMC Infectious Diseases*, 10(1), 1-12.

Figure 32. Product of the DMAC reaction with the flavan-3-ol terminal unit of proanthocyanidins. Adapted from Wallace, *et al.*[209].

Although there is no universally accepted method for the quantification of cranberry PACs, recent studies have proposed the use of DMAC assay as a standard method for cranberry[115,209] because it is inexpensive, rapid, and simple to perform, and it is less likely to undergo interferences from other sample components[73]. In fact, this method has been used to substantiate the French health claims from the AFSSA (Agence Française de Securité Sanitaire des Aliments) related to the bacterial anti-adhesion activity of cranberry juice concentrate and juice concentrate extract powder granted in 2004, cranberry juice cocktail in 2005, and fresh frozen and pureed cranberry in 2007[115].

4.2.4. Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is a biochemical technique used to detect the presence and to quantify peptides, proteins, antibodies, and hormones in a sample. Basically, ELISA consists of immobilizing an antigen on a solid surface and then complexing it with an antibody that is linked to an enzyme. Then, a substance is added to be converted by the enzyme into a detectable signal, commonly a color change in a chemical substrate (**Figure 33**).

[209] Wallace, T.C., & Giusti, M.M. (2010). Evaluation of parameters that affect the 4-dimethylaminocinnamaldehyde assay for flavanols and proanthocyanidins. *Journal of Food Science*, 75(7), C619-C625.

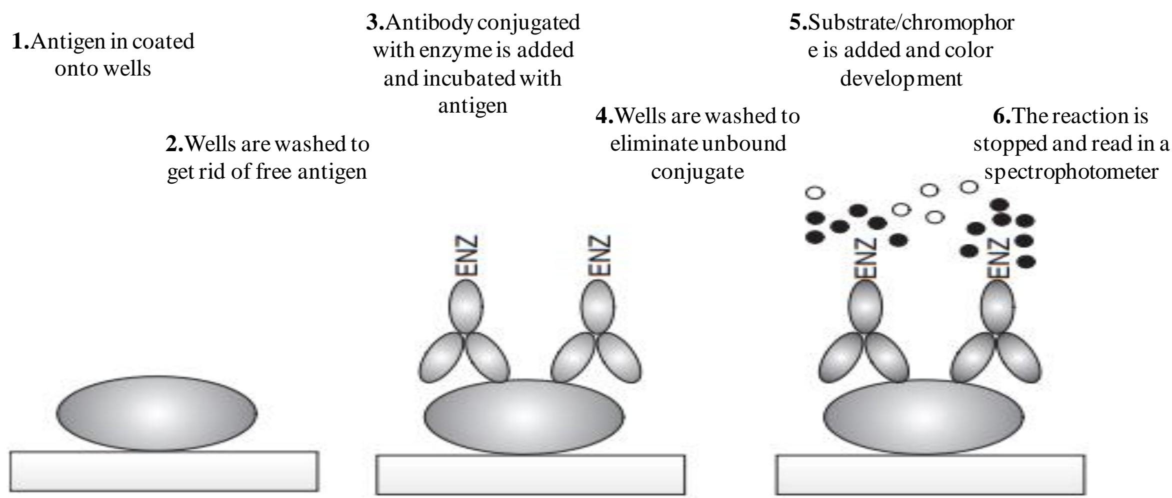


Figure 33. ELISA performance. Adapted from Crowther[210].

Since PCSK9 was discovered in 2003[100], many research groups have developed ELISAs for PCSK9 using different approaches, species, and/or antigens to produce the polyclonal antibodies, finding different results due to sample, methodology, antibody specificities, and the standard used for the absolute quantification of PCSK9[211]. Commercially specific kits are also available for PCSK9 quantification. In this Thesis, we carried out the quantikine human PCSK9 immunoassay which is a 4.5-hour solid-phase ELISA designed to measure in cell-culture supernates, cell lysates, serum, and plasma using natural PCSK9 to make the calibration curve. In the final step, the yellow color that appears after adding the stop solution is measured spectrophotometrically at 540 nm where the intensity of the color is directly proportional to the PCSK9 plasma concentration. Although the commercial kits are quite expensive, they present advantages such as being highly sensitive, accurate, reproducible, time saving, and specific. In addition, ELISA kits provide all the reagents and material necessary to perform the assay.

[210] Crowther, J. (2008). Enzyme Linked Immunosorbent Assay (ELISA). In: Molecular Biomethods Handbook. *Second Edition*, 657-682.

[211] Dubuc, G., Tremblay, M., Paré, G., Jacques, H., Hamelin, J., Benjannet, S., Boulet, L., Genest, J., Bernier, L., Seidah, N.G., & Davignon, J. (2010). A new method for measurement of total plasma PCSK9: Clinical applications. *Journal of Lipid Research*, 51(1), 140-149.

❖ FRACTIONATION AND PURIFICATION OF BIOACTIVE COMPOUNDS

The isolation of individual target compounds to test their biological activity is following an increasing trend. In this context, the necessity of isolating and purifying bioactive compounds becomes essential. Nevertheless, the complexity of each matrix makes it difficult to establish a universal methodology for purifying bioactive compounds from plants and plant-based foods. Therefore it frequently becomes necessary to divide the extract into various fractions containing compounds with similar polarity. To do so, chromatography has proved to be a powerful tool. In recent years, classical liquid-column chromatography, vacuum liquid chromatography, flash chromatography, and low-pressure chromatography have been extensively employed[212]. Currently, the most frequently used techniques are the so-called high-speed countercurrent chromatography (HSCCC), semipreparative high-performance liquid chromatography (semiprep-HPLC) and preparative-HPLC (prep-HPLC). All have been successfully used to separate and purify phenolic compounds from propolis[213].

Given that only semiprep-HPLC has been used in this Thesis, we will focus only on this technique. Semiprep-HPLC is a robust, versatile, and usually rapid technique which allows the fractionation of compounds from complex mixtures. The main difference with respect to other liquid chromatographic techniques is the diameter and length of the column. Early research demonstrated its capacity for isolating tocopherols and tocotrienols from different matrices[214]. Recently, Cádiz-Gurrea *et al.* demonstrated that semiprep-HPLC was a powerful technique to isolate

[212] Sarker, S.D., Latif, Z., & Gray, A.I. (2005). Natural product isolation. *Natural Products Isolation*, 1-25.

[213] Li, A., Xuan, H., Sun, A., Liu, R., & Cui, J. (2016). Preparative separation of polyphenols from water-soluble fraction of Chinese propolis using macroporous absorptive resin coupled with preparative high performance liquid chromatography. *Journal of Chromatography B*, 1012–1013, 42-49.

[214] Tai-Sun, S., & Godber, J.S. (1994). Isolation of four tocopherols and four tocotrienols from a variety of natural sources by semi-preparative high-performance liquid chromatography. *Journal of Chromatography A*, 678(1), 49-58.

phenolic compounds from *Theobroma cacao* extract[215]. However, prep-HPLC good resolution in one-step separation for crude extract may be difficult due to the complex composition. In addition, a large volume of solvent is necessary to get an acceptable weight of the isolated compound for certain purposes (e.g. *in vivo* assay requires grams) and therefore many cycles are often necessary.

❖ METHODOLOGIES FOR DETERMINING THE BIOACTIVITY

As mentioned above, bioactive compounds from natural sources have drawn the attention of the scientific community in the health area. Regarding nutraceutical and functional foods development, after the analytical identification of bioactive compounds from a sample, the next step is the determination of their *in vitro* bioactivity. In this regard, *in vitro* antioxidant and antibacterial activities have been studied in the present Thesis and will be examined in the following sections.

6.1. *In vitro* evaluation of antioxidant activity

The evaluation of antioxidant activity in foods, plants, and plant-derived foods is important for being associated with a notable health-protecting factor against diseases triggered by oxidative stress, including coronary heart disease, cancer, and neurodegenerative pathologies[55]. As no consensus exists on the most appropriate method for analyzing antioxidant activity, more than one *in vitro* chemical-based assay should be applied in order to gain more accurate information on the antioxidant potential of a sample[54].

6.1.1. 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) assay

The 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH) assay is based on the determination of the antiradical capacity by measuring the absorbance of the solutions of a stable DPPH$^{\bullet}$ at 516 nm after the addition of different quantities of the antiradical compound (**Figure 34**). The antiradical capacity is commonly expressed as

[215] Cádiz-Gurrea, M.L., Lozano-Sánchez, J., Contreras-Gámez, M., Legeai-Mallet, L., Fernández-Arroyo, S., & Segura-Carretero, A. (2014). Isolation, comprehensive characterization and antioxidant activities of *Theobroma cacao* extract. *Journal of Functional Foods*, 10, 485-498.

EC_{50} values, which indicate the concentration of the antiradical compound necessary to lower the initial $DPPH^{\bullet}$ absorbance to 50%[216].

Figure 34. Reaction of the stable radical DPPH with an antiradical compound (RH). Adapted from De Oliveira, *et al.*[216]

This method has found widespread applications in evaluating the antiradical capacity of pure natural compounds and of complex mixtures extracted from plants[217,218] and biological sources[219]. The extensive application of this assay can be attributed to factors such as ease of performance, speed, automatability, reproducibility, and usability at ambient temperature[201-221].

[216] De Oliveira, S., De Souza, G.A., Eckert, C.R., Silva, T.A., Sobral, E.S., Fávero, O.A., Ferreira, M.J.P., Romoff, P., & Baader, W.J. (2014). Evaluation of antiradical assays used in determining the antioxidant capacity of pure compounds and plant extracts. *Quimica Nova*, 37(3), 497-503.

[217] Bampouli, A., Kyriakopoulou, K., Papaefstathiou, G., Louli, V., Krokida, M., & Magoulas, K. (2014). Comparison of different extraction methods of *Pistacia lentiscus* var. chia leaves: Yield, antioxidant activity and essential oil chemical composition. *Journal of Applied Research on Medicinal and Aromatic Plants*, 1(3), 81-91.

[218] Bachrouch, O., Msaada, K., Wannes, W.A., Talou, T., Ksouri, R., Salem, N., Abderraba, M., & Marzouk, B. (2015). Variations in composition and antioxidant activity of Tunisian *Pistacia lentiscus* L. leaf essential oil. *Plant Biosystems*, 149(1), 38-47.

[219] Kedare, S.B., & Singh, R.P. (2011). Genesis and development of DPPH method of antioxidant assay. *Journal of Food Science and Technology*, 48(4), 412-422.

[220] Prymont-Przyminska, A., Zwolinska, A., Sarniak, A., Wlodarczyk, A., Krol, M., Nowak, M., de Graft-Johnson, J., Padula, G., Bialasiewicz, P., & Markowski, J. (2014). Consumption of strawberries on a daily basis increases the non-urate 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging activity of fasting plasma in healthy subjects. *Journal of Clinical Biochemistry and Nutrition*, 55(1), 48-55.

6.1.2. Trolox equivalent antioxidant capacity (TEAC)

The Trolox equivalent antioxidant capacity (TEAC) is based on the variation in the long-wavelength absorption (λ_{max} = 734 nm) of the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical ($ABTS^{\bullet+}$). The formation of $ABTS^{\bullet+}$, which is bluish green, stable, and has a UV-Vis spectrum, is inhibited in the presence of antioxidant compounds such as phenolic compounds, giving a colorless non-radical product. The generation of $ABTS^{\bullet+}$ requires a prior stoichiometric reaction (1:0.5) at room temperature, without light and for 12 to 16 hours (**Figure 35**).

Figure 35. Oxidation reaction of ABTS by potassium persulfate to generate $ABTS^{\bullet+}$ and its reaction with an antioxidant compound (AOH)[216].

This method has been used because it is the fastest one and it allows the analyses of both lipophilic and hydrophilic compounds[56]. Although some authors have noted that TEAC does not necessarily reflect the antioxidant effect of only one family

[221] Villaño, D., Fernández-Pachón, M.S., Moyá, M.L., Troncoso, A.M., & García-Parrilla, M.C. (2007). Radical scavenging ability of polyphenolic compounds towards DPPH free radical. *Talanta*, 71(1), 230-235.

of compounds[222], TEAC assay is a useful tool for determining unknown antioxidants in complex mixtures from foods such as fruits and vegetables[54], beverages, vegetable oils[223], and plant extracts[224].

Despite that the results found from the TEAC assay have been correlated to those of DPPH assay, research has demonstrated that TEAC offers better antioxidant capacity estimations in foods, particularly in fruits, vegetables, and their derivative beverages, thus, TEAC is a better choice than DPPH for plant-based foods rich in lipophilic, hydrophilic and highly pigmented contents[225].

6.2. *In vitro* evaluation of antibacterial activity

Innumerable *in vitro* studies illustrate how bioactive compounds such as phenolic compounds have antibacterial properties against several types of bacteria[61,226]. Generally, the processes involving bacterial infection are exposure to pathogens, surface adherence (i.e. influenced by surface hydrophobicity, among other factors), invasion, colonization and growth, and tissue damage that can result in diseases (**Figure 36**).

[222] Arts, M.J.T.J., Haenen, G.R.M.M., Voss, H., & Bast, A. (2004). Antioxidant capacity of reaction products limits the applicability of the Trolox Equivalent Antioxidant Capacity (TEAC) assay. *Food and Chemical Toxicology*, 42(1), 45-49.

[223] Pellegrini, N., Serafini, M., Colombi, B., Del Rio, D., Salvatore, S., Bianchi, M., & Brighenti, F. (2003). Total antioxidant capacity of plant foods, beverages and oils consumed in Italy assessed by three different *in vitro* assays. *The Journal of Nutrition*, 133(9), 2812-2819.

[224] Silva, E.M., Souza, J.N.S., Rogez, H., Rees, J.F., & Larondelle, Y. (2006). Antioxidant activities and polyphenolic contents of fifteen selected plant species from the Amazonian region. *Food Chemistry*, 101(3), 1012-1018.

[225] Chandra, S., Khan, S., Avula, B., Lata, H., Yang, M.H., Elsohly, M.A., & Khan, I.A. (2014). Assessment of total phenolic and flavonoid content, antioxidant properties, and yield of aeroponically and conventionally grown leafy vegetables and fruit crops: A comparative study. *Evidence-based Complementary and Alternative Medicine*, 2014.

[226] Foo, L.Y., Lu, Y., Howell, A.B., & Vorsa, N. (2000). The structure of cranberry proanthocyanidins which inhibit adherence of uropathogenic P-fimbriated *Escherichia coli in vitro*. *Phytochemistry*, 54(2), 173-181.

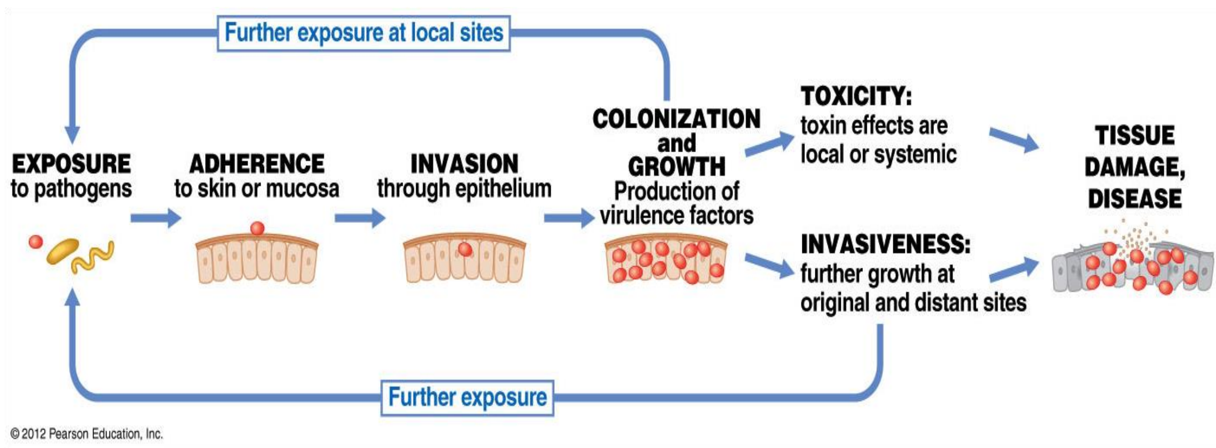


Figure 36. Scheme of bacterial pathogenesis. From Pearson Education, Inc.

In addition, bacteria such as *Escherichia coli* are capable of forming biofilms. In this case, the adhesion of bacteria to host surfaces is finally a key element in the formation of biofilms, which constitutes a protected mode of growth that on one hand allows bacteria to survive in hostile environments and on the other hand makes bacteria more resistant to host defenses or antibiotic treatments[227]. Hydrophobic interactions are crucial in the adherence of microorganisms to a wide variety of surfaces, facilitating biofilm formation due to bacterial adhesion[228]. For this reason, the study of bioactive compounds that can influence adherence and pathogenicity are attracting the attention of pharmaceutical, nutraceutical and functional food companies.

For the measurement of the **hydrophobic surface properties** of bacteria, the salt aggregation test (SAT) has been one of the most widely used. The SAT uses a salting-out agent (for instance ammonium sulfate) to induce the aggregation of cells for determining bacterial cell-surface hydrophobicity[229] followed by its spectrophotometric measurement at 540 nm. Although hydrophobicity measurement

[227] Ribet, D., & Cossart, P. (2015). How bacterial pathogens colonize their hosts and invade deeper tissues. *Microbes and Infection*, 17(3), 173-183.
[228] Tahmourespour, A., Kasra Kermanshahi, R., Salehi, R., & Nabinejad, A. (2008). The relationship between cell surface hydrophobicity and antibiotic resistance of Streptococcal strains isolated from dental plaque and caries. *Iranian Journal of Basic Medical Sciences*, 10(4), 251-255.
[229] Lindahl, M., Faris, A., Wadström, T., & Hjertén, S. (1981). A new test based on 'salting out' to measure relative hydrophobicity of bacterial cells. *BBA - General Subjects*, 677(3-4), 471-476.

depends considerably on the method used[230], the SAT method has proven to be rapid, simple, and effective to quantify bacterial cell-surface hydrophobicity[67,231].

One of the most widely used methods for determining the rate of adherence and subsequent **biofilm formation** is the tube method (TM). In this method, the bacterial film lining a culture tube is stained with a cationic dye and visually scaled, after which the optical density (OD) of the stained bacterial film is determined spectrophotometrically at 570 nm[232]. Despite that some researchers have concluded that other methods such as tissue culture plate are more accurate and reproducible[233], TM has the advantage of enabling researchers to rapidly analyze adhesion of multiple bacterial strains or growth conditions within each experiment[234].

❖ PLANT-BASED MATRICES UNDER STUDY

Following the workflow of nutraceutical and functional foods development presented in Section 1, *Pistacia lentiscus* and *Moringa oleifera* leaves and *Cucumis melo* varieties (melon) were chosen as potential sources of bioactive compounds for the extraction, characterization, and identification of relevant markers due to their traditional use in folk medicine or for being a common food included in the Mediterranean diet. In addition, nutraceuticals based on cranberry (*Vaccinium macrocarpon*) were selected to study their stability and the *in vitro* bioactivity of their bioactive compounds against uropathogenic *Escherichia coli* strains. Finally, canola oil enriched in different fatty acids served as a basis for carrying out *in vivo* studies.

[230] Qiao, G., Li, H., Xu, D., & Park, S.I. (2012). Modified a colony forming unit microbial adherence to hydrocarbons assay and evaluated cell surface hydrophobicity and biofilm production of *Vibrio scophthalmi*. *Journal of Bacteriology & Parasitology*, 2012.

[231] Wojnicz, D., Kucharska, A.Z., Sokól-Łętowska, A., Kicia, M., & Tichaczek-Goska, D. (2012). Medicinal plants extracts affect virulence factors expression and biofilm formation by the uropathogenic *Escherichia coli*. *Urological Research*, 40(6), 683-697.

[232] Stepanovic, S., Vukovic, D., Dakic, I., Savic, B., & Švabic-Vlahovic, M. (2000). A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of Microbiological Methods*, 40(2), 175-179.

[233] Mathur, T., Singhal, S., Khan, S., Upadhyay, D.J., Fatma, T., & Rattan, A. (2006). Detection of biofilm formation among the clinical isolates of staphylococci: An evaluation of three different screening methods. *Indian Journal of Medical Microbiology*, 24(1), 25-29.

[234] Djordjevic, D., Wiedmann, M., & McLandsborough, L.A. (2002). Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Applied and Environmental Microbiology*, 68(6), 2950-2958.

7.1. Natural sources of bioactive compounds

7.1.1. *Pistacia lentiscus*

Pistacia lentiscus L., commonly known as mastic tree which belongs to the family Anacardiaceae, is a shrub or small tree widely distributed in the Mediterranean basin where it grows wild. Traditionally, different parts of *P. lentiscus* have been used to treat stomach ache, dyspepsia, stomach ulcer, hypertension, eczema, diarrhea, and other pathologies[235].

Due to its great traditional potential, in recent years interest has grown regarding the health effect of this plant. Recent studies, both, *in vitro* and *in vivo,* have shown antioxidant[196,218], anti-inflammatory[236], antiulcerogenic[237], antimicrobial[238], anticancer[238], anti-diabetic, cytoprotective[236], and hypocholesterolemic activities[239] of extracts, mainly from the aboveground parts.

These effects are attributed to its composition in bioactive compounds. Probably, one of the most studied parts of *P. lentiscus* is mastic (a natural resin) highly rich in essential oils[240]. The most remarkable compounds from its fruits are

[235] Bozorgi, M., Memariani, Z., Mobli, M., Salehi Surmaghi, M.H., Shams-Ardekani, M.R., & Rahimi, R. (2013). Five pistacia species (*P. vera, P. atlantica, P. terebinthus, P. khinjuk, and P. lentiscus*): A review of their traditional uses, phytochemistry, and pharmacology. *The Scientific World Journal*, 2013.

[236] Remila, S., Atmani-Kilani, D., Delemasure, S., Connat, J., Azib, L., Richard, T., & Atmani, D. (2015). Antioxidant, cytoprotective, anti-inflammatory and anticancer activities of *Pistacia lentiscus* (Anacardiaceae) leaf and fruit extracts. *European Journal of Integrative Medicine*, 7(3), 274-286.

[237] Dellai, A., Souissi, H., Borgi, W., Bouraoui, A., & Chouchane, N. (2013). Antiinflammatory and antiulcerogenic activities of *Pistacia lentiscus* L. leaves extracts. *Industrial Crops and Products*, 49, 879-882.

[238] Mezni, F., Shili, S., Ben Ali, N., Larbi Khouja, M., Khaldi, A., & Maaroufi, A. (2015). Evaluation of *Pistacia lentiscus* seed oil and phenolic compounds for *in vitro* antiproliferative effects against BHK21 cells. *Pharmaceutical Biology*, 54, 747-751.

[239] Cheurfa, M., & Allem, R. (2015). Study of hypocholesterolemic activity of Algerian *Pistacia lentiscus* leaves extracts *in vivo*. *Revista Brasileira de Farmacognosia*, 25(2), 142-144.

[240] Koutsoudaki, C., Krsek, M., & Rodger, A. (2005). Chemical composition and antibacterial activity of the essential oil and the gum of *Pistacia lentiscus* Var. chia. *Journal of Agricultural and Food Chemistry*, 53(20), 7681-7685.

unsaturated fatty acids, mainly oleic and linoleic acids[241] and some sterols as β-sitosterol and campesterol[242]. On its behalf, mastic leaves are a rich source of essential oils[243] and phenolic compounds that constitute over 7.5% of dry weight, gallic acids and myricetin derivatives being the predominant ones[244].

7.1.2. *Cucumis melo*

Melon *(Cucumis melo,* Cucurbitaceae), a common fruit in the Spanish Mediterranean diet, and has been included in the so-called "five-a-day" healthy diet of fruit and vegetables[245].

Despite that their consumption is associated with summer time, it is currently possible to find melons year round. Specifically, the most consumed market varieties in Spain are Galia, Cantaloupe, and Piel de Sapo. Their consumption increased 15.9% in January 2015 compared with the previous year[246].

[241] Akdemir, O.F., Tilkat, E., Onay, A., Keskin, C., Bashan, M., Kilinç, F.M., Kizmaz, V., & Süzerer, V. (2015). Determination of the fatty acid composition of the fruits and different organs of lentisk (*Pistacia lentiscus* L.). *Journal of Essential Oil-Bearing Plants*, 18(5), 1224-1233.

[242] Trabelsi, H., Cherif, O. A., Sakouhi, F., Villeneuve, P., Renaud, J., Barouh, N., Boukhchina, S., & Mayer, P. (2012). Total lipid content, fatty acids and 4-desmethylsterols accumulation in developing fruit of *Pistacia lentiscus* L. growing wild in Tunisia. *Food Chemistry*, 131(2), 434-440.

[243] Negro, C., De Bellis, L., & Miceli, A. (2015). Chemical composition and antioxidant activity of *Pistacia lentiscus* essential oil from Southern Italy (Apulia). *Journal of Essential Oil Research*, 27(1), 23-29.

[244] Romani, A., Pinelli, P., Galardi, C., Mulinacci, N., & Tattini, M. (2002). Identification and quantification of galloyl derivatives, flavonoid glycosides and anthocyanins in leaves of *Pistacia lentiscus* L. *Phytochemical Analysis*, 13(2), 79-86.

[245] Moing, A., Aharoni, A., Biais, B., Rogachev, I., Meir, S., Brodsky, L., Allwood, J. W., Erban, A., Dunn, W.B., Kay, L., de Koning, S., de Vos, R.C.H., Jonker, H., Mumm, R., Deborde, C., Maucourt, M., Bernillon, S., Gibon, Y., Hansen, T.H., Husted, S., Goodacre, R., Kopka, J., Schjoerring, J.K., Rolin, D., & Hall, R.D. (2011). Extensive metabolic cross-talk in melon fruit revealed by spatial and developmental combinatorial metabolomics. *New Phytologist*, 190(3), 683-696.

[246] Boletin Mensual de Estadística. Junio. (2015). Ministerio de Agricultura, Alimentación y Medio Ambiente (MAGRAMA).

Traditionally, melon has been used for its diuretic and antihelminth activity but also for its effects in appetite suppression, weight loss, urinary-tract infections, constipation, and ulcers[247]. Its healthy effects have been associated with its low caloric content and its composition in vitamins and minerals. However, scant research has focused on minority bioactive compounds[248,249], and the complete composition is not yet well established.

Phytochemical studies have revealed that the edible portion of melon contains mainly phenolics[250,251] and organic acids[252] that allow its characteristic flavor[247]. In addition, melon, specifically the cantaloupe variety, has proved to be a rich source of SOD, which has shown antioxidant and anti-inflammatory properties[253].

[247] Ullah, N., Khan, S., Khan, A., Ahmad, W., Shah, Y., Ahmad, L., & Ullah, I. (2015). A prospective pharmacological review of medicinal herbs, *Cucumis melo* and *Berberis vulgaris*, commonly used in the treatment of renal diseases in Pakistan. *Acta Poloniae Pharmaceutica - Drug Research*, 72(4), 651-654.

[248] Ismail, H.I., Chan, K.W., Mariod, A.A., & Ismail, M. (2010). Phenolic content and antioxidant activity of cantaloupe (*Cucumis melo*) methanolic extracts. *Food Chemistry*, 119(2), 643-647.

[249] Norrizah, J.S., Hashim, S.N., Siti Fasiha, F., & Yaseer, S.M. (2012). ß-carotene and antioxidant analysis of three different rockmelon (*Cucumis melo* L.) cultivars. *Journal of Applied Sciences*, 12(17), 1846-1852.

[250] Yang, Y., Yu, Z., Xu, Y., & Shao, Q. (2012). Analysis of volatile compounds from oriental melons (*Cucumis melo* L.) using headspace SPME coupled with GC-MS. *Advanced Materials Research*, 554-556, 2102-2105.

[251] Feder, A., Burger, J., Gao, S., Lewinsohn, E., Katzir, N., Schaffer, A.A., Meir, A., Davidovich-Rikanat, R., Portnoy, V., Gal-On, A., Fei, Z., Kashi, Y., & Tadmor, Y. (2015). A Kelch domain-containing f-box coding gene negatively regulates flavonoid accumulation in muskmelon. *Plant Physiology*, 169(3), 1714-1726.

[252] Lignou, S., Parker, J.K., Baxter, C., & Mottram, D.S. (2014). Sensory and instrumental analysis of medium and long shelf-life Charentais cantaloupe melons (*Cucumis melo* L.) harvested at different maturities. *Food Chemistry*, 148, 218-229.

[253] Vouldoukis, I., Lacan, D., Kamate, C., Coste, P., Calenda, A., Mazier, D., Conti, M., & Dugas, B. (2004). Antioxidant and anti-inflammatory properties of a *Cucumis melo* L. extract rich in superoxide dismutase activity. *Journal of Ethnopharmacology*, 94(1), 67-75.

7.1.3. *Moringa oleifera*

Moringa oleifera (*M. oleifera)* is commonly known as "tree of life" or "miracle tree" because every part of the tree can be used for food, medication, and industrial purposes[254]. This cultivar is considered a fast-growing tree capable of germinating in practically any type of soil[255] in arid and semi-arid areas. Native to southern Asia, *M. oleifera* is currently cultivated throughout the tropics in Africa, India, Central and South America, and in Spain, specifically in the Canary Islands[256].

This plant supplies the necessary requirement of micro-nutrients for a healthy diet[255]. In particular, *M. oleifera* leaves contain: 30% protein, covering essential amino acids including the sulfur-containing amino acids[257]; 7% lipids, which include n-3 and n-6 fatty acids[258]; and 11% fiber, minerals, vitamins, essential oils, and phenolic compounds[254,259]. Not surprisingly, in some developing countries *M. oleifera* leaves, according to FAO, are used as fresh food or dried and ground into powder. In addition, some Non-Governmental Organizations (NGOs) such as "Agua de coco" are carrying out campaigns to furnish the social community kitchens and child population with supplements of *M. oleifera* leaves.

[254] Moyo, B., Masika, P.J., & Muchenje, V. (2012). Antimicrobial activities of *Moringa oleifera* Lam leaf extracts. *African Journal of Biotechnology*, 11(11), 2797-2802.
[255] Anwar, F., Latif, S., Ashraf, M., & Gilani, A.H. (2007). *Moringa oleifera*: A food plant with multiple medicinal uses. *Phytotherapy Research*, 21(1), 17-25.
[256] Godino, M., Arias, C., & Izquierdo, M.I. (2013). Interés forestal de la *Moringa oleifera* y posibles zonas de implantación en España. 6º Congreso forestal español. Edita: Sociedad Española de Ciencias Forestales. Vitoria-Gasteiz. 10-14 Junio, ISBN: 978-84-937964-9-5.
[257] Ferreira, P.M.P., Farias, D.F., Oliveira, J.T.D.A., & Carvalho, A.D.F.U. (2008). *Moringa oleifera*: Bioactive compounds and nutritional potential. *Revista de Nutricao*, 21(4), 431-437.
[258] Marrufo, T., Nazzaro, F., Mancini, E., Fratianni, F., Coppola, R., De Martino, L., Agostinho, A.B., & De Feo, V. (2013). Chemical composition and biological activity of the essential oil from leaves of *Moringa oleifera* Lam. cultivated in Mozambique. *Molecules*, 18(9), 10989-11000.
[259] Amaglo, N.K., Bennett, R.N., Lo Curto, R.B., Rosa, E.A.S., Lo Turco, V., Giuffrida, A., Curto, A. L., Crea, F., & Timpo, G.M. (2010). Profiling selected phytochemicals and nutrients in different tissues of the multipurpose tree *Moringa oleifera* L., grown in Ghana. *Food Chemistry*, 122(4), 1047-1054.

So far, ample research has demonstrated that its leaves have *in vitro* and *in vivo* therapeutic potential for hyperglycemia and hyperlipidemia[260], antioxidant, antimicrobial[258], antidiabetic[78], anti-inflammatory[261], and anticancer[262] effects. Despite that the mechanisms by which *M. oleifera* exerts its healthy effects remain to be clarified, research is available on the effect of certain bioactive compounds from *M. oleifera* leaves. In this sense, the glucosinolate from this plant, called glucomoringin, has shown anti-inflammatory[261] and antitumoral activities[263]. Flavonols and phenolic acids have been related to its anti-inflammatory, antioxidant, and antibacterial activity[264].

7.2. Nutraceuticals and functional foods

7.2.1. *Vaccinium macrocarpon*

Cranberry (*Vaccinium macrocarpon*) is a berry from an evergreen dwarf shrub native to North America. According to FAO's latest estimations, the global cranberry industry is dominated by production in the United States and Canada (FAO, 2012).

Different cranberries forms are available on the market: fresh, frozen, dried, dehydrated, concentrated or powdered. These fruits are commonly treated in order to counteract their tartness and can be used as juices, beverages, sauces, etc.. However, cranberries are also used by the food companies as colorants and preservatives and by the pharmaceutical companies to formulate capsules commercialized as dietary supplements.

[260] Mbikay, M. (2012). Therapeutic potential of *Moringa oleifera* leaves in chronic hyperglycemia and dyslipidemia: A review. *Frontiers in Pharmacology*, 3,24.

[261] Galuppo, M., Giacoppo, S., De Nicola, G.R., Iori, R., Navarra, M., Lombardo, G.E., Bramanti, P., & Mazzon, E. (2014). Antiinflammatory activity of glucomoringin isothiocyanate in a mouse model of experimental autoimmune encephalomyelitis. *Fitoterapia*, 95, 160-174.

[262] Jung, I.L., Lee, J.H., & Kang, S.C. (2015). A potential oral anticancer drug candidate, *Moringa oleifera* leaf extract, induces the apoptosis of human hepatocellular carcinoma cells. *Oncology Letters*, 10(3), 1597-1604.

[263] Brunelli, D., Tavecchio, M., Falcioni, C., Frapolli, R., Erba, E., Iori, R., Rollin, P., Barillari, J., Manzotti, C., Morazzoni, P., & D'Incalci, M. (2010). The isothiocyanate produced from glucomoringin inhibits NF-kB and reduces myeloma growth in nude mice *in vivo*. *Biochemical Pharmacology*, 79(8), 1141-1148.

[264] Razis, A.F.A., Ibrahim, M.D., & Kntayya, S.B. (2014). Health benefits of *Moringa oleifera*. *Asian Pacific Journal of Cancer Prevention*, 15(20), 8571-8576.

In general, American cranberry-based products have proven to have antioxidant activity, *in vitro* anti-cancer effects[265], *in vitro* and *in vivo* antibacterial activities[31,67] and properties to manage hypercholesterolemia, hypertension, and diabetes[266,267]. The bioactivity of this fruit has been associated with its composition in anthocyanins, proanthocyanidins (PACs), flavonol glycosides, low-molecular-weight phenolic acids, organic acids, and sugars[268].

Nutraceuticals based on cranberry are currently used mainly alone or in combination with antibiotics for preventing urinary-tract infections (UTIs) with satisfactory *in vivo* results[68,269] as a way to solve the problem associated to the increase in bacterial resistance to antibiotics and the selection of multiresistant bacterial flora[31].

Traditionally, the antibacterial activity of cranberry has been associated with its content in fructose, which may inhibit the adhesion of *E. coli* mediated by type 1 fimbriae and PACs with at least one A-type linkage that block the expression of p fimbriae in *E. coli*, when added to bacterial culture medium, thereby preventing adhesion[270]. Nevertheless, some authors highlight that there is a synergism between

[265] Kresty, L.A., Weh, K.M., Zeyzus-Johns, B., Perez, L.N., & Howell, A.B. (2015). Cranberry proanthocyanidins inhibit esophageal adenocarcinoma *in vitro* and *in vivo* through pleiotropic cell death induction and PI3K/AKT/mTOR inactivation. *Oncotarget*, 6(32), 33438-33455.

[266] Apostolidis, E., Kwon, Y., & Shetty, K. (2006). Potential of cranberry-based herbal synergies for diabetes and hypertension management. *Asia Pacific Journal of Clinical Nutrition*, 15(3), 433-441.

[267] Kim, M.J., Kim, J.H., & Kwak, H. (2014). Antioxidant effects of cranberry powder in lipopolysaccharide treated hypercholesterolemic rats. *Preventive Nutrition and Food Science*, 19(2), 75-81

[268] Seeram, N.P., Adams, L.S., Hardy, M.L., & Heber, D. (2004). Total cranberry extract versus its phytochemical constituents: antiproliferative and synergistic effects against human tumor cell lines. *Journal of Agricultural and Food Chemistry*, 52(9), 2512-2517.

[269] Foxman, B., Cronenwett, A.E.W., Spino, C., Berger, M.B., & Morgan, D.M. (2015). Cranberry juice capsules and urinary tract infection after surgery: Results of a randomized trial. *American Journal of Obstetrics and Gynecology*, 213(2), 194, 1-8.

[270] Nowak, M., de Graft-Johnson, J., Padula, G., Bialasiewicz, P., & Markowski, J. (2014). Consumption of strawberries on a daily basis increases the non-urate 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging activity of fasting plasma in healthy subjects. *Journal of Clinical Biochemistry and Nutrition*, 55(1), 48-55.

different compounds present in cranberry extracts[67,271] while other researchers call for studies focusing on bioactive compounds from cranberry instead of the entire fruit[272].

Despite the lack of an approved health claim related to PACs and antibacterial activity, an attempt to reach this endorsement was made in 2013[273]. Since then, many studies are emerging in order to accumulate scientific evidence to achieve that goal.

7.2.2. Canola oil

In Europe, rapeseed (*Brassica napus*) is a crop that contains high concentration of erucic acid (> 40%). After the intoxication that occurred in Spain in 1981, many Europeans associated canola oil with that oil and were reluctant to consume it. However, canola has become one of the most important oilseed crops in the world. In fact, according to the United States Department of Agriculture (USDA), canola oil, made from crushing canola seed, was the third-most-produced vegetable oil globally in 2008/09[274]. Although both canola and rapeseed belong to the cabbage and mustard family Brassicaceae, differences exist between them.

The regulated definition of canola is "seeds of the genus *Brassica* (*Brassica napus*, *Brassica rapa* or *Brassica juncea*) from which the oil shall contain less than 2% erucic acid in its fatty acid profile and the solid component shall contain less than 30 micromoles of any one glucosinolates per gram of air-dried, oil-free solid"[275].

[271] Laplante, K.L., Sarkisian, S.A., Woodmansee, S., Rowley, D.C., & Seeram, N.P. (2012). Effects of cranberry extracts on growth and biofilm production of *Escherichia coli* and *Staphylococcus* species. *Phytotherapy Research*, 26(9), 1371-1374.

[272] Hisano, M., Bruschini, H., Nicodemo, A.C., & Srougi, M. (2012). Cranberries and lower urinary tract infection prevention. *Clinics*, 67(6), 661-667.

[273] EFSA. (2013). http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/3326.pdf

[274] USDA. (2012). http://www.ers.usda.gov/topics/crops/soybeans-oil-crops/canola.aspx

[275] Lin, L., Allemekinders, H., Dansby, A., Campbell, L., Durance-Tod, S., Berger, A., & Jones, P.J. (2013). Evidence of health benefits of canola oil. *Nutrition Reviews*, 71(6), 370-385.

Canola oil has a healthy lipidic profile that includes 7% saturated fats, 32% PUFAs (21% linoleic acid and 11% α-linolenic acid), and 61% MUFAs[276]. When this profile is compared to that of olive oil, the latter contains 2-fold more content in saturated fats, less than half the content in polyunsaturated fats, and nearly 75% the content in monounsaturated fat. Although canola oil does not have a phenolic fraction; it is rich in plant sterols, which, as mentioned in Section 2.2, have been demonstrated to be effective in lowering cholesterol absorption. Several clinical trials have shown the benefits of canola oil consumption in the improvement of biomarkers beyond blood lipids[277,278]. Furthermore, trials testing the effect oleic and DHA enriched canola oils are emerging with promising results[96].

[276] Canola council of Canada. (2014). http://www.canolacouncil.org/publication-resources/print-resources/canola-oil-resources/comparison-of-dietary-fat-charts-pad-of-25-sheets/

[277] Gulesserian, T., & Widhalm, K. (2002). Effect of a rapeseed oil substituting diet on serum lipids and lipoproteins in children and adolescents with familial hypercholesterolemia. *Journal of the American College of Nutrition*, 21(2), 103-108.

[278] Senanayake, V.K., Pu, S., Jenkins, D.A., Lamarche, B., Kris-Etherton, P.M., West, S.G., Fleming, J.A., Liu, X., McCrea, C.E., & Jones, P.J. (2014). Plasma fatty acid changes following consumption of dietary oils containing n-3, n-6, and n-9 fatty acids at different proportions: Preliminary findings of the Canola Oil Multicenter Intervention Trial (COMIT). *Trials*, 15(1).

Experimental part.
Results and Discussion

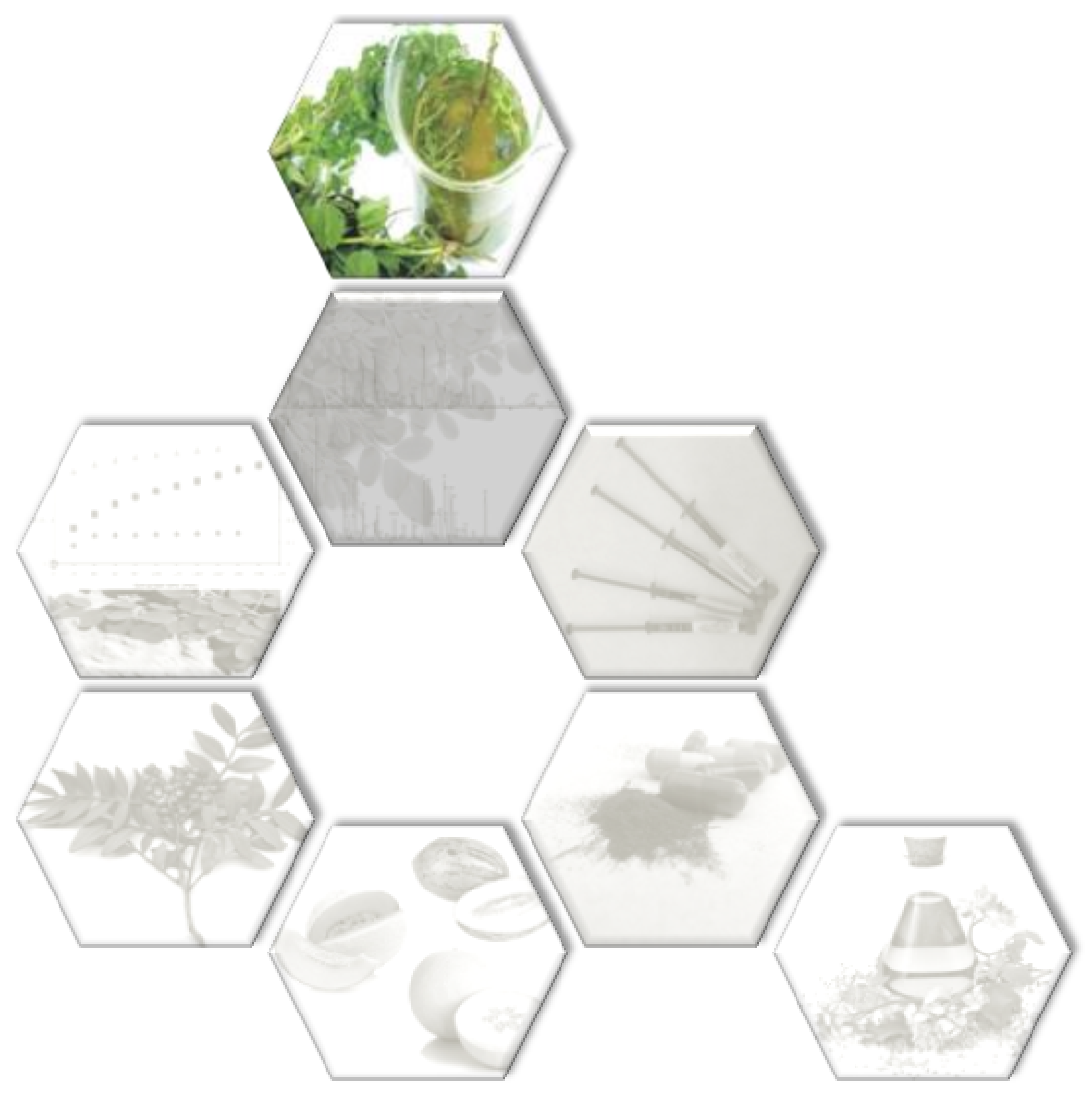
Section I.

“The search for new sources of bioactive compounds”

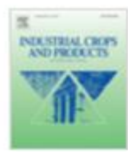
Chapter 1

Optimization of extraction method to obtain a phenolic compounds-rich extract from *Moringa oleifera* Lam leaves

Industrial Crops and Products, 2015, 66:246-254



Industrial Crops and Products
Volume 66, April 2015, Pages 246–254



Optimization of extraction method to obtain a phenolic compounds-rich extract from *Moringa oleifera* Lam leaves

C. Rodríguez-Pérez[a, b], R. Quirantes-Piné[b], A. Fernández-Gutiérrez[a, b], A. Segura-Carretero[a, b]

[a] Department of Analytical Chemistry, Faculty of Sciences, University of Granada, Avenida Fuentenueva s/n, 18071 Granada, Spain
[b] Research and Development Functional Food Centre (CIDAF), Health Science Technological Park, Avenida del Conocimiento s/n, Edificio BioRegión, 18016 Granada, Spain

Abstract

Moringa oleifera Lam. is considered one of the most useful trees in the World because every part of the Moringa tree can be used such as nutritional supplement, for medication, and industrial purposes. Conventional solid-liquid extraction and ultrasound-assisted extraction (UAE) were performed using different solvents and mixtures of solvents with water. The total phenolic content was determined using the Folin-Ciocalteu assay. UAE using ethanol:water (50:50) was the best extraction procedure, which allowed 47 ± 4 mg gallic acid equivalents (GAE)/g dry leaf to be obtained. In addition, high-performance liquid chromatography coupled to electrospray ionization quadropole-time of flight mass spectrometry (HPLC-ESI-QTOF-MS) was used to characterize the bioactive compounds in the resulting extract. Consequently, 59 compounds were tentatively characterized, phenolic acid derivatives and flavonoids being the most abundant. Furthermore, 30 of these compounds were tentatively identified for the first time in *M. oleifera* leaves. This study shows that leaves from *M. oleifera* are a good nutritional resource used as a nutritional supplement and may carry additional opportunities for food ingredient innovations, pharmaceutical and cosmetics products.

Keywords: maceration; ultrasound-assisted extraction; Folin-Ciocalteu; bioactive compounds; HPLC-ESI-QTOF-MS.

❖ Introduction

Moringa oleifera (Moringaceae), also known as "the tree of life", is mainly native to India and Africa. It is considered one of the most useful trees in the World because every part of the Moringa tree can be used for food, medication and industrial purposes (Moyo *et al.*, 2011). In particular, the leaves can be eaten fresh in salad, cooked, or stored as dried powder for many months without loss of nutritional value. For this reason, in some areas of Africa, various relief organizations are promoting consumption of its leaves as a nutritional supplement together with rice and other foods to prevent malnutrition in the poorest countries. Apart from treating malnutrition, in rural areas of Uganda, its leaves are used to treat a wide range of medical conditions such as HIV/AIDS-related symptoms, bronchitis, ulcers, malaria and fever, among others (Kasolo *et al.*, 2010).

Its benefits could be attributed to their composition of phenolic compounds. The search for new antioxidants and phenolics from herbal sources has garnered great attention in the last decade. In this regard, leaf extracts of *Moringa oleifera (M. oleifera)* have been reported to exhibit antioxidant activity both *in vitro* and *in vivo* due to their abundance of phenolic acids and flavonoids (Vongsak *et al.*, 2013). This fact, together with the possibility of the phenolic compounds interacting with other plant components, makes the extraction probably the most important step in sample pretreatment. The extraction of phenolic compounds depends greatly on the solvent's polarity, method and extraction time, which determine both the quantitative and qualitative composition of those compounds. The polarities of phenolic compounds vary significantly and it is difficult to develop a single method for optimal extraction of all phenolic compounds (García-Salas *et al.*, 2010). The total phenolic content determined from the same plant and its corresponding antioxidant activity may vary widely depending on the extraction conditions applied. Due to the aforementioned, the necessity of optimizing the extraction method for each sample prior to carrying out the analytical characterization seems indispensable. The optimal extraction method should be simple, rapid and economical.

Another point to keep in mind is that there are considerable variations in the nutritional value of *M. oleifera*, which depend on several factors like genetic background, environment and cultivation methods, as reported by Moyo *et al.* (Moyo *et al.*, 2011).

Previous phytochemical analyses of *M. oleifera* from different countries have shown that the leaves are particularly rich in potassium, calcium, phosphorous, iron, vitamins A and D, essential amino acids, as well as known antioxidants such as β-carotene, vitamin C, and flavonoids (Mbikay, 2012). However, *M. oleifera* from Madagascar (South Africa) has been little studied. Authors such as Moyo *et al.* have proclaimed the necessity of studying the nutritional value of *M. oleifera* of the South African ecotype (Moyo *et al.*, 2011).

For this reason, the purposes of this study were: 1) to determine the best way to extract the greatest amount of phenolics and other bioactive compounds from the leaves of *M. oleifera; 2*) to characterize the bioactive compounds using HPLC-ESI-QTOF-MS in order to provide an exhaustive compositional information.

❖ Materials and methods

Chemicals

HPLC–MS acetonitrile was purchased from Fisher (Thermo Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leicestershire, UK). Acetic acid of analytical grade (purity > 99.5%) was acquired from Fluka (Switzerland). Methanol and acetone used to extract the phenolic compounds from *M. oleifera* were purchased from Panreac (Barcelona, Spain). Ethanol, gallic acid and Folin-Ciocalteu reagent were from Sigma-Aldrich (Steinheim, Germany). Ultrapure water with a resistivity value of 18.2 MΩ was obtained from a Milli-Q system (Millipore, Bedford, MA, USA).

Plant material

The leaves of *M. oleifera* collected from Madagascar in 2010 were identified by the National Centre for environmental research (CNRE), Antanarivo, (Madagascar, Africa) where the voucher specimen was deposited. Branches from the tree were plucked by hand and laid on shelves 3 meters long, 30 cm wide and 50 cm apart in a dry place with good ventilation and in darkness. Two weeks later, the leaves were crushed with a stone mortar and the dust obtained was stored in darkness in a dry, fresh place until their treatment.

Methods for extracting phenolic compounds from *M. oleifera* leaves

Several extraction methods were performed using different percentages of different solvents and mixtures of solvent with water as shown in **Figure 1**. Conventional solid-liquid extraction (maceration) and ultrasound-assisted extraction (UAE) were tested as extraction methods.

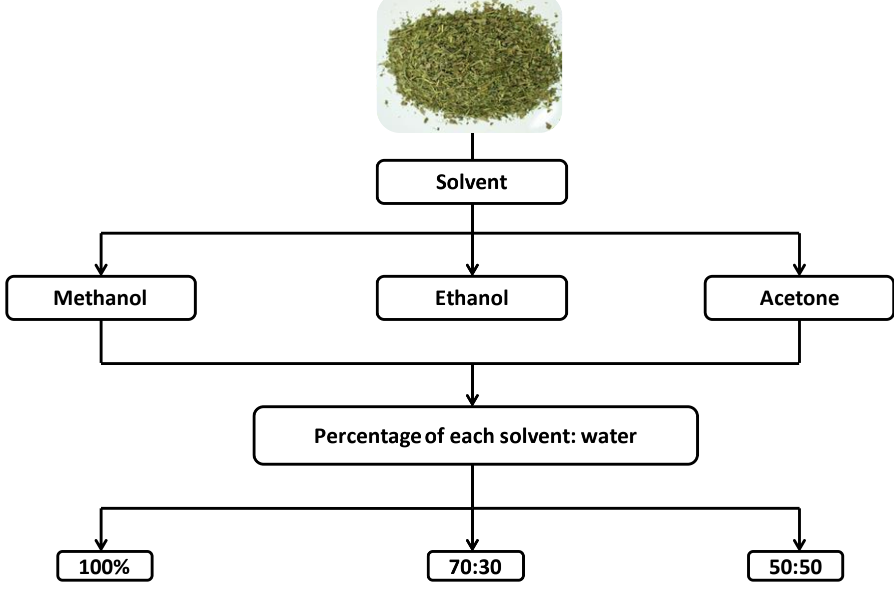


Figure 1. Percentage of different solvents and mixtures of solvents with water used for each method of extraction.

Conventional solid-liquid extraction

First, 0.5 g of plant material was extracted with 25 mL methanol:water (50:50, v:v). Maceration was carried out at different times (1, 3, 6 and 12 hours) at room

temperature in order to know the timeframe in which most of the compounds were extracted. There were no significant differences among the times of extraction with maceration. For this reason, the time selected for the other macerations was 1 hour.

After that, 0.5 g of plant material was extracted with 25 mL of the corresponding solvent for 1 h on a stir plate at room temperature. Then, samples were centrifuged for 10 min at 984 × g using a centrifuge to remove solids. Next, the solvent was evaporated using a rotary evaporator under vacuum at 40ºC, and the dried residue was redissolved in 2 mL of the corresponding solvent. These solutions were filtered through a 0.45-μm syringe filter and kept at -20ºC in amber bottles to avoid degradation until analysis.

Each extraction was done in triplicate.

Ultrasound-assisted extraction (UAE)

Afterwards, ultrasound-assisted extraction was carried out as described by Rodríguez-Pérez *et al.*, with some modifications (Rodríguez-Pérez *et al.*, 2013b). First, 0.5 g of each *M. oleifera* extract was extracted using 25 mL of different solvents in a sonicator Branson B3510 for 15 min at room temperature. Then, the samples were centrifuged for 10 min at 984 × g using a centrifuge to remove solids. After centrifugation, the pellets were extracted with fresh solvent under the same conditions in such a way that 4 consecutive extractions were made. The supernatants were dried in a rotary evaporator under vacuum at 40ºC, and the dried residue was redissolved in 2 mL of methanol, ethanol or acetone. These solutions were filtered through a 0.45-μm syringe filter and kept at -20ºC in amber bottles to avoid degradation until analysis.

Each extraction was done in triplicate.

Determination of total phenolic content

The total phenolic content of the obtained extracts was determined using the Folin-Ciocalteu assay (Herrero *et al.*, 2011) with some modifications. The total volume of the reaction mixture was reduced to 1 mL. 600 μL of water and 10 μL of sample were mixed, to which 50 μL of undiluted Folin–Ciocalteu reagent was

subsequently added. After 10 min, 150 µL of 2% (w/v) Na_2CO_3 were added and the volume was made up to 1.0 mL with water. After 2 h of incubation at room temperature in darkness, 200 µL of the mixture was transferred into a well of a microplate. The absorbance was measured at 760 nm using a Synergy Mx Monochromator-Based Multi-Mode Micro plate reader, by Bio-Tek Instruments Inc., (Winooski, VT) and compared to a gallic acid calibration curve (5 to 150 µg/mL) elaborated in the same manner. The total phenolic content was calculated as mean ±SD (n=6) and expressed as mg of gallic acid (GAE) per g of dry leaves.

Chromatographic separation

HPLC analyses were carried out using an Agilent 1200 Series Rapid Resolution LC system (Agilent Technologies, Palo Alto, CA, USA), equipped with a vacuum degasser, a binary pump, an autosampler, and a diode array detector (DAD). The column used for the chromatographic separation was a Zorbax Eclipse Plus C_{18} (1.8 µm, 150 mm × 4.6 mm) (Agilent Technologies, Palo Alto, CA, USA). Acidified water (0.5% formic acid, v/v) and acetonitrile were used as mobile phases A and B, respectively. The gradient was programmed as follows: 0 min, 5% B; 10 min, 35% B; 65 min, 95% B; 67 min, 5% B; and finally a conditioning cycle of 3 min under the initial conditions. The flow rate was set at 0.50 mL/min throughout the gradient and the effluent from the HPLC column was split using a T-type splitter before being introduced into the mass spectrometer (split ratio = 1:2) to provide a stable spray and, consequently, reproducible results. The injection volume in the HPLC system was 10 µL.

ESI-QTOF-MS analyses

The HPLC system was coupled to a micrOTOF-Q II mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with electrospray ionization (ESI) operating in negative mode. Detection was carried out considering a mass range of 50–1100 *m/z* and using a capillary voltage of + 4000 V, a dry gas temperature of 210°C, a dry gas flow of 8.0 L/min, a nebulizer pressure of 2.0 bar, and spectra rate of 1 Hz.

Moreover, automatic MS/MS experiments were performed using nitrogen as the collision gas, adjusting the collision energy values as follows: *m/z* 100, 20 eV; *m/z* 500, 25 eV; *m/z* 1000, 30 eV.

To attain the mass accuracy necessary to identify the compounds, external instrument calibration was used. For this, the calibrant used was sodium formate clusters consisting of 5 mM sodium hydroxide and water: 2-propanol 1:1 (v/v) with 0.2% formic acid. This calibrant was injected at the beginning of the run using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, IL, USA) directly connected to the interface, equipped with a Hamilton syringe (Reno, NV, USA).

The accuracy of the mass data for the molecular ions was controlled by the newest DataAnalysis 4.0 software (Bruker Daltonik), which provided a list of possible elemental formulas by using the SmartFormula™ editor. The Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double-bond equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (sigma value) for increased confidence in the suggested molecular formula.

Statistical analysis

Quantitative data are represented as mean ± SD. To evaluate the differences at a 95% confidence level ($p \leq 0.05$), a one-way analysis of variance (ANOVA) was followed by Tukey's test using Origin (version Origin Pro 8 SR0, Northampton, MA, USA).

❖ Results and discussion

Extraction methods and phenolic content

The extraction of phenolic compounds is demanding due to their chemical structures. Their interactions with other food components are not fully understood and many factors, such as solvent composition, time of extraction, temperature, pH, solid-to-liquid ratio and particle size, may significantly influence the solid–liquid extraction

(Dent *et al.*, 2013). Consequently, several extraction procedures were tested including maceration and UAE using methanol, ethanol, acetone and 70:30 and 50:50 mixtures with water as extracting solvents. The use of solvent mixtures was found to enhance the extraction yields by improving the solubility and increasing interaction of the targeted analyte with the extraction solvent (Mustafa and Turner, 2011).

Table 1 shows the total phenolic compounds obtained by the Folin-Ciocalteu assay, expressed in mg of GAE per g of dry leaves, using conventional extraction (maceration) and UAE with different solvents and solvent-water mixtures.

The results showed that using 100% organic solvent, the quantity of phenolic compounds extracted by both methods is lower than using the solvent mixed with water. The exception was UAE with 100% methanol, which allowed the extraction of 24 ± 2 mg GAE/g dry leaf. It is no wonder that these results were obtained since 100% methanol has previously been used to extract phenolic compounds from other plant leaves, obtaining good results (Amessis-Ouchemoukh *et al.,* 2014; Rodríguez-Pérez *et al.*, 2013a).

As reported by Proestos and Komaitis, a decline in the quantity of phenolic compounds was noted with decreasing polarity of the solvent (Proestos and Komaitis, 2006). In this way, the lowest total phenolic content values were obtained by using 100% acetone with either maceration or UAE. However, there were no significant differences between maceration using 100% ethanol, methanol and acetone and using UAE with 100% ethanol and 100% acetone.

A higher total phenolic content was obtained with UAE than using maceration. It could be due to using ultrasounds can disrupt plant cell walls with a subsequent increase in solvent penetration, which helps in obtaining a higher extraction yield (Vinatoru, 2001). For this reason this technique seems to be the best choice to extract phenolic compounds from *M. oleifera* leaves. On the other hand, a 50% concentration of each solvent was found to be the most efficient under these parameters. There were no significant differences between UAE using either methanol:water (50:50) or

ethanol:water (50:50). Dent *et al.* reported that hydroalcoholic mixtures of ethanol are possibly the most suitable solvent systems for the extraction of sage polyphenols due to the different polarities of the bioactive constituents and the acceptability of this solvent system for human consumption (Dent *et al.*, 2013). Furthermore, other authors investigated the influence of solvents on the quantity of extracted phenolic acids and concluded optimal extraction capacity was achieved with a 30 to 60 % aqueous solution of ethanol (Wang *et al.*, 2004). For all these reasons, UAE with ethanol: water (50:50) was the selected extraction method for characterizing the phenolic compounds from *M. oleifera* leaves and preparing an effective *M. oleifera* leaf extract in order to develop further pharmaceutical and/or nutraceutical products.

The final extraction was carried out for three consecutive times in order to obtain the greatest quantity of phenolic compounds. The extraction was done in triplicate and each extract was directly analyzed.

Table 1. Total phenolic content from extracts obtained by one conventional solid-liquid extraction (maceration) and by four successive UAE extractions using different solvents. Et: Ethanol; Me: Methanol; Ac: Acetone. The numbers correspond to the organic solvent percentage.

Extraction number	Solvent	Maceration Total phenolic content (mg GAE/ g dry leaf)	UAE Total phenolic content (mg GAE/ g dry leaf)
1st	**Et70**	24.3 ± 0.3	12.6 ± 0.2
2nd	**Et70**	--	4.4 ± 0.4
3th	**Et70**	--	1.2 ± 0.5
4th	**Et70**	--	0.8 ± 0.3
Total		**24.3 ± 0.3** $^{e, i}$	**19 ± 1** ^{b}
1st	**Et50**	27 ± 2	24 ± 3
2nd	**Et50**	--	18.4 ± 0.4
3th	**Et50**	--	4.6 ± 0.5
4th	**Et50**	--	0.29 ± 0.07
Total		**27 ± 2** $^{e, f, i, j}$	**47 ± 4** ^{a}
1st	**Et100**	3.82 ± 0.09	1.7 ± 0.2
2nd	**Et100**	--	2.84 ± 0.06
3th	**Et100**	--	2.2 ± 0.4
4th	**Et100**	--	1.9 ± 0.2
Total		**3.82 ± 0.09** $^{h, k, l}$	**8.6 ± 0.7** ^{c}
1st	**Me70**	20 ± 1	27 ± 2
2nd	**Me70**	--	4.2 ± 0.2
3th	**Me70**	--	1.22 ± 0.01
4th	**Me70**	--	0.24 ± 0.06
Total		**20 ± 1** ^{g}	**33 ± 3** ^{d}
1st	**Me50**	27 ± 3	22 ± 1
2nd	**Me50**	--	11 ± 1
3th	**Me50**	--	9.2 ± 0.2
4th	**Me50**	--	3.25 ± 0.02
Total		**27 ± 3** $^{b, e, i}$	**46 ± 3** ^{a}
1st	**Me100**	3.6 ± 0.3	12 ± 1
2nd	**Me100**	--	5.8 ± 0.6
3th	**Me100**	--	4.5 ± 0.4
4th	**Me100**	--	1.39 ± 0.08
Total		**3.6 ± 0.3** $^{h, k}$	**23 ± 2** ^{e}
1st	**Ac70**	26 ± 2	16.7 ± 0.4
2nd	**Ac70**	--	5.12 ± 0.06
3th	**Ac70**	--	3.18 ± 0.07
4th	**Ac70**	--	0.84 ± 0.03
Total		**26 ± 2** $^{e, f, i, j, m}$	**25.8 ± 0.5** $^{d, f}$
1st	**Ac50**	26 ± 2	28 ± 1
2nd	**Ac50**	--	4.2 ± 0.7
3th	**Ac50**	--	0.84 ± 0.05
4th	**Ac50**	--	0.16 ± 0.02
Total		**26 ± 2** $^{e, f, i, j, m}$	**33 ± 2** $^{d, g}$
1st	**Ac100**	2.0 ± 0.2	1.83 ± 0.04
2nd	**Ac100**	--	1.4 ± 0.1
3th	**Ac100**	--	0.98 ± 0.01
4th	**Ac100**	--	1.10 ± 0.09
Total		**2.0 ± 0.2** $^{h, k, l}$	**5.3 ± 0.2** $^{c, h}$

Data are expressed as mean ± standard deviation. Mean values with different superscript letters are significantly different ($p < 0.05$).

Compound identification

The base peak chromatograms of the *M. oleifera* leaves extracted with ethanol:water (50:50) resulting from the HPLC-ESI-QTOF-MS method described above are shown in **Figure 2**, where the peaks are numbered according to their elution order. The compounds were tentatively identified by interpreting their MS and MS/MS spectra determined by QTOF-MS and also considering data from the literature and open-access mass-spectra databases such as Metlin and MassBank. **Table 2** summarizes the MS data of the compounds tentatively identified. A total of 59 compounds were tentatively characterized; among them, amino acids, one nucleoside, organic acids, phenolic acid and derivatives, thyoglicosides, lignans and flavonoids. Thirty of these compounds were tentatively identified for the first time in *M. oleifera* leaves.

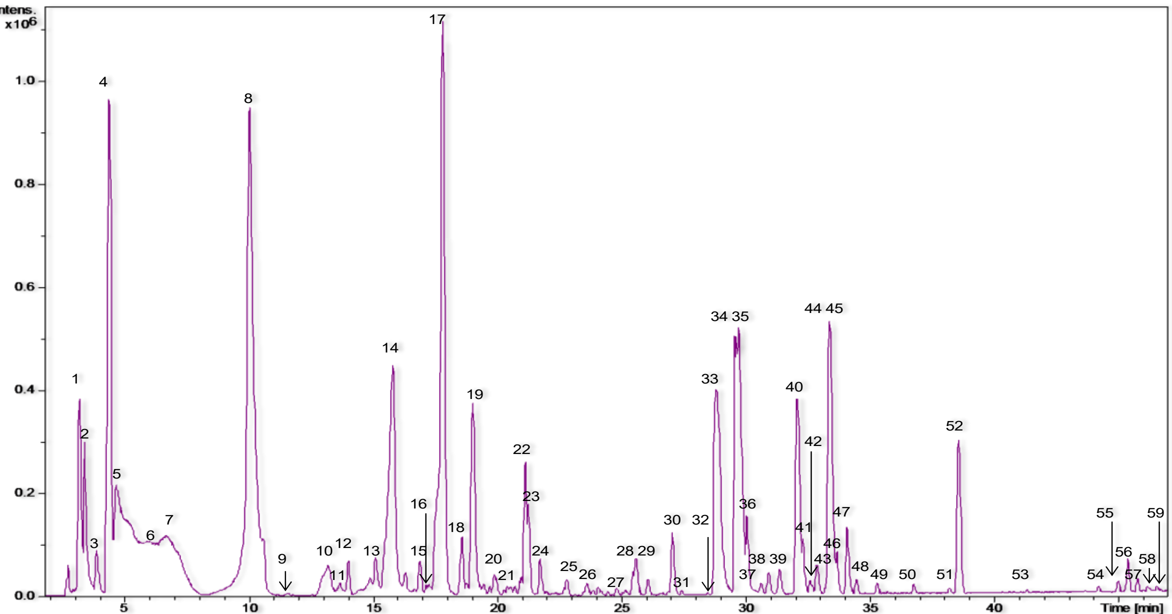


Figure 2. Base-Peak Chromatogram (BPC) of *M. oleifera* leaves by HPLC-ESI-QTOF-MS analysis in the negative ion mode.

Table 2. HPLC-ESI-QTOF-MS data of the compounds identified in *Moringa oleifera* leaf extracted by UAE with ethanol:water (50:50)

Peak[a]	RT	m/z experimental	Molecular formula	m/z calculated	Error (ppm)	mSigma Value	Fragments[b]	Compound
Aminoacids								
5	4.79	130.0872	$C_6H_{13}NO_2$	130.0874	1.1	5.3	128.0367 (100%)	Leu/Ile[c]
10	13.19	164.0719	$C_9H_{11}NO_2$	164.0717	1.4	4.6	147.0456 (100%)	Phenilalanine
15	16.89	203.0829	$C_{11}H_{12}N_2O_2$	203.0826	1.6	1.3	116.0507 (100%); 142.0656 (40.4%); 117.0535 (10.6%)	L-tryptophan
Nucleosides								
9	11.6	266.0896	$C_{10}H_{13}N_5O_4$	266.0895	0.6	32	134.0470 (100%)	Adenosine
Organic acids								
1	3.27	195.0521	$C_6H_{12}O_7$	195.051	5.4	1.4	129.0196 (100%); 99.0073 (10%)	Gluconic acid
2	3.47	191.057	$C_7H_{12}O_6$	191.0561	4.9	2.4	127.0398 (100%)	Quinic acid
3	3.95	133.0145	$C_4H_6O_5$	133.0142	1.7	6.8	115.0048 (100%)	Malic acid
4	4.44	191.0205	$C_6H_8O_7$	191.0197	4.1	8.4	111.0085 (100%); 129.0222 (5.3%)	Citric acid
42	32.55	187.0979	$C_9H_{16}O_4$	187.0976	1.9	9.6	125.0868 (100%); 126.1023 (11.5%); 123.0817 (10%); 169.0885 (10%)	Azelaic acid
52	38.49	201.1136	$C_{10}H_{18}O_4$	201.1132	1.9	52.2	183.1012 (100%); 139.1120 (69%)	Sebacic acid
55	44.87	327.2184	$C_{18}H_{32}O_5$	327.2177	2.2	2.6	211.1347 (100%); 171.1027 (84.6%)	Trihydroxyoctadecadienoic acid isomer 1
56	45.27	327.2184	$C_{18}H_{32}O_5$	327.2177	2.2	3	211.1350 (100%); 229.1463 (54.3%); 171.1027 (18%)	Trihydroxyoctadecadienoic acid isomer 2
57	45.67	327.2182	$C_{18}H_{32}O_5$	327.2177	1.5	6	171.1034 (100%); 211.1358 (96.2%)	Trihydroxyoctadecadienoic acid isomer 3
58	46.06	327.2182	$C_{18}H_{32}O_5$	327.2177	1.5	7.9	211.1337 (30%)	Trihydroxyoctadecadienoic acid isomer 4
59	46.42	327.2182	$C_{18}H_{32}O_5$	327.2177	1.5	1.3	211.1349 (100%); 171.1034 (34.3%)	Trihydroxyoctadecadienoic acid isomer 5

Table 2. *(Continued)*

Peak[a]	RT	m/z experimental	Molecular formula	m/z calculated	Error (ppm)	mSigma Value	Fragments[b]	Compound
Phenolic acids and derivatives								
11	13.76	353.0876	$C_{16} H_{18} O_9$	353.0878	0.5	40.1	179.0372 (100%); 191.0568 (87.3%); 135.0453 (49.2%)	Caffeoylquinic acid isomer 1
12	14.03	353.0878	$C_{16} H_{18} O_{10}$	353.0878	0	39.8	179.0357 (100%); 191.0564 (59.8%); 135.0452 (33.8%)	Caffeoylquinic acid isomer 2
13	15.12	353.0883	$C_{16} H_{18} O_{11}$	353.0878	1.4	22.7	191.0570 (100%); 179.0358 (71.9%)	1-Caffeoylquinic acid
14	15.81	353.0879	$C_{16} H_{18} O_{12}$	353.0878	0.1	0.6	191.0567 (100%); 179.0355 (70.3%); 135.0452 (31.3%)	3-Caffeoylquinic acid
16	17.18	337.0927	$C_{16} H_{18} O_8$	337.0929	0.7	47.7	163.0373 (100%); 177.0227 (70.8%); 173.0466 (70%); 119.0492 (40.9%)	Coumaroylquinic acid isomer 1
17	17.72	337.0935	$C_{16} H_{18} O_8$	337.0929	1.7	5.4	163.0401 (100%); 119.0505 (37.5%); 191.0559 (8.1); 173.0456 (10%)	Coumaroylquinic acid isomer 2
18	18.59	367.1036	$C_{17} H_{20} O_9$	367.1035	0.4	5.1	193.0513 (100%); 134.0373 (32%)	Feruloylquinic acid isomer 1
19	19.02	353.088	$C_{16} H_{18} O_9$	353.0878	0.7	3.7	173.0461 (100%); 179.0357 (80.2%); 191.0566 (73.5%); 135.0454 (44.6)	4-Caffeoylquinic acid
20	19.87	337.093	$C_{16} H_{18} O_8$	337.0929	0.3	6.2	173.0459 (100%); 191.0567 (22.4%); 163.0398 (21.1 %); 119.0505 (16.4%)	Coumaroylquinic acid isomer 3
22	21.13	337.093	$C_{16} H_{18} O_8$	337.0929	0.3	2.3	173.0467 (100%); 163.0400 (25.5%); 119.0503 (10%)	4-*p*-Coumaroylquinic acid
24	21.73	367.1036	$C_{17} H_{20} O_9$	367.1035	0.1	8.7	193.0510 (70%); 134.0375 (12%)	Feruloylquinic acid isomer 2

Table 2. *(Continued)*

Peak[a]	RT	m/z experimental	Molecular formula	m/z calculated	Error (ppm)	mSigma Value	Fragments[b]	Compound
Thioglycosides (Glucosinolates)								
6	6.05	570.0956	$C_{20}H_{29}NO_{14}S_2$	570.0957	0.2	3	96.9594 (20%); 328.0861 (10%)	Glucomoringin isomer 1
7	6.72	570.095	$C_{20}H_{29}NO_{14}S_2$	570.0957	1.2	6.1	96.9598 (20%); 328.0856 (10%)	Glucomoringin isomer 2
8	10.08	570.0964	$C_{20}H_{29}NO_{14}S_2$	570.0957	1.3	2	96.9595 (20.1%); 328.0856 (6%)	Glucomoringin isomer 3
Lignans								
25	22.77	521.201	$C_{26}H_{34}O_{11}$	521.2028	3.5	16.4	359.1487 (100%)	Isolariciresinol glycoside/lariceresinol glycoside isomer 1
27	25.06	519.1872	$C_{26}H_{32}O_{11}$	519.1872	0.1	44	357.1358 (100%); 151.0406 (43.9%)	Pinoresinol/epipinoresinol glycoside
28	25.45	521.2028	$C_{26}H_{34}O_{11}$	521.2018	1.9	3.4	341.1408 (100%); 179.0529 (30%)	Isolariciresinol glycoside/lariceresinol glycoside isomer 2
31	27.37	523.2165	$C_{26}H_{36}O_{11}$	523.2185	3.8	21.6	361.1640 (100%); 199.0980 (27%)	Secoisolariciresinol glycoside
Flavonoids								
21	20.69	609.1456	$C_{27}H_{30}O_{16}$	609.1461	0.9	14.3	447.0927 (100%); 285.0419 (40%); 179.0389 (10%)	Kaempferol diglycoside
23	21.25	593.1508	$C_{27}H_{30}O_{15}$	593.1512	0.6	5.4	593.1518 (100%); 473.1083 (22%); 353.0370 (10%)	Multiflorin B
26	23.84	625.1428	$C_{27}H_{30}O_{17}$	625.141	2.9	35.2	463.0873 (100%); 301.0362 (53.6%)	Quercetin diglycoside
29	25.55	431.0973	$C_{21}H_{20}O_{10}$	431.0984	2.6	3	311.0572 (100%); 341.0982 (13%); 283.0620 (10%)	(Vitexin) Apigenin glucoside isomer 1
30	27.02	431.0977	$C_{21}H_{20}O_{10}$	431.0984	1.6	2.3	311.0567 (100%); 341.0675 (52.2%); 431.0982 (13%)	(Vitexin) Apigenin glucoside isomer 2
32	28.22	463.0873	$C_{21}H_{20}O_{12}$	463.0882	2	44.2	300.0276 (100%); 301.0363 (56.7%)	Quercetin glycoside

Table 2. *(Continued)*

Peak[a]	RT	m/z experimental	Molecular formula	m/z calculated	Error (ppm)	mSigma Value	Fragments[b]	Compound
33	28.78	463.0876	$C_{21} H_{20} O_{12}$	463.0882	1.4	5	300.0288 (100%); 301.0350 (55%); 463.0884 (20%)	Quercetin 3-O-glucoside
34	29.24	549.0896	$C_{24} H_{22} O_{15}$	549.0886	1.8	2.2	300.0288 (100%); 301.0353 (57.9%); 463.0883 (5.6%)	Quercetin malonylglucoside isomer 1
35	29.55	549.088	$C_{24} H_{22} O_{15}$	549.0886	1.1	2.9	300.0276 (100%); 301.0345 (61.7%); 505.0983 (63.2%)	Quercetin malonylglucoside isomer 2
36	30	607.1299	$C_{27} H_{28} O_{16}$	607.1305	0.8	1.3	505.0989 (100%); 463.0885 (81%); 301.0353 (36%); 300.0285 (31%);	Quercetin-hydroxy-methylglutaroyl glycoside
37	30.57	589.1206	$C_{27} H_{26} O_{15}$	589.1199	1.2	15.9	301.0365 (90%); 463.0897 (73.5%); 300.0290 (65%)	Quercetin triacetylgalactoside
38	30.87	505.0982	$C_{23} H_{22} O_{13}$	505.0988	1	4.6	300.0291 (100%); 301.0317 (54%)	Quercetin-acetyl-glycoside isomer 1
39	31.32	505.0985	$C_{23} H_{22} O_{13}$	505.0988	0.5	3.7	300.0288 (100%); 301.0351 (50.4%); 463.0874 (10%)	Quercetin-acetyl-glycoside isomer 2
40	32	447.0931	$C_{21} H_{20} O_{11}$	447.0933	0.5	4.7	284.0342 (100%); 285.0399 (40%)	Kaempferol 3-O-glucoside
41	32.25	477.1031	$C_{22} H_{22} O_{12}$	477.1038	1.6	0.9	314.0445 (100%); 315.0488 (23%)	Isorhamnetin 3-O-Glucoside
43	32.8	489.1036	$C_{23} H_{22} O_{12}$	489.1038	0.4	2.8	284.0337 (100%); 285.0409 (53.4%)	Kaempferol acetyl glycoside isomer 1
44	33.32	489.1037	$C_{23} H_{22} O_{12}$	489.1037	0.3	4.6	284.0337 (100%); 285.0409 (53.4%)	Kaempferol acetyl glycoside isomer 2

Table 2. *(Continued)*

Peak[a]	RT	m/z experimental	Molecular formula	m/z calculated	Error (ppm)	mSigma Value	Fragments[b]	Compound
45	33.32	533.0938	$C_{24} H_{22} O_{14}$	533.0937	0.3	2.5	284.0338 (100%); 285.0407 (54%)	Kaempferol malonylglucoside
46	33.6	591.1358	$C_{27} H_{28} O_{15}$	591.1355	0.4	9.7	489.1034 (100%); 285.0413 (80%); 447.0937 (49.8%); 529.1360 (32.4%); 284.0330 (30%)	Kaempferol glycoside-hydroxy-methylglutarate
47	34.02	519.1142	$C_{24} H_{24} O_{13}$	519.1144	0.5	4.7	315.0502 (100%); 314.0443 (94%); 300.0271 (10%)	Isorhamnetin-acetil-glycoside
48	34.4	531.1134	$C_{25} H_{24} O_{13}$	531.1144	1.9	15.5	300.0287 (100%); 301.0352 (51.4%); 463.0873 (18.8%)	Quercetin derivative
49	35.21	489.1032	$C_{23} H_{22} O_{12}$	489.1038	1.3	11.6	284.0337 (100%); 285.0401 (34.2%)	Kaempferol acetyl glycoside isomer 3
50	36.7	301.0358	$C_{15} H_{10} O_{7}$	301.0354	1.3	5	151.0038 (100%); 178.9987 (40.2%); 121.0296 (18.5%), 107.0144 (10%)	Quercetin
51	38.1	515.1184	$C_{25} H_{24} O_{12}$	515.1195	2.2	8.8	284.0328 (100%); 285.0405 (70%)	Kaempferol-di-acetyl-rhamnoside
53	41.22	285.0402	$C_{15} H_{10} O_{6}$	285.0405	0.9	17.1	285.0407 (100%); 219.0231 (10%); 131.0142 (6.3%);	Kaempferol
54	44.08	573.061	$C_{28} H_{30} O_{13}$	573.0614	0.7	3.9	300.0280 (100%); 301.0360 (65.4%); 463.0897 (41.2%)	Quercetin derivative

[a] Peak numbers assigned according to the overall elution order, [b] Numbers in brackets show the relative abundance of each MS/MS fragment expressed in percentage, [c] Amino acids are denoted by the three letter code: isoleucine, lle, leucine, Leu.

Amino acids and nucleosides

M. oleifera leaves are a good source of amino acids (Moyo *et al.*, 2011). This method is not designed to identify this type of compound; however, it allowed characterization of three amino acids: leucine or isoleucine (peak 5), phenylalanine (peak 10) and tryptophan (peak 15). In addition, the nucleoside adenosine at RT 11.6 was identified in this sample. All of them were previously described in *M. oleifera* leaves and their fragments were compared with the Metlin database.

Organic acids

Eleven organic acids were tentatively identified in the leaves of *M. oleifera,* 8 of them being tentatively characterized for the first time in this sample. In this way, peak 1, which presented two main fragments at m/z 129 and m/z 99, was characterized as gluconic acid. Gluconic acid is an abundant mild organic acid available in plants, which is derived from the glucose by a simple oxidation reaction. In general, this organic acid is sold for use in the formulation of food, pharmaceutical and hygienic products (Ramachandran *et al.*, 2006). Its two main fragments were compared with the Metlin database.

Peak 2 presented a characteristic fragment at m/z 127 and was tentatively identified as quinic acid, which has been previously identified as one of the major compounds in *M. oleifera* leaves (Mbikay, 2012). Peaks 3 and 4 were tentatively identified as malic acid and citric acid, respectively. These two compounds have not been extensively described in *M. oleifera* leaves. Malic acid presented a fragment at m/z 115 due to loss of a water molecule, while citric acid had a major fragment at m/z 111 due to $[M\text{-}H\text{-}CO_2\text{-}2H_2O]^-$. Citric acid is used as a natural food preservative and both, together with other organic acids and compounds such as glucose, sucrose and some vitamins present in *M. oleifera* extracts, produce protective effects in renal tissue against DMBA-induced toxicity, which acts as a potent carcinogen by generating various reactive metabolic intermediates leading to oxidative stress (Sharma and Paliwal, 2012).

Peak 42 was characterized as azelaic acid, a 9-carbon saturated dicarboxylic acid found naturally in some plants which takes part in plant systemic immunity and is involved in priming defenses (Jung *et al.*, 2009). It main fragment at m/z 125 was due to the loss of one of the acid groups and further dehydration. This organic acid was tentatively identified for the first time in leaves of *M. oleifera.* Another compound that was tentatively identified in these leaves was sebacid acid (peak 52). Its MS/MS spectrum showed two fragments at m/z 183 and 139, the same reported in the Metlin database. Sebacic acid belongs to the medium-chain dicarboxylic acids, naturally occurring in higher plants, and some authors affirm that it is a promising alternative energy substrate (Mingrone *et al.*, 2013). This organic acid is soluble in water and some studies suggest this compound could help improve glucose metabolism in type 2 diabetes, presenting a high potential as a natural food ingredient for patients with this illness (Mingrone *et al.*, 2013).

In addition, five isomers of 9, 12, 13-trihydroxyoctadecadienoic acid (peaks 55 to 59) were also tentatively characterized for the first time in this sample. They presented two main fragments at m/z 211 and 229 due to C12-C13 bond cleavage and subsequent dehydration of the formed product ion, according to Rodríguez-Pérez *et al.* (Rodríguez-Pérez *et al.*, 2013b).

Phenolic acid and derivatives

Phenolic acids are aromatic secondary plant metabolites, widely spread throughout the plant kingdom. Eleven phenolic acids and derivatives were tentatively characterized in the present sample, 6 of them being identified for the first time in *M. oleifera* leaves and in the Moringaceae family.

Among them, 5 isomers of caffeoylquinic acid (peaks 11, 12, 13, 14 and 19) were characterized. In this way, and according with their fragmentation pattern, it was possible to distinguish between 1-caffeoylquinic acid (peaks 13) and 3-caffeoylquinic acid (peak 14) which were tentatively identified in agreement with Clifford *et al.* (Clifford *et al.* 2005). These compounds are part of the *M. oleifera* composition as reported previously (Amaglo *et al.*, 2010; Kashiwada *et al.*, 2012). Their main

fragments at m/z 179, 191 and 135 were the same as previously described by (Jaiswal *et al.*, 2013).

In addition, 2 isomers of feruloylquinic acid (peaks 18 and 24) were tentatively characterized for the first time in *M. oleifera* leaves according with Jaiswal et al. (Jaiswal *et al.* 2010). These compounds presented two different fragments at m/z 134 and 193. The fragment at m/z 134 corresponded to $[M\text{-}CH_3\text{-}CO_2\text{-}H]^-$ from ferulic acid while the fragment at m/z 193 corresponded to loss of the ferulic acid.

On the other hand, peak 19 was tentatively identified as 4-caffeoylquinic acid, a compound previously described in *M. oleifera* (Bennett et al., 2003), according to Clifford *et al.*, who described its fragments and used them to discriminate among different caffeoylquinic acids. Furthermore, peaks 16, 17 and 20 showed an $[M–H]^-$ at *m/z* 337 and were tentatively identified as isomers of coumaroylquinic acid. As well as peak 22 was characterized as 4-*p*-coumaroylquinic acid according to Clifford *et al.* (Clifford *et al.*, 2003). These compounds were tentatively characterized for the first time in *M. oleifera* leaves and the Moringaceae family.

The coumaroylquinic acid isomers (peaks 16, 17 and 20) and 4-caffeoylquinic acid were apparently some of the more abundant phenolic acids and derivative compounds in *M. oleifera* leaf extracts. Some hydroxycinnamates such as caffeic, ferulic or ρ-coumaric acids have been shown to protect LDL particles against oxidation *in vitro*, which is a key step in the formation of atherosclerotic plaques (Guy *et al.*, 2009).

Thioglycosides (glucosinolates)

Glucosinolates are natural compounds present in species of the order *Brassicales* and the precursors of bioactive isothiocyanates. In recent years, they have been studied mainly for their chemopreventive as well as novel chemotherapeutic properties (Galuppo *et al.*, 2013). In this regard, 3 isomers of glucomoringin (peaks 6, 7 and 8) were tentatively identified in *M. oleifera* leaves. Their fragmentation pattern

is shown in **Figure 3**. Glucosinolates have usually been found in the seeds of certain plants. Their identification in leaves is less common due to plant age and environmental factors having significant effects on the level of glucosinolates that may also affect their distribution among plant organs (Fahey *et al.*, 2001). The same author (Fahey *et al.*, 2001) explained the attention paid to these metabolites due to their cancer-preventive potential, primarily as inducers of phase 2 enzymes but with potential antiproliferative, apoptosis-promoting, redox regulatory and phase 1 enzyme inhibiting roles as well. Other authors have described glucomoringin as a bioactive compound that can reduce myeloma growth in nude mice (Brunelli *et al.*, 2010).

Figure 3. Fragmentation pattern for glucomoringin.

Lignans

Lignans are one of the major phytostrogens found in plants, which also act as antioxidant compounds. Although they have been widely described in plants, lignans have not been found before in *M. oleifera* leaves, nor in the Moringaceae family. In this regard, 4 lignans, which showed low intensities in the chromatogram, were tentatively characterized for the first time in *M. oleifera* leaves (peaks 25, 27, 28 and 31). Peak 27 was tentatively characterized as pinoresinol or epipinoresinol glycoside. This compound showed a fragment at m/z 357 due to loss of the glycoside moiety. Peaks 25 and 28 were tentatively identified as lariceresinol glycoside or isolariceresinol glycoside isomers. Their two main fragments at m/z 341 and 179 are due to loss of the glycoside moiety (m/z 179) while the rest of the molecule corresponds to the fragment at m/z 341, whereas the compound at RT 22.77 showed a fragment at m/z 359 after the neutral loss of the glycoside moiety. The fragmentation

pattern of these compounds is displayed in **Figure 4**. In addition, peak 31 was tentatively characterized as secolariciresinol glycoside. Its main fragment at m/z 361 corresponds to the loss of the lariciresinol moiety.

Flavonoids

As expected, the predominant group of phenolic compounds in *M. oleifera* leaves was the flavonoid group, with kaempferol and quercetin derivatives being the most predominant flavonoids in *M. oleifera* leaves. Thus, 26 different flavonoids were tentatively characterized in this sample, with 12 of them being tentatively identified for the first time in this plant.

Kaempferol and kaempferol derivatives have been previously described in *M. oleifera* leaves (Coppin *et al.*, 2013). In this regard, the compound at RT 40.85 was tentatively identified as kaempferol, which presented its typical main ion at m/z 285, while peak 40 was tentatively characterized as kaempferol 3-*O*-glucoside, which presented the expected fragments at 284 and 285. On the other hand, some kaempferol derivatives were also tentatively identified in the present sample (peaks 21, 43, 44, 45, 46, 49 and 51). These compounds were tentatively characterized for the first time in *M. oleifera* leaves. In this regard, peak 21 was tentatively identified as kaempferol diglycoside, which presented different fragments at m/z 447 corresponding to the kaempferol glycoside, at m/z 285 due to loss of the kaempferol moiety, and at 179 due to the other glycoside moiety. The others compounds were tentatively identified as isomers of kaempferol acetyl glycoside (peaks 43, 44 and 49), kaempferol malonylglycoside (peak 45), kaempeferol glycoside-hydroxy-methylglutarate (peak 46) and kaempferol diacetyl rhamnoside (peak 51). All of them presented the characteristic fragments of the kaempferol core at m/z 284 and 285. As well as, fragmentation pattern of peak 46 is displayed in **Figure 4b**.

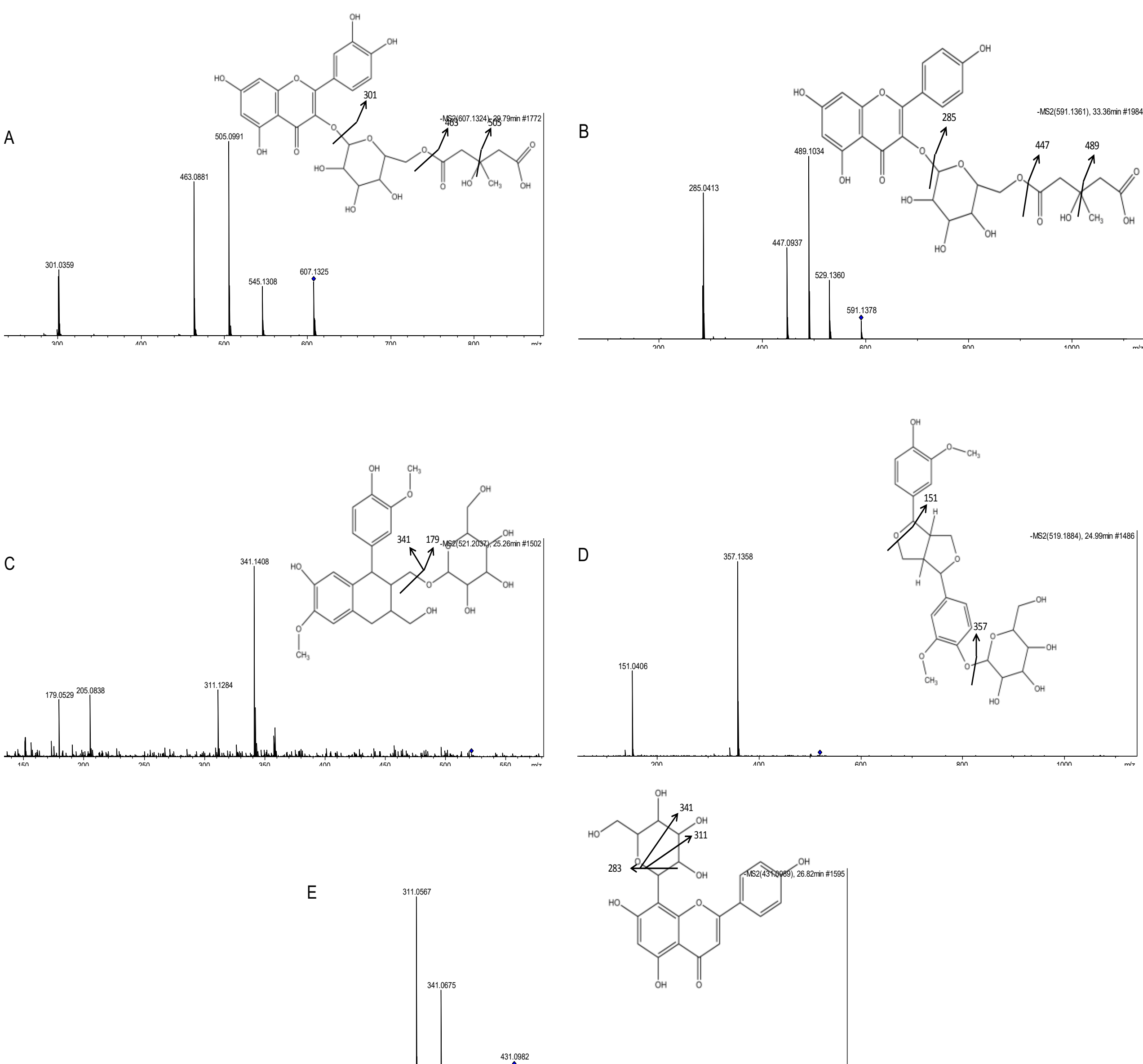


Figure 4. Fragmentation pattern for (A) quercetin hydroxy methylglutaroyl glycoside; (B) kaempferol hydroxy methylglutaroyl glycoside; (C) isolariciresinol/lariciresinol glycoside; (D) pinoresinol/epipinoresinol glycoside and (E) apigenin glycoside (vitexin).

Epidemiological studies have shown an inverse relationship between kaempferol intake and cancer, and it has been demonstrated that kaempferol alters several different mechanisms in the regulation of cancer cells. Not only is kaempferol a potent promoter of apoptosis but it also modifies a host of cellular signaling pathways. At the molecular level, kaempferol has been reported to modulate a number of key elements in cellular signal transduction pathways linked to apoptosis, angiogenesis, inflammation, and metastasis (Berger *et al.*, 2013; Chen and Chen, 2013; Chen *et al.*, 2013).

According to some authors, kaempferol derivatives together with quercetin derivatives are the main flavonoids in *M. oleifera* leaves (Coppin *et al.*, 2013). Quercetin is a ubiquitous flavonoid that was also tentatively identified in this sample (peak 50). This compound showed its typical fragments, according to the Metlin database, at m/z 151, 178, 121 and 107. Apart from quercetin, eleven quercetin derivatives were tentatively characterized in *M. oleifera* leaves. Four of them (32, 33, 34 and 35) have been previously described in *M. oleifera* leaves (Bennett *et al.*, 2003; Coppin *et al.*, 2013; Karthivashan *et al.*, 2013; Kashiwada *et al.*, 2012) as quercetin glycoside, quercetin 3-*O*-glucoside (Ablajan, K. *et al.* 2006), quercetin malonylglycoside and quercetin-hydroxy-methylglutaroyl glycoside (**Figure 4a**). As shown in **Figure 2**, these three compounds were among those that showed more intensity in the BPC.

The other five compounds (peaks 37, 38, 39, 48 and 54) were tentatively characterized for the first time in *M. oleifera* leaves as quercetin triacetylglycoside, isomers of quercetin acetyl glycoside and two unspecified quercetin derivatives, respectively. All these compounds showed common fragments at m/z 300 and 301 due to the quercetin core and at 463 corresponding to quercetin glycoside.

Quercetin represents the main dietary flavonoid that has emerged as a molecule possessing multiple properties that are all directed at ameliorating pathological conditions associated with degenerative diseases due to its antioxidant and anti-

inflammatory properties, which have also been associated with the prevention and therapy of cardiovascular diseases and cancer (Russo *et al.*, 2012).

As well, apart from the aforementioned, other flavonoids were characterized in *M. oleifera* leaves. Peak 23 was identified as multiflorin B. This bioactive compound with all its fragments has been previously characterized in *M. oleifera* leaves (Karthivashan *et al.*, 2013). Compounds 29 and 30 were tentatively characterized as isomers of apigenin glycoside, commonly known as vitexin. These compounds and their fragments have also been previously identified in *M. oleifera* leaves (Karthivashan *et al.*, 2013) and its fragmentation patterns are available in **Figure 4e**. These two compounds showed good intensity in the BPC (**Figure 2**), being some of the most intense peaks in the chromatogram. The importance of those compounds derives from the fact that apigenin has gained particular attention in recent years as a beneficial and health promoting agent because of its low intrinsic toxicity. Specifically, vitexin and isovitexin, naturally occurring *C*-glycosylated derivatives of apigenin, have been shown to possess potent anti-diabetic, anti-Alzheimer's, and anti-inflammatory activities (Choi *et al.*, 2014).

Moreover, two isohramnetin glycosides were tentatively identified (peaks 41 and 47). Despite peak 47 being acetylated, both of them showed the same fragments at m/z 314 and 315 characteristic of isorhamnetin. In this regard, peak 41 was characterized as isohramnetin 3-O-glucoside. A mass difference of 162 and the significantly higher abundance of the $[Y_0 - H]^{-\bullet}$ ion at *m/z* 314 than that of the Y_0^- ion at *m/z* 315 clearly revealed the structure of methoxylated flavonoid-3-*O*-glucoside, according to Sriseadka *et al.* (Sriseadka *et al.* 2012).

Flavonoids such as quercetin and kaempferol have been shown to be more powerful antioxidants than traditional vitamins (Coppin *et al.*, 2013). For all these reasons, flavonoids may offer many opportunities as innovative food ingredients and for commercial development due to the health benefits they provide, which are being studied by many authors.

❖ Conclusions

After studying two different extraction procedures, UAE using ethanol:water (50:50, v/v) produced an extract of *M. oleifera* leaves with the largest amount of phenolic compounds. HPLC-ESI-QTOF-MS is a powerful technique which allowed us to determine that leaves from *M. oleifera* are a good source of bioactive compounds, flavonoids and phenolic acid derivatives being the most abundant of these bioactive compounds. This study has shown that leaves from *M. oleifera* could be a good source of bioactive compounds if used as a nutritional supplement overall in those developing countries where this tree is autochthonous, as well offering new opportunities for food companies to develop nutraceuticals, cosmetic and pharmaceutical products.

Acknowledgments

This work was supported by projects AGL2011-29857-C03-02 and AP2010-1551 (Spanish Ministry of Science and Innovation), as well as P09-CTS-4564, P10-FQM-6563 and P11-CTS-7625 (Andalusian Regional Government Council of Innovation and Science). The authors are grateful to the Spanish Ministry of Science and Innovation for a "Personal técnico de apoyo" grant PTA2012-6956-E. We would like also to thank to the non-governmental organization “Agua de coco” for providing us the samples to analyze.

References

Ablajan, K., Anliz, Z., Shang, X.Y., He, J.M., Zhang, R.P., Shi, J.G. 2006. Structural characterization of flavonol 3,7-di-O-gylcosides and determination of the glycosilation position by using negative ion electrospray ionization tandem mass spectrometry. J Mass Spectrom. 41(3), 352-360.

Amaglo, N.K., Bennett, R.N., Lo Curto, R.B., Rosa, E.A.S., Lo Turco, V., Giuffrida, A., Curto, A.L., Crea, F., Timpo, G.M., 2010. Profiling selected phytochemicals and nutrients in different tissues of the multipurpose tree *Moringa oleifera* L., grown in Ghana, Food Chem. 122, 1047-1054. doi: 10.1016/j.foodchem.2010.03.073.

Amessis-Ouchemoukh, N., Abu-Reidah, I.M., Quirantes-Piné, R., Rodríguez-Pérez, C., Madani, K., Fernández-Gutiérrez, A., Segura-Carretero, A., 2014. Tentative Characterisation of Iridoids, Phenylethanoid Glycosides and Flavonoid Derivatives from *Globularia alypum* L. (Globulariaceae) Leaves by LC-ESI-QTOF-MS, Phytochem. Anal. .

Bennett, R.N., Mellon, F.A., Foidl, N., Pratt, J.H., Dupont, M.S., Perkins, L., Kroon, P.A., 2003. Profiling glucosinolates and phenolics in vegetative and reproductive tissues of the multi-purpose trees *Moringa oleifera* L. (Horseradish tree) and *Moringa stenopetala* L. J. Agric. Food Chem. 51, 3546-3553. doi: 10.1021/jf0211480.

Berger, A., Venturelli, S., Kallnischkies, M., Böcker, A., Busch, C., Weiland, T., Noor, S., Leischner, C., Weiss, T.S., Lauer, U.M., Bischoff, S.C., Bitzer, M., 2013. Kaempferol, a new nutrition-derived pan-inhibitor of human histone deacetylases, J. Nutr. Biochem. 24, 977-985.

Brunelli, D., Tavecchio, M., Falcioni, C., Frapolli, R., Erba, E., Iori, R., Rollin, P., Barillari, J., Manzotti, C., Morazzoni, P., D'Incalci, M., 2010. The isothiocyanate produced from glucomoringin inhibits NF-kB and reduces myeloma growth in nude mice in vivo, Biochem. Pharmacol. 79, 1141-1148.

Chen, A.Y., Chen, Y.C., 2013. A review of the dietary flavonoid, kaempferol on human health and cancer chemoprevention, Food Chem. 138, 2099-2107.

Chen, H.J., Lin, C.M., Lee, C.Y., Shih, N.C., Peng, S.F., Tsuzuki, M., Amagaya, S., Huang, W.W., Yang, J.S., 2013. Kaempferol suppresses cell metastasis via inhibition of the ERK-p38-JNK and AP-1 signaling pathways in U-2 OS human osteosarcoma cells, Oncol. Rep. 30, 925-932.

Choi, J.S., Nurul Islam, M., Yousof Ali, M., Kim, E.J., Kim, Y.M., Jung, H.A., 2014. Effects of C-glycosylation on anti-diabetic, anti-Alzheimer's disease and anti-inflammatory potential of apigenin, Food and Chemical Toxicology 64, 27-33.

Clifford, M.N., Knight, S., Kuhnert, N., 2005. Discriminating between the six isomers of dicaffeoylquinic acid by LC-MSn, J. Agric. Food Chem. 53, 3821-3832.

Clifford, M.N., Johnston, K.L., Knight, S., Kuhnert, N. 2003. Hierarchical scheme for LC-MS^n identification of chlorogenic acids. J. Agric. Food Chem 51, 2900−2911.

Coppin, J.P., Xu, Y., Chen, H., Pan, M.H., Ho, C.T., Juliani, R., Simon, J.E., Wu, Q., 2013. Determination of flavonoids by LC/MS and anti-inflammatory activity in *Moringa oleifera*, Journal of Functional Foods 5, 1892-1899.

Dent, M., Dragovic-Uzelac, V., Penic, M., Brñic, M., Bosiljkov, T., Levaj, B., 2013. The effect of extraction solvents, temperature and time on the composition and mass fraction of polyphenols in dalmatian wild sage (*Salvia officinalis* L.) extracts, Food Technology and Biotechnology 51, 84-91.

Fahey, J.W., Zalcmann, A.T., Talalay, P., 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants, Phytochemistry 56, 5-51.

Galuppo, M., De Nicola, G.R., Iori, R., Dell'Utri, P., Bramanti, P., Mazzon, E., 2013. Antibacterial activity of glucomoringin bioactivated with myrosinase against two important pathogens affecting the health of long-term patients in hospitals, Molecules 18, 14340-14348.

García-Salas, P., Morales-Soto, A., Segura-Carretero, A., Fernández-Gutiérrez, A., 2010. Phenolic-compound-extraction systems for fruit and vegetable samples, Molecules 15, 8813-8826.

Guy, P.A., Renouf, M., Barron, D., Cavin, C., Dionisi, F., Kochhar, S., Rezzi, S., Williamson, G., Steiling, H., 2009. Quantitative analysis of plasma caffeic and ferulic acid equivalents by liquid chromatography tandem mass spectrometry, Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences 877, 3965-3974.

Herrero, M., Temirzoda, T.N., Segura-Carretero, A., Quirantes, R., Plaza, M., Ibáñez, E., 2011. New possibilities for the valorization of olive oil by-products, Journal of Chromatography A 1218, 7511-7520.

Jaiswal, R., Karaköse, H., Rühmann, S., Goldner, K., Neumüller, M., Treutter, D., Kuhnert, N., 2013. Identification of phenolic compounds in plum fruits (*Prunus salicina* L. and *Prunus domestica* L.) by high-performance liquid chromatography/tandem mass spectrometry and characterization of varieties by quantitative phenolic fingerprints, J. Agric. Food Chem. 61, 12020-12031.

Jaiswal, R., Sovdat, T., Vivan, F., Kuhnert, N. 2010. Profiling and characterization by LC-MS^n of the chlorogenic acids and hydroxycinnamoylshikimate esters in Maté (Ilex paraguariensis). J. Agric. Food Chem. 58, 5471-5484.

Jung, H.W., Tschaplinski, T.J., Wang, L., Glazebrook, J., Greenberg, J.T., 2009. Priming in systemic plant immunity, Science 324, 89-91.

Karthivashan, G., Tangestani Fard, M., Arulselvan, P., Abas, F., Fakurazi, S., 2013. Identification of bioactive candidate compounds responsible for oxidative challenge from hydro-ethanolic extract of *Moringa oleifera* leaves, J. Food Sci. 78, C1368-C1375.

Kashiwada, Y., Ahmed, F.A., Kurimoto, S., Kim, S.Y., Shibata, H., Fujioka, T., Takaishi, Y., 2012. New a-glucosides of caffeoyl quinic acid from the leaves of *Moringa oleifera* Lam, Journal of Natural Medicines 66, 217-221.

Kasolo, J.N., Bimenya, G.S., Ojok, L., Ochieng, J., Ogwal-Okeng, J.W., 2010. Phytochemicals and uses of *Moringa oleifera* leaves in Ugandan rural communities, Journal of Medicinal Plants Research 4, 753-757.

Mbikay, M., 2012. Therapeutic potential of *Moringa oleifera* leaves in chronic hyperglycemia and dyslipidemia: A review, Frontiers in Pharmacology 3 MAR.

Mingrone, G., Castagneto-Gissey, L., Macé, K., 2013. Use of dicarboxylic acids in type 2 diabetes, Br. J. Clin. Pharmacol. 75, 671-676.

Moyo, B., Masika, P.J., Hugo, A., Muchenje, V., 2011. Nutritional characterization of Moringa (*Moringa oleifera* Lam.) leaves, African Journal of Biotechnology 10, 12925-12933.

Mustafa, A., Turner, C., 2011. Pressurized liquid extraction as a green approach in food and herbal plants extraction: A review, Anal. Chim. Acta 703, 8-18.

Proestos, C., Komaitis, M., 2006. Ultrasonically assisted extraction of phenolic compounds from aromatic plants: Comparison with conventional extraction technics, J. Food Qual. 29, 567-582.

Ramachandran, S., Fontanille, P., Pandey, A., Larroche, C., 2006. Gluconic acid: Properties, applications and microbial production, Food Technology and Biotechnology 44, 185-195.

Rodríguez-Pérez, C., Quirantes-Piné, R., Amessis-Ouchemoukh, N., Khodir, M., Segura-Carretero, A., Fernández-Gutierrez, A., 2013a. A metabolite-profiling approach allows the identification of new compounds from *Pistacia lentiscus* leaves, J. Pharm. Biomed. Anal. 77, 167-174.

Rodríguez-Pérez, C., Quirantes-Piné, R., Fernández-Gutiérrez, A., Segura-Carretero, A., 2013b. Comparative characterization of phenolic and other polar compounds in Spanish melon cultivars by using high-performance liquid chromatography coupled to electrospray ionization quadrupole-time of flight mass spectrometry, Food Res. Int. 54, 1519-1527.

Russo, M., Spagnuolo, C., Tedesco, I., Bilotto, S., Russo, G.L., 2012. The flavonoid quercetin in disease prevention and therapy: Facts and fancies, Biochem. Pharmacol. 83, 6-15.

Sharma, V., Paliwal, R., 2012. Chemo protective role of *Moringa oleifera* and its isolated saponin against DMBA induced tissue damage in male mice: A histopathological analysis, International Journal of Drug Development and Research 4, 215-228.

Sriseadka, T., Wongpornchai, S., Rayanakorn, M. 2012. Quantification of flavonoids in black rice by liquid chromatography-negative electrospray ionization tandem mass spectrometry. J. Agric. Food Chem. 60(47), 11723-11732.

Vinatoru, M., 2001. An overview of the ultrasonically assisted extraction of bioactive principles from herbs, Ultrason. Sonochem. 8, 303-313.

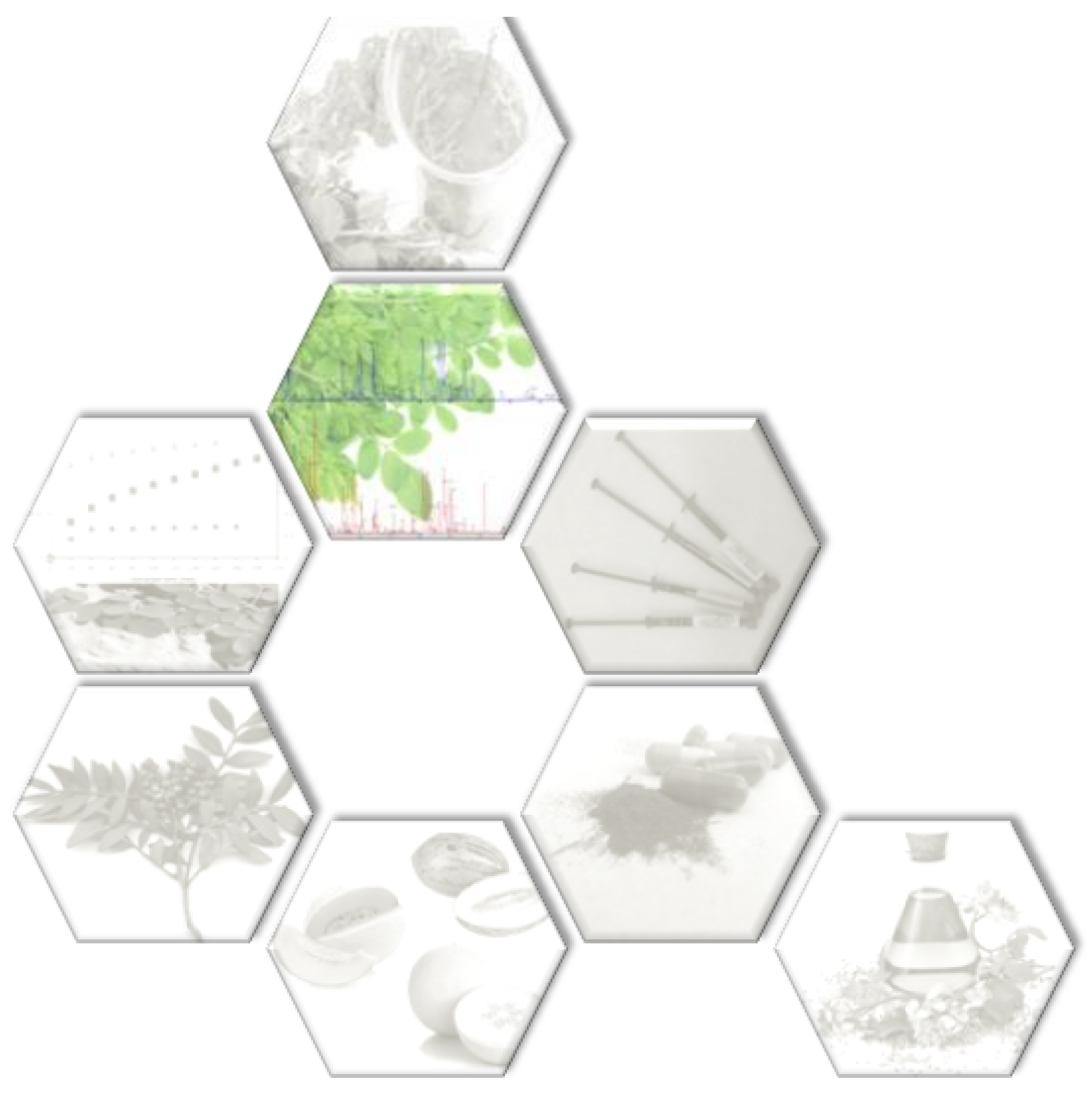
Vongsak, B., Sithisarn, P., Mangmool, S., Thongpraditchote, S., Wongkrajang, Y., Gritsanapan, W., 2013. Maximizing total phenolics, total flavonoids contents and antioxidant activity of *Moringa oleifera* leaf extract by the appropriate extraction method, Ind. Crops Prod. 44, 566-571. doi: 10.1016/j.indcrop.2012.09.021.

Wang, H., Provan, G.J., Helliwell, K., 2004. Determination of rosmarinic acid and caffeic acid in aromatic herbs by HPLC, Food Chem. 87, 307-311.

Chapter 2

Optimization of microwave-assisted extraction and pressurized liquid extraction of phenolic compounds from *Moringa oleifera* leaves by multi-response surface methodology

Electrophoresis, 2016 (Accepted)



ELECTROPHORESIS

Optimization of microwave-assisted extraction and pressurized liquid extraction of phenolic compounds from *Moringa oleifera* leaves by multi-response surface methodology

Rodríguez-Pérez, C.[a,b]; Gilbert-López, B.[c], Mendiola, J.A.[c]; Quirantes-Piné, R.[b]; Segura-Carretero, A.[a,b]; Ibáñez, E.[c]

[a] *Department of Analytical Chemistry, Faculty of Sciences, University of Granada, Avenida Fuentenueva s/n, 18071 Granada, Spain*

[b] *Research and Development Functional Food Centre (CIDAF), Health Science Technological Park, Avenida del Conocimiento s/n, Edificio BioRegión, 18016 Granada, Spain*

[c] *Foodomics Laboratory, Bioactivity and Food Analysis Department, Institute of Food Science Research (CIAL-CSIC), Campus de Cantoblanco, Calle Nicolás Cabrera 9, 28049 Madrid, Spain*

Abstract

This work aims at studying the optimization of microwave-assisted extraction (MAE) and pressurized liquid extraction (PLE) by multi-response surface methodology (RSM) to test their efficiency towards the extraction of phenolic compounds from *M. oleifera* leaves. The extraction yield, total phenolic content (TPC), total flavonoid content (TF), DPPH scavenging method and trolox equivalent antioxidant capacity (TEAC) assay were considered as response variables while effects of extraction time, percentage of ethanol, and temperature were studied. Extraction time of 20 min, 42% ethanol and 158°C were the MAE optimum conditions for achieving extraction yield of 26 ± 2%, EC_{50} 15 ± 2 µg/mL, 16 ± 1 Eq Trolox/100 g dry leaf, 5.2 ± 0.5 mg Eq quercetin/g dry leaf, and 86 ± 4 mg GAE/g dry leaf. Regarding PLE, the optimum conditions that allowed extraction yield of 56 ± 2%, EC_{50} 21 ± 3 µg/mL, 12 ± 2 mmol Eq Trolox/100 g dry leaf, 6.5 ± 0.2 mg Eq quercetin/g dry leaf, and 59 ± 6 mg GAE/g dry leaf were 128°C, 35% of ethanol, and 20 min. PLE enabled the extraction of phenolic compounds with a higher number of hydroxyl-type substituents such as kaempferol diglycoside and its acetyl derivatives and those that are sensitive to high

temperatures (glucosinolates or amino acids) while MAE allowed better recoveries of kaempferol, quercetin, and their glucosides derivatives.

Keywords: HPLC-ESI-QTOF-MS, Microwave-assisted extraction, *Moringa oleifera*, Pressurized liquid extraction, Response-surface methodology.

❖ Introduction

During the last few years researchers have shown growing interest in *Moringa oleifera* Lam. (*M. oleifera*) leaves due to its potential for preventing or treating diabetes, cardiovascular diseases [1], and cancer [2], among other pathologies. Such beneficial properties have been related mainly to leaf composition in phenolic compounds such as flavonols. Furthermore, these compounds have major industrial applications in many fields such as cosmetics, pharmaceutics or agriculture. Therefore, there is a trend based on the environmentally friendly extraction methods for phenolic compounds by using technologies which are safe and effective while providing improved yields and minimizing the impact of the quality of the end-product.

Different extraction techniques such as ultrasound-assisted extraction [3], solid-liquid extraction [3,4], pressurized hot-water extraction (PHWE) [5], soxhlet extraction [4] or supercritical fluid extraction (SFE) [6] have been used to extract bioactive compounds from *M. oleifera* leaves. However, pressurized liquid extraction (PLE) and microwave-assisted extraction (MAE) have not previously been employed. The recovery of bioactive compounds by these extraction techniques is a promising trend in the field of nutraceutical and functional-food development. Both extractions techniques provide shorter extraction times due to the increase of the analyte solubility in the extraction media when surface tension and solvent viscosity decreases, improving the extraction efficiency [7]. Nevertheless, differences in the action mechanism should be pointed out. On one hand, PLE, which is also referred to in the literature as pressurized fluid extraction (PFE) or accelerated solvent extraction (ASE), is a technique that involves the use of pressurized solvents at high temperatures under conditions that allow solvents to be maintained in the liquid state during the extraction process [8]. Pressure is applied inside the extraction cells to achieve the high temperatures. For instance, compounds such as flavonoids and other phenolic compounds [9, 10] or fatty acids [11] have been extracted from different plant leaves by PLE. On the other hand, MAE has been also used for the same extraction purpose [9,12] with the difference that MAE is based on the use of electromagnetic radiation at

frequencies from 0.3 to 300 GHz. This allows rapid heating of solvent and suspension due to the transformation of rapid energy delivery [13]. The non-ionizing electromagnetic energy is applied directly to the raw material. This heating acts on the molecules by ionic conduction and dipole rotation, inducing a migration of dissolved ions. This increases solvent penetration into the matrix, thereby facilitating the recovery of compounds of interest [14].

Due to the complexity and the chemical characteristic of plant extracts, the optimization of the extraction procedure is a critical step to produce high-added-value extracts. However, most previous studies have used the one-factor-at-a-time approach, in which only one factor is variable at a time while all others are kept constant. This approach is time-consuming, expensive, and does not allow the possible effects between variables to be evaluated [5]. These disadvantages can be overcome by using response-surface methodology (RSM), which is a compilation of mathematical and statistical techniques based on the fit of a polynomial equation to the experimental data, in order to describe the behavior of a data set with the aim of making statistical predictions [15].

Despite that the guaranteed availability of bioactive compounds in *M. oleifera* leaves, the extraction process requires working on the most cost-effective feasible process that allows the highest extraction efficiency. Therefore, the goal of this study was to optimize the effect of solvent composition, extraction time, and temperature on the extraction yield, total phenolic content (TPC), total flavonoids (TF), and antioxidant activity from *Moringa oleifera* leaves by using technologies such as MAE and PLE combined with green solvents. In addition, the effect of those parameters on the metabolite profiling was evaluated by high-performance liquid chromatography coupled to electrospray ionization quadrupole time-of-flight mass spectrometry (HPLC-ESI-QTOF-MS).

❖ Material and methods

Plant material and reagents

The leaves of *M. oleifera* collected from Madagascar in 2010 were identified by the National Center for environmental research (CNRE), Antanarivo, (Madagascar, Africa) where the voucher specimen was deposited. Branches from the tree were removed by hand and laid on shelves 3 m long, 30 cm wide and 50 cm apart in a well-ventilated dry place in darkness at room temperature. Two weeks later, the leaves were crushed with a stone mortar and the resulting powder was stored in darkness in a dry, cool place until treatment.

Absolute ethanol for extractions was purchased from TechniSolv (VWR International, France). HPLC–MS acetonitrile was purchased from Fisher (Thermo Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leicestershire, UK). Ethanol, gallic acid, apigenin, folin–Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), ABTS [2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonate)], trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), potassium persulfate, quercetin and aluminum chloride were from Sigma–Aldrich (Steinheim, Germany). Sea sand was from Panreac (Barcelona, Spain). Ultrapure water with a resistivity value of 18.2 MΩ was obtained from a Milli-Q system (Millipore, Bedford, MA, USA).

Extraction methods

Microwave-assisted extraction (MAE)

MAE extraction was carried out by a microwave extraction reactor (Anton Paar GmbH, Graz, Austria) composed by a Monowave 300 operating at a maximum power of 850 W with a frequency of 2,455 MHz, and an autosampler MAS 24. The system can be operated at a maximum pressure of 30 bar over the sample vial, which depends on the solvent composition and volume, and on the working temperature. One gram of dried *M. oleifera* leaves was placed into 20-mL extraction cells. It is commonly know that the extraction yield strongly depends on the solvent employed. In this study, two

different green solvents (water and ethanol) and their mixtures were used for the experiment. Ten milliliters of different water, ethanol:water, and ethanol concentrations were used for the extraction. After cooling, samples were centrifuged at 5000 rpm for 15 min and the supernatant was collected. Samples extracted with ethanol 100% were dried under N_2, samples extracted with ethanol and water mixtures were firstly dried under N_2 and then freeze-dried in a freeze-dryer (Lyobeta, Telstar, Terrassa, Spain), while those extracted with water were directly freeze-dried.

Pressurized Liquid Extraction (PLE)

PLE extraction of *M. Oleifera* leaves was performed using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA), equipped with a solvent controller. The equipment has been exhaustively described elsewhere [16]. One gram of dried *M. oleifera* leaves was mixed with 2 g of sea sand and placed into an 11-mL volume extraction cells. Samples were extracted under the following conditions: the time was fixed at 20 min, pressure: 10 MPa (1500 psi), heat-up time: 5 min; static time: 5 min; flush volume: 60%; purge: N2, 60 s; number of cycles: 1. For removing the solvent, the abovementioned processes were used.

Determination of total phenolic content (TPC)

The total phenolic content of the resulting extracts was determined using the Folin–Ciocalteu assay [3], measuring the absorbance at 760 nm. Briefly, 600 µL of water and 10 µL of sample were mixed, to which 50 µL of undiluted Folin–Ciocalteu reagent was subsequently added. After 10 min, 150 µL of 2% (w/v) Na_2CO_3 were added and the volume was made up to 1 mL with water. After 2 h of incubation at room temperature in darkness, 200 µL of the mixture was transferred into a microplate well. The absorbance was measured at 760 nm using a Synergy Mx Monochromator-Based Multi-Mode Microplate reader, by Bio-Tek Instruments Inc. (Winooski, VT, USA) and compared to a gallic acid calibration curve (5 to 150 µg/mL) prepared in the same manner. The total phenolic content was calculated as mean ± SD ($n = 6$) and expressed as mg of Eq gallic acid (GAE) per g of dry leaves. The TPC of *M. oleifera*

leaves' extracts was measured by a Synergy Mx Monochromator-Based Multi-Mode Micro plate reader, by Bio-Tek Instruments Inc. (Winooski, VT, USA) by using 96-well polystyrene microplates.

Determination of total flavonoids (TF) content

The total flavonoid content was measured by the aluminum chloride colorimetric modifying assay of Woisky *et al.* [17]. First, 240 μL of methanolic reference solutions of quercetin (from 4 to 14 μg/mL) and 100 μL of each sample, and then 60 μL of $AlCl_3$ 8 mM, were added to each well. After 30 min of incubation in darkness, the absorbance was measured at 425 nm. The total flavonoid content was calculated as mean ± SD ($n = 6$) and results were expressed as mg of Eq quercetin/g of dry leaves. The TF content was measured with the aforementioned apparatus.

Antioxidant activity assays

The antioxidant activity was measured by DPPH scavenging method following Brand-Williams *et al.* [18]. Briefly, 20 μL of each extract at different concentrations (ethanol 100% extracts: 40-200 ppm; water 100% and ethanol:water mixtures extract: 20-70 ppm) were added into a 980 μL of DPPH diluted solution (50 μmol/L). The mixture was incubated 1 h at room temperature in darkness, and then, 200 μL of the mixture was transferred to a well of the microplate, and the absorbance was measured at 516 nm. A calibration curve was calculated in order to determine the DPPH concentration remaining in each well, and then the percentage of the remaining DPPH was represented against the extract concentration to determine the amount of sample necessary to decrease the initial DPPH concentration by 50% or EC_{50}. Measurements were made in triplicate.

The trolox equivalent antioxidant capacity (TEAC) assay was conducted using the modified method proposed elsewhere [19]. This antioxidant assay is based on the reduction of the radical cation of 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonate) (ABTS) by antioxidants. In brief, the ABTS radical cation ($ABTS^{+\bullet}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration)

and keeping the mixture in darkness at room temperature for 16 h before use. For the assay, the $ABTS^{+\bullet}$ solution was diluted with water until reaching an absorbance value of 0.70 (± 0.02) at 734 nm. For the spectrophotometric assay, 300 µL of the $ABTS^{+}$ solution and 30 µL of the extract were mixed and measured immediately after 5 min. The readings were performed at 734 nm and 25ºC. The result of each sample was then compared with a standard curve made from the corresponding readings of trolox (0.5–30 µM in the microplate wells). Measurements were made in triplicate.

The antioxidant activity was measured with the aforementioned apparatus and calculated as mean ± SD ($n = 3$) and data were expressed as EC_{50} (µg/mL) and mmol Eq Trolox/100 g of dry leaves, respectively.

RSM experimental designs

Extraction conditions were optimized using response surface methodology (RSM) in order to determine the relationship between the responses selected (extract yield, total flavonoids, total phenolic content, and antioxidant activity) and the independent variables such as percentage of solvent (0-100% ethanol), extraction time (3-20 min) and temperature (50-180°C). In the case of MAE, a box-behnken design with 3 central points was used. For PLE extraction, the effect of solvent concentration and temperature were studied using a full factorial experimental design at three levels (3^2). The range and central point values of four independent variables used in these studies are summarized in **Table 1** (PLE) and **Table 2** (MAE).

Optimal PLE and MAE extraction conditions were estimated by multiple linear regressions using Statgraphics Centurion XVI software (Statpoint Technologies, Warrenton, Virginia, USA).

Table 1. PLE 3-level factorial design (3^2) of the two-level and five-variable and observed responses under different experimental conditions. Predicted and observed values of each individual response

Run	Independent variables		Investigated responses				
	Solvent (%)	**T (°C)**	**Yield (%)**	**DPPH (EC_{50} µg/mL)**	**TEAC (mmol Eq Trolox/100 g dry leaf)**	**TF (mg Eq quercetin/g dry leaf)**	**TPC (mg Eq GAE/g dry leaf)**
1	50	115	44.51	27.81	12.85	8.08	72.63
2	0	50	38.15	31.43	13.22	3.59	60.67
3	0	115	43.77	26.29	12.60	4.50	62.04
4	0	180	63.82	29.05	14.37	4.28	69.38
5	50	50	40.39	20.93	13.15	7.24	69.37
6	50	115	42.14	25.51	12.63	7.33	69.93
7	50	180	33.96	36.85	10.41	7.50	70.55
8	100	50	9.76	91.04	2.31	8.08	29.07
9	100	115	47.76	68.33	4.23	8.71	51.79
10	100	180	21.19	85.88	2.76	7.48	45.79
11	50	115	46.65	35.98	11.60	7.86	75.63

Optimized desirability 0.8567. Extraction conditions: 128°C; 35% ethanol, 20 min

Response	Predicted	Observed
Yield (%)	49.3966	56 ± 2
DPPH (EC_{50} µg/mL)	22.0509	21 ± 3
TEAC (mmol Eq Trolox/100 g dry leaf)	13.4024	12.3 ± 1.6
TF (mg Eq quercetin/g dry leaf)	7.1695	6.5 ± 0.2
TPC (mg Eq GAE/g dry leaf)	75.629	59 ± 6

Table 2. MAE Box-behnken design of the three-level and three-variable and observed responses under different experimental conditions. Predicted and observed values of each individual response

	Independent variables			Investigated responses				
Run	**Solvent (%)**	**T (°C)**	**t (min)**	**Yield (%)**	**DPPH (EC_{50} µg/mL)**	**TEAC (mmol Eq Trolox/100 g dry leaf)**	**TF (mg Eq quercetin/g dry leaf)**	**TPC (mg Eq GAE/g dry leaf)**
1	50	115	11.5	19.44	27.67	14.12	6.28	67.02
2	0	50	11.5	17.02	48.88	11.65	2.13	47.21
3	100	50	11.5	5.72	55.95	3.45	10.40	41.16
4	0	180	11.5	21.26	45.81	12.84	1.61	59.76
5	100	180	11.5	14.24	140.99	1.34	4.12	23.69
6	0	115	3	17.03	37.46	12.76	2.38	53.98
7	100	115	3	9.68	44.13	7.01	13.86	63.87
8	50	115	11.5	19.00	30.59	14.66	6.01	68.08
9	0	115	20	15.25	41.68	14.48	2.35	51.78
10	100	115	20	11.18	43.04	2.52	9.93	59.06
11	50	50	3	21.68	22.55	13.33	6.27	67.01
12	50	180	3	17.71	22.65	12.46	5.19	80.69
13	50	50	20	22.31	23.25	13.12	6.21	63.93
14	50	180	20	24.34	19.72	13.01	7.52	105.70
15	50	115	11.5	16.58	23.13	13.44	6.43	72.27

Optimized desirability 0.78. Extraction conditions: 158°C; 42% ethanol, 20 min

Response	**Predicted**	**Observed**
Yield (%)	23.2463	25.75 ± 2.26
DPPH (EC_{50} µg/mL)	17.2265	15 ± 2
TEAC (mmol Eq Trolox/100 g dry leaf)	14.4373	16.4 ± 1.1
TF (mg Eq quercetin/g dry leaf)	6.3292	5.2 ± 0.5
TPC (mg Eq GAE/g dry leaf)	91.992	86 ± 4

Verification of the predicted models

To verify the suitability of the quadratic equations for predicting the optimal response values, the verification experiments for each type of extraction were carried out under optimal conditions. The extractions with the optimal values were made in triplicate.

Statistical analysis

The differences were evaluated at a 95% confidence level ($p \leq 0.05$) between optimal values obtained by PLE and MAE, using a one-way analysis of variance (ANOVA) followed by Tukey's test using Origin (version Origin Pro 8 SR0, Northampton, MA, USA).

Characterization of PLE and MAE *M. oleifera* leaves extracts by HPLC-ESI-QTOF-MS

Analyses were made using an Agilent 1200 series HPLC (Santa Clara, CA, USA). Compounds were separated at room temperature using a Zorbax Eclipse Plus C_{18} rapid resolution column (1.8 µm, 150 × 4.6 mm) (Agilent Technologies, Palo Alto, CA, USA) according to the method used previously [3]. The system was coupled to a 6540 Agilent Ultra-High-Definition (UHD) Accurate-Mass QTOF LC/MS (Palo Alto, CA, USA) equipped with an ESI interface operating in negative ionization mode. To maintain mass accuracy during the run time, continuous infusion of a reference mass solution containing ions m/z 112.985587 (trifluroacetate anion) and 1033.988109 (trifluroacetic adduct of hexakis (1H, 1H, 3H-tetrafluoropropoxy)phosphazine or HP-921) was used. The data were processed using MassHunter Workstation Software (Version B.06.00 Qualitative Analysis, Agilent Technologies, Santa Clara, CA, USA). Compound characterization was performed by generation of the candidate formula with a mass accuracy limit of 10 ppm and also considering their retention time (RT), tandem mass spectrometry (MS^2) data and literature.

The optimal MAE and PLE extracts were injected at the same concentration (5 mg/mL). For the comparison of the compounds presented in both extracts, the relative area was calculated by using apigenin (0.05 mg/mL) as internal standard in order to compensate for the potential variations in the instrumental analysis and between different extracts obtained under optimal MAE and PLE conditions.

❖ Results and discussion

Analysis of multi-response surfaces

RSM was employed to maximize extract yield, TPC, TF, and antioxidant activity through the optimization of three independent variables, namely the extraction temperature, extraction time, and water:ethanol ratio in the solvent mixture at three levels. The results are presented in **Table 1** and **2** corresponding to PLE and MAE, respectively.

The type of solvent used for the extractions was one of the most thoroughly investigated factors. Thus, ethanol and water are the most appropriate solvents used in the food and nutraceutical industry, because they are easily removed from the final product and they increase the efficiency of the extraction of phenolic acids and flavonoids [20]. Ethanol effectively extracts flavonoids and their glycosides, catechols, and tannins from raw plant materials but the solubility of these compounds can be enhanced by using a mixed solvent over a limited compositional range [21]. An ANOVA for each response was performed in order to fit and optimize the statistical models corresponding to the Derringer's desirability function, which allows the simultaneous optimization of several responses. **Figure 1** shows the 3D plots of the response surface for the effects of the solvent percentage and temperature on the overall desirability in MAE (**Figure 1a**) and PLE (**Figure 1b**) extractions. The numerical ranges specified were set to minimize DPPH. The program was designed to maximize extraction yield, TPC, TF, and TEAC.

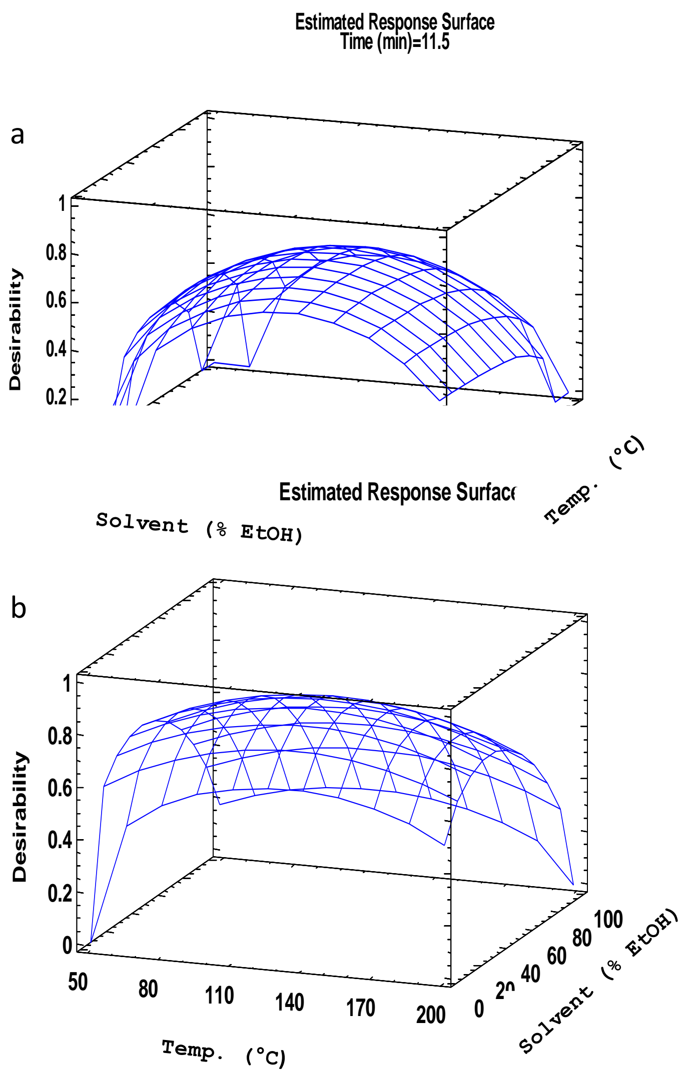


Figure 1. 3D plots of response surface for the effects of solvent (% ethanol in the mixture) and temperature on the overall desirability in MAE (a) and PLE (b) extractions.

The optimum MAE conditions given by the model were as follows: 158ºC, 42% ethanol, and 20 min extraction time. The optimal conditions and predicted values were determined on the basis of the desirability function, which was 0.78 (**Figure 1a**). **Tables 1** and **2** present the optimum extraction responses, which will be discussed throughout this section. In the case of PLE, the optimum values provided by the function were temperature of 128ºC and 35% ethanol. For this experiment, the extraction time was fixed at 20 min, taking into account the optimal extraction time in MAE extraction and based on the effective results from previous research in

recovering phenolic compounds from leaf matrices [22, 23]. In this case, the optimization desirability for PLE was 0.86 (**Figure 1b**), the optimum extraction responses being displayed in **Table 1**.

Recently, research on the extraction of bioactive compounds from *M. oleifera* leaves by ultrasound-assisted extraction (UAE) has shown that the most efficient solvent for extracting phenolic compounds was close to 50% ethanol [3]. In comparison to our previous experiments, the TPC of *M. oleifera* leaves found under optimum PLE conditions was significantly higher ($p < 0.05$) than using UAE. In addition, the extraction of moringa leaves at optimum conditions of MAE (**Table 2**), gave 2-fold higher values of TPC than using UAE [3]. Other studies based on traditional extraction (maceration) concluded that the use 70% ethanol gave the best results [4]. However, no studies are available regarding the optimum values for recovering phenolic compounds from *M. oleifera* leaves via MAE and PLE extractions.

Experiments performed under the optimum conditions provided results closer to the predicted ones (**Tables 1** and **2**). This means that the experimental validation was consistent with the mathematical description of the model, indicating the suitability of RSM in optimizing the extraction of phenolic compounds from *M. oleifera* leaves. Tukey's *post hoc* test showed that PLE provided a significantly higher extraction yield than did MAE. Recently, Taamalli *et al.* concluded, after optimizing extraction conditions, that extraction yields from olive leaves were higher when using PLE compared to MAE [7]. Despite that high temperatures are reported to improve extraction efficiency because of enhanced diffusion rates and solubility of analytes in solvents [24], the extraction yield was higher using a temperature of 128°C (PLE) than when using 158°C (MAE). The flavonoid content was also significantly higher in PLE extracts (6.5 ± 0.2 *vs.* 5.2 ± 0.5). It is important to bear in mind that the effect of temperature should not be generalized since it strongly depends on the typology of the compounds. In this sense, flavonoids are reportedly temperature sensitive and MAE extraction was carried out using higher temperatures than for PLE, which could have

been unfavorable to prevent degradation and oxidation of flavonoid derivatives. Tukey's *post hoc* test also confirmed that the TPC and antioxidant activity were higher in MAE extracts. The increment of TPC, previously observed in other plant matrices, could be attributed to the high pressure achieved in the plant cell that could lead to the breaking down of cell walls and the release of phenolic compounds [25]. Following the same trend as TPC, antioxidant activity measured by TEAC was higher in *M. oleifera* leaves extracted under MAE than under PLE conditions. Antioxidant activity measured by DPPH was also apparently better under MAE conditions. However, despite TEAC and DPPH are both based on electron transfer and involve the reduction of a colored oxidant, there were no significant differences between the EC_{50} values found by both extraction methods. Some authors have highlighted that TEAC allows the measurement of both hydrophilic and lipophilic compounds while DPPH has a limitation for interpreting the role of hydrophilic antioxidants in plant samples [26]. In this regard, high temperature decreases the viscosity of the solvent, thereby enhancing its penetration into the matrix and resulting in an improved extraction process of both, polar and less polar compounds [14]. This could explain the higher TEAC values obtained under MAE conditions. In agreement with the aforementioned results, some authors have affirmed that the increased extraction temperature resulted in higher TEAC values [27,28].

Chemical characterization of the extract yielded by MAE and PLE

As mentioned, extraction is a crucial step for recovering bioactive compounds from plant material. MAE and PLE, apart from being considered green technologies, present some advantages such as reduction in extraction time, energy savings, and increased extraction yield compared with traditional extraction [7].

It is commonly known that extracts yielded by different extraction techniques can be completely different in terms of chemical composition and therefore, a complete characterization is necessary to evaluate the complete potential of the different extraction techniques [7]. **Figure 2** shows the base-peak chromatogram

extracted by MAE (A) and PLE (B) resulting from the HPLC–ESI–QTOF–MS method described above. Each peak was numbered according to its elution order. A total of 62 compounds were tentatively characterized in both extracts. In addition, **Table 3** summarizes the MS data of the compounds tentatively identified considering their MS and MS/MS spectra determined by QTOF–MS and data from the literature. For the acquisition of chemical-structure information and data from the literature, the following databases were employed: SciFinder Scholar (http://scifinder.cas.org), MassBank (http://massbank.jp), and METLIN Metabolite Database (http://metlin.scripps.edu).

Figure 2. Base-Peak Chromatogram (BPC) of *M. oleifera* leaves extracted under optimal MAE conditions (a) and extracted under optimal PLE conditions (b) analyzed by HPLC-ESI-QTOF-MS in negative ion mode.

Table 3. HPLC-ESI-QTOF-MS data of the compounds identified in *Moringa oleifera* leaf extracted by MAE and PLE at optimum conditions

Peak[a]	RT	m/z experimental	Molecular formula	Error (ppm)	Fragments[b]	Compound	Relative Area[c]	
							MAE	PLE
Aminoacids								
5	4.56	128.0354	$C_5H_7NO_3$	1.3	128.0358 (100); 82.0299 (39.29); 52.0188 (16.42)	Pyroglutamic acid	0.06	0.15
10	10.32	164.071	$C_9H_{11}NO_2$	4.5	103.0554 (100); 72.0093 (80); 147.0455 (16.16)	L-Phenilalanine	0.11	0.31
16	13.04	203.0824	$C_{11}H_{12}N_2O_2$	1	116.0507 (100%); 142.0656 (40.4%)	L-tryptophan isomer	ND	0.02
17	13.33	203.0819	$C_{11}H_{12}N_2O_2$	3.7	116.0588 (100); 74.0251 (37.1); 142.067 (15.12); 117.0536 (10)	L-tryptophan isomer	ND	0.15
Nucleosides								
9	8.6	266.0896	$C_{10}H_{13}N_5O_4$	0.6	134.0475 (100); 59.014 (10.5)	Adenosine	0.07	0.09
Organic acids								
1	3.06	195.0514	$C_6H_{12}O_7$	2	75.0096 (100); 59.0163 (81.2); 71.0137 (11)	Gluconic acid	1.94	1.33
2	3.26	191.0574	$C_7H_{12}O_6$	2	127.0402 (100)	Quinic acid	2.3	1.68
3	3.75	133.0139	$C_4H_6O_5$	2.9	71.0144 (100); 72.9936 (49.68); 115.0045 (17.33); 59.0147 (12.22)	Malic acid	0.03	0.03
4	4.25	191.0193	$C_6H_8O_7$	2	87.0089 (100); 111.0088 (72.69); 85.0297 (48.62); 57.0348 (48.19)	Citric acid isomer	0.18	0.06

Table 3. *(Continued)*

Peak[a]	RT	m/z experimental	Molecular formula	Error (ppm)	Fragments[b]	Compound	Relative Area[c] MAE	PLE
6	5.45	191.0209	$C_6 H_8 O_7$	6.3	87.0088 (100); 111.0089 (68.63); 57.0347 (52.23)	Citric acid isomer	ND	0.24
58	42.86	327.2161	$C_{18} H_{32} O_5$	4.7	211.1351 (100); 171.1027 (92)	Trihydroxyoctadecadienoic acid isomer	0.13	0.09
59	43.32	327.2168	$C_{18} H_{32} O_5$	2.7	211.1350 (100); 229.1452 (54.3); 171.1027 (10)	Trihydroxyoctadecadienoic acid isomer	0.22	0.18
60	43.72	327.2161	$C_{18} H_{32} O_5$	4.8	171.102 (100); 211.1357 (89.63)	Trihydroxyoctadecadienoic acid isomer	0.15	0.11
61	47.1	307.1898	$C_{18} H_{28} O_4$	5.5	139.1129 (100); 171.139 (52)	Methylgingerol isomer	0.1	0.12
62	47.36	307.1897	$C_{18} H_{28} O_4$	5.9	139.113 (100); 171.1387 (45.3)	Methylgingerol isomer	0.07	0.08
					Phenolic acids and derivatives			
12	11.51	315.1059	$C_{14} H_{20} O_8$	8.5	153.056 (100); 123.0453 (74.06)	Vanillin glucoside	0.04	0.26
13	12.12	353.0888	$C_{16} H_{18} O_9$	2.7	191.0601 (100); 135.0481 (72.39); 179.0386 (64.45)	Caffeoylquinic acid isomer	1.65	1.66
14	12.58	353.0883	$C_{16} H_{18} O_9$	1.3	191.059 (100); 135.0471 (72.72); 179.0372 (63.66)	Caffeoylquinic acid isomer	0.3	0.23
15	12.84	353.087	$C_{16} H_{18} O_9$	2.1	191.0568 (100); 135.0452 (65.36); 179.0354 (55.95)	Caffeoylquinic acid isomer	ND	0.02
18	13.83	337.0949	$C_{16} H_{18} O_8$	5.9	163.0429 (100); 119.0526 (95.86); 191.0576 (12.26)	Coumaroylquinic acid isomer	ND	3.13

Table 3. *(Continued)*

Peak[a]	RT	m/z experimental	Molecular formula	Error (ppm)	Fragments[b]	Compound	Relative Area[c]	
							MAE	PLE
19	14.06	337.0936	$C_{16}H_{18}O_8$	2.2	163.0427 (100); 119.0522 (96.34); 191.0572 (12.08)	Coumaroylquinic acid isomer	0.56	ND
20	14.8	367.1041	$C_{17}H_{20}O_9$	1.7	134.0365 (100); 193.0495 (92.73)	Feruloylquinic acid isomer	0.97	0.15
21	15.22	353.0884	$C_{16}H_{18}O_9$	1.7	173.047 (100%); 135.0467 (95.77); 191.0581 (89.43); 179.0361 (82.54)	4-Caffeoylquinic acid	1.65	1.11
24	17.14	337.0905	$C_{16}H_{18}O_8$	7.1	173.0462 (48.89)	4-*p*-Coumaroylquinic acid	0.56	0.91
26	17.98	367.1025	$C_{17}H_{20}O_9$	2.7	173.0462 (100); 134.0375 (34.76); 193.0512 (19.51)	Feruloylquinic acid isomer	0.22	0.1
27	18.71	355.0741	$C_{16}H_{16}O_8$	9.4	--	Caffeoylshikimic acid	0.09	ND
Thioglycosides (Glucosinolates)								
7	6.92	570.0956	$C_{20}H_{29}NO_{14}S_2$	0.2	96.962 (100); 328.0888 (10)	Glucomoringin isomer	ND	16.61
8	7.52	570.0963	$C_{20}H_{29}NO_{14}S_2$	1.2	96.9624 (100); 328.0876 (12)	Glucomoringin isomer	ND	8.04
Lignans								
28	19.23	521.2013	$C_{26}H_{34}O_{11}$	3	359.1514 (100)	Isolariciresinol glycoside/lariciresinol glycoside	0.21	0.1
31	21.76	519.1833	$C_{26}H_{32}O_{11}$	7.5	151.0406 (100); 357.1358 (48.27)	Pinoresinol/epipinoresinol glycoside	0.02	0.04
34	24.13	523.2155	$C_{26}H_{36}O_{11}$	5.6	361.1666 (100)	Secoisolariciresinol glycoside	0.07	0.02

Table 3. *(Continued)*

Peak[a]	RT	m/z experimental	Molecular formula	Error (ppm)	Fragments[b]	Compound	Relative Area[c]	
							MAE	PLE
Flavonoids								
11	10.65	595.1363	$C_{26} H_{28} O_{16}$	9.8	--	Quercetin sambubioside/ Quercetin-3-vicianoside	0.06	ND
22	15.49	625.1385	$C_{27} H_{30} O_{17}$	4	463.0875 (100); 301.0360 (52)	Quercetin diglycoside	0.29	0.25
23	17.11	609.1425	$C_{27} H_{30} O_{16}$	5.9	447.0925 (100); 285.042 (38)	Kaempferol diglycoside	0.29	1.56
25	17.63	593.1498	$C_{27} H_{30} O_{15}$	2.3	593.1519 (100); 473.1118 (20.54); 353.0691 (16.27)	Multiflorin B	0.53	0.74
29	20.34	625.1365	$C_{27} H_{30} O_{17}$	7.2	463.087 (100); 301.0360 (52.1)	Quercetin diglycoside	0.11	0.05
30	20.7	447.0918	$C_{21} H_{20} O_{11}$	3.3	284.0338 (100); 285.040 (20)	Kaempferol 3-*O*-glucoside isomer	0.08	0.02
32	22.14	431.0974	$C_{21} H_{20} O_{10}$	2.3	311.0561 (100); 283.0626 (17.77)	(Vitexin) Apigenin glucoside isomer	0.42	0.38
33	23.68	431.0974	$C_{21} H_{20} O_{10}$	2.2	311.0532 (100); 283.0621 (13.63); 341.068 (13.55)	(Vitexin) Apigenin glucoside isomer	0.49	0.5
35	25.39	463.0896	$C_{21} H_{20} O_{12}$	2.9	300.0336 (100); 301.0399 (57.32); 271.0265 (24.75)	Quercetin 3-*O*-glucoside	5.04	4.27
36	26.14	549.0856	$C_{24} H_{22} O_{15}$	5.4	300.029 (100); 301.0366 (18.09)	Quercetin malonylglucoside	ND	2.86
37	26.77	607.1289	$C_{27} H_{28} O_{16}$	3	300.028 (100); 301.0352 (90.43); 463.0885 (87.76); 505.0984 (83.89)	Quercetin-hydroxy-methylglutaroyl glycoside	0.14	0.21
38	27.36	589.1173	$C_{27} H_{26} O_{15}$	4.4	301.0353 (100); 300.0279 (77.23); 463.0889 (54.32)	Quercetin triacetylgalactoside	0.03	0.02
39	27.63	505.0976	$C_{23} H_{22} O_{13}$	2.3	300.0328 (100); 301.0388 (53.93)	Quercetin-acetyl-glycoside isomer	ND	0.05
40	28.16	505.0984	$C_{23} H_{22} O_{13}$	2.3	300.0305 (100); 301.0343 (69.65)	Quercetin-acetyl-glycoside isomer	3.46	1.00
41	29	447.0935	$C_{21} H_{20} O_{11}$	0.6	284.0374 (100); 285.0429 (49.69); 255.0332 (48.81); 227.0365 (37.98)	Kaempferol 3-*O*-glucoside isomer	3.39	2.84
42	29.15	477.1031	$C_{22} H_{22} O_{12}$	1.6	314.0443 (100); 315.05 (12.39)	Isorhamnetin 3-*O*-Glucoside	0.71	0.53

Table 3. *(Continued)*

Peak[a]	RT	m/z experimental	Molecular formula	Error (ppm)	Fragments[b]	Compound	Relative Area[c]	
							MAE	PLE
43	29.62	489.102	$C_{23}H_{22}O_{12}$	3.8	284.0338 (100); 285.0408 (76.24); 255.0308 (15.22)	Kaempferol-acetyl-glycoside isomer	ND	0.07
44	29.79	489.1037	$C_{23}H_{22}O_{12}$	3.2	284.0366 (100); 285.0436 (86.98); 255.0319 (10)	Kaempferol-acetyl-glycoside isomer	ND	0.07
45	30.02	505.0985	$C_{23}H_{22}O_{13}$	2.2	300.0268 (100); 301.0353 (16.48)	Quercetin-acetyl-glycoside isomer	0.48	ND
46	30.2	489.1054	$C_{23}H_{22}O_{12}$	3.1	284.035(100); 285.0412 (12.05)	Kaempferol-acetyl-glycoside isomer	ND	1.2
47	30.66	591.1327	$C_{27}H_{28}O_{15}$	2.1	489.1029 (100); 285.0399 (75.16); 447.094 (50.2); 284.0325 (20)	Kaempferol -glycoside-hydroxy-methylglutarate	0.04	0.04
48	31.02	519.1142	$C_{24}H_{24}O_{13}$	0.5	314.0458 (100); 315.0509 (26.39); 271.0269 (11.52)	Isorhamnetin-acetyl-glycoside isomer	ND	0.08
49	31.21	489.0991	$C_{23}H_{22}O_{12}$	5.1	284.0333 (100); 285.0392 (38.32); 255.0304 (27.48)	Kaempferol-acetyl-glycoside isomer	0.07	ND
50	31.57	531.1137	$C_{25}H_{24}O_{13}$	1.3	300.0279 (100); 301.0348 (55.67); 463.0870 (15)	Quercetin derivative	0.34	0.06
51	32.39	489.1025	$C_{23}H_{22}O_{12}$	2.8	284.0329 (100); 285.0387 (38.75); 255.0296 (29.14); 277.0347 (16.38)	Kaempferol-acetyl-glycoside isomer	1.84	0.16
52	32.86	519.1126	$C_{24}H_{24}O_{13}$	3.5	314.042 (100); 315.0471 (27.05)	Isorhamnetin-acetyl-glycoside isomer	0.23	ND
53	33.83	301.0352	$C_{15}H_{10}O_{7}$	0.6	151.0039 (100); 178.9985 (25.57); 121.0294 (24.4), 107.0135 (21.68%)	Quercetin	0.68	0.07
54	35.54	515.1182	$C_{25}H_{24}O_{12}$	2.4	284.0321 (100); 285.0387 (54.83); 255.0297 (15.63)	Kaempferol-di-acetyl-rhamnoside	0.03	ND
55	35.69	621.1611	$C_{32}H_{30}O_{13}$	0.4	284.0341 (100); 285.039 (27)	Kaempferol derivative	ND	0.67
56	38.7	285.0396	$C_{15}H_{10}O_{6}$	0.4	285.0425 (100); 229.0515 (4.8); 151.0041 (4.72)	Kaempferol	0.28	0.04
57	42.06	573.158	$C_{28}H_{30}O_{13}$	5.9	300.0301 (100); 301.0372 (50.56); 463.0915 (20.89)	Quercetin derivative	1	0.08
Total area							1.89×10^{8}	1.09×10^{8}

[a] Peak numbers assigned according to the overall elution order; [b] Numbers in brackets show the relative abundance of each MS/MS fragment expressed in percentage; [c] Relative area (calculated using apigenin as internal standard); ND, non-detected.

In a recent study, we characterized in depth, via HPLC-ESI-QTOF-MS, the composition of *M. oleifera* leaves extracted by an optimized ultrasound-assisted extraction [3], which has been used as a basis for identifying the compounds detected in the current study. The characterization allowed the grouping of different compounds such as essential amino acids, one nucleoside, organic acids, phenolic acids and derivatives, glucosinolates, lignans, and flavonoids.

It can be noted on the HPLC profiles that the extraction techniques do not induce large selectivity since no major differences of the extract fingerprints were detected. Extracts obtained under MAE and PLE conditions showed a similar qualitative composition. Optimum PLE conditions allowed 55 compounds to be characterized, while by the MAE extraction system, 49 compounds were tentatively characterized. The most remarkable qualitative differences can be seen in **Table 3**, which displays the retention time (RT), experimental m/z of negative molecular ions, molecular formula, mass error, main MS^2 fragments, and the relative abundance or each compound. In general, the extraction of amino acids was lower in the MAE extract; in fact the essential amino acid L-tryptophan was not detected and the extraction of L-phenylalanine was two-fold higher under PLE conditions. In addition, no thioglycosides isomers (glucomoringin) were detected in MAE extracts. This finding agrees with previous studies that ascribed temperatures above 100ºC as the main factor in glucosinolate degradation [29,30]. The optimal temperature in MAE extraction (158ºC) was much higher than in PLE (128ºC), and this probably caused the thermal degradation of glucomoringin isomers.

In addition, 10 organic acids were characterized in *M. oleifera* leaves. Apparently, both MAE and PLE conditions showed similar efficiency extracting this group of compounds. Nevertheless, MAE demonstrated higher extract recovery; over 15% of organic acids *vs.* PLE extract that extracted over 6% could be related to an increase of their solubility under these extraction conditions.

Phenolic acids are aromatic secondary metabolites that constitute an important group of phytochemicals in *M. oleifera* leaves. Their identification can be difficult due to their structural complexity, polarity, and their capacity of existing not only as free, esterified, glycosylated or polymeric compounds but also complexed with proteins, carbohydrates, lipids or other plant compounds [31]. However, both MAE and PLE extractions enabled the detection of 11 phenolic acids derivatives. PLE showed the best recoveries for vanillin glucoside, coumaroylquinic acid isomer (peak 18), 4-*p*-coumaroylquinic acid, and caffeoylquinic acid isomer (peak 15). Previous research has revealed that when the temperature is increased from 125ºC to 150ºC, nearly 20% of vanillin is lost [32]. By contrast, feruloylquinic acid isomers (peaks 20 and 26) were two-fold higher under MAE conditions, and 4-caffeoylquinic acid was also higher under these conditions. Moreover, an isomer of coumaroylquinic acid (RT 14.06) and caffeoylshikimic acid was detected only under MAE extraction conditions.

Regarding lignans, although these compounds have been previously characterized in *M. oleifera* leaves, their concentration is very low compared to those of other bioactive compounds such as flavonoids. However, it bears mentioning that MAE seemed to extract isolariciresinol glycoside/lariciresinol glycoside and secoisilariciresinol glycoside better than did PLE.

On the other hand, flavonoids have been demonstrated to exert positive effects on human health. These phytochemicals are the major bioactive constituents of *M. oleifera* leaves, led by quercetin and kaempferol derivatives, which are reported to be more powerful antioxidants than traditional vitamins are [33]. In addition, flavonoids from *M. oleifera* leaves also present anti-inflammatory effects [33]. Therefore, the selection of the extraction method is a critical step that should be carefully selected. In this sense, quercetin malonylglucoside (peak 36), quercetin-acetyl-glycoside isomer (peak 40), most of kaempferol acetyl glycoside isomers (peaks 43, 44 and 46), isorhamnetin-acetyl-glycoside isomer (peak 48), and kaempferol derivative (peak 55) were detected only in PLE extract, while quercetin sambubioside (peak 11), an isomer of quercetin-acetyl-glycoside (peak 45), an isomer of kaempferol acetyl glycoside

(peak 49), isorhamnetin-acetyl-glycoside (peak 52), and kaempferol-di-acetyl-rhamnoside (peak 54) were detected in *M. oleifera* MAE extract but not in PLE extract. Consequently, it is necessary to adjust the extraction method depending of the target compounds which want to be analyzed.

Quantitatively, the present results show that quercetin, kaempferol, quercetin-3-*O*-glucoside, and both kaempferol-3-*O*-glucoside isomers and kaempferol acetyl glycoside isomer (RT 32.39) were also better extracted with MAE. As aforementioned, the use of higher temperatures can be beneficial for increasing solubility of compounds due to the increased intermolecular interaction in the solvent [14]. Previous reports have noted that compounds which have a greater number of hydroxyl groups are more easily degraded under high extraction temperatures [32]. This could be the case of kaempferol diglycoside, which was less efficiently extracted under higher temperature extraction conditions (MAE). However, the recovery of quercetin diglycoside, which has one more hydroxyl group in its skeleton, was similar under both extraction conditions. Coppin *et al.* observed that the flavonoid malonyl derivatives are not thermally stable and can easily lose malonyl groups to yield their respective flavonoid glycosides [33]. Our results showed that quercetin malonylglucoside was not detected in MAE extract, whereas quercetin-3-*O*-glucoside (peak 36) recovery was higher under the same conditions, a finding that reinforces the aforementioned observation.

Despite that not all compounds followed the same trend, it is worth mentioning that other glycosylated compounds such as quercetin-hydroxy-methylglutaroyl glycoside (peak 37), quercetin-acetyl-glycoside isomers (peaks 39 and 40), kaempferol acetyl glycoside isomers (peaks 43, 44 and 46), isorhamnetin-acetyl-glycoside isomer (peak 48) or kaempferol derivative (peak 55) were mainly or only detected under PLE conditions (128ºC). As can be seen in **Figure 2** and **Table 3**, flavonoids quercetin and kaempferol were better extracted under MAE conditions than under PLE. Thus, under optimal MAE conditions which implied using 42% of ethanol, the flavonol skeleton was prone to dissolve into ethanol. However, under PLE extraction conditions using

35% of ethanol, attached sugars had relatively high affinity with water due to their polarity and most of them were better extracted.

Conclusions

The current work shows that multi-RSM can be successfully used to optimize the extraction of bioactive compounds from *M. oleifera* leaves by MAE and PLE for maximizing TPC, TF, and antioxidant activity. Extraction yield under optimal PLE conditions was two-fold higher than MAE. Although TF content was also higher in *M. oleifera* PLE extract, the antioxidant activity and TPC were higher in MAE extract. In general, MAE and PLE seem to be good choices to extract bioactive compounds. Nevertheless, the extraction method should be selected depending on the target compounds to be isolated. In this sense, MAE conditions were not as suitable as those of PLE for extracting phenolic compounds having a higher number of hydroxyl-type substituents (kaempferol diglycoside and its acetyl derivatives or malonyl, hydroxyl or acetyl glycosylated of quercetin) and those that are sensitive to high temperatures (glucosinolates or amino acids). On the contrary, MAE allowed better recoveries of kaempferol, quercetin, and their glucosides derivatives. Despite that MAE and PLE have proven to be user friendly and techniques that provide short extraction times; optimal PLE conditions required less organic solvent and lower temperature than MAE for extracting bioactive compounds from *M. oleifera* leaves.

Acknowledgments

This work was supported by projects AGL2011-29857-C03-02, P09-CTS-4564, P10-FQM-6563 and P11-CTS-7625 (Andalusian Regional Government Council of Innovation and Science) and AGL2014-53609-P (MINECO, Spain) for financial support. The authors are grateful to the Spanish Ministry of Economy and Competitiveness (MINECO) for a FPU fellowship AP2010-1551 (Spanish Ministry of Science and Innovation) (C. Rodríguez-Pérez), and a grant “Personal técnico de apoyo" PTA2012-6956-E (R. Quirantes-Piné). We would like also to thank to the non-governmental organization “Agua de coco” for providing us the samples to analyze.

B.G.L. thanks MINECO (Ministerio de Economía y Competitividad) for her Juan de la Cierva postdoctoral research contract. We thank Anton-paar GmbH for access to Monowave EXTRA instrument.

Conflicts of interest

The authors declare no competing financial interest.

References

[1] Mbikay, M. *Front. Pharmacol.* 2012, 3, 1-12.

[2] Sreelatha, S., Jeyachitra, A., Padma, P.R., *Food Chem. Toxicol.* 2011, 49, 1270-1275.

[3] Rodríguez-Pérez, C., Quirantes-Piné, R., Fernández-Gutiérrez, A., Segura-Carretero, A., *Ind. Crops Prod.* 2015, 66, 246-254.

[4] Vongsak, B., Sithisarn, P., Gritsanapan, W., *Altern. Med.* 2013, 44, 566-571.

[5] Matshediso, P.G., Cukrowska, E., Chimuka, L., *Food Chem.* 2015, 172, 423-427.

[6] Zhao, S., Zhang, D., *Sep. Purif. Technol.* 2013, 118, 497-502.

[7] Taamalli, A., Arráez-Román, D., Barrajón-Catalán, E., Ruíz-Torres, V., Pérez-Sánchez, A., Herrero, M., Ibáñez, E., Micol, V., Zarrouk, M., Segura-Carretero, A., Fernández-Gutiérrez, A., *Food Chem. Toxicol.* 2012, 50, 1817-1825.

[8] Herrero, M., Sánchez-Camargo, A.D.P., Cifuentes, A., Ibáñez, E. *TrAC Trends Anal. Chem.* 2015, 71, 26-38.

[9] Borrás-Linares, I., Arráez-Román, D., Herrero, M., Ibáñez, E., Segura-Carretero, A., Fernández-Gutiérrez, A., *Journal of Chromatography A*. 2011, 1218, 7682-7690.

[10] Xynos, N., Papaefstathiou, G., Gikas, E., Argyropoulou, A., Aligiannis, N., Skaltsounis, A., *Sep. Purif. Technol.* 2014, 122, 323-330.

[11] Jacques, R.A., Dariva, C., de Oliveira, J.V., Caramão, E.B., *Anal. Chim. Acta.* 2008, 625, 70-76.

[12] Borrás-Linares, I., Stojanovic, Z., Quirantes-Piné, R., Arráez-Román, D., Švarc-Gajic, J., Fernández-Gutiérrez, A., Segura-Carretero, A., *Int. J. Mol. Sci*. 2014, 15, 20585-20606.

[13] Bouras, M., Chadni, M., Barba, F.J., Grimi, N., Bals, O., Vorobiev, E., *Ind. Crops Prod*. 2015, 77, 590-601.

[14] Routray, W., Orsat, V., *Food. Bioprocess Technol.* 2012, 5, 409-424.

[15] Bezerra, M.A., Santelli, R.E., Oliveira, E.P., Villar, L.S., Escaleira, L.A. 2008, 76, 965-977.

[16] Ibáñez, E., Herrero, M., Martín-Álvarez, P.J., Señoráns, F.J., Reglero, G., Cifuentes, A., *ACS Symp. Ser.* 2006, 926, 65-78.

[17] Woisky, R.G., Salatino, A., *J. Apic. Res.* 1998, 37, 99-105.

[18] Brand-Williams, W., Cuvelier, M.E., Berset, C., *LWT - Food Sci. Technol.* 1995, 28, 25-30.

[19] Morales-Soto, A., García-Salas, P., Rodríguez-Pérez, C., Jiménez-Sánchez, C., Cádiz-Gurrea, M.D.L.L., Segura-Carretero, A., Fernández-Gutiérrez, A., *Food Res. Int.* 2014, 58, 35-46.

[20] Moraes, M.N., Zabot, G.L., Prado, J.M. Meireles, M.A., *Food and Public Health.* 2013, 3, 195-214.

[21] Spigno, G., Tramelli, L., De Faveri, D.M., *J. Food Eng.* 2007, 81, 200-208.

[22] Kraujalis, P., Venskutonis, P.R., Ibáñez, E., Herrero, M., *J. Supercrit. Fluids*. 2015, 104, 234-242.

[23] Herrero, M., Plaza, M., Cifuentes, A., Ibáñez, E., *J. Chromatogra. A.* 2010, 1217, 2512-2520.

[24] Santos, D.T., Veggi, P.C., Meireles, M.A.A., *J. Food Eng.* 2012, 108, 444-452.

[25] Nayak, B., Dahmoune, F., Moussi, K., Remini, H., Dairi, S., Aoun, O., Khodir, M., *Food Chem.* 2015, 187, 507-516.

[26] Wojdylo, A., Oszmianski, J., Czemerys, R., *Food Chem.* 2007, 105, 940-949.

[27] Cha, K.H., Kang, S.W., Kim, C.Y., Um, B.H., Na, Y.R., Pan, C.H., *J. Agric. Food Chem.* 2010, 58, 4756-4761.

[28] Mendes, M., Carvalho, A.P., Magalhães, J.M.C.S., Moreira, M., Guido, L., Gomes, A.M., Delerue-Matos,C. *Innov. Food sci. & Emerg. Technol.* 2016, 33, 319-326.

[29] Oerlemans, K., Barrett, D.M., Suades, C.B., Verkerk, R., Dekker, M., *Food Chem.* 2006, 95, 19-29.

[30] Ares, A.M., Bernal, J., Nozal, M.J., Turner, C., Plaza, M., *Food Res. Int.* 2015, 76, 498-505.

[31] Luthria, D.L., Mukhopadhyay, S., *J. Agric. Food Chem.* 2006, 54, 41-47.

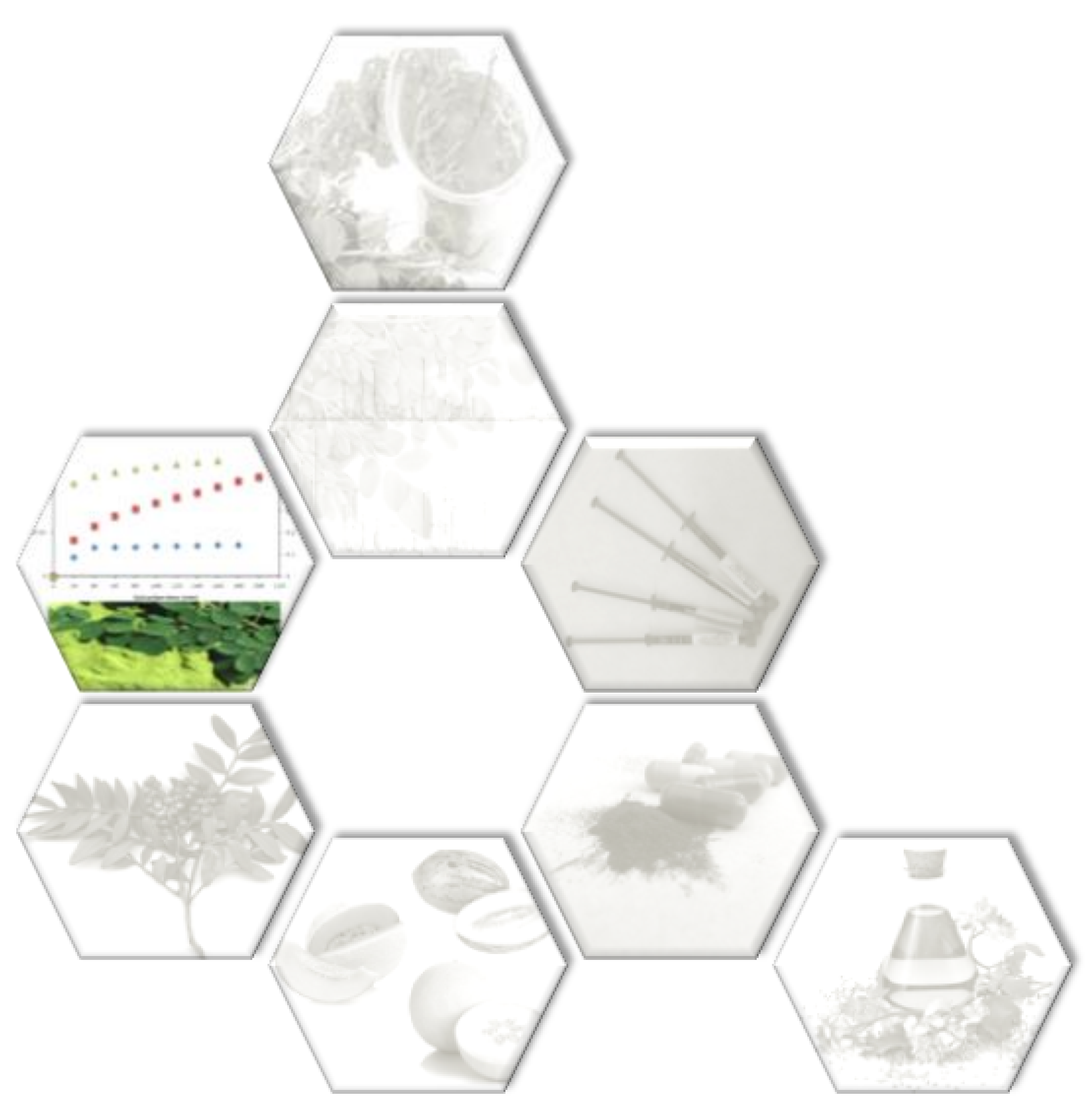
[32] Liazid, A., Palma, M., Brigui, J., Barroso, C.G., *J. Chromatogr. A.* 2007, 1140, 29-34.

[33] Coppin, J.P., Xu, Y., Chen, H., Pan, M., Ho, C., Juliani, R., Simon, J.E., Wu, Q., J. *Funct Foods.* 2013, 5, 1892-1899.

Chapter 3

Green downstream processing using supercritical fluid, CO_2-expanded ethanol and pressurized hot water extractions for recovering bioactive compounds from *Moringa oleifera* leaves

Journal of Supercritical Fluids, 2016 (Accepted)



The Journal of Supercritical Fluids

Available online 17 May 2016

In Press, Accepted Manuscript — Note to users

Green downstream processing using supercritical carbon dioxide, CO_2-expanded ethanol and pressurized hot water extractions for recovering bioactive compounds from *Moringa oleifera* leaves

C. Rodríguez-Pérez[a, b], J.A Mendiola[c], R. Quirantes-Piné[b], E. Ibañez[c], A. Segura-Carretero[a, b]

[a] Department of Analytical Chemistry, Faculty of Sciences, University of Granada, Avenida Fuentenueva s/n, 18071 Granada, Spain

[b] Research and Development Functional Food Centre (CIDAF), Health Science Technological Park, Avenida del Conocimiento s/n, Edificio BioRegión, 18016 Granada, Spain

[c] Foodomics Laboratory, Bioactivity and Food Analysis Department, Institute of Food Science Research (CIAL-CSIC), Campus de Cantoblanco, Calle Nicolás Cabrera 9, 28049 Madrid, Spain

Received 27 February 2016, Revised 14 May 2016, Accepted 16 May 2016, Available online 17 May 2016

Abstract

A green platform based on a three-step downstream processing included the use of supercritical carbon dioxide ($ScCO_2$), carbon dioxide-expanded ethanol (CXE) and pressurized hot water (PHWE) extractions has been developed to obtain different fractions from *Moringa oleifera* leaves. Each process was optimized based on the extraction yield. Optimal extracts were functionally characterized by measuring total phenolic (TPC) and total flavonoid contents (TF). The antioxidant activity by TEAC assay was also tested. Moreover, a chemical characterization of the extracts was performed by GC-MS and HPLC–ESI–QTOF–MS. The extraction yields of the optimized process were 3.1, 29.5 and 30.2% respectively. The extraction of total phenolics was higher in PHWE than in CXE extract (62.4 vs. 20.3 GAE/g leaves) while TF showed the opposite (3.8 *vs.* 9.6 mg quercetin/g leaves respectively). $ScCO_2$ fraction was rich in fatty acids such as α-linoleic acid and alkanes. CXE fraction was richer in glycosylated flavonoids than PHWE while the latest fraction was richer in phenolic acids which could explain the higher antioxidant activity of this fraction (13.4 g dry leaves) compared to CXE (6 mmol Eq trolox/100 g dry leaves).

Keywords: downstream processing, green compressed fluids, carbon dioxide extraction, gas expanded liquids, pressurized hot water extraction, *Moringa oleifera*.

❖ Introduction

Nowadays, the demand of the functional foods, nutraceutical, pharmaceutical and cosmetic industries of new sources of bioactive compounds is increasing together with the public interest in herbal medicines and natural products. To achieve this goal, green technologies are emerging as the best alternative due to its reduction of energy consumption, allowing the use of alternative solvents, and because they ensure safe and high-quality extracts [1,2]. In this regard, extraction with compressed fluids such as supercritical fluid extraction (SFE) or pressurized liquid extractions (PLE) using ethanol, water or CO_2 as green solvents is being successfully used for the extraction of nutraceuticals from plant matrices [3-7]. Supercritical fluids are defined as substances with physicochemical properties intermediate between gases and liquids at temperature and pressure above their critical points. Among available supercritical fluids, CO_2 (critical point: 7.4 MPa and 32ºC) is the most commonly used to extract volatiles and essential oils from plants due to its low polarity, mild critical conditions, non-flammability, availability with high purity at relatively low cost, and easiness to be removed from the extract leaving no residues [8,11,12]. However, the use of co-solvents becomes sometimes necessary to increase the extraction efficiency of SFE when the target compounds present mid-polarity [9,13]. In this regard, ethanol (concentrations from 1 to 10%) is the most employed because it is easily available in high purity, has a low cost, is completely biodegradable and GRAS (Generally Recognized as Safe) for the food industry; moreover, it favors the extraction of polyphenols and flavonols, among other bioactive compounds, from plant matrices [2]. The use of a co-solvent at higher concentration allows working in the region of gas-expanded liquids (GXLs) [14], which are mixed solvents composed of an organic solvent and a compressible gas, usually CO_2. This approach has proven to decrease interfacial tension, to reduce viscosity and to improve diffusivity compared to pure solvent, thus producing an enhancement of mass transfer in a similar way that $scCO_2$ [15]. Despite the great potential that GXLs have demonstrated, only a few studies have

been described in the literature focused on the extraction of valuable compounds (mainly pigments and fatty acids) from algae [8,16,17].

On the other hand, PLE has been extensively employed for the extraction of phenolic compounds, lignans, carotenoids, among other bioactive compounds from plants [4-6,18]. There are many excellent reviews which explain the fundamentals and applications of PLE [9,19,20]. Briefly, PLE uses liquid solvents at elevated temperatures, above their atmospheric boiling point, and controlled pressure enhancing the solubility and mass transfer properties [19]. When the solvent employed is pure water this technique is so-called pressurized hot water extraction (PHWE) or subcritical water extraction (SWE). The main parameter that affects the extraction is the temperature due to the change in the physicochemical properties of water at high temperatures. This change can be positive since can affect the selectivity of the water as a solvent (by change on the dielectric constant of water and therefore on its polarity) [21] or negative since large exposition at high temperatures can cause thermal decomposition of labile compounds [22].

Nevertheless, bioactive compounds have been traditionally extracted depending on the target family group adapting, thus, the most suitable extraction system for each group of compounds and comparing two or several extraction techniques. Recent trends in the development of green processes for extracting bioactive compounds focus on the idea of developing a platform involving multiple sequential processes in order to exhaust the sample. In this way, the downstream processing emerged as a successful way to obtain valuable compounds from different fractions of plant materials using consecutive extraction techniques over the same matrix [16].

Moringa oleifera Lam (*M. oleifera*), commonly known as moringa, has been used in traditional medicine [23,24]. Apart from its high potential as antioxidant, other studies *in vivo* have revealed the remarkable activity of moringa leaves as antidiabetic [25], anticarcinogenic [26,27], hepato-protective [28], hypotensive and cardioprotective, among other activities. In addition, *M. oleifera* leaves have been

orally tested as antifungal agent [29]. Its bioactivity is related to its chemical composition. Particularly, moringa leaves have proved to be a complex matrix that includes essential oils, fatty acids, aminoacids and derivatives, organic acids, phenolic acid and derivatives, thioglycosides, lignans and flavonoids [23,30,31]. Few studies have been conducted on the use of advanced green technologies for the extraction of bioactive compounds from *M. oleifera* leaves [5,30,31]. Besides, no extraction studies for downstream processing of bioactive compounds from moringa or moringa leaves are available. Then, the main objective of the present work was to develop a green platform, based on the use of compressed fluids, to extract the main valuable bioactive compounds from *M. oleifera* leaves in order to achieve different fractions with different composition. Thus, a three-step downstream processing has been studied including the use of supercritical carbon dioxide, CO_2-expanded ethanol and pressurized hot water extraction. An in depth chemical and functional characterization of the different fractions has been carried out in order to better understand the applicability and possibilities of the different extraction processes integrated in the platform.

❖ Materials and methods

Samples and chemicals

The leaves of *M. oleifera* collected from Madagascar in 2010 were identified by the National Center for environmental research (CNRE), Antanarivo, (Madagascar, Africa) where the voucher specimen was deposited. Branches from the tree were plucked by hand and laid on shelves 3 m long, 30 cm wide and 50 cm apart in a dry place with good ventilation and in darkness. Two weeks later, the leaves were crushed with a stone mortar and the dust obtained was stored in darkness in a dry, fresh place until their treatment.

Ethanol absolute was purchased from TechniSolv (VWR International, France), sea sand was from Panreac (Barcelona, Spain), HPLC–MS acetonitrile was purchased from Fisher (Thermo Fisher Scientific UK, Bishop Meadow Road, Loughborough,

Leicestershire, UK), hexane from VWR and acetic acid of analytical grade (purity > 99.5%) was acquired from Fluka (Switzerland). Distilled water with a resistance of 18.2 MΩ was deionized in a Milli-Q system (Bedford, MA, USA).

Downstream processing

The green compressed fluids platform was designed using a home-made supercritical fluid extractor that consists in two pumps for CO_2 and ethanol PU2080 from Jasco (Tokyo, Japan), a home-built oven and a manual back pressure regulator LF-540 from Pressure Tech (Hadfield, United Kingdom).

For the extraction, 1 g of sample was mixed with 1 g of sea sand, and glass wool was packed at both ends of the extractor to stop entrainment of the sample. Three consecutive steps, in increasing order of polarity, were carried out namely supercritical CO_2 ($ScCO_2$), carbon dioxide-expanded ethanol (CXE) and pressurized hot water extraction (PHWE). The extraction vessel were weighed before and after each extraction to determine the extraction yield, expressed as the percentage of the mass extracted to the dry mass of *M. oleifera* leaves loaded into the extraction vessel.

Step 1. The first step consisted on a SFE of the non-polar fraction using $ScCO_2$. A constant flow rate of premier quality CO_2 (Carburos metálicos, Madrid, Spain) was established at 2 mL/min and temperatures from 40 to 60°C and pressure from 10 MPa to 20 MPa were tested, leading to six experimental combinations which were done in duplicate. In order to fix the extraction time, a kinetic study was performed at the midpoint of the experimental conditions (50°C, 15 MPa) for 180 min. During this time, the extraction yield was measured every 20 min and extracts were collected in 15 mL-falcon tubes protected from light that were placed in an ice bath. Finally the extracts were stored under -80°C to prevent their degradation until analysis.

Step 2. In order to increase the polarity of the extract, a carbon dioxide-expanded ethanol (CXE) extraction was carried out. The extraction was performed in the residual biomass from the first step. In this step, a low extraction pressure was

selected (7 MPa), temperature was fixed at 50ºC and the co-solvent pump was operated at 1, 1.2 and 1.4 mL/min to obtain mixtures containing 50, 60 and 70% of ethanol (w/w) respectively. Each extraction was done in duplicate. In this experiment a kinetic study was also carried out at the central point of the experimental conditions, that is, 1.2 mL/min (ethanol 60%), collecting the extract every 20 min for a total extraction time of 200 min and using the biomass remained from the previous step Extracts by CXE were collected in 100 mL-bottles protected from light which were placed in an ice bath during the dynamic extractions. Solvent from all extracts was removed under a stream of N_2. Finally the extracts were stored under -80°C to prevent their degradation until analysis.

Step 3. The last step involved the use of Pressurized Hot Water Extraction (PHWE) to extract the most polar compounds from the residue from the previous two extractions. In this step, pressure was maintained at 7 MPa. After performing a kinetic study at 125°C for 160 min, samples were freeze-dried in a freeze-dryer (Lyobeta, Telstar, Terrassa, Spain) and weighed; the extraction time was selected based on these results. Temperatures from 50 to 200°C were tested. Extracts by PHWE were collected in 100 mL-bottles protected from light which were placed in an ice bath during the dynamic extractions. Once the samples were freeze-dried, the extracts were stored under -80°C to prevent their degradation until analysis.

After optimizing all the extraction methods, the optimal extraction conditions were reproduced in triplicate.

Total phenolic content (TPC) and total flavonoid content (TFC)

The total phenolic content (TPC) of the obtained extracts was determined using the Folin–Ciocalteu assay [31] measuring the absorbance at 760 nm, and was calculated as mean ± SD and expressed as mg of Eq gallic acid per g of dry leaves. The total flavonoid content (TFC) was measured by means of the aluminum chloride colorimetric method proposed by Woisky *et al.* [32] with some modifications. Briefly, 240 µL of methanolic reference solutions of quercetin (from 4 to 14 µg/mL) and 100

µL of each sample, following by 60 µL of $AlCl_3$ 8 mM were added into each well. Then, the absorbance was measured at 425 nm after 30 min of incubation in darkness, The TF content was calculated as mean ± SD and results were expressed as mg of Eq quercetin/g of dry leaves.

All measurements were made in triplicate by a Synergy Mx Monochromator-Based Multi-Mode Micro plate reader, by Bio-Tek Instruments Inc. (Winooski, VT, USA) by using 96-well polystyrene microplates.

Trolox equivalent antioxidant capacity (TEAC) assay

In order to determine the antioxidant activity of the more polar extracts, the trolox equivalent antioxidant capacity assay was carried out according with the modified method proposed by Morales-Soto et al. [33]. This assay is based on the variation in the long wavelength absorption (λ_{max} = 734 nm) scavenging of the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical ($ABTS^{\bullet}$) converting it into a colorless product in the presence of antioxidant compounds such as phenolic compounds. The antioxidant activity was calculated as mean ± SD and data were expressed as mmol Eq Trolox/100 g of dry leaves respectively. Measurements were made in triplicate by the abovementioned Micro plate reader.

Extract composition

Gas chromatography-mass spectrometry analysis of moringa extracts

A GCMS-QP2010 plus system (Shimadzu, Kyoto, Japan) equipped with a DB-5ms column (30 m × 0.25 mm I.D. × 0.25 µm df, Quadrex Corporation, Woodbridge, CT) was used. The separation was performed according with the method proposed by Sánchez-Camargo *et al.* [34]. The data collection and handling were performed using the GCMS solution (ver. 2.50 SU3, Shimadzu) software. The system was equipped with a commercial mass spectral database (Wiley) that was employed to identify the separated compounds as well as using their respective linear retention indices (LRIs).

For determination of the LRIs, a hydrocarbon mixture ranging from C_8 to C_{30} was employed, and it was analyzed under the same experimental conditions as the samples.

HPLC-ESI-QTOF-MS characterization of moringa extracts

Analyses were carried out by an Agilent 1200 series HPLC (Santa Clara, CA, USA) equipped with a binary pump, an autosampler and DAD. Separation was made at room temperature using a Zorbax Eclipse Plus C_{18} rapid resolution column (1.8 µm, 150 × 4.6 mm I.D.) (Agilent Technologies, Palo Alto, CA, USA), according to the method previously described in Rodríguez-Pérez *et al.* [31]. The compounds detection was carried out using a quadrupole time-of-flight (QTOF) mass spectrometer (Agilent 6540) equipped with Jet Stream dual electrospray ionization (ESI) interface operating in negative ionization mode. To maintain mass accuracy during the run time, a reference mass solution containing ions m/z 112.985587 (trifluroacetate anion) and 1033.988109 (trifluroacetic adduct of hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine or HP-921) was continuously infused. Automatic MS/MS experiments were performed adjusting the collision energy values as follows: *m/z* 100, 20 eV; *m/z* 500, 30 eV; *m/z* 1000, 35 eV; and using nitrogen as the collision gas. Data acquisition in profile mode was governed *via* MassHunter Workstation Software (Agilent Technologies). Data analysis was performed on MassHunter Qualitative Analysis Version B.06.00 (Agilent Technologies).

Statistical analysis

A one-way analysis of variance (ANOVA) following by Tukey's test using Origin Pro 8 SR0 (OriginLab Corporation, Northampton, MA, USA) was done in order to evaluate the statistical differences at 95% confidence level ($p \leq 0.05$) between each studied extraction parameter.

❖ Results and discussion

A green processing platform based on the use of compressed fluids ($scCO_2$, ethanol and water) was developed and optimized in our laboratory as a downstream

process to recover the maximum amount and variety of bioactive compounds from *M. oleifera* leaves. To do so, three sequential steps were designed considering an increasing order of polarity of the solvents: SFE with CO_2, GXL with ethanol and PHWE with water.

Extraction kinetics

Figure 1 shows the overall extraction curves obtained for the three kinetic extraction processes in terms of mg extracted versus time. As can be seen, the curves profiles are quite different, not only in terms of amount extracted but also in its shape.

Several authors have previously described adjusted mathematical models for extraction kinetics [35,36]. Extraction curves are characterized by three time periods: a constant extraction rate period (CER), characterized by the extraction of the solute contained in the surface of the particles, that is, easily accessible. The mass transfer in this step is controlled by convection. A falling extraction rate period (FER), in which most of the easily accessible solute has been extracted and mass transfer starts to be controlled by diffusion. And, finally a diffusion controlled rate period (DCR), in which the easily extractable solute has been completely removed and the extraction process is controlled by the diffusion of the solvent inside the particles and the diffusion of solute + solvent to the surface. Those periods have been marked with different dashed lines in **Figure 1** for the three extraction processes considered in the present work.

In the SFE (50°C and 15 MPa) kinetics extraction curve (**Figure 1, top**) more than 60% of the total extractable material was extracted in the first 20 min. The rate of extraction was 1.12 mg/min in the CER period. Meanwhile, in the FER period the rate decreased to 0.55 mg/min and finally in the last period (DCR) the extraction rate decreased to 0.03 mg/min. After 60 min, the extraction recovery was 92.33% (data not shown), thus, 60 minutes were considered enough to extract most of the valuable material and therefore was the time selected for further studies.

A similar extraction curve can be observed for CXE 50ºC, 7 MPa and 60% EtOH (**Figure 1, middle**), but in this case the extraction can be considered more efficient. The first period extracted the 35% of the total extractable material at a rate of 2.07 mg/min in 20 min, then the second period showed a slightly higher extraction rate than SFE, in this particular case, 0.62 mg/min, and, finally, the diffusion period had a faster extraction rate than SFE, 0.27 mg/min. The optimal extraction time, that allowed extracting 87.91% of the total weight (data not shown), was established as 160 min.

In **Figure 1, bottom**, the kinetics for PHWE at 125ºC and 7 MPa is shown. As can be seen, only two periods could be appreciated. This was the faster extraction of the three. In the CER period more than 80% of the total extractable material was obtained at a rate of 14.67 mg/min in 30 min. No FER period was found and, finally, the diffusion controlled rate was 0.44 mg/min. The kinetics study revealed an optimal extraction time of 60 min that allowed close to 90% of the total weight (data not shown).

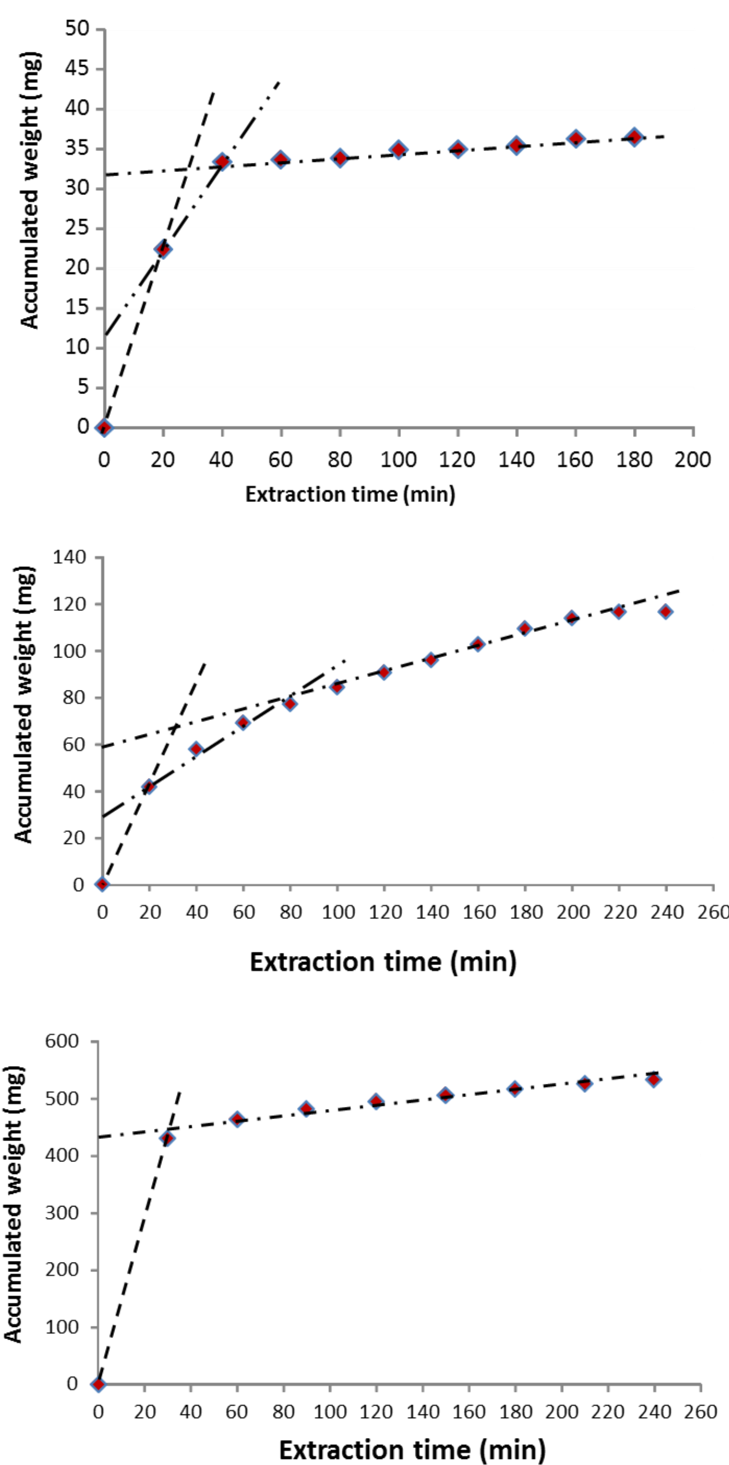


Figure 1. Kinetics of the three different steps evaluated at midpoint of the corresponding experimental conditions: a) SFE 50°C and 15 MPa; b) CXE 50ºC, 7 MPa and 60% EtOH; c) PHWE 125ºC and 7 MPa. Different dashed lines indicate different periods of extraction: – – (CER), – ·· – (FER), – · – (DCR) (see text for explanation).

Optimization of supercritical CO_2 extraction of moringa leaves (Step 1)

Most works devoted to the extraction of bioactive compounds from vegetable matrices by SFE have concentrated in temperatures within 40-60ºC to avoid degradation of thermal labile compounds; therefore, conditions selected to perform the study were: 40 and 60ºC at 10 and 20 MPa and 50ºC at 15 MPa. The different experiments provided extraction yields ranging from 13.7 mg/g to 31 mg/g (corresponding to 1.37%-3.1% w/w), which are displayed in **Figure 2a**. Compared to others research focus on the extraction of essential oils from this matrix by conventional extraction methods such as hydrodistillation [37] scCO2 resulted in higher oil extraction yields.

The effect of temperature and pressure on the extraction yield by $scCO_2$ has been widely studied [7,38]. Theoretically, the extraction yield should be enhanced significantly with increasing pressure at a constant temperature leading to a higher fluid density, thus, increasing the solubility of the oil. This effect can be observed in our experiments (**Figure 2a**) where at constant temperature of, both, 40 and 60ºC, the higher extraction yields were reached at higher pressure. A recent study has shown similar results keeping constant the temperature at 30ºC and testing pressures from 15 to 35 MPa, being the last one that allowed higher extraction yields [39]. In contrast, the effect of temperature is not so straightforward, generally, increasing the temperature at a constant pressure, promotes two opposite effects; on one hand, it reduces the solvent power of CO_2 by a decrease of the density, and, on the other hand, it increases the vapor pressure of solutes which can be more easily transferred to the supercritical phase. The balanced effect on the solubility of the solute in the supercritical solvent depends on the operating pressure. In **Figure 2a**, it can be seen that increasing the temperature from 40 to 60°C at low pressure (10 MPa), there is a decrease in fluid density and, therefore, the extraction yields are lower at 60°C. But, at slightly higher pressure (15 and 20 MPa), the increase in the vapor pressure prevails, thus the solubility increases with the temperature. In our particular conditions, there were no significant differences between 50ºC and 15 MPa and 60ºC and 20 MPa

which provided the higher extraction yields. In order to minimize the operational cost, 50ºC and 15 MPa conditions were selected as the most appropriate for the first step in the downstream processing of moringa leaves. These results are quite in agreement with a previous study that concluded that 60ºC was the optimal temperature to extract oil from *M. oleifera* leaves by SFE [30].

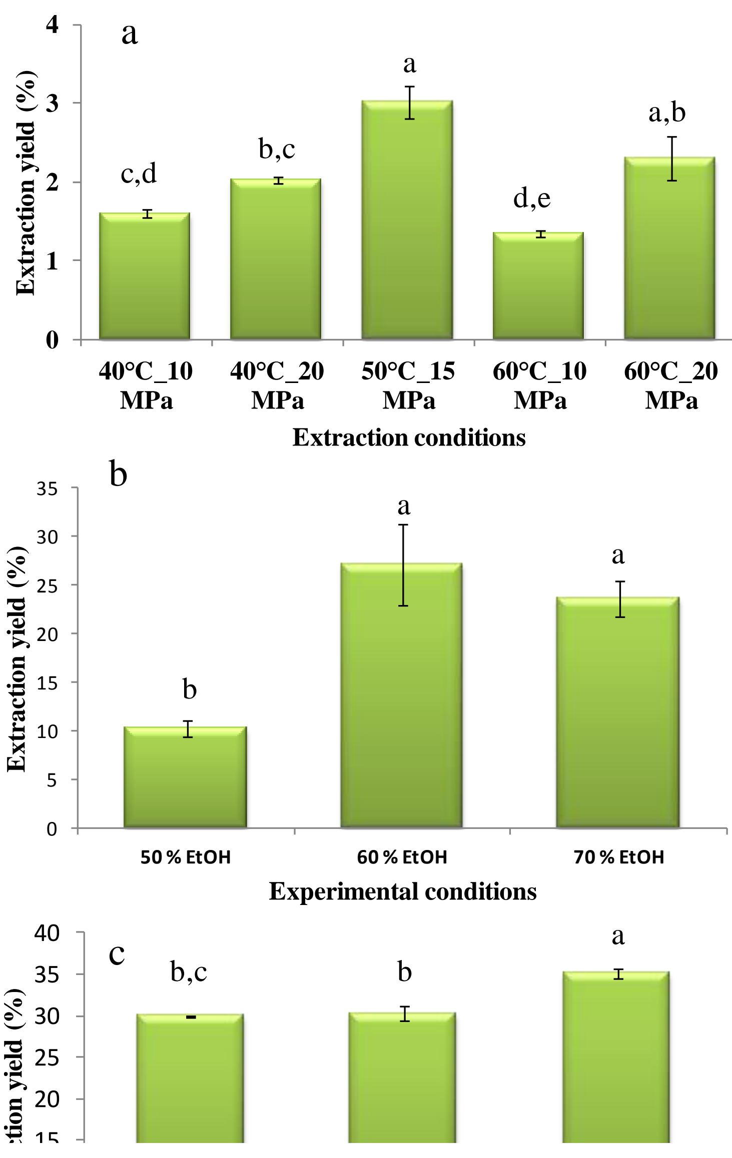


Figure 2. Extraction recoveries at the different experimental conditions tested for: a) SFE; b) CXE and c) PHWE. Mean values with different superscript letters are significantly different ($p < 0.05$).

.

Optimization of carbon dioxide expanded ethanol (CXE) extraction of moringa leaves (Step 2)

Once fixed the optimal extraction parameters (50ºC and 15 MPa for 60 min) in the previous step, the same temperature (50ºC) was used to avoid the heating or cooling of the system, allowing minimizing energy consumption. As mentioned in previous section, 160 min were established as extraction time for this second step.

Many bioactive compounds such as phenolic or glycosidic compounds, among others are poorly soluble in CO_2, conditioning their extraction. However, the use of polar solvent during the extraction with carbon dioxide has been demonstrated to increase the solubility of phenolic compounds [40]. The main industrial advantages that GXL presents are two: it allows working at low pressures (compared to scCO2) and using less amount of organic solvent (compared to PLE) [15].

Results obtained by studying the different extraction conditions with CO_2 expanded ethanol (50, 60 and 70% w/w) are displayed in **Figure 2b**. Significant differences were found among the use of 50 and 60% of ethanol (see **Figure 2b**), being 60% of ethanol the one that allowed the highest extraction yield, near 30%. Our results showed that even using 50% of ethanol the extraction yield is more than two-fold higher that using only $scCO_2$. In spite that there are not researches conducted to extract bioactive compounds in plant leaves by GXL, some studies carried out on microalgae demonstrated that the extraction yield (%, w/w) for GXL increased by increasing ethanol fraction (%) in the solvent up to 50% [17] and up to 70% [15]. This could be explained by the enhancement of the solubility of solid and liquids solutes in the presence of higher concentrations of organic solvents.

Compared to conventional extraction systems, CXE has proven to be more efficient for the extraction of compounds from *M. oleifera* leaves compared to conventional extraction system such as maceration, percolation, squeezing, decoction and soxhlet extractions [42]. In addition, GXL advantages include recovery and recycle of both the organic compound and CO_2 through depressurization, which is less energy intensive than standard separation techniques, moderate operating pressures

compared to the use of supercritical CO_2, and enhanced transport rates and reaction rates, compared to pure organic solvents in which the difficult separation of the solvent residue as well as harsh environmental impacts must be highlighted [42].

Optimization of PHWE extraction of moringa leaves (Step 3)

The last step was performed under pressurized conditions using water at different temperatures (50, 125 and 200ºC) as a solvent. In PHWE, pressure is usually varied from 1 to 8 MPa to maintain water in its liquid phase at the extraction temperature [20]. In our experiments, the pressure was maintained at 7 MPa and the extraction time was fixed at 60 min.

Temperature is the main factor that affects the extraction efficiency and selectivity in PHWE [20,21]. As expected, maximum temperature tested (200ºC) was proposed as the optimum condition in PHWE in terms of extraction yield. However, for the extraction of thermolabile compounds, high temperatures may cause the degradation of extracts. Matshediso *et al.* [5] noticed that increasing the temperature resulted in increased concentrations of flavonols that could be explained for the decreasing of polarity of water with the temperature, thus becoming less polar and more ready to solubilize medium polarity compounds. The same authors concluded that the optimum temperature for extracting flavonols by PHWE was 100ºC and that at temperatures higher than 150ºC, the content of flavonoids decreased drastically [5]. A principal component analysis (PCA) carried out extracting compounds from *M. oleifera* leaves by PHWE at 50, 100 and 200ºC, revealed differential clustering of samples extracted at different temperatures, being samples extracted at 200ºC less stable than the others [22]. Thus, in order to achieve a compromise between the quantity and the quality of *M. oleifera* leaves extracts, the optimal temperature was fixed at 125ºC.

Thus, the final selected conditions were: step 1, 10 MPa, 50ºC and 60 min; step 2, 7 MPa, 60% EtOH, 50ºC and 160 min and step 3, 7 MPa, 100% water, 125ºC and 60 min. Finally, a total extraction time of 280 min allowed obtained different fractions

rich in different bioactive compounds from *M. oleifera* leaves with different polarities which were consecutively analyzed.

Total phenolic compounds and total flavonoids content

To have a preliminary overview on the composition of *M. oleifera* leaves extracts, total phenolic compounds and total flavonoids content were determined and their results are depicted in **Figure 5**. In this regard, CXE fraction allowed the extraction of less content of total phenolics (20.3 ± 0.3 mg GAE/g of dry leaves) compared to PHWE extract (62.4 ± 1.3 mg GAE/g of dry leaves). The opposite trend was found for total flavonoids content, in which CXE resulted the most suitable extraction technique for flavonoids (9.6 ± 0.2 Eq quercetin/g of dry leaves) compared to PHWE (3.8 ± 0.1 Eq quercetin/g of dry leaves). Results will be discussed later together with the characterized compounds.

Compound characterization by GC-MS

The volatile profiles of the fractions obtained by $scCO_2$ and CXE are displayed in **Figure 3**. **Table 1** summarizes the compounds identified in the different fractions and their relative contributions, their retention times and linear retention indices (LRIs) employed to confirm their identity. In addition, the identification of those compounds whose retention index was not confirmed in databases was carried out comparing their mass spectra from NIST/EPA/NIH databases and the WILEY library. Besides, alkanes were identified comparing their retention time with the standards. Despite the chemical composition of both fractions analyzed by GC-MS presented similar profiles, some variations in the qualitative and quantitative composition can be seen (**Table 1**). The results revealed a total of 17 and 20 compounds for SFE and CXE respectively. In general, volatile fractions were composed by alcohols (linalool, eudsm-11-en-4α, 6α-diol, 1-hexacosanol), ketones (pyran-4-one derivative and, 1-cyclohexyl-ethanone), organic and fatty acids (palmitic acid, stearic cid, oleic acid and, 3-hydroxyoctadecanoic acid), esters (heptyl isobutyrate, octadecanal, methyl octadecanoate, ethyl octadecanoate, methyl nonadecanoate, methyl eicosanoate,

Trimethylsilyl icosanoate, ethyl eicosinoate, ethyl behenate and methyl 23-oxotetracosanoate), and alkanes (nonadecane, octacosane, nonacosane and tricontane) according to their nature. In general, CXE was more suitable for extracting ketones and esters compared to SFE. The most abundant compounds in both extracts were the alkanes named octacosane, nonacosane and tricontane. These results are consistent with those presented by [29,30,40] who found alkanes the most abundant volatile compounds in moringa leaves. If compared to conventional extraction techniques such as soxhlet or distillation, it can be seen that SFE is more selective extraction technique, allowing less extraction of compounds than the abovementioned techniques probably due to the CO_2 pulling of some compounds [28,29]. In addition to be a greener and faster technique than conventional extraction, the selectivity of SFE allowed obtaining extracts with fewer chromatographically interfering components. Beside these majority compounds, minor amount of fatty acids were found in moringa leaves. Authors such as Saini et al. [43] extracted 15 fatty acids from the leaves of *M. oleifera* from Indian cultivars by solid-liquid extraction using chloroform and methanol. They concluded that fatty acids composition of moringa could enhance the health benefits of moringa-based products. However, the selection of the extraction technique has to be a result of a compromise between the efficiency of extraction, the type and amount of target compounds and the cost, time, safety and degree of automation.

Figure 3. Volatile profiles of the fractions obtained by $scCO_2$ (a) and CXE (b) from GC-MS analysis.

Table 1. Identified compounds by GC-MS and percentage relative to total peak area in SFE and CXE extracts.

ID	RT (min)	Calculated LRI[a]	References LRI	Proposed compound	Formula	% Area[e]	
						SFE	CXE
1	9.413	1138	1099 [b,c,d]	linalool	$C_{10}H_{18}O$	ND	0.19
2	14.433	1241	1248 [b,c,d]	Heptyl isobutyrate	$C_6H_{12}O_2$	ND	0.89
3	17.482	1436	-- [c,]	pyran-4-one derivative	$C_6H_8O_4$	ND	3.15
4	23.972	1578	-- [c,d]	1-Cyclohexyl-ethanone	$C_8H_{14}O$	ND	2.95
5	37.727	1874	1807 [b,c,d]	Eudesm-11-en-4α, 6α-diol	$C_{15}H_{26}O$	0.53	0.21
6	38.103	1900	1900 [b,c,d]	Nonadecane	$C_{13}H_{28}$	0.17	ND
7	40.872	1999	1990 [b,c,d]	Palmitic acid	$C_{16}H_{32}O_2$	0.13	0.22
8	41.114	2138	2091 [b,c,d]	Octadecanal	$C_{18}H_{36}O$	0.70	8.90
9	41.358	2164	2128 [b,c,d]	Methyl octadecanoate	$C_{19}H_{38}O_2$	ND	2.51
10	41.526	2182	2194 [b,c,d]	Ethyl octadecanoate	$C_{20}H_{40}O_2$	1.92	15.24
11	42.059	2244	2228 [b,c,d]	Methyl nonadecanoate	$C_{20}H_{40}O_2$	0.46	ND
12	42.526	2291	2178 [b,c,d]	Oleic acid	$C_{16}H_{34}O_2$	ND	1.24
13	42.539	2309	2332[b,c,d]	Methyl eicosanoate	$C_{21}H_{42}O_2$	3.41	ND
14	42.844	2343	2385 [c,d]	Trimethylsilyl icosanoate	$C_{23}H_{48}O_2Si$	6.29	1.32
15	43.286	2398	2200[c,d]	Stearic acid	$C_{18}H_{36}O_2$	ND	0.33
16	43.481	2430	2400[c,d]	Ethyl eicosinoate	$C_{22}H_{44}O_2$	2.30	1.98
17	43.879	2484	2482 [b,c,d]	palmitoylglycerol	$C_{19}H_{38}O_4$	7.54	1.55
18	43.946	2497	2430 [b,c,d]	3-hydroxyoctadecanoic acid	$C_{18}H_{36}O_3$	0.66	ND
19	44.103	2509	2574 [b,c,d]	Ethyl behenate	$C_{24}H_{48}O_2$	1.60	ND
20	45.403	2812	-- [c]	Methyl 23-oxotetracosanoate	$C_{25}H_{48}O_3$	1.37	4.92

Table 1. *(Continued)*

21	45.809	2842	2800[c,d]	Octacosane	$C_{28}H_{58}$	6.79	4.18
22	46.652	2889	2526[c,d]	Non-identified	$C_{22}H_{44}O_2$	0.75	0.81
23	46.962	2941	2900 [b,c,d]	Nonacosane	$C_{29}H_{60}$	19.07	9.49
24	48.218	2998	2884 [b,c,d]	1-Hexacosanol	$C_{26}H_{54}O$	0.10	0.23
25	48.930	3066	3000[b,c,d]	Tricontane	$C_{30}H_{62}$	33.67	15.47

[a] Linear Retention Indices (LRI) on DB-5 MS capillary column; [b]Peak identified based on Kovats Index; [c] Peak identified based on Wiley library; [d] Peak identified based on literature; [e] Relative area (percent of total peak area: 1.21 x 10^8 for SFE; 9.26 x 10^7 for CXE); ND, non-detected.

Compound characterization by HPLC-ESI-QTOF-MS

Moringa leaves fractions obtained in the second (CXE) and third (PHWE) steps of the sequential process were analyzed by using HPLC–ESI–QTOF–MS in negative ionization mode, in order to characterize the bioactive compounds present in this matrix. Peak identification was performed by generation of the candidate formula with a mass accuracy limit of 10 ppm and also considering their MS and MS/MS spectra determined by QTOF–MS and data from the literature. For the acquisition of chemical structure information and data from literature, the following databases were consulted: SciFinder Scholar (http://scifinder.cas.org), MassBank (http://massbank.jp), and METLIN Metabolite Database (http://metlin.scripps.edu).

Figure 4 shows the base peak chromatograms (BPCs) at the same intensity of each moringa leaves fractions extracted under optimal conditions, where each peak was numbered according to its elution order. Furthermore, **Table 2** shows the overall results: retention time (RT), experimental m/z of negative molecular ions ($[M - H]^-$), molecular formula, mass error, main MS^2 fragments and the relative abundance of each compound (normalized using apigenin as internal standard). A total of 50 compounds were tentatively characterized in both fractions including essential amino acids, nucleosides, organic acids, phenolic acids and derivatives, thioglycosides, lignans and flavonoids.

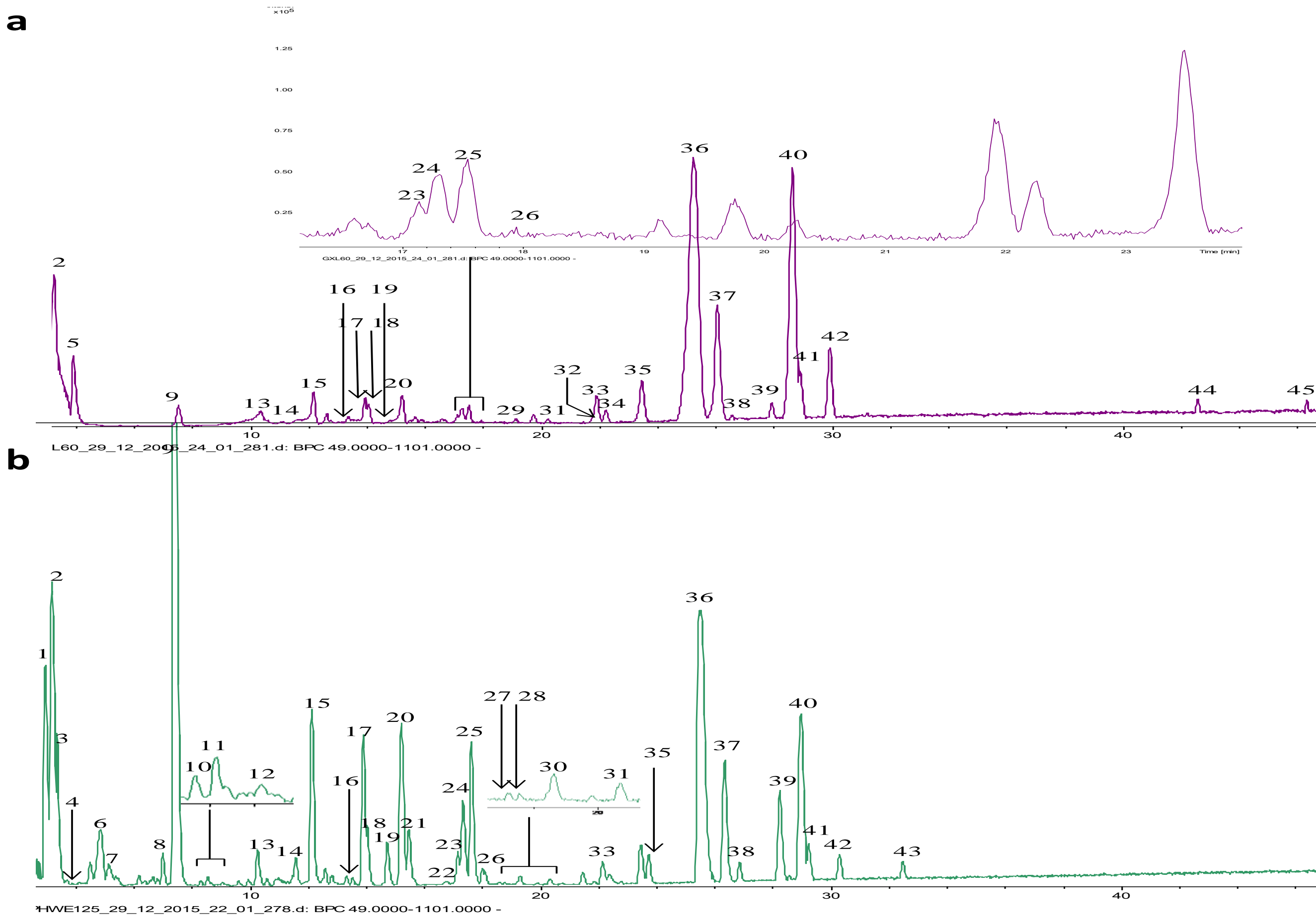


Figure 4. Base-Peak Chromatogram (BPC) of *M. oleifera* leaves fractions obtained by CXE (a) and PHWE (b) analyzed by HPLC-ESI-QTOF-MS in negative ionization mode.

Table 2. HPLC-ESI-QTOF-MS data of the compounds identified in *Moringa oleifera* leaf extracted by CXE 60% and PHWE 125°C.

Peak[a]	RT	$[M - H]^-$	Molecular formula	Error (ppm)	Fragments[b]	Compound	Area[c]	
							CXE	PHWE
Aminoacids								
13	10.31	164.0708	$C_9H_{11}NO_2$	5.3	103.0555 (100); 72.0095 (93.05); 77.0399 (31.001); 147.0451 (19.42)	L-phenylalanine	0.21	0.24
16	13.37	203.081	$C_{11}H_{12}N_2O_2$	8	116.0503 (100)	L-tryptophan	0.06	0.05
Nucleosides								
6	4.92	150.0418	$C_5H_5N_5O$	2	135.0310 (100)	Guanine isomer	ND	0.59
8	7.01	150.0415	$C_5H_5N_5O$	4.3	135.0323 (100)	Guanine isomer	ND	0.17
10	8.38	266.0896	$C_{10}H_{13}N_5O_4$	0.5	134.0435 (100)	Adenosine	ND	0.03
11	8.62	282.0823	$C_{10}H_{13}N_5O_5$	6.7	150.0441 (100); 133.0172 (27.5)	Guanosine	ND	0.05
Organic acids								
1	3.05	195.0517	$C_6H_{12}O_7$	3.5	75.0099 (100); 59.0148 (25.73); 71.0148 (10)	Gluconic acid	ND	1.53
2	3.26	191.0578	$C_7H_{12}O_6$	8.6	127.0387 (100)	Quinic acid isomer	1.39	2.36
3	3.45	191.0556	$C_7H_{12}O_6$	2.6	127.0397 (100)	Quinic acid isomer	ND	0.82
4	3.78	133.0134	$C_4H_6O_5$	6.2	71.015 (100); 72.9942 (45.12); 115.0037 (1.26); 59.0151 (10.36)	Malic acid	ND	0.04

Table 2. *(Continued)*

Peak[a]	RT	$[M - H]^-$	Molecular formula	Error (ppm)	Fragments[b]	Compound	Area[c]	
							CXE	PHWE
7	5.22	191.0192	$C_6 H_8 O_7$	2.7	87.0092 (100); 111.0083 (65.44)	Citric acid	ND	0.2
44	42.5	327.2169	$C_{18} H_{32} O_5$	4.7	211.1349 (100); 229.1484 (45.06); 171.1024 (33.95)	Trihydroxyoctadecadienoic acid	0.24	ND
45	46.23	307.1919	$C_{18} H_{28} O_4$	1.3	139.1129 (100); 171.139 (52)	Methylgingerol	0.18	ND
46	47.36	309.2062	$C_{18} H_{30} O_4$	3.2	171.1038 (100); 71.0499 (71.07); 291.1988 (66.36); 137.0981 (43.45); 185.1187 (31.47); 251.1678 (30.44)	Hydroperoxy-octadecatrienoic acid isomer	0.17	ND
47	48.85	309.2062	$C_{18} H_{30} O_4$	0.1	171.1035 (100); 291.1988 (65); 251.1668 (20)	Hydroperoxy-octadecatrienoic acid isomer	0.15	ND
48	57.63	293.2128	$C_{18} H_{30} O_3$	2	183.1398 (100); 171.103 (75.3); 121.1023 (63.26); 275.2024 (59.95); 293.213 (19.93)	9-Hydroxyoctadeca-10,12,15-trienoic acid	0.78	ND
49	57.77	293.2126	$C_{18} H_{30} O_3$	1.4	183.1374 (100); 171.1009 (26.67); 121.102 (23.69): 275.1965 (22.21); 293..2067 (13.26)	13-Hydroxyoctadeca-9,11,15-trienoic acid isomer	0.45	ND
50	58.08	293.2135	$C_{18} H_{30} O_3$	4.4	275.2034 (100); 235.1716 (75.82); 59.0137 (61.89); 195.1397 (59.64); 293.2137 (53.02)	13-Hydroxyoctadeca-9,11,15-trienoic acid isomer	1.55	ND

Table 2. *(Continued)*

Peak[a]	RT	[M - H]⁻	Molecular formula	Error (ppm)	Fragments[b]	Compound	Area[c]	
							CXE	PHWE
12	9.12	353.087	$C_{16} H_{18} O_9$	2.3	191.0613 (100); 135.0478 (83.55); 179.036 (78.81)	Caffeoylquinic acid isomer	ND	0.02
14	11.56	315.1081	$C_{14} H_{20} O_8$	1.3	153.0501 (100)	Vanillin glucoside	0.03	0.17
15	12.18	353.0887	$C_{16} H_{18} O_9$	2.6	173.0467 (100); 135.0463 (97.07); 191.0557 (94.36); 179.0339 (80.61)	Caffeoylquinic acid isomer	0.27	1.28
17	13.95	337.0913	$C_{16} H_{18} O_8$	4.6	119.0548 (100); 163.0443 (92.29)	Coumaroylquinic acid isomer	0.17	0.91
18	14.08	337.0922	$C_{16} H_{18} O_8$	2.2	119.0519 (100); 163.0424 (94.2); 191.0588 (30.29)	Coumaroylquinic acid isomer	0.12	0.34
19	14.85	367.1019	$C_{17} H_{20} O_9$	4.3	134.0408 (100); 193.0541 (67.82)	Feruloylquinic acid isomer	0.04	0.28
20	15.26	353.0885	$C_{16} H_{18} O_9$	2.1	135.0502 (100); 191.062 (90.19); 173.0511 (88.98); 179.041 (55.14)	Caffeoylquinic acid isomer	0.23	1.28
23	17.2	337.0926	$C_{16} H_{18} O_8$	0.8	173.0482 (100); 191.0589 (72.97); 119.0517 (36.87); 163.0422 (30.92)	Coumaroylquinic acid isomer	0.06	0.19
24	17.36	337.0923	$C_{16} H_{18} O_8$	1.6	173.0509 (100); 119.0543 (16.59); 163.0446 (13.36)	Coumaroylquinic acid isomer	0.13	0.61
26	18.02	367.1031	$C_{17} H_{20} O_9$	1	173.0476 (100); 134.0395 (37.57); 193.0524 (17.22)	Feruloylquinic acid isomer	0.03	0.1
28	18.78	335.077	$C_{16} H_{16} O_8$	0.8	161.0252 (100); 133.0301 (13.62)	Caffeoylshikimic acid	ND	0.01

Table 2. *(Continued)*

Peak[a]	RT	$[M - H]^-$	Molecular formula	Error (ppm)	Fragments[b]	Compound	Area[c]	
							CXE	PHWE
Thioglycosides (Glucosinolates)								
5	3.98	570.0956	$C_{20} H_{29} N O_{14} S_2$	0.1	96.9635 (100); 259.0111 (19.41); 328.0808 (17.85)	Glucomoringin isomer	0.5	ND
9	7.46	570.0961	$C_{20} H_{29} N O_{14} S_2$	0.8	96.9637 (100); 259.0111 (19.6); 328.0808 (18.04)	Glucomoringin isomer	0.14	7.56
Lignans								
29	19.03	521.2019	$C_{26} H_{34} O_{11}$	1.9	359.1487 (100)	Isolariciresinol glycoside/lariciresinol glycoside isomer	0.03	ND
32	21.81	521.2029	$C_{26} H_{34} O_{11}$	0.1	359.1483 (100)	Isolariciresinol glycoside/lariciresinol glycoside isomer	0.17	ND
Flavonoids								
21	15.54	625.1397	$C_{27} H_{30} O_{17}$	2	463.0865 (100); 301.0351 (44)	Quercetin diglycoside isomer	ND	0.38
22	16.79	607.1291	$C_{27} H_{28} O_{16}$	2.3	463.0875 (90); 301.0352 (35); 300.0285 (28)	Quercetin-hydroxy-methylglutaroyl glycoside isomer	ND	0.04
25	17.65	593.1507	$C_{27} H_{30} O_{15}$	0.8	593.1536 (100); 473.1096 (74.5); 353.0659 (72.38); 383.0785 (15.47)	Multiflorin B isomer	0.14	1.06
27	18.6	593.1466	$C_{27} H_{30} O_{15}$	7.7	593.143 (100); 473.1022 (77.13); 353.0616 (75.62)	Multiflorin B isomer	ND	0.02
30	19.32	593.15	$C_{27} H_{30} O_{15}$	2	593.1475 (100); 473.1082 (20.63); 353.0681 (18.68)	Multiflorin B isomer	ND	0.07
31	20.34	625.1391	$C_{27} H_{30} O_{17}$	3	463.0873 (100); 301.0362 (54)	Quercetin diglycoside isomer	0.03	0.04

Table 2. *(Continued)*

Peak[a]	RT	$[M - H]^-$	Molecular formula	Error (ppm)	Fragments[b]	Compound	Area[c]	
							CXE	PHWE
33	22.16	431.0971	$C_{21}H_{20}O_{10}$	3	311.0568 (100); 283.0628 (16.08)	(Vitexin) Apigenin glucoside isomer	0.25	0.18
34	23.33	431.0978	$C_{21}H_{20}O_{10}$	1.3	311.054 (100); 283.0597 (53.39); 341.0632 (10)	(Vitexin) Apigenin glucoside isomer	0.1	0.11
35	23.73	431.0972	$C_{21}H_{20}O_{10}$	2.7	311.06 (100); 283.0644 (47.25); 341.0703 (39.69)	(Vitexin) Apigenin glucoside isomer	0.38	0.23
36	25.39	463.0885	$C_{21}H_{20}O_{12}$	0.7	300.0336 (100); 301.0392 (55.033); 271.0261 (30.29)	Quercetin 3-*O*-glucoside	5.06	3.99
37	26.34	505.0982	$C_{23}H_{22}O_{13}$	1	300.028 (100); 301.0343 (43.4)	Quercetin-acetyl-glycoside isomer	1.24	1.08
38	26.85	607.1294	$C_{27}H_{28}O_{16}$	1.7	463.0874 (50); 301.0353 (13); 300.0275 (10)	Quercetin-hydroxy-methylglutaroyl glycoside isomer	0.05	0.16
39	28.23	505.0988	$C_{23}H_{22}O_{13}$	0.1	300.0333 (100); 301.0375 (64.16)	Quercetin-acetyl-glycoside isomer	0.13	0.8
40	28.95	447.0956	$C_{21}H_{20}O_{11}$	5.1	284.0332 (100); 25.0283 (61.1); 285.0357 (56.54)	Kaempferol 3-*O*-glucoside	2.97	1.65
41	29.15	477.1044	$C_{22}H_{22}O_{12}$	1.3	314.0442 (100); 315.0498 (29.4)	Isorhamnetin 3-*O*-Glucoside	0.33	0.26
42	30.29	489.1026	$C_{23}H_{22}O_{12}$	2.5	284.0344 (100); 285.0415 (53.4%); 255.0315 (17.52)	Kaempferol acetyl glycoside isomer	0.62	0.2
43	32.45	489.1043	$C_{23}H_{22}O_{12}$	0.9	284.0308 (100); 285.0364 (32.94); 255.0285 (32.52)	Kaempferol acetyl glycoside isomer	ND	0.15
Total area							6.6×10^7	9.5×10^7

[a] Peak numbers assigned according to the overall elution order; [b] Numbers in brackets show the relative abundance of each MS/MS fragment expressed in percentage; [c] Relative area (calculated using apigenin as internal standard); ND, non-detected.

By analyzing the chromatograms (**Figure 4**), it is possible to assess the qualitative differences between both extracts. As expected, the use of pressurized water at 125ºC allowed obtaining a fraction richer in more polar compounds such as polar organic acids (gluconic, quinic, malic and citric acids), nucleosides, which were only detected in this fraction, and phenolic acids derivatives (caffeoylquinic acid isomers, feruloylquinic acid isomers, coumaroylquinic acid isomers and vanillin glucoside) than CXE fraction. Contrary, CXE fraction was more effective extracting less polar flavonoids, low-polar organic acids (peaks 44-50) and lignans (isolariciresinol glycoside/lariciresinol glycoside isomers).

While essential amino acids were extracted in a similar way in steps 2 and 3, total phenolic acids and total thioglycosides were much better extracted by pressurized water. The polar nature of water enabled a major extraction of phenolic acids which were nearly 3-fold higher in PHWE extract than in CXE extract. These results are in agreement with the results obtained for TPC, a measure commonly used to determine the concentration of hydroxyl groups in plant extracts. The same behavior was observed for thioglycosides that have proven to be very extractable under different extraction conditions [31,44]. In fact, these compounds have been detected even in CXE extract.

Leaves of moringa are recognized as a rich source of flavonoids [31,45]. Thus, it was not surprising that the most abundant group of bioactive compounds extracted were flavonoids, being quercetin and kaempferol derivatives the main ones. Concretely, the highest total flavonoids content was found in the CXE extract that allowed obtaining almost 2-fold higher recoveries of flavonoids than PHWE. These data are in accordance with the quantitative results obtained by means of the colorimetric assay. As aforementioned, flavonoids are practically insoluble in pure CO_2, but sufficiently soluble in CO_2-ethanol to enable their separation from high molecular mass and/or more polar components [46]. In addition, glycosylated flavonoids have a low melting point and a high enthalpy of fusion compared to the aglycons that makes easier their solubility in organic solvents such as ethanol [47]. A

recent study has concluded that the use of 50% ethanol as co-solvent allowed the extraction of polar compounds such as flavonoids compared to CO_2-SFE [48]. Moreover, despite CXE extract was richer in organic acids, it should be mentioned that its highest content was owing to the extraction of organic acids so-called oxylipins (peaks 44, 46-50) and an a fatty aliphatic alcohol (peak 45) which were only extracted under CXE conditions and that contributed over 71% of the total organic acids extracted in this step. On the other hand, small quantities of lignans were only detected in CXE extract.

Antioxidant activity

The reducing power of the CXE and PHWE of *M. oleifera* leaves extracts revealed that the extract obtained by pressurized water at 125ºC was more effective reducing activity (13.4 ± 0.7 mmol Eq trolox/100 g dry leaves) than the obtained by CO_2 expanded ethanol (6 ± 0.3 mmol Eq trolox/100 g dry leaves). The differences for the reducing activity of different extracts could be attributed to the varied quantity of phenolic acids derivatives contents in each extract. In fact, PHWE extract also showed the highest TPC. In this regard, previous studies have indicated a linear relationship between total phenolics and antioxidant activity [49,50]. The current results evidenced that the use of pressurized water at mid-temperatures is a powerful extraction technique that allows obtaining antioxidant-rich extracts.

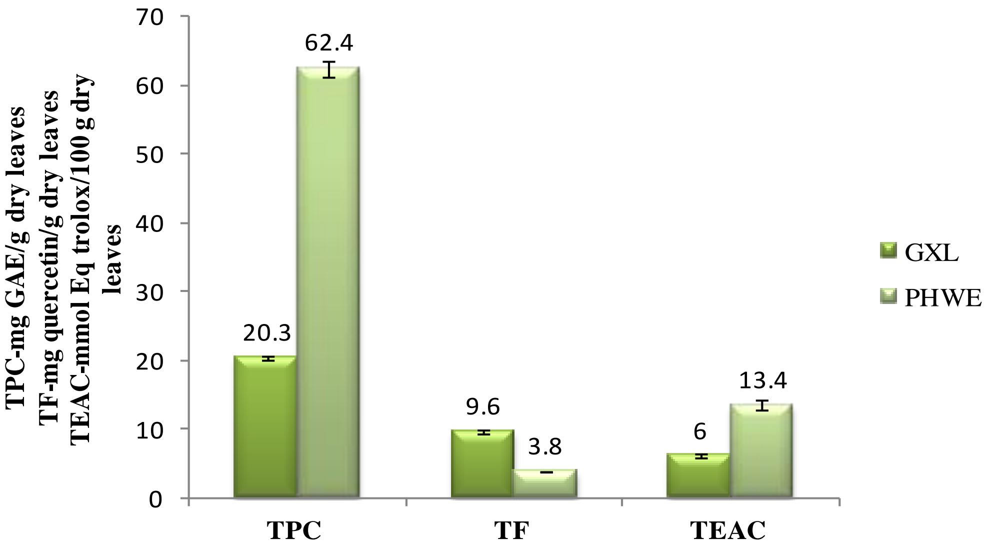


Figure 5. Total phenolic content (TPC), total flavonoid content (TF) and antioxidant activity of the fraction obtained under optimal conditions of CXE and PHWE. Data are expressed as mean ± standard deviation and mean values are provided on top of each bar.

❖ Conclusions

The current research represents the first approach to the development of a green platform for downstream processing of *M. oleifera* leaves, focused on obtaining different fractions rich in bioactive compounds. It has been demonstrated that supercritical fluids extraction, carbon dioxide-expanded ethanol and pressurized hot water extraction are green and sustainable extraction methods that allow the recovery of a wide range of bioactive compounds, from alcohols and fatty acids to phenolic acids and flavonoids. Under optimized conditions, a process based on three steps has been performed considering the use of 1) $scCO_2$ at 15 MPa and 50 C, 2) 60% ethanol in CXE at 7 MPa, and 3) water at 125ºC and 7 MPa. Under these conditions, different fractions with a varied composition of bioactive compounds were achieved, thus serving as a basis for further research for nutraceutical and cosmeceutical companies interested in the complete recovery of bioactive compounds from leaves of *M. oleifera*.

Acknowledgments

This work was supported by projects AGL2014-53609-P and AGL2011-29857-C03-02 (Spanish Ministry of Economy and Competitiveness (MINECO), as well as P09-CTS-4564, P10-FQM-6563 and P11-CTS-7625 (Andalusian Regional Government Council of Innovation and Science). The authors are grateful to the for a FPU fellowship AP2010-1551 (Spanish Ministry of Science and Innovation) (C. Rodríguez-Pérez), and a grant "Personal técnico de apoyo" PTA2012-6956-E (R. Quirantes-Piné). We would like also to thank to the Non-Governmental Organization "Agua de coco" for providing us the samples to analyze.

References

[1] M. Herrero, E. Ibáñez, Green processes and sustainability: An overview on the extraction of high added-value products from seaweeds and microalgae, J. Supercrit.l Fluids. 96 (2015) 211-216.

[2] C. Rodríguez-Pérez, C. Jiménez-Sánchez, J. Lozano-Sánchez, R. Quirantes-Piné, A. Segura-Carretero, Emerging green technologies for the extraction of phenolic compounds from medicinal plants, in: J.N. Govil (Ed.), Recent Progress in Medicinal Plants. Vol. 41. Analytical and Processing Techniques, Studium Press LLC, USA, 2016, pp. 81-104.

[3] M. Herrero, A.D.P. Sánchez-Camargo, A. Cifuentes, E. Ibáñez, Plants, seaweeds, microalgae and food by-products as natural sources of functional ingredients obtained using pressurized liquid extraction and supercritical fluid extraction, TrAC Trends Anal. Chem. 71 (2015) 26-38.

[4] I. Borrás-Linares, D. Arráez-Román, M. Herrero, E. Ibáñez, A. Segura-Carretero, A. Fernández-Gutiérrez, Comparison of different extraction procedures for the comprehensive characterization of bioactive phenolic compounds in *Rosmarinus officinalis* by reversed-phase high-performance liquid chromatography with diode array detection coupled to electrospray time-of-flight mass spectrometry, J. Chromatogr. A. 1218 (2011) 7682-7690.

[5] P.G. Matshediso, E. Cukrowska, L. Chimuka, Development of pressurised hot water extraction (PHWE) for essential compounds from *Moringa oleifera* leaf extracts, Food Chem. 172 (2015) 423-427.

[6] A. Taamalli, D. Arráez-Román, E. Barrajón-Catalán, V. Ruiz-Torres, A. Pérez-Sánchez, M. Herrero, E. Ibáñez, V. Micol, M. Zarrouk, A. Segura-Carretero, A. Fernández-Gutiérrez, Use of advanced techniques for the extraction of phenolic compounds from Tunisian olive leaves: Phenolic composition and cytotoxicity against human breast cancer cells, Food Chem. Toxicol. 50 (2012) 1817-1825.

[7] S. Zhao, D. Zhang, Supercritical fluid extraction and characterisation of *Moringa oleifera* leaves oil, Sep. Purif. Technol. 118 (2013) 497-502.

[8] J.A. Mendiola, M. Herrero, M. Castro-Puyana, E. Ibáñez, Supercritical fluid extraction, RSC Green Chem. (2013) 196-230.

[9] M. Herrero, M. Castro-Puyana, J.A. Mendiola, E. Ibáñez, Compressed fluids for the extraction of bioactive compounds, TrAC Trends Anal. Chem. 43 (2013) 67-83.

[10] E. Ghasemi, F. Raofie, N.M. Najafi, Application of response surface methodology and central composite design for the optimisation of supercritical fluid extraction of essential oils from *Myrtus communis* L. leaves, Food Chem. 126 (2011) 1449-1453.

[11] S.M. Pourmortazavi, S.S. Hajimirsadeghi, Supercritical fluid extraction in plant essential and volatile oil analysis, J. Chromatogr. A. 1163 (2007) 2-24.

[12] M. Herrero, M. Castro-Puyana, J.A. Mendiola, E. Ibáñez, Compressed fluids for the extraction of bioactive compounds, TrAC Trends Anal. Chem. 43 (2013) 67-83.

[13] R.J. Hwang, J. Ortiz, Mitigation of asphaltics deposition during CO_2 flood by enhancing CO_2 solvency with chemical modifiers, Org. Geochem. 31 (2000) 1451-1462.

[14] G.R. Akien, M. Poliakoff, A critical look at reactions in class I and II gas-expanded liquids using CO_2 and other gases, Green Chem. 11 (2009) 1083-1100.

[15] F.A. Reyes, J.A. Mendiola, E. Ibáñez, J.M. Del Valle, Astaxanthin extraction from *Haematococcus pluvialis* using CO 2-expanded ethanol, J. Supercrit. Fluids. 92 (2014) 75-83.

[16] B. Gilbert-López, J.A. Mendiola, J. Fontecha, L.A.M. Van Den Broek, L. Sijtsma, A. Cifuentes, M. Herrero, E. Ibáñez, Downstream processing of *Isochrysis galbana*: a step towards microalgal biorefinery, Green Chem. 17 (2015) 4599-4609.

[17] M. Golmakani, J.A. Mendiola, K. Rezaei, E. Ibáñez, Expanded ethanol with CO_2 and pressurized ethyl lactate to obtain fractions enriched in γ-Linolenic acid from *Arthrospira platensis* (Spirulina), J. Supercrit. Fluids. 62 (2012) 109-115.

[18] S. Santoyo, M. Herrero, F.J. Señorans, A. Cifuentes, E. Ibáñez, L. Jaime, Functional characterization of pressurized liquid extracts of *Spirulina platensis*, Eur. Food Res. Technol. 224 (2006) 75-81.

[19] A. Mustafa, C. Turner, Pressurized liquid extraction as a green approach in food and herbal plants extraction: A review, Anal. Chim. Acta. 703 (2011) 8-18.

[20] C.C. Teo, S.N. Tan, J.W.H. Yong, C.S. Hew, E.S. Ong, Pressurized hot water extraction (PHWE), J. Chromatogr. A. 1217 (2010) 2484-2494.

[21] E. Ibáñez, A. Kubátová, F.J. Señoráns, S. Cavero, U. Reglero, S.B. Hawthorne, Subcritical water extraction of antioxidant compounds from rosemary plants, J. Agric. Food Chem. 51 (2003) 375-382.

[22] B.S. Khoza, L. Chimuka, E. Mukwevho, P.A. Steenkamp, N.E. Madala, The effect of temperature on pressurised hot water extraction of pharmacologically important metabolites as analysed by UPLC-qTOF-MS and PCA, Evid. -Based Complement. Altern. Med. 2014 (2014).

[23] B. Posmontier, The medicinal qualities of *Moringa oleifera*, Holistic Nurs. Pract. 25 (2011) 80-87.

[24] F. Anwar, S. Latif, M. Ashraf, A.H. Gilani, *Moringa oleifera*: A food plant with multiple medicinal uses, Phytother. Res. 21 (2007) 17-25.

[25] H.D. Yassa, A.F. Tohamy, Extract of *Moringa oleifera* leaves ameliorates streptozotocin-induced diabetes mellitus in adult rats, Acta Histochem. 116 (2014) 844-854.

[26] L. Berkovich, G. Earon, I. Ron, A. Rimmon, A. Vexler, S. Lev-Ari, *Moringa Oleifera* aqueous leaf extract down-regulates nuclear factor-kappaB and increases cytotoxic effect of chemotherapy in pancreatic cancer cells, BMC Complement. Altern. Med. 13 (2013).

[27] I.L. Jung, J.H. Lee, S.C. Kang, A potential oral anticancer drug candidate, *Moringa oleifera* leaf extract, induces the apoptosis of human hepatocellular carcinoma cells, Oncol. Lett. 10 (2015) 1597-1604.

[28] G. Karthivashan, P. Arulselvan, S.W. Tan, S. Fakurazi, The molecular mechanism underlying the hepatoprotective potential of *Moringa oleifera* leaves extract against acetaminophen induced hepatotoxicity in mice, J. Funct. Foods. 17 (2015) 115-126.

[29] P. Chuang, C. Lee, J. Chou, M. Murugan, B. Shieh, H. Chen, Anti-fungal activity of crude extracts and essential oil of *Moringa oleifera* Lam, Bioresour. Technol. 98 (2007) 232-236.

[30] S. Zhao, D. Zhang, Supercritical fluid extraction and characterisation of *Moringa oleifera* leaves oil, Sep. Purif. Technol. 118 (2013) 497-502.

[31] C. Rodríguez-Pérez, R. Quirantes-Piné, A. Fernández-Gutiérrez, A. Segura-Carretero, Optimization of extraction method to obtain a phenolic compounds-rich extract from *Moringa oleifera* Lam leaves, Ind. Crops Prod. 66 (2015) 246-254.

[32] R.G. Woisky, A. Salatino, Analysis of propolis: Some parameters and procedures for chemical quality control, J. Apic. Res. 37 (1998) 99-105.

[33] A. Morales-Soto, P. García-Salas, C. Rodríguez-Pérez, C. Jiménez-Sánchez, M.D.L.L. Cádiz-Gurrea, A. Segura-Carretero, A. Fernández-Gutiérrez, Antioxidant capacity of 44 cultivars of fruits and vegetables grown in Andalusia (Spain), Food Res. Int. 58 (2014) 35-46.

[34] A.D.P. Sánchez-Camargo, A. Valdés, G. Sullini, V. García-Cañas, A. Cifuentes, E. Ibáñez, M. Herrero, Two-step sequential supercritical fluid extracts from rosemary with enhanced anti-proliferative activity, J. Funct. Foods. 11 (2014) 293-303.

[35] E.L. Oliveira, A.J. Silvestre, C.M. Silva, Review of kinetic models for supercritical fluid extraction, Chem. Eng. Res. Design. 89 (2011) 1104-1117.

[36] C.G. Pereira, M.A.A. Meireles, Supercritical fluid extraction of bioactive compounds: fundamentals, applications and economic perspectives, Food Bioprocess Tech. 3 (2010) 340-372.

[37] T. Marrufo, F. Nazzaro, E. Mancini, F. Fratianni, R. Coppola, L. De Martino, A.B. Agostinho, V. De Feo, Chemical composition and biological activity of the

essential oil from leaves of *Moringa oleifera* Lam. cultivated in Mozambique, Molecules. 18 (2013) 10989-11000.

[38] H.A. Martinez-Correa, F.A. Cabral, P.M. Magalhães, C.L. Queiroga, A.T. Godoy, A.P. Sánchez-Camargo, L.C. Paviani, Extracts from the leaves of *Baccharis dracunculifolia* obtained by a combination of extraction processes with supercritical CO2, ethanol and water, J. Supercrit. Fluids. 63 (2012) 31-39.

[39] P.F. Martins, M.M.R. De Melo, C.M. Silva, Techno-economic optimization of the subcritical fluid extraction of oil from *Moringa oleifera* seeds and subsequent production of a purified sterols fraction, J. Supercritical Fluids. 107 (2016) 682-689.

[40] L. Wang, C.L. Weller, Recent advances in extraction of nutraceuticals from plants, Trends Food Sci. Technol. 17 (2006) 300-312.

[41] B. Vongsak, P. Sithisarn, S. Mangmool, S. Thongpraditchote, Y. Wongkrajang, W. Gritsanapan, Maximizing total phenolics, total flavonoids contents and antioxidant activity of *Moringa oleifera* leaf extract by the appropriate extraction method, Ind. Crops Prod. 44 (2013) 566-571.

[42] E. Siougkrou, A. Galindo, C.S. Adjiman, On the optimal design of gas-expanded liquids based on process performance, Chemical Engineering Science. 115 (2014) 19-30.

[43] R.K. Saini, N.P. Shetty, P. Giridhar, GC-FID/MS analysis of fatty acids in Indian cultivars of *Moringa oleifera*: Potential sources of PUFA, JAOCS J Am Oil Chem Soc. 91 (2014) 1029-1034.

[44] C. Waterman, D.M. Cheng, P. Rojas-Silva, A. Poulev, J. Dreifus, M.A. Lila, I. Raskin, Stable, water extractable isothiocyanates from *Moringa oleifera* leaves attenuate inflammation in vitro, Phytochem. 103 (2014) 114-122.

[45] B. Vongsak, P. Sithisarn, W. Gritsanapan, Simultaneous determination of crypto-chlorogenic acid, isoquercetin, and astragalin contents in *Moringa oleifera* leaf extracts by TLC-densitometric method, Evid. -Based Complement. Altern. Med. 2013 (2013).

[46] O.J. Catchpole, J.B. Grey, K.A. Mitchell, J.S. Lan, Supercritical antisolvent fractionation of propolis tincture, J. Supercrit. Fluids. 29 (2004) 97-106.

[47] L. Chebil, C. Humeau, J. Anthony, F. Dehez, J.-. Engasser, M. Ghoul, Solubility of flavonoids in organic solvents, J. Chem. Eng. Data. 52 (2007) 1552-1556.

[48] A.R.M. Costa, L.A.P. Freitas, J. Mendiola, E. Ibáñez, *Copaifera langsdorffii* supercritical fluid extraction: Chemical and functional characterization by LC/MS and in vitro assays, J. Supercrit. Fluids. 100 (2015) 86-96.

[49] A. Djeridane, M. Yousfi, B. Nadjemi, D. Boutassouna, P. Stocker, N. Vidal, Antioxidant activity of some algerian medicinal plants extracts containing phenolic compounds, Food Chem. 97 (2006) 654-660.

[50] D. Kim, S.W. Jeong, C.Y. Lee, Antioxidant capacity of phenolic phytochemicals from various cultivars of plums, Food Chem. 81 (2003) 321-326.

Chapter 4

A metabolite-profiling approach allows the identification of new compounds from *Pistacia lentiscus* leaves

Journal of Pharmaceutical and Biomedical Analysis, 2013, 77:167-174

Journal of Pharmaceutical and Biomedical Analysis

Volume 77, 15 April 2013, Pages 167–174

A metabolite-profiling approach allows the identification of new compounds from *Pistacia lentiscus* leaves

C. Rodríguez-Pérez[a, b], R. Quirantes-Piné[a, b], N. Amessis-Ouchemoukh[c], K. Madani[c], A. Segura-Carretero[a, b], A. Fernández-Gutierrez[a, b]

[a] Department of Analytical Chemistry, Faculty of Sciences, University of Granada, Fuentenueva s/n, E 18071 Granada, Spain

[b] Functional Food Research and Development Center, Health Science Technological Park, Avenida del Conocimiento s/n, E-18100 Granada, Spain

[c] Faculty of Nature and Life Sciences, 3bs Laboratory, A. Mira University, Bejaia 06000, Algeria

Abstract

Pistacia lentiscus L. (*P. Lentiscus*), commonly known as Mastic tree or lentisk, is a Mediterranean evergreen shrub widely used in traditional medicine to treat such diseases as eczema, diarrhoea, and throat infections. Furthermore, other properties are currently attributed to *P. lentiscus*, such as antioxidant capacity, hepatoprotective action, and anti-inflammatory effects. High-performance liquid chromatography with diode array coupled to electrospray ionization mass spectrometry (HPLC-ESI-QTOF-MS) was used for the comprehensive characterization of methanol extract from *P. lentiscus* leaves. After the optimisation of the HPLC-ESI-QTOF-MS method and the use of the negative ionization mode, 46 different compounds were identified, 20 of which were tentatively characterized for the first time in *P. Lentiscus* leaves. The majority of the compounds were quantified. Flavonoids, phenolic acids and their derivatives were the most abundant compounds, those with the highest concentrations being myricetin glycoside (6216.13 mg/kg of plant), catechin (3354.78 mg/kg of plant), β-glucogallin (2214.461 mg/kg of plant), and quercitrin gallate (1160 mg/kg of plant). The importance of the knowledge of plants is increasing and our study may help in the future to formulate nutraceutical preparations and will provide the basis for new investigation into activities of the various compounds found in *P. lentiscus.*

Keywords: *Pistacia lentiscus*, metabolite profiling, phenolic compounds, quantification, HPLC-DAD-ESI-MS.

❖ Introduction

Pistacia lentiscus L. (*P. Lentiscus*), commonly known as Mastic tree or lentisk, is an evergreen Mediterranean shrub belonging to the Anacardiaceae family. It is a traditional medicinal plant of Mediterranean area commonly used by rural populations in Algeria, where it is endemic. It grows wild also in Turkey, Morocco, France, Spain, Italy, and Greece. In the folk medicine, the entire shrub is utilized, and some of its benefits were described as early as the 15th-16th centuries [1].

Historically, this plant has been widely used, the composition of its berries, essential oil, and mastic-gum resin being more studied than that of the leaves [2,3]. The essential oil from the berries is used to rub on the body as well as to cook in time of scarcity. Recently, studies on the composition of essential oil from leaves of this plant have reported antimutagenic and antibacterial activities [4]. Berries from *P. lentiscus* are rich in anthocyanins, which confer antioxidant capacity and induce autophagy, a mechanism to enhance chemoprevention [5]. In the same way, the resin from this plant has exhibited anti-inflammatory and antioxidant properties in patients with Crohn's disease [6]. The leaves are applied in traditional medicine for treatment of such diseases as eczema, diarrhoea, and throat infections especially of infusions form [7].

P. lentiscus leaves contain different types of secondary metabolites. Among these, the most abundant compounds in *P. lentiscus* leaves are reportedly flavonoids, which have displayed a powerful antioxidant capacity [8] as well as hepatoprotective, anti-inflammatory, and anticancer effects [9,10,11]. Thus, the characterization of bioactive compounds from *P. lentiscus* leaf extracts has relevance for developing nutraceuticals and dietary supplements.

A prerequisite for investigating the bioavailability and biochemical effect of any dietary phytochemical is to know the qualitative and quantitative composition as well as occurrence of the metabolite group in a given plant species. Metabolomics is a growing field of analytical chemistry that focuses on identifying small metabolites and

has become a powerful tool in agriculture and food science [12]. Within metabolomics, metabolite profiling can be defined as an analytical method for relative quantification of a number of metabolites from biological samples, restricting itself to a certain range of compounds or even to screening a pre-defined number of members of a compound class [13].

In this way, mass spectrometric methods are by the far the most widely used in the field of metabolomics. Specifically, LC-DAD-MS with electrospray ionization (ESI) is being used increasingly for screening botanical metabolites [14]. In this sense, the characterization of polyphenols and other polar compounds from *P. lentiscus* has been profitably performed by RP-HPLC with spectrophotometric detection coupled to MS. This technique, using electrospray ionization (ESI) as an interface, is a powerful method because of its highly efficient resolution and characterization of a wide range of polar compounds, allowing the separation of compounds by polarity differences. The mass accuracy and true isotopic pattern provided by QTOF-MS for both precursor and fragment ions enabled the determination of many well-known compounds present in *P. lentiscus* by facilitating additional information key for determining the elemental composition.

The aim of this study is to present a complete and exhaustive analysis of a methanol extract from *P. lentiscus* leaves. High performance liquid chromatography (HPLC) with mass spectrometry (MS) was used to characterize the polyphenolic fraction and other related compounds. Their structures were studied and proposed by electrospray ionization tandem mass spectrometry (ESI-MS/MS). These bioactive metabolites were submitted to a quantitative analysis that may help in the future to design nutraceutical formulations and will be the basis for new research into activities of the various compounds found in *P. lentiscus.*

❖ Materials and methods

Plant material

The leaves of *P. lentiscus* were harvested in April 2009, in remote areas in the suburbs of Aboudaou, City of Bejaia, Algeria. The samples were identified in the Botany laboratory of the University of Bejaia, where the voucher specimen was deposited. The *P. lentiscus* leaves were air-dried in the shade at room temperature. After drying, the plant material was ground to a fine powder (diameter < 250 µm) using an electric mill (IKAR A11 basic, Germany) and 4 g of this powder was exhaustively extracted by maceration with 50 mL of methanol, at room temperature for 24 h. The solution was filtered and concentrated to dryness under reduced pressure in a rotary evaporator (40°C). Finally, the sample was stored at −20ºC to avoid any possible degradation until used.

Chemicals

All chemicals were of analytical reagent grade and used as received. Standards of kaemperol-3-glucoside, (+)-catechin, quercetin-3-O-glucoside, luteolin, myricetin, gallic acid and syringic acid were from Sigma-Aldrich (Steinheim, Germany). Methanol used for the extraction of the phenolic compounds from the *P. lentiscus*, were from Panreac (Barcelona, Spain), acetonitrile from Lab-Scan (Dublin, Ireland) and acetic acid from Sigma-Aldrich (Steinheim, Germany). Distilled water with a resistance of 18.2 MΩ was deionized in a Milli-Q system (Bedford, MA, USA).

Sample preparation

Of the *P. lentiscus* extract, 1 mg was dissolved in 1 mL methanol, vortex-mixed for 1 min in a G560E Vortex-genie 2 (scientific Industries, Bohemia, NY, USA), filtered with a cellulose acetate syringe filter (0.2 µm pore size), and injected directly into the HPLC system.

Chromatographic separation

HPLC analyses were made using an Agilent 1200 Series Rapid Resolution LC system (Agilent Technologies, Palo Alto, CA, USA), equipped with a vacuum degasser, an autosampler, a binary pump and a Diode Array Detector (DAD). The column used for the chromatographic separation was a Zorbax Eclipse Plus C_{18} (1.8 µm, 150 x 4.6 mm) (Agilent Technologies, Palo Alto, CA, USA).

The chromatographic parameters were optimized for the best resolution, sensitivity, analysis time, and peak shape. Finally, the compounds from the *P. lentiscus* leaf extract were separated at room temperature with a gradient elution program at a flow rate of 0.8 mL/min. The gradient elution was conducted using water with 0.25% of acetic acid as eluent A and methanol as eluent B. The following multi-step gradient was applied: 0 min, 5% B; 10 min, 40% B; 25 min, 51% B; 35 min, 68% B; 38 min, 95% B; and finally a conditioning cycle of 7 min with the same conditions for the next analysis. The injection volume in the HPLC system was 10 µL. The compounds separated were monitored with DAD, and peak spectra were recorded between 190 and 450 nm.

ESI-QTOF-MS conditions

HPLC system was coupled to micrOTOF-Q II (Bruker Daltonik, Bremen, Germany) equipped with an ESI interface operating in negative ion mode, considering a mass range of 50–1100 *m/z* and using a capillary voltage of + 4000 V; dry gas temperature, 210ºC; dry gas flow, 8.0 L/min; nebulizer pressure, 2.0 bar; and spectra rate 1 Hz. The flow delivered into the MS detector from HPLC was split using a flow splitter 0.5 for stable electrospray ionization and reproducible results. Moreover, automatic MS/MS experiments were performed adjusting the collision energy values as follows: m/z 100, 20 eV; m/z 500, 25 eV; m/z 1000, 30 eV; and using nitrogen as the collision gas. For the necessary mass accuracy to identify compounds, external instrument calibration was used. For this, the calibrant used was sodium acetate clusters consisting of 5 mM sodium hydroxide and water:2-propanol 1:1 (v/v) with

0.2% of acetate acid was injected at the beginning of the run using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, Illinois, USA) directly connected to the interface, equipped with a Hamilton syringe (Reno, Nevada, USA).

The accuracy of the mass data for the molecular ions was controlled by Data Analysis 4.0. Software (Bruker Daltonik) offering a list of possible elemental formulas by using the GenerateMolecularFormula™ Editor. The Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double-bond equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (sigma value) for increased confidence in the suggested molecular formula.

❖ Results and discussion

Optimization of the HPLC-ESI-QTOF-MS method

The HPLC-ESI-QTOF-MS method was optimized using the *P. lentiscus* extract. The optimum parameters were selected according to the following criteria: resolution, analysis time, and peak shape.

The initial conditions consisted of a gradient elution using aqueous acetic acid 0.5% (v/v) as mobile phase A and acetonitrile as phase B at a flow rate of 0.5 mL/min. The following initial gradient was applied: 0 min, 95% B; 50 min, 5% B; and 55 min, 95% B. With 5 µL of sample injected, the starting conditions were held for 5 min.

The mobile phases, gradient, flow rate, and injection volume were optimized. The optimization began by testing the mobile phases A and B to determine which provided the best separation of the compounds. First, methanol and acetonitrile as well as different mixtures of the two (50:50 and 25:75, v/v) were tested as eluent B while maintaining the composition of eluent A (acidified water with 0.5% of acetic acid). The results are summarized in **Figure 1**. The resulting chromatograms displayed great differences in the separation of the studied compounds when different mobile phases B were used. The differences in the resolution, consequences of the different eluting power of acetonitrile and methanol, were especially remarkable in the separation of

polar compounds, which was better when using methanol 100% than acetonitrile or mixtures of acetonitrile/methanol as eluent B. In this way, these compounds were best separated and had the best resolution being important for a subsequent quantification. Therefore, methanol was selected as the organic phase. The next step was to test different percentages of acetic acid to acidify the Milli-Q water for mobile phase A. The acetic acid concentration was varied from 0.25 to 2.0% (0.25%, 0.5%, 0.75%, 1%, 1.5%, and 2%), corresponding to pH values from 2.67 to 3.08. When using 0.25% of acetic acid as mobile phase A, the intensity and peak shape of compounds was significantly better. After the selection of water with 0.25% acetic acid and MeOH as mobile phases A and B, respectively, four different gradients were tested, of which the best results were achieved with the multistep linear gradient detailed in "Materials and Methods", corresponding to a final gradient of 45 min with an optimal chromatographic resolution. Flow rates ranging from 0.2 to 0.8 mL/min were evaluated. The best compromise in terms of efficiency, resolution, and analysis time was found with a flow rate of 0.8 mL/min. Lastly, different injection volumes were tested: 1, 2.5, 5, 7.5, and 10 μL. The best chromatographic resolution was attained with a volume of 10 μL.

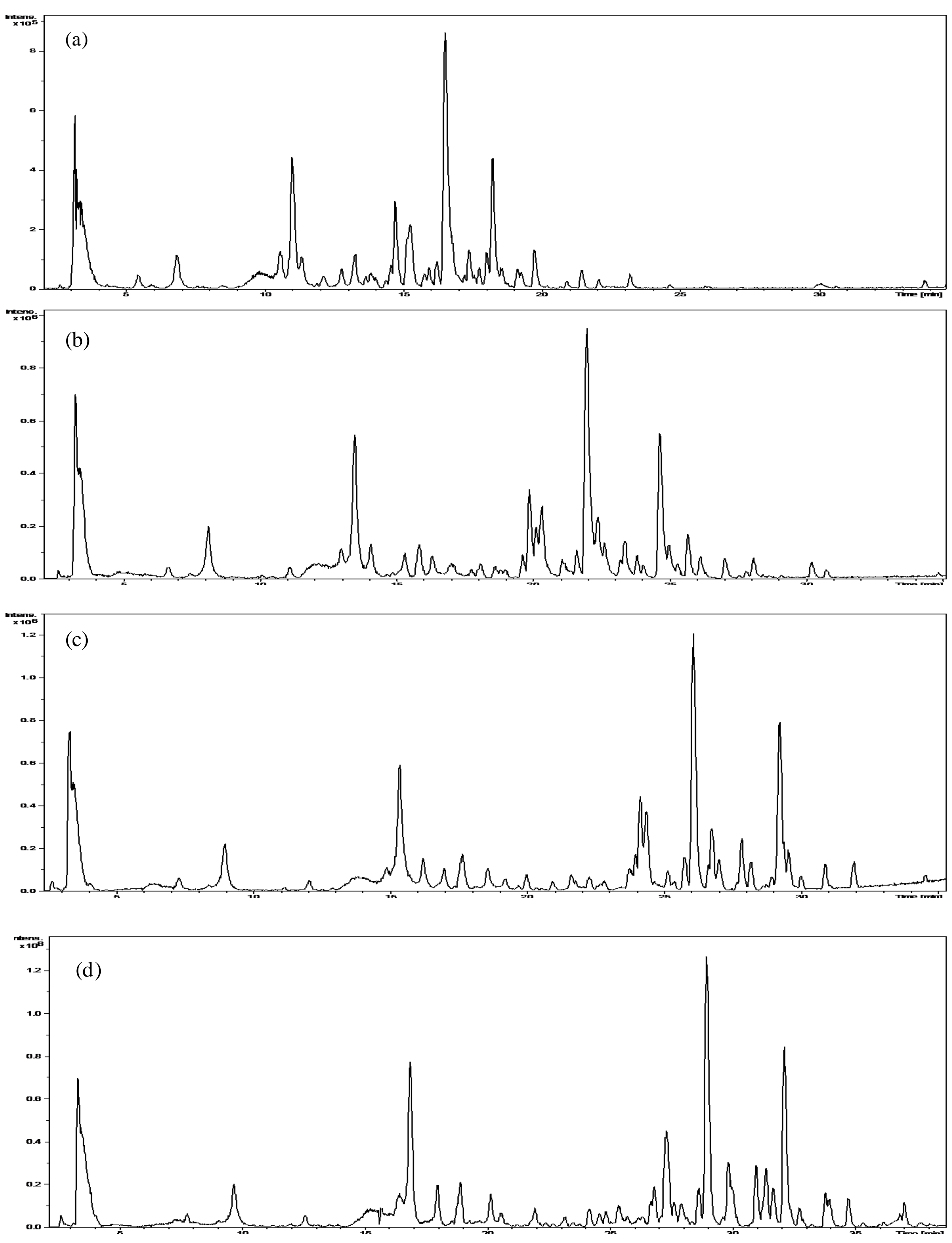


Figure 1. Comparison of the base peak chromatograms (BPC) obtained by HPLC using different mobile phase B: (a), acetonitrile 100%; (b), methanol:acetonitrile 50:50 (v/v); (c), methanol:acetonitrile 25:75 (v/v); (d), methanol 100%. The mobile phase A was water with 0.5% acetic acid.

Assessment of the HPLC-ESI-QTOF-MS method

The sensitivity of the method was studied by defining the limits of detection (LODs) and limits of quantification (LOQs) for individual compounds in standard solutions (Table 1). LOD and LOQ were, respectively, set at S/N = 3 and S/N = 10, where S/N is the signal-to-noise ratio. Intraday and interday precisions values were measured to evaluate the repeatability of the method. *Pistacia lentiscus* extract was injected (n=3) during the same day, to determine the intraday precision, for 3 consecutive days to estimate the interday precision (n=8). The relative standard deviations (RSDs) of analysis time and peak area were determined. Intraday repeatability of the method developed (for all the analytes) was from 0.21% to 2.07%, whereas the interday repeatability was from 3.39% to 12.72%.

Table 1. Analytical parameters of the method

Analyte	**RSD interday (n=8)**	**RSD intraday (n=3)**	**LOD (µg/mL)**	**LOQ (µg/mL)**	**Calibration range (µg/mL)**	**Calibration equations**	**R^2**
Kaemperol-3- glucoside	8.20%	2.07%	0.004 ± 0.001	0.012 ± 0.004	0.05-1	y = 0,5491x + 0,0399	0,9969
(+)-Catechin	3.39%	0.21%	0.017 ± 0.004	0.055 ± 0.013	1-15	y = 0,114x + 0,1196	0.9944
Quercetin-*3*-*O*-glucoside	9.59%	0.44%	0.006 ± 0.001	0.047 ± 0.01	0.25-10	Y = 0,3392x + 0,042	0,9984
Luteolin	8.60%	0.24%	0.003 ± 0.001	0.01 ± 0.002	0.05-1	y = 0,4317x + 0,0103	0.9991
Myricetin	8.04%	0.73%	0.02 ± 0.01	0.06 ± 0.02	0.25-25	y = 0,1814x + 0,0449	0,9953
Gallic acid	12.72%	0.98%	0.12 ± 0.04	0.40 ± 0.12	LOQ-1	y = 0,0062x + 0,0002	0.9944

Compound identification

The base peak chromatogram of *P. lentiscus* leaves resulting from the HPLC-ESI-QTOF method previously developed is shown in **Figure 2**. The compounds characterized are listed in **Table 2**, identified with the numbers 1-46 according to their elution order. The compounds identified were classified into four groups: flavonoids, hydroxycinnamic acid derivatives, phenolic acid derivatives, and other polar compounds. All these compounds were identified by interpreting their MS and MS/MS spectra obtained by QTOF-MS and also taking into account the data from the literature.

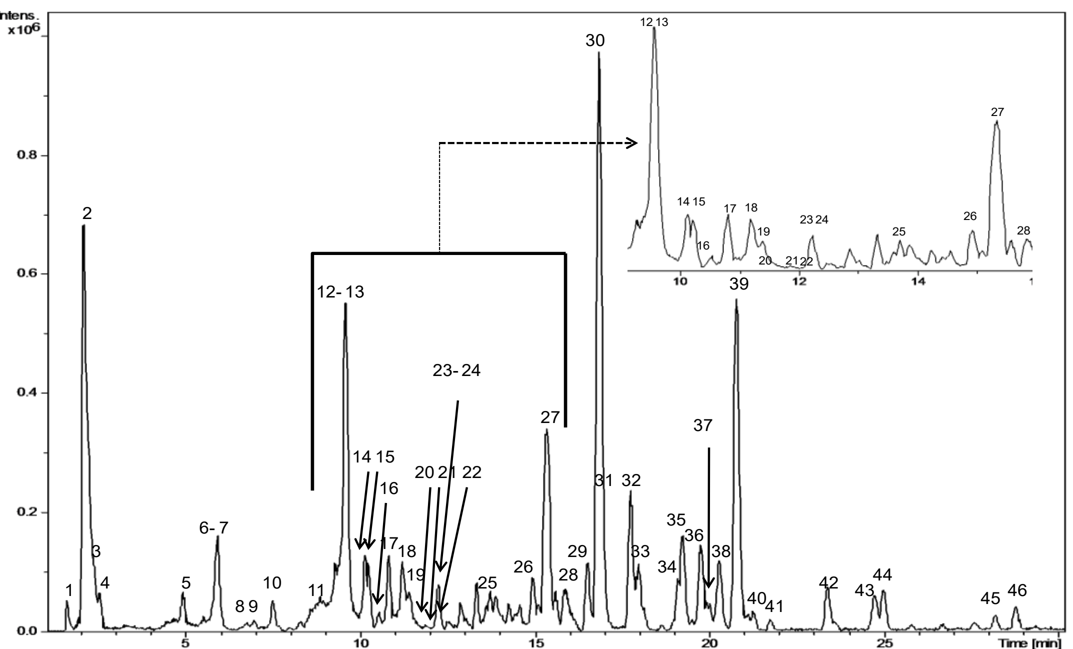


Figure 2. Base peak chromatogram (BPC) of *P. lentiscus* by HPLC-ESI-TOF-MS in the negative ion mode. Peak labeling represents the identified compounds.

Table 2. HPLC-ESI-TOF-MS data of the compounds identified

Peak[a]	RT (min)	m/z experimental	Molecular formula	m/z calculated	Error (ppm)	mSigma value	Tolerance (ppm)	MS/MS Fragments	Proposed compound	References
Flavonoids										
10	7.56	305.0677	$C_{15}H_{14}O_7$	305.0667	3.3	16.4	15	125.0237 (100.0), 167,0237 (31,2), 219,0675 (31,2)	D-Gallocatechin	[27]
15	10.30	289.0727	$C_{15}H_{14}O_6$	289.0718	3.3	5.6	15	245.0825 (100.0), 203.0691 (85.2), 109.0291 (84.8), 125.0245 (78.9)	Catechin *	[15, 28]
23	12.64	417.0823	$C_{20}H_{18}O_{10}$	417.0827	1.0	22.6	15	284.0339 (100.0)	Kaempferol arabinoside (Isomer 1)	[29]
26	15.05	391.1992	$C_{25}H_{28}O_4$	391.1915	19.8	27.9	20	161.0461 (61.2)	Glabrol	[30]
27	15.42	479.0846	$C_{21}H_{20}O_{13}$	479.0831	3.2	5.5	15	316.0246 (61.5), 317.0288 (22.3)	Myricetin galactoside	[15]
28	15.96	615.1000	$C_{28}H_{24}O_{16}$	615.0992	1.4	28.2	15	317.0293 (100.0), 463.0909 (7.6)	Myricetin galloyl rhamnopyranoside (isomer 1)	[27]
29	16.57	449.0732	$C_{20}H_{18}O_{12}$	449.0725	1.5	13.2	15	316.0243 (100.0), 317.0288 (30.1)	Myricetin O-xyloside	[31]
30	16.91	463.0927	$C_{21}H_{20}O_{12}$	463.0882	9.7	6.3	15	316.0242_(91.2)	Myricetin O-rhamnoside (isomer 1)	[27, 15]
32	17.85	463.0899	$C_{21}H_{20}O_{12}$	463.0882	3.6	15.4	15	316.0229 (100)	Myricetin O-rhamnoside (isomer 2)	[15, 27]
33	18.05	463.0875	$C_{21}H_{20}O_{12}$	463.0882	1.6	22.2	15	301.0352 (42.0)	Quercetin glycoside (isomer 1)	[32, 15]
34	19.20	433.0816	$C_{20}H_{18}O_{11}$	433.0776	9.2	7.1	15	300.0284 (100.0), 301.0356 (37.0)	Quercetin arabinopyranoside	[15, 32]
35	19.33	463.0908	$C_{21}H_{20}O_{12}$	463.0882	5.5	9.5	15	301.0350 (100.0)	Quercetin glycoside (isomer 2)	[32, 15]

Table 2. *(Continued)*

Peak[a]	RT (min)	m/z experimental	Molecular formula	m/z calculated	Error (ppm)	mSigma value	Tolerance (ppm)	MS/MS Fragments	Proposed compound	References
37	20.09	615.1066	$C_{28}H_{24}O_{16}$	615.0992	12.1	16.1	15	317.0332 (17.8)	Myricetin galloyl rhamnopyranoside (isomer 2)	[27]
38	20.39	615.0973	$C_{28}H_{24}O_{16}$	615.0992	3.0	10.9	15	317.0307 (19.3)	Myricetin galloyl rhamnopyranoside (isomer 3)	[27]
39	20.89	447.0952	$C_{21}H_{20}O_{11}$	447.0933	4.2	4.5	15	285.0421 (3.7)	Kaempferol-3-glucoside *	[32]
41	21.89	417.0817	$C_{20}H_{18}O_{10}$	417.0827	2.5	23.4	15	284.0337 (100.0)	Kaempferol arabinoside (isomer 2)	[32]
42	23.50	447.0963	$C_{21}H_{20}O_{11}$	447.0933	6.8	17.3	15	285.0424 (100.0)	Kaempferol glycoside	[32]
43	24.87	599.1042	$C_{28}H_{24}O_{15}$	599.1101	0.1	26.1	15	301.0373 (48.2)	Quercitrin-O-gallate	[33]
44	25.10	431.0994	$C_{21}H_{20}O_{10}$	431.0984	2.4	7.2	15	285.0399 (100.0)	Kaempferol rhamnoside	[34]
46	28.88	285.0413	$C_{15}H_{10}O_6$	285.0405	3.1	20.7	15	203.0739 (100.0), 123.0477 (61.9), 109.0323 (56.1)	Luteolin *	[28]
					Phenolic acids and derivatives					
2	2.18	331.0152	$C_{13}H_{16}O_{10}$	331.0671	2.7	25.2	15	169.0143 (100.0), 151.0049 (45.7)	β-Glucogallin (isomer 1)	[35]
5	4.97	331.0688	$C_{13}H_{16}O_{10}$	331.0671	5.4	25.6	15	169.0154 (100.0)	β-Glucogallin (isomer 2)	[35]
6	5.97	343.0687	$C_{14}H_{16}O_{10}$	343.0671	4.8	36.5	15	191.0567 (100.0)	Galloyl quinic acid (isomer 1)	[15, 36]
8	6.94	331.0682	$C_{13}H_{16}O_{10}$	331.0671	3.5	33.8	15	169.0148 (100.0), 125.0234 (9.2)	β-Glucogallin (isomer 3)	[35]
9	7.04	315.0749	$C_{13}H_{16}O_9$	315.0722	8.7	31.6	20	153.0200 (54.3), 109.0283 (25.1)	Gentisic acid glycoside	[37]

Table 2. *(Continued)*

Peak[a]	RT (min)	m/z experimental	Molecular formula	m/z calculated	Error (ppm)	mSigma value	Tolerance (ppm)	MS/MS Fragments	Proposed compound	References
11	9.20	285.0662	$C_{12}H_{14}O_8$	285.0616	16.1	22.4	25	153.0194 (41.0)	Uralenneoside	[38]
12	9.65	343.0696	$C_{14}H_{16}O_{10}$	343.0671	7.3	6.8	15	191.0573 (100.0)	Galloyl quinic acid (isomer 2)	[15, 36]
13	9.65	495.0840	$C_{21}H_{20}O_{14}$	495.0933	18.2	4.7	15	343.0708 (100.0), 169,0166 (41,4), 191.0585 (39.3)	Digalloyl quinic acid (isomer 1)	[15, 36]
14	10.20	495.0812	$C_{21}H_{20}O_{14}$	495.0780	6.4	5.9	15	343.0708 (100.0), 191.0592 (54.0), 169.00168 (41.6)	Digalloyl quinic acid (isomer 2)	[15, 36]
17	10.89	183.0312	$C_8H_8O_5$	183.0299	7	19.9	15	124.0169 (71.2), 139.0056 (43.0), 111.0085 (17.8)	Gallic acid methyl ester	[27]
18	11.29	647.0933	$C_{28}H_{24}O_{18}$	647.0890	6.6	14	15	495.0785 (100.0)	Pistafolin A (isomer 1)	[15, 36]
19	11.34	647.0865	$C_{28}H_{24}O_{18}$	647.0890	3.9	29.1	15	495.0760 (100.0), 343.0770 (19.8)	Pistafolin A (isomer 2)	[36, 15]
24	12.64	647.0727	$C_{28}H_{24}O_{18}$	647.0890	25.7	42.3	15	343.0718 (39.4), 495.0762 (100.0)	Pistafolin A (isomer 3)	[36, 15]
					Other polar compounds					
1	1.66	201.0239	$C_{11}H_6O_4$	201.0193	2.7	65.6	50	157.0341 (100.0)	Xanthotoxol	[39]
3	2.41	173.0436	$C_7H_{10}O_5$	173.0455	11.5	6.55	15	137.0256 (100.0), 111.0434 (72.1), 93.0319 (65.5)	Shikimic acid (isomer 1)	[40]
4	2.58	173.0433	$C_7H_{10}O_5$	173.0455	12..8	8.8	15	137.0258 (100.0), 93.0324 (79.6)	Shikimic acid (isomer 2)	[40]
7	5.97	191.0577	$C_7H_{12}O_6$	191.0561	7.5	4.8	15	127.0413 (6.6),	Quinic acid (isomer 1)	[36, 15]
16	10.62	443.1937	$C_{21}H_{32}O_{10}$	443.1923	3.2	27	15	101.0236 (5.7), 340.8748 (8.0)	Penstemide	[24]

Table 2. *(Continued)*

Peak[a]	RT (min)	m/z experimental	Molecular formula	m/z calculated	Error (ppm)	mSigma value	Tolerance (ppm)	MS/MS Fragments	Proposed compound	References
20	11.69	293.1231	$C_{12}H_{22}O_8$	293.1242	3.6	76.2	15	131.0726 (100)	methyl glucopyranosyloxy pentanoic acid	--
21	11.99	293.1208	$C_{12}H_{22}O_8$	293.1242	11.6	76.2	15	131.0710 (67.1)	methyl glucopyranosyloxy pentanoic acid	--
22	12.33	445.2122	$C_{21}H_{34}O_{10}$	445.2079	9.7	48.0	15	401.0214 (19.5), 282.9553 (21.4)	Sacranoside A	[41]
25	13.83	381.1797	$C_{16}H_{30}O_{10}$	381.1766	8.1	23.4	15	249.1325 (100.0)	Everlastoside C	[21]
31	16.93	465.2312	$C_{21}H_{38}O_{11}$	465.2341	0.9	20.2	15	405.2127 (100.0), 179.0574 (37.7), 161.0471 (17.7)	Cymal 3	[42]
36	19.92	431.2295	$C_{21}H_{36}O_9$	431.2287	1.9	12.5	15	371.2086 (100.0), 161.0445 (48.1)	Neorehmannioside	[43]
40	21.36	349.0923	$C_{17}H_{18}O_8$	349.0929	1.8	3.3	15	165.0395 (100.0)	feruloyl-γ-quinide	[44]
Lignans										
45	28.35	521.2222	$C_{30}H_{34}O_8$	521.2181	7.9	65.1	15	167.1117 (100.0), 293.0890 (28.4), 149.0458 (20.0)	Benzoylgomisin H	[45]

[a]Peak numbers assigned according to the overall elution order, *Compounds characterized by standard

Flavonoids

Twenty peaks were identified as flavonoids, most of them previously reported in *P. lentiscus* or other plants belonging to Anacardiaceae family. Peaks 15, 39, and 46 were identified as (+)-catechin, kaempferol-3-glucoside, and luteolin respectively, by comparison with authentic standards. As expected in RP-HPLC, myricetin glycosides eluted first because of the larger number of hydroxyl groups, followed by quercetin glycosides and kaempferol glycosides. In this group of compounds, the most frequent fragmentation corresponded to the loss of the glycoside moiety. This was true of peak 10, identified as D-gallocatechin, as well as peaks 29, 30, and 32 which were characterized as myricetin xyloside and myricetin rhamnoside isomer 1 and isomer 2 respectively, and peaks 33, 35, and 34, characterized as quercetin glycoside isomer 1 and isomer 2, and quercetin arabinopyranoside, respectively. Compound 42 (m/z 447) was characterized as kaempferol glycoside and compound 44 corresponded to kaempferol rhamnoside. Compound 27 was characterized as myricetin galactoside with fragments of 316 and 317 described in literature [15] while peaks 28, 37 and 38 (m/z 615) were characterized as myricetin galloyl rhamnopyranoside isomers. Peaks 37 and 38 had fragment with m/z 317 corresponding with the loss of myricetin group. In addition, peak 21 had another fragment, m/z 463, which corresponded to the loss of galloyl group.

The loss of pentafuranose moiety was another typical fragmentation in some flavonids. This occurred in peaks 23 and 41, both with m/z 417, which were characterized as kaempferol arabinoside isomer 1 and isomer 2, respectively.

Peak 43 MS/MS spectrum gave a fragment at m/z 301 corresponding to the loss of the gallic acid rhamnoside moiety. This was characterized as quercitrin gallate, being described in *P. lentiscus* for the first time. Certain healthy benefits have been reported, such as the prevention of diabetic complications and improvement of the intestinal inflammatory response [16].

Peak 26 (m/z 391) had a representative fragment at m/z 161 corresponding to the loss of one flavone ring with the methylated chain. It is present in the plant

kingdom, but it had never before been described in *P. lentiscus*. This was characterized as glabrol, which is a bioactive flavanone which has been reported to decrease the cholesterol absorption [17] and it is associated with the modulation of the expression of a group of genes that regulate glucose and lipid metabolism [18].

Phenolic acid and derivatives

Thirteen compounds were identified as phenolic acids or derivatives in the *P. lentiscus* leaves extract. Gentisic acid glycoside (peak 9) showed an MS^2 spectrum with fragments at m/z 153 and 109 in accordance with the loss of glycose group followed by a decarboxylation of gentisic acid. This compound has been found in *P. lentiscus* for the first time.

Peaks 2, 5, and 8 were designated as β-glucogallin isomers. All of these compounds had a common fragment at m/z 169, corresponding to the loss of the glucose moiety, while peak 2 also showed another fragment at m/z 151 due to the subsequent dehydration of the gallic acid moiety. In the case of peak 8, it presented an additional fragment at 125, corresponding to the decarboxylation of the galloyl group. These compounds have been described in other plants from the Anacardiaceae family, but this was characterized in *P. lentiscus* leaves for the first time.

Compounds 6 and 12 presented a molecular ion at m/z 343 and a fragment at m/z 191. Therefore, they were described as isomers of galloyl quinic acid and the fragment was due to the loss of gallic acid. One peak was characterized as methyl gallate (peak 17). Its MS^2 spectra provided two majority fragments at m/z 139 and 125 corresponding to the loss of a CO_2 molecule and the subsequent elimination of a methyl group. Compounds 18, 19, and 24 with a molecular ion at m/z 647 were identified as pistafolin A isomers. The fragments at m/z 343 and 495 have been previously reported in *P. lentiscus* as successive losses of two molecules of gallic acid. Similarly, compounds 13 and 14 were reported in *P. lentiscus* as digalloyl quinic acid with m/z 495 and they presented fragments at m/z 343, 191 and 169 corresponding to two successive losses of gallic acid ($[M-152-H]^-$ and $[M-304-H]^-$), and the fragment of gallic acid, respectively. MS/MS spectrum of compound 11 showed fragments at m/z

153 due to the loss of xylose residue. This compound was identified as uralenneoside, which has never been described in *P. lentiscus*, nor in any other member of the Anacardiaceae family, but it has been reported in other plants.

Other polar compounds

Besides the previously mentioned phenolic compounds identified, with this method it was possible to identify other 10 polar compounds present in this fraction characterized by HPLC-ESI-QTOF-MS. All of the compounds were described in *P. lentiscus* leaves for the first time, except for peak 7, which corresponded to quinic acid. It presented the largest fragment, at m/z 127 ([M-H-CO-2H_2O]$^-$).

Compound at m/z 521 was proposed as benzoylgomisin H. The fragmentation pattern of this compound is shown in **Figure 3a**. This lignan has never been characterized in *P. lentiscus* and it has a great potential antioxidant capacity [19]. Compounds eluted at RT 2.41 and 2.58 min were elucidated as shikimic acid isomers. The fragment at m/z 93 has been previously described in the literature [20] as [M-H-CO_2-H_2O]$^-$. The peak with a retention time of 13.83 min (m/z 381) was assigned to everlastoside c, and its MS/MS spectrum showed one fragment at m/z 249. This compound as well as its fragmentation pattern had previously been described in the plant kingdom [21]. Peaks 20 and 21, with a molecular ion at m/z 293, were tentatively proposed as methyl glucopyranosyloxy pentanoic acid isomers. The fragmentation pathway proposed for this compound consisted of the loss of the aliphatic chain, leaving a fragment corresponding to m/z 131. Peak with a RT 16.93 (m/z 465) was proposed as cymal 3, the fragments of which were 405, 179 and 161. The fragments corresponding to the loss of glucose (179) followed by dehydration (161). Consecutively breaking of the glucose ring was produced as a [M-H-60] fragment according to the proposed fragmentation pattern by Gao *et al.* [22]. The fragmentation pattern is shown in **Figure 3b**. Compound with RT 21.36 min was tentatively described as feruloyl-γ-quinide, a lactone with a fragment at m/z 165 corresponding to the loss of feruloyl moiety. Peak 22 (m/z 445) was characterized as sacranoside A. The MS^2 spectrum of this glicosylated terpene presented a fragment at m/z 282 and 401.

The fragmentation pathway is represented in **Figure 3d**. The compound eluting at 19.92 min was proposed as the carotenoid neorehmannioside, which showed fragments at m/z 371 and 161, corresponding to the loss of four methyl groups followed by a cleavage of the glucosidic bond.

The compound 1 was identified as xanthotoxol, a coumarin which had not previously been described in *P. lentiscus*. It presented a fragment at m/z 157 consistent with the loss of CO_2. This coumarin has been used to treat Alzheimer's disease, to inhibit human cytochrome P450, and to retard the formation of fibril and β-amyloid [23]. Besides, iridoid penstemide was characterized for the first time in *P. lentiscus* on basis of breaking up the molecule described in **Figure 3c**, which produced the fragments at m/z 101 and 340. It reportedly has anti-inflammatory and antitumor properties [24].

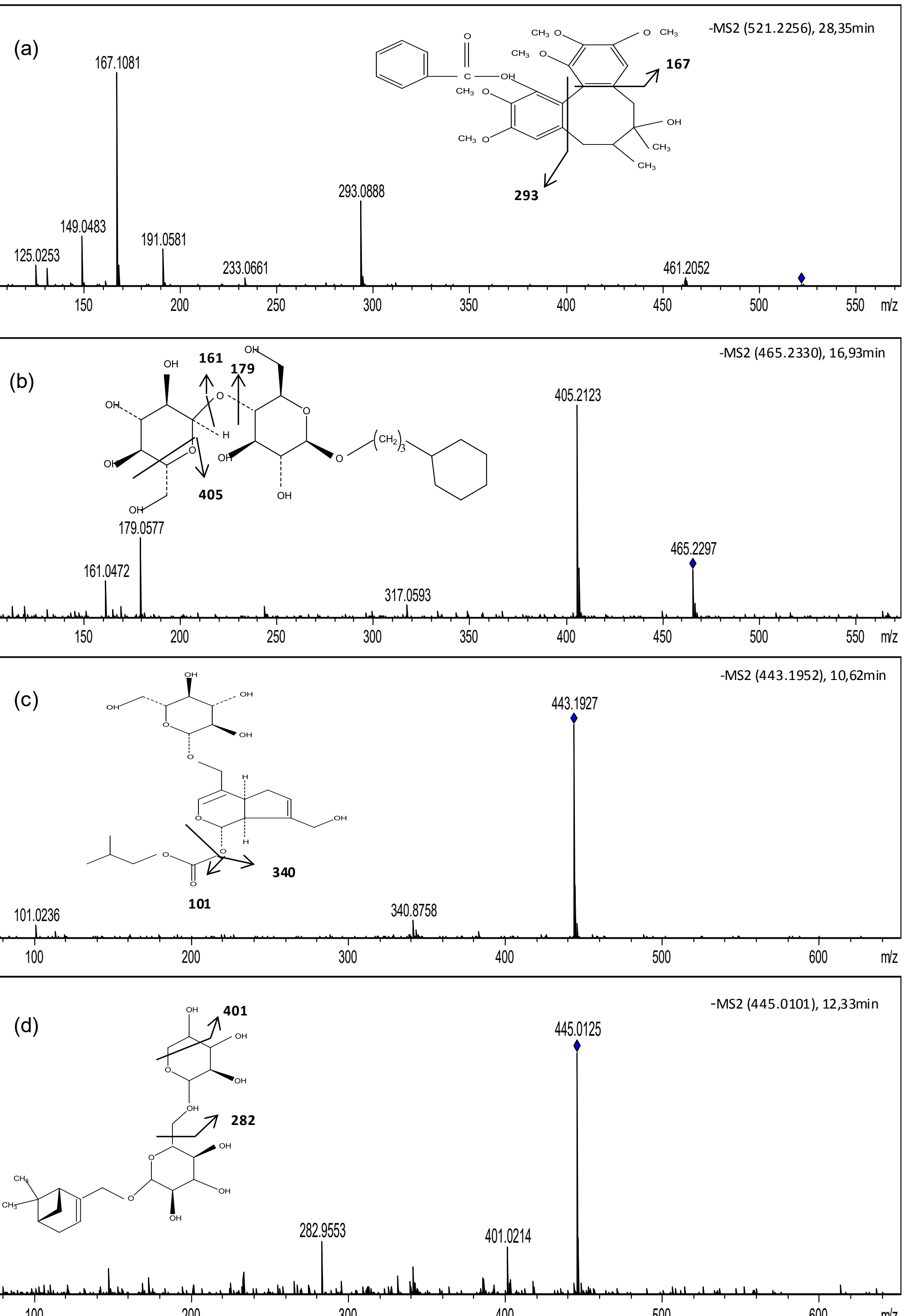


Figure 3. Fragmentation pattern for (a) benzoylgomisin H; (b) cymal 3; (c) penstemide and (d) sacranoside A

Quantification of phenolic compounds in *P. lentiscus* extract

Six standard calibration graphs for quantifying the main compounds found in *P. lentiscus* extract were prepared using the following commercial standards: kaempferol-3-glucoside, (+)-catechin, quercetin-3-O-glucoside, luteolin, myricetin and gallic acid. All calibration curves presented good linearity between different concentrations. The calibration showed a strong correlation between peak areas and analyte concentrations, and regression coefficients were higher than 0.99 in all cases (**Table 1**).

Quantification with HPLC-MS indicates the area of the peak for each m/z ratio but the mass data have great variability and can change over the course of several days, depending on the instrument, or even within the same day. The chosen internal standard (syringic acid) met all our requirements both in terms of its retention time, which did not interfere with other metabolites, and in terms of compensating for potential variations in instrumental analysis.

Catechin, luteolin and kaempferol-*3-O*-glucoside were quantified by the calibration curves determined from their respective commercial standards. The other compounds, which had no commercially available standards, were tentatively quantified with other compounds having similar structures. Trigalloyl quinic acid, gallic acid methyl ester, as well as β-glucogallin and its isomer were quantified using the gallic acid calibration curve; kaempferol arabinoside and the isomer of kaempferol glucoside were quantified using the kaempferol-*3-O*-glucoside calibration curve; myricetin glycoside and its isomers were quantified using a myricetin calibration curve; and quercetin glucoside and its isomers were quantified with the quercetin-*3-O*-glucoside calibration curve. The quantitative results are presented in **Table 3**. Myricetin glycoside isomers, quercitrin gallate, catechin and β-glucogallin were the compounds with the highest concentrations in *P. lentiscus* leaves. The high concentration of these compounds makes the plant worthy of further research, since the properties previously described as therapeutic could lead to the development of new therapies to treat diabetes and diabetic complications, to improve inflammatory

response, to strengthen neuroprotection in neurodegenerative diseases, and to help alleviate aspects of the ageing process [16,25,26].

Table 3. Phenolic compounds in *P. lentiscus* leaves methanol extract expressed in mg/kg of plant (n = 3).

Peak number	Phenolic compounds	mg/kg plant
2	β-glucogallin	296 ± 9
8	β-glucogallin isomer	2214.461 ± 15.55
15	Catechin	3354.78 ± 36.81
17	Gallic acid methyl ester	298.576 ± 7.63
18	Pistafolin A	59.631 ± 1.29
23	Kaempferol glycoside	114.93 ± 4.83
27	Myricetin galactoside	1650.197 ± 16.01
28	Myricetin galloyl rhamnopyranoside (isomer 2)	276.892 ± 6.82
38	Myricetin galloyl rhamnopyranoside (isomer 3)	67.346 ± 6.28
29	Myricetin O-xylosde	449.188 ± 4.81
30	Myricetin O-rhamnoside (isomer 1)	6216.125 ± 157.79
32	Myricetin O-rhamnoside (isomer 2)	986.654 ± 12.19
33	Quercetin glycoside (isomer 1)	117.468 ± 1.89
35	Quercetin arabinopyranoside	367.556 ± 5.80
37	Myricetin glycoside	489.882 ± 5.64
39	Kaempferol-3-glucoside	37.203 ± 0.89
42	Kaempferol glycoside isomer	78.548 ± 1.28
43	Quercitrin O-gallate	1160.002 ± 2.3
46	Luteolin	100.954 ± 0.05

❖ Conclusions

A powerful HPLC-ESI-QTOF-MS method has been developed and validated in order to characterize *P. lentiscus* leaves in methanol extract. This method is of particular importance in the metabolic profiling of plants due to the diversity of phytochemicals that a plant usually contains. This method allowed the tentative identification of 46 compounds on the basis of their chromatographic retention, MS and MS/MS spectra in negative ionization mode and data from the literature. The most representative the groups were flavonoids, phenolic acids and derivatives, among other polar compounds. To our knowledge, 20 of these compounds are tentatively identified in *P. lentiscus* leaves for the first time. The importance of the knowledge of plants is increasing and our study may help in the future design of a formulation of nutraceutical preparations and will serve as the base of new research into activities of the various compounds found in *P. lentiscus.*

Acknowledgements

This work was supported by the project AGL2011-29857-C03-02 and AP2010-1551 (Spanish Ministry of Science and Innovation), as well as P09-CTS-4564, P10-FQM-6563 and P11-CTS-7625 (Andalusian Regional Government Council of Innovation and Science). The authors are also grateful to Danone Institute for a grant.

References

[1] S. Jarić, M. Mitrović, L. Djurdjević, O. Kostić, G. Gajić, D. Pavlović, P. Pavlović, Phytotherapy in medieval Serbian medicine according to the pharmacological manuscripts of the Chilandar Medical Codex (15-16th centuries), J. Ethnopharmacol. 137 (2011) 601-619, doi: 10.1016/j.jep.2011.06.016.

[2] S. Paraschos, P. Magiatis, P. Gousia, V. Economou, H. Sakkas, C. Papadopoulou, A.L. Skaltsounis, Chemical investigation and antimicrobial properties of mastic water and its major constituents, Food Chem. 129 (2011) 907-911, doi: 10.1016/j.foodchem.2011.05.043.

[3] H. Trabelsi, O.A. Cherif, F. Sakouhi, P. Villeneuve, J. Renaud, N. Barouh, S. Boukhchina, P. Mayer, Total lipid content, fatty acids and 4-desmethylsterols accumulation in developing fruit of *Pistacia lentiscus* L. growing wild in Tunisia, Food Chem. 131 (2012) 434-440, doi: 10.1016/j.foodchem.2011.08.083.

[4] F.B. Douissa, N. Hayder, L. Chekir-Ghedira, M. Hammami, K. Ghedira, A.M. Mariotte, M.G. Dijoux-Franca, New study of the essential oil from leaves of *Pistacia lentiscus* L. (Anacardiaceae) from Tunisia, Flavour Fragrance J. 20 (2005) 410-414, doi: 10.1002/ffj.1445.

[5] L. Longo, F. Platini, A. Scardino, O. Alabiso, G. Vasapollo, L. Tessitore, Autophagy inhibition enhances anthocyanin-induced apoptosis in hepatocellular carcinoma, Mol. Cancer Ther. 7 (2008) 2476-2485, doi: 10.1158/1535-7163.MCT-08-0361.

[6] A. Gioxari, A.C. Kaliora, A. Papalois, G. Agrogiannis, J.K. Triantafillidis, N.K. Andrikopoulos, Pistacia lentiscus resin regulates intestinal damage and inflammation in trinitrobenzene sulfonic acid-induced colitis, J. Med. Food. 14 (2011) 1403-1411, doi: 10.1089/jmf.2010.0240.

[7] A. Markovics, I. Cohen, H. Muklada, T.A. Glasser, L. Dvash, E.D. Ungar, H. Azaizeh, S.Y. Landau, Consumption of *Pistacia lentiscus* foliage alleviates coccidiosis in young goats, Vet. Parasitol. (2011).

[8] G. Piluzza, S. Bullitta, Correlations between phenolic content and antioxidant properties in twenty-four plant species of traditional ethnoveterinary use in the Mediterranean area, Pharm. Biol. 49 (2011) 240-247, doi: 10.3109/13880209.2010.501083.

[9] S. Janakat, H. Al-Merie, Evaluation of hepatoprotective effect of *Pistacia lentiscus*, *Phillyrea latifolia* and *Nicotiana glauca*, J. Ethnopharmacol. 83 (2002) 135-138, doi: 10.1016/S0378-8741(02)00241-6.

[10] J. Qiao, A. Li, X. Jin, J. Wang, Mastic alleviates allergic inflammation in asthmatic model mice by inhibiting recruitment of eosinophils, Am. J. Resp. Cell Mol. Biol. 45 (2011) 95-100, doi: 10.1165/rcmb.2010-0212OC.

[11] A. Esmat, F.A. Al-Abbasi, M.M. Algandaby, A.Y. Moussa, R.M. Labib, N.A. Ayoub, A.B. Abdel-Naim, Anti-inflammatory activity of *Pistacia khinjuk* in different

experimental models: Isolation and characterization of its flavonoids and galloylated sugars, J. Med. Food. 15 (2012) 278-287, doi: 10.1089/jmf.2011.0099.

[12] J.M. Cevallos-Cevallos, D.B. Futch, T. Shilts, S.Y. Folimonova, J.I. Reyes-De-Corcuera, GC-MS metabolomic differentiation of selected citrus varieties with different sensitivity to citrus huanglongbing, Plant Physiol. Biochem. 53 (2012) 69-76, doi: 10.1016/j.plaphy.2012.01.010.

[13] O. Fiehn, Metabolomics - The link between genotypes and phenotypes, Plant Mol. Biol. 48 (2002) 155-171, doi: 10.1023/A:1013713905833.

[14] J.M. Harnly, S. Bhagwat, L.Z. Lin, Profiling methods for the determination of phenolic compounds in foods and dietary supplements, Anal. Bioanal. Chem. 389 (2007) 47-61, doi: 10.1007/s00216-007-1424-7.

[15] A. Romani, P. Pinelli, C. Galardi, N. Mulinacci, M. Tattini, Identification and quantification of galloyl derivatives, flavonoid glycosides and anthocyanins in leaves of *Pistacia lentiscus* L, Phytochem. Anal. 13 (2002) 79-86, doi: 10.1002/pca.627.

[16] B. Romier, Y.J. Schneider, Y. Larondelle, A. During, Dietary polyphenols can modulate the intestinal inflammatory response, Nutr. Rev. 67 (2009) 363-378, doi: 10.1111/j.1753-4887.2009.00210.x.

[17] Y. Zeng, J.X. Song, X.C. Shen, Herbal remedies supply a novel prospect for the treatment of atherosclerosis: A review of current mechanism studies, Phytother. Res. 26 (2012) 159-167, doi: 10.1002/ptr.3587.

[18] M. Kuroda, Y. Mimaki, S. Honda, H. Tanaka, S. Yokota, T. Mae, Phenolics from *Glycyrrhiza glabra* roots and their PPAR-γ ligand-binding activity, Bioorg. Med. Chem. 18 (2010) 962-970, doi: 10.1016/j.bmc.2009.11.027.

[19] Y.W. Choi, S. Takamatsu, S.I. Khan, P.V. Srinivas, D. Ferreira, J. Zhao, I.A. Khan, Schisandrene, a dibenzocyclooctadiene lignan from *Schisandra chinensis*: Structure-antioxidant activity relationships of dibenzocyclooctadiene lignans, J. Nat. Prod. 69 (2006) 356-359, doi: 10.1021/np0503707.

[20] P. Flores, P. Hellín, J. Fenoll, Determination of organic acids in fruits and vegetables by liquid chromatography with tandem-mass spectrometry, Food Chem. 132 (2012) 1049-1054, doi: 10.1016/j.foodchem.2011.10.064.

[21] M. Gómez-Romero, A. Segura-Carretero, A. Fernández-Gutiérrez, Metabolite profiling and quantification of phenolic compounds in methanol extracts of tomato fruit, Phytochemistry. 71 (2010) 1848-1864, doi: 10.1016/j.phytochem.2010.08.002.

[22] Q. Gao, U. Nilsson, L.L. Ilag, C. Leck, Monosaccharide compositional analysis of marine polysaccharides by hydrophilic interaction liquid chromatography-tandem mass spectrometry, Anal. Bioanal. Chem. 399 (2011) 2517-2529, doi: 10.1007/s00216-010-4638-z.

[23] C. Xiao, Z.G. Song, Z.Q. Liu, Synthesis of methyl-substituted xanthotoxol to clarify prooxidant effect of methyl on radical-induced oxidation of DNA, Eur. J. Med. Chem. 45 (2010) 2559-2566, doi: 10.1016/j.ejmech.2010.02.044.

[24] M. Domínguez, J.G. Avila, A. Nieto, C.L. Céspedes, Anti-inflammatory activity of *Penstemon gentianoides* and *Penstemon campanulatus*, Pharm. Biol. 49 (2011) 118-124, doi: 10.3109/13880209.2010.503708.

[25] S. Mandel, M.B.H. Youdim, Catechin polyphenols: Neurodegeneration and neuroprotection in neurodegenerative diseases, Free Radic. Biol. Med. 37 (2004) 304-317, doi: 10.1016/j.freeradbiomed.2004.04.012.

[26] S. Bastianetto, Y. Dumont, Y. Han, R. Quirion, Comparative neuroprotective properties of stilbene and catechin analogs: Action via a plasma membrane receptor site?, CNS Neurosci. Ther. 15 (2009) 76-83, doi: 10.1111/j.1755-5949.2008.00074.x.

[27] A.J. Hou, L.Y. Peng, Y.Z. Liu, Z.W. Lin, H.D. Sun, Gallotannins and related polyphenols from *Pistacia weinmannifolia*, Planta Med. 66 (2000) 624-626, doi: 10.1055/s-2000-8633.

[28] J. Vaya, S. Mahmood, Flavonoid content in leaf extracts of the fig (*Ficus carica* L.), carob (*Ceratonia siliqua* L.) and pistachio (Pistacia lentiscus L.), Biofactors. 28 (2006) 169-175.

[29] R.S. Compagnone, A.C. Suarez, L. Sciacovelli, F. Delle Monache, Arabinofuranoside flavonoids from *Tapirira guianensis*, Bol. Soc. Chil. Quim. 42 (1997) 445-448.

[30] M.S. Buchanan, A.R. Carroll, G.A. Fechner, A. Boyle, M. Simpson, R. Addepalli, V.M. Avery, P.I. Forster, G.P. Guymer, T. Cheung, H. Chen, R.J. Quinn, Small-molecule inhibitors of the cancer target, isoprenylcysteine carboxyl methyltransferase,

from Hovea parvicalyx, Phytochemistry. 69 (2008) 1886-1889, doi: 10.1016/j.phytochem.2008.04.011.

[31] I.O. Vvedenskaya, N. Vorsa, Flavonoid composition over fruit development and maturation in American cranberry, *Vaccinium macrocarpon* Ait., Plant Sci. 167 (2004) 1043-1054, doi: 10.1016/j.plantsci.2004.06.001.

[32] S.A. Kawashty, S.A.M. Mosharrafa, M. El-Gibali, N.A.M. Saleh, The flavonoids of four Pistacia species in Egypt, Biochem. Syst. Ecol. 28 (2000) 915-917, doi: 10.1016/S0305-1978(99)00113-1.

[33] M.S. Marzouk, F.M. Soliman, I.A. Shehata, M. Rabee, G.A. Fawzy, Flavonoids and biological activities of *Jussiaea repens*, Nat. Prod. Res. 21 (2007) 436-443, doi: 10.1080/14786410600943288.

[34] M. Nawwar, S. Hussein, N. Ayoub, A. Hashim, R. El-Sharawy, U. Lindequist, M. Harms, K. Wende, Constitutive phenolics of *Harpephyllum caffrum* (Anacardiaceae) and their biological effects on human keratinocytes, Fitoterapia. 82 (2011) 1265-1271, doi: 10.1016/j.fitote.2011.08.014.

[35] R. Niemetz, G.G. Gross, Gallotannin biosynthesis: β-glucogallin: Hexagalloyl 3-O-galloyltransferase from *Rhus typhina* leaves, Phytochemistry. 58 (2001) 657-661, doi: 10.1016/S0031-9422(01)00300-4.

[36] M.C. Baratto, M. Tattini, C. Galardi, P. Pinelli, A. Romani, F. Visioli, R. Basosi, R. Pogni, Antioxidant activity of galloyl quinic derivatives isolated from *P. lentiscus* leaves, Free Radic. Res. 37 (2003) 405-412, doi: 10.1080/1071576031000068618.

[37] F. Ieri, M. Innocenti, L. Andrenelli, V. Vecchio, N. Mulinacci, Rapid HPLC/DAD/MS method to determine phenolic acids, glycoalkaloids and anthocyanins in pigmented potatoes (*Solanum tuberosum* L.) and correlations with variety and geographical origin, Food Chem. 125 (2011) 750-759, doi: 10.1016/j.foodchem.2010.09.009.

[38] D.Q. Yu, Y. Chen, X.T. Liang, Structural chemistry and biological activities of natural products from Chinese herbal medicines - Part II, Res. Commun. Mol. Pathol. Pharmacol. 108 (2000) 393-436.

[39] Y. Xie, W. Zhao, T. Zhou, G. Fan, Y. Wu, An efficient strategy based on MAE, HPLC-DAD-ESI-MS/MS and 2D-prep-HPLC-DAD for the rapid extraction,

separation, identification and purification of five active coumarin components from radix angelicae dahuricae, Phytochem. Anal. 21 (2010) 473-482, doi: 10.1002/pca.1222.

[40] B. Avula, Y.H. Wang, T.J. Smillie, I.A. Khan, Determination of shikimic acid in fruits of illicium species and various other plant samples by LC-UV and LC-ESI-MS, Chromatographia. 69 (2009) 307-314, doi: 10.1365/s10337-008-0884-z.

[41] S. Nakamura, X. Li, H. Matsuda, K. Ninomiya, T. Morikawa, K. Yamaguti, M. Yoshikawa, Bioactive constituents from Chinese natural medicines. XXVI. Chemical structures and hepatoprotective effects of constituents from roots of *Rhodiola sachalinensis*, Chem. Pharm. Bull. 55 (2007) 1505-1511, doi: 10.1248/cpb.55.1505.

[42] T. Hibi, S. Aoki, K. Oda, S. Munemasa, S. Ozaki, O. Shirai, Y. Murata, N. Uozumi, Purification of the functional plant membrane channel KAT1, Biochem. Biophys. Res. Commun. 374 (2008) 465-469, doi: 10.1016/j.bbrc.2008.07.026.

[43] S. Fu, D. Arráez-Roman, A. Segura-Carretero, J.A. Menéndez, M.P. Menéndez-Gutiérrez, V. Micol, A. Fernández-Gutiérrez, Qualitative screening of phenolic compounds in olive leaf extracts by hyphenated liquid chromatography and preliminary evaluation of cytotoxic activity against human breast cancer cells, Anal. Bioanal. Chem. 397 (2010) 643-654, doi: 10.1007/s00216-010-3604-0.

[44] K. Redeuil, C. Smarrito-Menozzi, P. Guy, S. Rezzi, F. Dionisi, G. Williamson, K. Nagy, M. Renouf, Identification of novel circulating coffee metabolites in human plasma by liquid chromatography-mass spectrometry, J. Chromatogr. A. 1218 (2011) 4678-4688, doi: 10.1016/j.chroma.2011.05.050.

[45] W.H. Ma, J.C. He, P.S. Duan, T. Han, C.J. Zheng, L.P. Qin, Dibenzocyclooctane lignans from the stems of *Schisandra bicolor*, Biochem. Syst. Ecol. 38 (2010) 1156-1159, doi: 10.1016/j.bse.2010.10.011.

Chapter 5

Comparative characterization of phenolic and other polar compounds in Spanish melon cultivars by using high-performance liquid chromatography coupled to electrospray ionization quadrupole-time of flight mass spectrometry

Food Research International, 2013, 54:1519-1527

Food Research International

Volume 54, Issue 2, December 2013, Pages 1519–1527

Comparative characterization of phenolic and other polar compounds in Spanish melon cultivars by using high-performance liquid chromatography coupled to electrospray ionization quadrupole-time of flight mass spectrometry

C. Rodríguez-Pérez, R. Quirantes-Piné, A. Fernández-Gutiérrez, A. Segura-Carretero

Department of Analytical Chemistry, Faculty of Sciences, University of Granada, Fuentenueva s/n, E 18071 Granada, Spain

Functional Food Research and Development Center, Health Science Technological Park, Avenida del Conocimiento s/n, Edificio BioRegión, 18016 Granada, Spain

Abstract

Melon (*Cucumis melo L.*), belonging to the Cucurbitaceae family, is a significant source of phytochemicals which provide human health benefits. High-performance liquid chromatography coupled to electrospray ionization mass spectrometry quadropole-time of flight (HPLC-ESI-QTOF-MS) was used for the comprehensive characterization of 14 extracts from 3 Spanish varieties of melon (Galia, Cantaloupe, and Piel de Sapo). A total of 56 different compounds were tentatively identified, including: amino acids and derivatives, nucleosides, organic acids, phenolic acids and derivatives, alcohols, flavonoids, lignans, and other polar compounds. Of these, 25 were tentatively characterized for the first time in *C. melo* varieties. Principal-component analysis (PCA) was applied to gain an overview of the distribution of the melon varieties and to clearly separate the different varieties. The result of the PCA for the negative mode was evaluated. The variables most decisive to discriminate among varieties included 12 of the metabolites tentatively identified.

Keywords: *Cucumis melo*, characterization, phenolic compounds, tandem mass spectrometry, PCA.

❖ Introduction

Research on fruits and vegetables is increasing because of their benefits to human health. Indeed, seem to be that fruit consumption protect against chronic diseases including obesity, cardiovascular disease, and certain types of cancer (Jansen *et al.*, 2011; Boeing *et al.*, 2012). These effects have been attributed to the content in bioactive compounds.

Melon is one of the most widely cultivated and consumed fruits in the world. In Spain, more than 800,000 tons were produced in 2011, with a cultivation area exceeding 28,000 Ha (Seoane Spielgelberg, Rábade Rodríguez, & López Pérez, 2013), with Almería (Andalusia) being one of the main producer provinces. Spanish consumption was around 9.05 Kg per person in 2012. Melon (*Cucumis melo L.*), belongs to the family Cucurbitaceae, commonly known as cucurbits or the gourd family. In Spain, the most widely produced *Cucumis melo* groups are Cantalupensis and Inodorus, and the most consumed market varieties are Galia and Cantaloupe, belonging to Cantalupensis group, and Piel de Sapo, belonging to the Inodorus group.

Fruits, together with vegetables, represent the major source of phytochemicals and other compounds such as amino acids and fatty acids (Poiroux-Gonord, Bidel, Fanciullino, Gautier, Lauri-López, & Urban, 2010). In this sense, melon is a significant source of phytochemicals, mainly polyphenols and other antioxidants, which provide potential health benefits, especially aiding the cardiovascular system (Maietti *et al.*, 2012). Its chemical composition depends on the cultivar, environmental conditions, and also on the stage of fruit maturity (Villanueva, Tenorio, Esteban, & Mendoza, 2004).

In this context, some melon varieties have been studied in depth, mainly by gas chromatography, providing a thorough knowledge of the volatile composition, which is the major determinant of melon-fruit quality perceived by consumers (Bernillon *et al.*, 2012; Biais *et al.*, 2009; Kourkoutas, Elmore, & Mottram, 2006; Obando-Ulloa, Ruiz, Monforte, & Fernández-Trujillo, 2010; Pang, Chen, Hu, Zhang, & Wu, 2012).

By this technique, more than 240 volatile compounds have been tentatively identified in Galia or Cantaloupe melons while only 42 compounds have been tentatively identified in Piel de Sapo variety (Obando-Ulloa *et al.*, 2010). Other techniques such as quantitative nuclear magnetic resonance spectroscopy (^{1}H-NMR) or liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) have been applied to characterize the polar fraction of different melon varieties (Biais *et al.*, 2009; De Marino, Festa, Zollo, & Iorizzi, 2009; Moing *et al.*, 2011; Villanueva *et al.*, 2004), although the phytochemical composition of the polar fraction of these three typical Spanish varieties has not yet been completely characterized.

In the present work, using HPLC-ESI-QTOF-MS, we offer a complete and exhaustive characterization of 14 extracts from 3 different Spanish melon varieties (Galia, Cantaloupe, and Piel de Sapo), collected in different ripening periods. Furthermore, PCA was applied to the data set to evaluate the compounds responsible for discriminating between the varieties.

❖ Experimental

Chemicals

HPLC-MS acetonitrile and methanol were purchased from Fisher (Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leics, UK). Acetic acid of analytical grade (purity > 99.5%) was acquired from Fluka (Switzerland). Distilled water with a resistance of 18.2 MΩ was deionized in a Milli-Q system (Bedford, MA, USA).

Melon samples

Fourteen samples of three different varieties of melon (Galia Cantaloupe, and Piel de Sapo) were collected in 2011 from May to September from El ejido, Almería, Andalusia (Spain). After the harvest, the samples were washed with distilled water and transported directly to the lab under refrigeration conditions at 4ºC. The samples were stored at the same temperature until its treatment.

Sample treatment

Fresh melon samples were peeled and the seeds were removed. Later, 1.5 kg of the edible portion were crushed and placed on the lyophilizer shelf (Christ Alpha 1-2 LD Freeze dryer, Shropshire, UK), which was pre-cooled at -50ºC for 1 h at 1 mbar and then were frozen at -20ºC until analyze. The lyophilized samples were stored at -20ºC until analyzed. Afterwards, 0.5 g of lyophilized melons were extracted using 15 mL of 80:20 (v/v) methanol/H_2O, sonicated for 15 min and centrifugated at 984 × g and the supernatant was collected in a round-bottom flask. Next, the solvent was evaporated by using a rotary evaporator under vacuum at 40ºC, and the dried residue was redissolved in 2 mL of 80:20 (v/v) methanol/H_2O. Finally, the extract was filtered through a 0.2 µm syringe filter and analyzed.

Chromatographic separation

HPLC analyses were carried out using an Agilent 1200 Series Rapid Resolution LC system (Agilent Technologies, Palo Alto, CA, USA), equipped with a vacuum degasser, a binary pump, an autosampler, and a diode array detector (DAD). The column used for the chromatographic separation was a Zorbax Eclipse Plus C_{18} (1.8 µm, 150 mm × 4.6 mm) (Agilent Technologies, Palo Alto, CA, USA). Acidified water (0.5% acetic acid, v/v) and acetonitrile were used as mobile phases A and B, respectively. The gradient was programmed as follows: 0 min, 5% B;10 min, 30% B; 12 min, 33% B; 16 min, 38% B; 19 min, 50% B; 22 min, 95% B; 24 min 5% B, and finally a conditioning cycle of 10 min with the initial conditions. The flow rate was set at 0.80 mL/min throughout the gradient and the effluent from the HPLC column was split using a T-type splitter before being introduced into the mass spectrometer (split ratio = 1:3) to provide a stable spray and consequently reproducible results. The injection volume in the HPLC system was 10 µL.

ESI-QTOF-MS analyses

HPLC system was coupled to a micrOTOF-Q II mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with electrospray ionization (ESI) operating in

the negative mode. The detection was made considering a mass range of 50–1100 *m/z* and using a capillary voltage of + 4000 V, a dry gas temperature of 210ºC, a dry gas flow of 8.0 L/min, a nebulizer pressure of 2.0 bar, and spectra rate of 1 Hz. Moreover, automatic MS/MS experiments were performed using nitrogen as the collision gas and adjusting the collision energy values as follows: *m/z* 100, 20 eV; *m/z* 500, 25 eV; *m/z* 1000, 30 eV.

For the necessary mass accuracy to identify compounds, external instrument calibration was used. For this, the calibrant used was sodium acetate clusters consisting of 5 mM sodium hydroxide and water: 2-propanol 1:1 (v/v) with 0.2% of acetic acid. This calibrant was injected at the beginning of the run using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, IL, USA) directly connected to the interface, equipped with a Hamilton syringe (Reno, NV, USA). The accuracy of the mass data for the molecular ions was controlled by Data Analysis 4.0. Software (Bruker Daltonik) offering a list of possible elemental formulas by using the GenerateMolecularFormula™ Editor. The Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double-bond equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (sigma value) for increased confidence in the suggested molecular formula.

Principal component analysis

The data were evaluated with ProfileAnalysis for PCA after Pareto scaling (Bruker Daltonik, Bremen, Germany). The LC–MS data were prepared for PCA using FindMolecularFeature (FMF) peak finder algorithm and advanced bucketing. The LC–MS was integrated from 2-22 min in time and 49-1001 *m/z* in mass.

❖ Results and discussion

Compound identification

The base peak chromatograms of the three varieties of melon resulting from the HPLC-ESI-QTOF-MS method described above are shown in **Figure 1**, where the peaks are numbered according to their elution order. The compounds were tentatively identified by interpretation of their MS and MS/MS spectra determined by QTOF-MS and also considering the data from the literature and open-access mass-spectra databases such as Metlin and MassBank. **Table 1** summarizes the MS data of the compounds tentatively identified, including experimental and calculated *m/z* for the molecular formula provided, error, sigma value, and the main fragments obtained by MS/MS, as well as the proposed compound for each peak. The compounds characterized may be classified into different groups: amino acids and derivatives, nucleosides, organic acids, phenolic acids and derivatives, alcohols, flavonoids, lignans, and other polar compounds.

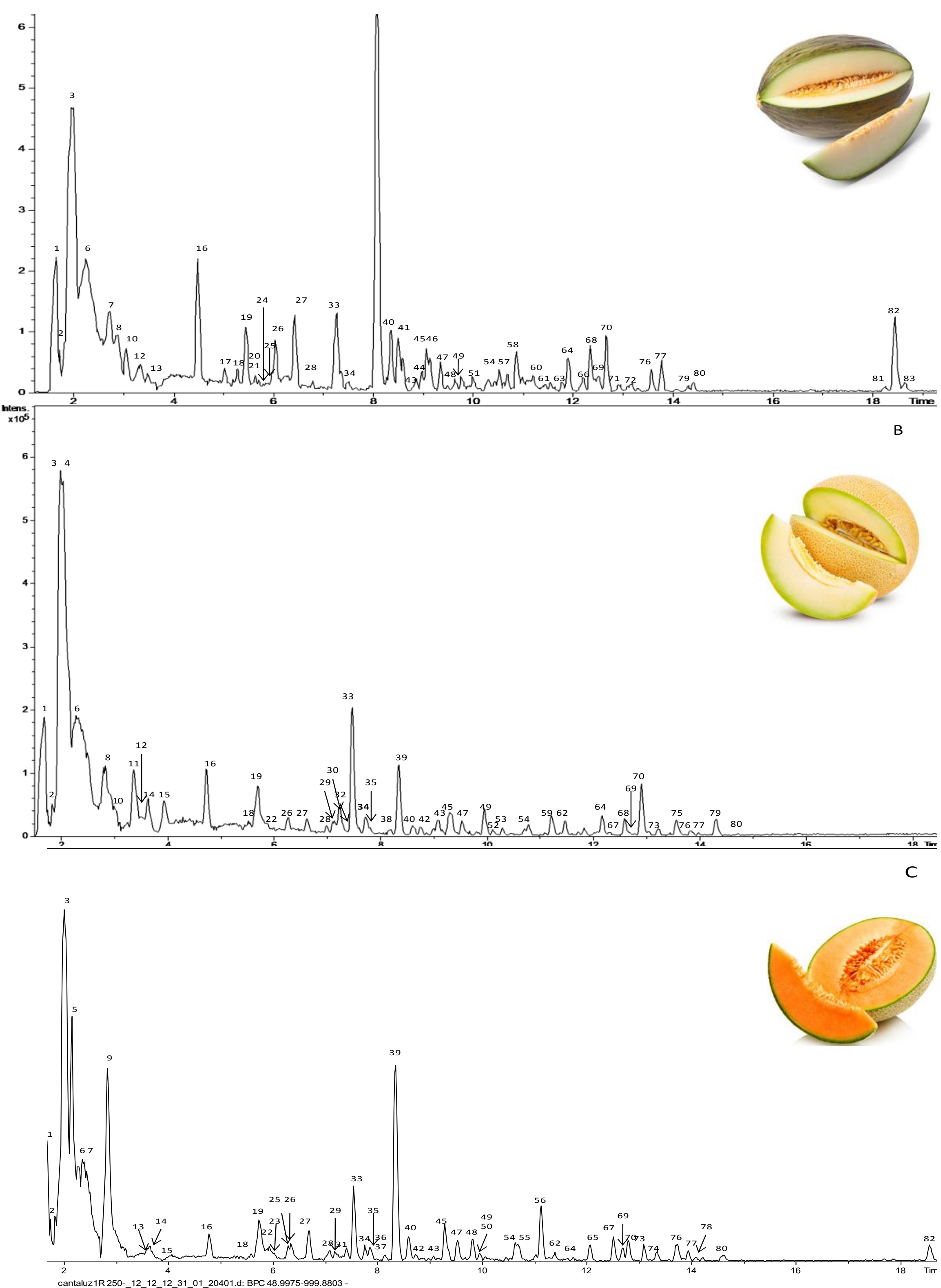


Figure 1. Base-peak chromatogram (BPC) of *C. melo* by HPLC-ESI-TOF-MS in the negative ion mode for (a) Piel de Sapo, (b) Galia and (c) Cantaloupe varieties.

Table 1. HPLC-ESI-TOF-MS data of the compounds identified.

Peak[a]	RT (min)	m/z experimental	Molecular formula	m/z calculated	Error (ppm)	mSigma value	MS/MS Fragments[b]	Proposed compound	Variety		
									Piel de Sapo	Galia	Cantaloupe
Amino acids and derivatives											
2	1.88	145.0629	$C_5H_{10}N_2O_3$	145.0619	6.9	6.9	127.0504 (100%)	Glutamine	+	+	+
9	**2.85**	**290.0880**	$C_{11}H_{17}NO_8$	**290.0881**	**0.4**	**18.5**	**128.0338 (100%), 200.0574 (31.2%)**	**Pyroglutamic acid hexoside**	**-**	**-**	**+**
13	3.66	180.0667	$C_9H_{11}NO_3$	180.0666	0.1	12.3	119.0497 (100%), 163.0412 (52.8%)	Tyrosine	+	-	+
14	3.70	292.1393	$C_{12}H_{23}NO_7$	292.1402	3.1	4.3	130.0873 (100%)	Hexose-leucine isomer 1	-	+	+
15	4.08	292.1381	$C_{12}H_{23}NO_7$	292.1402	7.1	7.6	130.0871 (100%)	Hexose-leucine isomer 2	-	-	+
19	5.47	164.0724	$C_9H_{11}NO_2$	164.0717	4.1	3.3	147.0462 (100%); 148.0519 (15.7%); 103.0555 (13%); 151.3955 (10.1%)	Phenylalanine	+	+	+
22	5.94	326.1233	$C_{15}H_{21}NO_7$	326.1245	3.7	9.1	164.0717 (100%)	hexose-phenylalanine	-	+	+
29	**7.09**	**203.0843**	$C_{11}H_{12}N_2O_2$	**203.0826**	**8.5**	**9.9**	**116.0503 (100%)**	**Tryptophan isomer 1**	**-**	**+**	**+**
33	7.51	203.0848	$C_{11}H_{12}N_2O_2$	203.0826	8.8	38.1	116.0514 (100%); 142.0660 (41.7%); 117.0564 (15.6%)	Tryptophan isomer 2	+	+	+
20	5.65	217.1192	$C_9H_{18}N_2O_4$	217.1194	0.8	5.7	130.0881 (100%), 187.1106 (45.4%)	Isoleucyl-serine	+	-	-
38	8.18	235.1075	$C_{12}H_{16}N_2O_3$	235.1088	5.6	14.8	217.1011 (100%)	Alanyl-phenylalanine	-	+	-
41	8.60	243.1712	$C_{12}H_{24}N_2O_3$	243.1714	0.7	13.3	199.1793 (100%)	Leucyl-isoleucine	+	-	-
58	10.86	358.1974	$C_{16}H_{29}N_3O_6$	358.1984	2.7	29.6	323.1626 (100%); 340.1845 (22.8%); 130.0876 (15.6%); 279.1797 (12.4%)	Asp-Leu-Leu/ Asp-Ile-Ile[c]	+	-	-
61	11.42	392.1803	$C_{19}H_{27}N_3O_6$	392.1827	6.1	12.5	357.1418 (100%)	Asp-Leu-Phe/ Asp-Ille-Phec	+	-	-

Table 1. *(Continued)*

Peak[a]	RT (min)	m/z experimental	Molecular formula	m/z calculated	Error (ppm)	mSigma value	MS/MS Fragments[b]	Proposed compound	Variety		
									Piel de Sapo	Galia	Cantaloupe
Nucleosides											
12	3.60	243.0624	$C_9H_{12}N_2O_6$	243.0623	0.6	10.1	110.0260 (100%); 152.0364 (37%)	Uridine	+	+	-
16	**4.78**	**282.0842**	$C_{10}H_{13}N_5O_5$	**282.0844**	**0.7**	**3.9**	**150.0426 (100%); 133.0158 (10.2%)**	**Guanosine**	+	+	+
Organic acids											
6	2.38	191.0207	$C_6H_8O_7$	191.0197	1	7.1	111.0091 (100%)	Citric acid isomer 1	+	+	+
7	2.75	191.0201	$C_6H_8O_7$	191.0197	2.1	2.3	111.0088 (100%)	Citric acid isomer 2	+	+	+
8	**2.85**	**191.0209**	$C_6H_8O_7$	**191.0197**	**6.2**	**3.5**	**111.0081 (100%)**	**Citric acid isomer 3**	+	-	-
39	**8.35**	**293.1249**	$C_{12}H_{22}O_8$	**293.1242**	**2.6**	**5.6**	**131.0710 (100%); 113.0208 (9%)**	**Hydroxybutanoic acid ethyl ester hexoside isomer 1**	+	+	+
40	8.36	293.1271	$C_{12}H_{22}O_8$	293.1242	10	7.7	131.0707 (100%); 113.0401 (10%)	Hydroxybutanoic acid ethyl ester hexoside isomer 2	+	+	+
76	13.68	187.0988	$C_9H_{16}O_4$	187.0976	6.6	10.8	125.0977 (100%)	Azelaic acid	+	+	+
81	18.21	327.2155	$C_{18}H_{32}O_5$	325.2177	6.6	16.6	211.1382 (52.9%)	Trihydroxyoctadecadienoic acid isomer 1	+	-	-
82	**18.51**	**327.2172**	$C_{18}H_{32}O_5$	**327.2177**	**1.7**	**11.8**	**211.1335 (79.1%); 229.1455 (58.6%); 171.1035 (26.5%)**	**9,12,13-Trihydroxyoctadeca dienoic acid**	+	-	+
83	18.58	327.2166	$C_{18}H_{32}O_5$	325.2177	3.5	13.3	171.1035 (52.9%)	Trihydroxyoctadecadienoic acid isomer 2	+	-	-

Table 1. *(Continued)*

Peak[a]	RT (min)	m/z experimental	Molecular formula	m/z calculated	Error (ppm)	mSigma value	MS/MS Fragments[b]	Proposed compound	Variety		
									Piel de Sapo	Galia	Cantaloupe
Phenolic acids and derivatives											
18	5.59	325.094	$C_{15}H_{18}O_8$	325.0929	3.3	27.3	164.0722 (100%)	p-Coumaric acid hexoside	+	+	+
21	5.72	315.0744	$C_{13}H_{16}O_9$	315.0722	7.2	21.15	152.0107 (100%); 153.0191 (70.2%); 108.0166 (30%)	Gentisic acid hexoside isomer 1	+	-	-
23	6.04	315.0705	$C_{13}H_{16}O_9$	315.0722	5.3	15	153.0192 (100%); 109.0286 (15.7%)	Gentisic acid hexoside isomer 2	-	-	+
24	6.22	315.0707	$C_{13}H_{16}O_9$	315.0722	4.6	7.7	152.0121 (100%); 153.0181 (86.1%); 108.0230 (70.6%)	Gentisic acid hexoside isomer 3	+	-	-
27	**6.69**	**475.1475**	$\mathbf{C_{20}H_{28}O_{13}}$	**475.1457**	**3.8**	**4.8**	**167.0352 (100%)**	**Vanillic acid dihexoside**	+	+	+
31	7.41	329.0877	$C_{14}H_{18}O_9$	329.0878	0.4	13.7	167.0358 (100%)	Vanillic acid hexoside	-	-	+
35	7.86	473.1654	$C_{21}H_{30}O_{12}$	473.1664	2.3	15	179.0725 (100%)	Coelovirin A/ Coelovirin B isomer 1	-	+	+
37	8.15	299.0755	$C_{13}H_{16}O_8$	299.0772	5.7	19.7	137.0222 (100%)	Hydroxybenzoic acid hexoside	-	-	+
42	8.75	473.1656	$C_{21}H_{30}O_{12}$	473.1664	0.4	19.9	179.0733 (100%)	Coelovirin A/ Coelovirin B isomer 2	-	+	+
50	9.95	193.0509	$C_{10}H_{10}O_4$	193.0506	1.4	3.6	134.0341 (100%)	Ferulic acid	-	-	+

Table 1. *(Continued)*

Peak[a]	RT (min)	m/z experimental	Molecular formula	m/z calculated	Error (ppm)	mSigma value	MS/MS Fragments[b]	Proposed compound	Variety		
									Piel de Sapo	Galia	Cantaloupe
Lignans											
48	9.80	537.1990	$C_{26}H_{34}O_{12}$	537.1978	0.5	24.1	327.1239 (100%); 195.0675 (57.7%); 179.0588 (23.5%); 489.1820 (17.7%)	Citrusin A isomer 1	+	-	+
55	10.62	537.2017	$C_{26}H_{34}O_{12}$	537.1978	7.4	16.9	327.1245 (100%); 195.0669 (40.9%); 179.0574 (37.9%); 161.0451 (27%)	Citrusin A isomer 2	-	-	+
64	11.91	521.2059	$C_{26}H_{34}O_{11}$	521.2028	5.9	104.7	359.1433 (100%)	Isolariciresinol hexoside/lariciresinol hexoside	-	-	+
Esters											
69	12.68	173.0833	$C_8H_{14}O_4$	173.0819	7.7	10.8	131.0722 (100%)	Butanedioldiacetate isomer 1	+	+	+
71	**13.15**	**173.083**	$\mathbf{C_8H_{14}O_4}$	**173.0819**	**6**	**15.1**	**131.0712 (100%)**	Butanedioldiacetate isomer 2	**+**	**-**	**-**
77	13.92	173.1184	$C_9H_{18}O_3$	173.1183	0.6	5	127.1130 (55%)	Methyl-4-butoxybutanoate	+	+	+
Flavonoids											
59	11.12	595.1704	$C_{27}H_{32}O_{15}$	595.1668	5.9	10.5	287.0561 (100%)	Eriodictyol rutinoside	-	+	-
67	12.56	607.1672	$C_{28}H_{32}O_{15}$	607.1668	0.6	24.6	299.0597 (100%); 145.5485 (46.5%)	Diosmetin rutinoside	-	+	-
70	**12.78**	**609.184**	$\mathbf{C_{28}H_{34}O_{15}}$	**609.1825**	**2.5**	**8.9**	**301.0734 (100%); 145.2260 (8,2%)**	**Hesperidin**	+	+	+

Table 1. *(Continued)*

Peak[a]	RT (min)	m/z experimental	Molecular formula	m/z calculated	Error (ppm)	mSigma value	MS/MS Fragments[b]	Proposed compound	Variety		
									Piel de Sapo	Galia	Cantaloupe
Other polar compounds											
3	2.03	341.1108	$C_{12}H_{22}O_{11}$	341.1089	8.2	9.7	165.0408 (100%); 113.0224 (21.2%); 161.0490 (8.4%); 101.0218 (6.5%)	Sucrose isomer 1	+	+	+
4	2.14	341.1095	$C_{12}H_{22}O_{11}$	341.1089	1.7	10	165.0417 (100%); 113.0245 (16.6%); 119.0358 (9.4%); 101.0272 (8.3%)	Sucrose isomer 2	-	+	-
11	**3.38**	**341.1081**	$C_{12}H_{22}O_{11}$	**341.1089**	**2.5**	**29.2**	**165.0417 (100%); 113.0245 (25%)**	**Sucrose isomer 3**	**-**	**+**	**-**
25	6.29	218.1039	$C_9H_{17}NO_5$	218.1034	2.2	8.6	146.0825 (100%)	Pantothenic acid	+	-	+
26	**6.34**	**380.1547**	$C_{16}H_{23}N_5O_6$	**380.1576**	**7.4**	**21.1**	**146.0835 (23.2%); 308.1354 (12.1%)**	**Zeatin hexoside**	**+**	**+**	**+**
43	**9.07**	**401.1461**	$C_{18}H_{26}O_{10}$	**401.1453**	**1.9**	**9.8**	**161.0440 (100%); 179.0587 (93%)**	**Benzyl alcohol-hexose-pentose isomer 1**	**+**	**+**	**+**
45	9.28	401.1466	$C_{18}H_{26}O_{10}$	401.1453	3.1	24.4	161.0446 (100%); 179.0597 (88%)	Benzyl alcohol hexose-pentose isomer 2	+	+	+
47	9.53	385.1843	$C_{19}H_{30}O_8$	385.1868	6.4	16.1	153.0925 (100%); 161.0505 (33.2%); 113.0225 (23.4%)	Vomifoliol hexoside (roseoside A)	+	+	+
52	10.10	589.2521	$C_{29}H_{42}O_{14}$	589.2502	3.2	17.8	161.0503 (13.1%); 179.0548 (100%); 367.2113 (70.3%)	Serrulatoside	-	+	+
54	10.50	415.1627	$C_{19}H_{28}O_{10}$	415.1610	4.3	9.8	149.2253 (100%)	Phenethyl alcohol hexose-pentose	+	+	+
80	14.67	329.1378	$C_{19}H_{22}O_5$	329.1394	4.9	14.4	211.1339 (54.9%); 229.1449 (43.6%); 171.1016 (11.3%)	Gibberellin A7	+	+	+

Table 1. *(Continued)*

Peak[a]	RT (min)	m/z experimental	Molecular formula	m/z calculated	Error (ppm)	mSigma value	MS/MS Fragments[b]	Proposed compound	Variety		
									Piel de Sapo	Galia	Cantaloupe
36	8.13	475.1832	$C_{21}H_{32}O_{12}$	475.1821	2.4	32.2	167.0353 (100%)	Cistanoside E	-	-	+

[a] Peak numbers assigned according to the overall elution order; Compounds in bold corresponding to the metabolites which constituted the major difference between the three varieties.
[b] Numbers in brackets show the relative abundance of each MS/MS fragment expressed in percentage. [c] Amino acids are denoted by the three letter code: Aspartic acid, Asp, isoleucine, lle, leeucine, Leu, phenylalanine, Phe.

Amino acids and derivatives

Several amino acids and derivatives were tentatively identified in the different melon varieties, most of them previously reported in *Cucumis melo* or Cucurbitaceae family (Bernillon *et al.*, 2012; Gonda *et al.*, 2010; Lamikanra, Chen, Banks, & Hunter, 2000; Moing *et al.*, 2011). In this way, single amino acids such as glutamine (peak 2), tyrosine (peak 13), phenylalanine (peak 19) and tryptophan (peaks 29 or 33) were found in these melon samples. These compounds generally presented a common fragmentation pattern due to the consecutive losses of ammonia and carbon dioxide. These amino acids were found in the three melon varieties except tyrosine, which was found only in the varieties Piel de Sapo and Cantaloupe.

Three dipeptides were also detected, namely, isoleucyl-serine (peak 20) and leucyl-isoleucine (peak 41) in Piel de Sapo, and alanyl-phenylalanine (peak 38) in the variety Galia. Isoleucyl-serine presented different fragments due to the loss of the formaldehyde group from serine (*m/z* 187) and the loss of the serine amino acid (*m/z* 130), while leucyl-isoleucine and alanyl-phenylalanine produced only one product ion due to decarboxylation and dehydration, respectively. Leucyl-isoleucine has previously been characterized in other fruits (Özcan & Şenyuva, 2006). Three amino acid derivatives were also characterized in Galia and Cantaloupe: pyroglutamic acid hexoside (peak 9), fructose-leucine (peaks 14 or 15), and fructose-phenyalanine (peak 22). The fructoside derivatives of leucine and phenylalanine presented a common fragmentation pattern consistent with the cleavage of the glycosidic bond which produced fragments at *m/z* 130 and 164, respectively. MS/MS spectrum of peak 9 showed two fragments at *m/z* 128 and 200 due to the losses of the hexoside moiety and the rupture of pyroglutamic acid. It is the first time in which these compounds have been characterized in *C. melo* or Cucurbitaceae family.

In addition to these compounds, two tripeptides were detected only in Piel de Sapo: peak 58 (Asp-leu-leu/Asp-Ile-Ile) and peak 61 (Asp-leu-phe/ Asp-Ile-phe). Peak 58 presented different fragments at *m/z* 340, 323, 279, and 130. This fragmentation pattern matched the dehydration (*m/z* 340) followed by the loss of one

amine terminal group (*m/z* 323), further decarboxylation (*m/z* 279), and finally the loss of leucine or isoleucione residue (*m/z* 130). The other tripeptide found in Piel de Sapo (peak 61) had a fragment at *m/z* 357 due to the loss of water and one amine terminal group. The main fragments were compared with the Metlin database.

Nucleosides

Two nucleosides, namely uridine and guanosine, were tentatively identified in the melon samples. Peak 12, found in Piel de Sapo and Galia, was characterized as uridine, showing a fragment at *m/z* 110 consistent with the uracil group (Kang, 2012). This nucleoside has been previously described in other varieties of melon (Cohen *et al.*, 2012). On the other hand, peak 16 was characterized as guanosine and presented a major fragment at *m/z* 150 corresponding to $[M\text{-}H\text{-pentose}]^-$ as well as another at *m/z* 133 due to the loss of the amino group. This nucleoside, which appeared in the three varieties, has been previously characterized in Cucurbitaceae family (Iswaldi, Gómez-Caravaca, Lozano-Sánchez, Arráez-Román, Segura-Carretero, & Fernández-Gutiérrez, 2013) but not in melon.

Organic acids

Organic acids, together with sugars and amino acids, are a widely represented group in melon samples that significantly contribute to the overall aroma of melon (Lignou, Parker, Oruna-Concha, & Mottram, 2013). One of the main organic acids that are accumulated in most of the fruits, including melon fruit is citric acid (Cohen *et al.*, 2012). Three citric acid isomers were found in all the varieties (peaks 6, 7, and 8) presenting a fragment at *m/z* 111 due to $[M\text{-}H\text{-}CO_2\text{-}2H_2O]^-$.

Compounds 39 and 40 were characterized as isomers of hydroxybutanoic acid ethyl ester-hexoside. This compound has been previously described in other melon varieties together with its fragment at *m/z* 131 and its presence has been associated with amino acids such as alanine, glutamine, isoleucine, phenylalanine, tryptophan or tyrosine (Bernillon *et al.*, 2012).

Peak 76 was characterized as azelaic acid, a 9-carbon saturated linear dicarboxylic acid found naturally in some plants (Vinholes *et al.*, 2011), especially in seed (Bondia-Pons *et al.*, 2013). Its fragment at *m/z* 125 is due to the loss of one of the acid groups and further dehydration, and it has been found in Cucurbitaceae (Dan & Thakur, 1995) but is the first time that this compound has been reported in *C. melo*.

Peak 82 was characterized as 9,12,13-trihydroxyoctadeca dienoic acid. This organic acid, tentatively identified for the first time in melon, was characterized mainly in the variety Piel de Sapo and also, in less intensity, in the variety Cantaloupe. It compound presented two main fragments at *m/z* 211 and 229 due to the C12-C13 bond cleavage and the subsequent dehydration of the formed product ion. Similarly, peaks 81 and 83 were characterized as trihydroxyoctadeca dienoic acid isomers, which presented an individual fragment at *m/z* 211 and at *m/z* 171, respectively.

Phenolic acids and derivatives

The predominant group of phenolic antioxidants in the melon samples studied was composed of phenolic acids, which may be further classified into hydroxybenzoic, hydroxycinnamic, and phenolic acid derivatives. Several hydroxycinnamic acids and derivatives were characterized in the samples under study such as ferulic acid (peak 50) and p-coumaric acid hexoside (peak 18). P-coumaric acid-hexoside was found in the three melon varieties and its fragment at *m/z* 164 has been previously described in Cucurbitaceae (Abu-Reidah, Arráez-Román, Quirantes-Piné, Fernández-Arroyo, Segura-Carretero, & Fernández-Gutiérrez, 2012). On the other hand, ferulic acid, which appeared only in the variety Cantaloupe, showed a fragment at *m/z* 134 corresponding to $[M\text{-}H\text{-}CH_3\text{-}CO_2]^-$. Other authors have previously identified ferulic acid derivatives in Escrito melon (Moing *et al.*, 2011).

Concerning hydroxybenzoic acid derivatives, isomers of gentisic acid hexoside (peaks 21, 23 or 24) and hydroxybenzoic acid hexoside (peak 37) were found in the melon samples analyzed. Gentisic acid hexoside isomers presented a fragment at *m/z* 153 matching with the gentisic acid moiety. These isomers were found in Piel de Sapo and Cantaloupe and, although these compounds are widely distributed in the plant

kingdom, they are described here in *C. melo* (and Cucurbitaceae) for the first time. Hydroxybenzoic acid hexoside, present only in Cantaloupe, has been previously described in other melon varieties together with its fragmentation pattern (Bernillon *et al.*, 2012).

Furthermore, three phenolic acid derivatives were characterized, namely vanillic acid dihexoside (peak 27), vanillic acid hexoside (peak 31), and coelovirin A or B isomers (peaks 35 or 42). In this group of compounds, the most frequent fragmentation corresponded to the loss of the hexoside moiety as in the case of coelovirin a or b with a fragment at *m/z* 179, as reported in the literature for other plants (Huang, Li, Shi, Mo, Wang, & Yang, 2004), but this is the first time in which it has been reported in Cucurbitaceae. The hexoside and dihexoside derivatives of vanillic acid both presented a fragment at *m/z* 167 due to the vanillic acid moiety. The dihexoside derivative appeared in the three varieties studied and has been previously characterized in other melon varieties (Bernillon *et al.*, 2012; Moing *et al.*, 2011) while the monohexoside has not been described before in *C. melo*. Cantaloupe was the variety that presented the most compounds from this group, while Piel de Sapo was the poorest in phenolic acid derivatives.

Lignans

Three lignans were characterized in Cantaloupe and one of them also appeared in Piel de Sapo: two citrusin A isomers (peaks 48 and 55) and isolariciresinol hexoside or lariciresinol hexoside (peak 64). The proposed fragmentation pattern of citrusin A is shown in **Figure 2a**.

Compound 55 showed the precursor ion and a fragment ion at *m/z* 521 and 359, respectively. The fragment ion corresponds to lariciresinol or isolariciresinol after the neutral loss of the hexose moiety. This compound has been described in some Cucurbitaceae species (Abu-Reidah, Arráez-Román, Quirantes-Piné, Fernández-Arroyo, Segura-Carretero, & Fernández-Gutiérrez, 2012) but not in melon fruit.

Figure 2. Fragmentation pattern for (a) citrusin A, (b) hesperidin, and (c) serrulatoside.

Esters

Melon aroma, determined by unique combinations of aroma-active compounds, strongly depends on the variety (Pang *et al.*, 2012). Among other compounds, alcohols take part in the aroma profile, which has been extensively investigated, especially by using gas chromatography. The method applied here enabled the identification of three alcohols in all three varieties.

Peaks 69 and 71 were characterized as isomers of butanedioldiacetate. These compounds presented a fragment at *m/z* 131, which has previously been described in other melon varieties (Moing *et al.*, 2011). The MS/MS spectrum of compound 77 showed a fragment at *m/z* 127 due to [M-HCOOH]. This compound was characterized as methyl-4-butoxybutanoate, which has been previously described in muskmelon (Xiao, Zhao, Wang, Dong, & Jiao, 2010).

Flavonoids

Flavonoids are the most common and widely distributed group in plant phenolic compounds, which are usually very effective antioxidants. Different flavonoids were found mainly in the variety Galia. Two of these, eriodictyol rutinoside and hesperidin, had never before been described in the family Cucurbitaceae or the species *C. melo*. Eriodictyol rutinoside (peak 59) showed a fragment at *m/z* 287 due to the loss of the rutinoside group. Hesperidin (Peak 70), the only flavonoid detected in all the three varieties of melon studied, presented the fragmentation pattern depicted in **Figure 2b** and it has been characterized in other fruits (Kuroyanagi *et al.*, 2008). Peak 67 has been tentatively identified as diosmetin rutinoside, since this compound and its fragment at *m/z* 299 have been previously reported in Cucurbitaceae (Siciliano, De Tommasi, Morelli, & Braca, 2004).

Other polar compounds

Besides the abovementioned compounds tentatively identified, another 12 polar compounds were tentatively identified from different families found in the study samples.

According to Biais *et al.* (Biais *et al.* 2010), the most important properties of melon for organoleptic quality and consumer acceptance are the aroma profile and the sucrose level. In this regard, 3 sucrose isomers (peak 3, 4, and 11), which showed fragments at *m/z* 101, 113, 119, and 161, were characterized on the basis of their previously described fragmentation pattern (Taylor, March, Longerich, & Stadey,

2005). Galia was the variety which contained all three of the sucrose isomers. In addition, one vitamin (peak 25) was also characterized in Piel de Sapo and Cantaloupe as pantothenic acid. This vitamin (B_5) with an hexose group has been previously described in melon fruit (Bernillon *et al.*, 2012; Moing *et al.*, 2011) and its assignment is consistent with the presence of the fragment at *m/z* 146 due to $[M-H-CO-CO_2]^-$, as described by Gómez-Romero (Gómez-Romero, Segura-Carretero, & Fernández-Gutiérrez, 2010). A phenolic compound derivative was characterized in the variety Cantaloupe as cistanoside E (peak 36) on the basis of its MS/MS spectrum, where a fragment at *m/z* 167 appeared due to the cleavage of the bond between the phenolic group and the dihexoside moiety.

Two isomers of benzyl alcohol-hexose-pentose (peaks 43 and 45) have previously been described in other melon varieties (Bernillon *et al.*, 2012) as well as their fragment at *m/z* 161 which has been reported in other fruits (Moco, Vervoort, Moco, Bino, De Vos, & Bino, 2007). Related to these compounds, peak with RT 10.50 was tentatively identified as phenethyl alcohol-hexose-pentose with a fragment at *m/z* 149, corresponding, to the pentose group of the hexose-pentose moiety. This compound has never before been described in *C. melo* or Cucurbitaceae but has been characterized in other fruits (Kuang, Xia, Yang, Zhang, & Li, 2009).

The iridoid serrulatoside was found in the variety Galia (peak 52) and has previously been reported in other plants (Bazylak, Rosiak, & Shi, 1996), although not in *C. melo* or in any other member of Cucurbitaceae. Its fragmentation pattern is shown in **Figure 2c**.

A cyclohexanone hexoside was found and characterized as roseoside A (peak 47). This compound presented two main fragments, at *m/z* 153 and 161, the first being due to the loss of the cyclohexanone moiety and the second one to the loss of the glycose moiety. This compound has been found in the plant kingdom (Champavier, Comte, Vercauteren, Allais, & Chulia, 1999; Iha, Matsunami, Otsuka, Kawahata, Yamaguchi, & Takeda, 2012) but not previously in the Cucurbitaceae family.

Finally, in the three varieties studied, two hormones were tentatively identified as gibberellin A7 (peak 80) and zeatin hexoside (peak 26). Gibberellin A7 presented fragments at *m/z* 211 and 229, in agreement with the fragmentation pattern recorded in MassBank database. In the case of zeatin hexoside, its fragments at *m/z* 308 and 146 were due to the loss of the hydrocarbon chain and the subsequent loss of the hexoside moiety from the product ion. Both are described in Cucurbitaceae as plant-growth-regulating hormones that play an essential role in the plant growth and development (Pimenta Lange *et al.*, 2013; Sidik, Hashim, Mohamad, & Abdullah, 2012).

Multivariate data analysis

For an overview of the distribution of the melon varieties and to explore chemical markers contributing to the classification, a PCA was performed. Firstly, a peak-finder algorithm was applied to combine ions belonging to one compound, such as common adducts, isotopes, and charge states. In this way, features were determined using LC-MS chromatograms acquired from each of the 14 melon samples. Then, the LC-MS data were prepared for the statistical analysis by generating a so-called variable or bucket table. The buckets represent pairs of retention-time and *m/z* values in such a way that their intensities are listed in the bucket table for each sample and a PCA was calculated based on this table.

The PCA scores plot (PC1 vs. PC2) shows a clear separation of the three melon varieties (**Figure 3a**). Piel de sapo is separated from the others on the first PC and galia and cantaloupe on the second. This plot also revealed that the varieties Cantaloupe and Galia are quite similar in composition but very different with respect to Piel de Sapo.

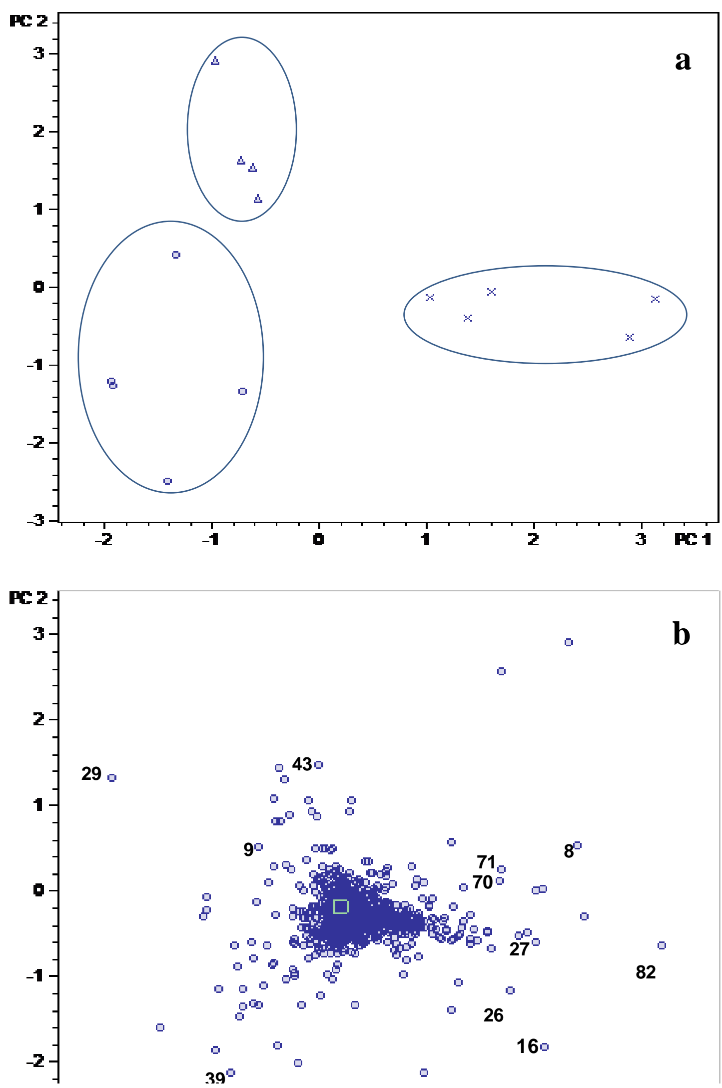


Figure 3. Principal Component Analysis to the composition of the three varieties: a) PCA scores plot (PC1 vs. PC2); Symbol shape corresponds to different varieties (cross = Piel de Sapo; triangle = Cantaloupe; circle = Galia); b) PCA loadings plot. Numbers identify the compounds reported in **Table 1** in bold.

The loadings plot shows how principal components are related to the pairs of retention time and m/z value in such a way that the pairs that are far away from the central cloud are responsible for the variance within the data set. The orientation of these points in the loadings plot corresponds to the distribution of analyses in scores plot. Therefore, the PCA loadings plot (**Figure 3b**) enabled to identify several compounds as being mainly responsible for the observed sample differentiation among

varieties, as shown in bold in **Table 1**. Thus, 8 tentatively identified metabolites constituted the major difference between Piel de Sapo and the other two varieties (Galia and Cantaloupe), as reflected in **Figure 3**; these were hexoside and dihexoside derivatives of vanillic acid, one of the isomers of citric acid, and butanodioldiacetate, zeatin hexoside, 9,12,13-trihydroxyoctadeca-10,15-dienoic acid, guanosine, and hydroxybutanoic acid ethyl ester-hexoside. These compounds presented a significantly higher intensity in Piel de Sapo samples than in the other varieties, while the isomer of citric acid was found only in this variety, in agreement with previous research demonstrating the variation in the citrate content of the fruit depending on planting dates and cultivars (Vallone *et al.*, 2013).

Although Galia and Cantaloupe showed fewer differences between them, 4 compounds proved to be discriminative. Among these, tryptophan or one of its isomers as well as an isomer of benzyl primeveroside were found in both varieties but not in Piel de Sapo samples, presenting higher intensity in Cantaloupe. On the other hand, pyroglutamic acid hexoside was characterized only in Cantaloupe whereas hesperidin was found in both Galia and Cantaloupe, but with significantly higher intensity in the Galia variety. Some of these compounds (vanillic acid dihexoside, hydroxybutanoic acid ethyl ester-hexoside, and benzyl alcohol hexose-pentose), which might be hexoside precursors of volatile compounds (Kilic, Kollmannsberger, & Nitz, 2005), have previously been described as differentiators of two varieties from the Cantalupensis group (Bernillon *et al.*, 2012).

As previously reported, the chemical composition of melon and other fruits is strongly influenced by the physiological state of the plants and by the environmental parameters as well as by the genotype. Thus, the PCA results in this study revealed genotype to be the parameter which most influences the phytochemical composition, as found by other authors (Liu *et al.*, 2004; Manohar & Murthy, 2012).

❖ Conclusions

In the present work, HPLC-ESI-QTOF-MS has been confirmed to be a powerful analytical technique for separating and detecting phenolic and other polar compounds in *Cucumis melo* varieties. This is the first available qualitative characterization of the Spanish varieties Galia, Cantaloupe, and Piel de Sapo, by this technique. With this method, 56 compounds were tentatively identified on the basis of their chromatographic retention, MS data, and MS/MS fragmentation pattern. The most representative groups of compounds tentatively identified were amino acids and derivatives, phenolic acid and derivatives, and organic acids, although nucleosides, alcohols, lignans, flavonoids and other polar compounds were also characterized. Of these compounds, 25 have been tentatively identified for the first time in *C. melo*. Preliminary PCA has shown a clear separation among varieties, although further research is needed to confirm the differentiation among the varieties studied. This work provides a better understanding of the distinction between those different Spanish melon varieties. The importance of knowledge concerning fruit composition is increasing due to their high consumption, and our study may help to improve the data in food-composition tables.

Acknowledgements

This work was supported by the project AGL2011-29857-C03-02 and AP2010-1551 (Spanish Ministry of Science and Innovation), as well as P09-CTS-4564, P10-FQM-6563 and P11-CTS-7625 (Andalusian Regional Government Council of Innovation and Science). The authors are also grateful to Danone Institute for a grant.

References

Abu-Reidah, I. M., Arráez-Román, D., Quirantes-Piné, R., Fernández-Arroyo, S., Segura-Carretero, A., & Fernández-Gutiérrez, A. (2012). HPLC-ESI-Q-TOF-MS for a comprehensive characterization of bioactive phenolic compounds in cucumber whole fruit extract. *Food Research International, 46*(1), 108-117.

Bazylak, G., Rosiak, A., & Shi, C.Y. (1996). Systematic analysis of glucoiridoids from *Penstemon serrulatus* Menz. by high-performance liquid chromatography with pre-column solid-phase extraction. *Journal of Chromatography A, 725*(1), 177-187.

Bernillon, S., Biais, B., Deborde, C., Maucourt, M., Cabasson, C., Gibon, Y., Hansen, T. H., Husted, S., de Vos, R. C. H., Mumm, R., Jonker, H., Ward, J. L., Miller, S. J., Baker, J. M., Burger, J., Tadmor, Y., Beale, M. H., Schjoerring, J. K., Schaffer, A. A., Rolin, D., Hall, R. D., & Moing, A. (2012). Metabolomic and elemental profiling of melon fruit quality as affected by genotype and environment. *Metabolomics,* 1-21.

Biais, B., Allwood, J. W., Deborde, C., Xu, Y., Maucourt, M., Beauvoit, B., Dunn, W. B., Jacob, D., Goodacre, R., Rolin, D., & Moing, A. (2009). 1H NMR, GC-EI-TOFMS, and data set correlation for fruit metabolomics: Application to spatial metabolite analysis in melon. *Analytical Chemistry, 81*(8), 2884-2894.

Boeing, H., Bub, A., Ellinger, S., Haller, D., Kroke, A., Leschik-Bonnet, E., Müller, M.J., Oberritter, H., Schulze, M., Stehle, P., & Watzl, B. (2012). Critical review: Vegetables and fruit in the prevention of chronic diseases. *Eur. J. Nutr,* 51(6), 637-663.

Bondia-Pons, I., Barri, T., Hanhineva, K., Juntunen, K., Dragsted, L. O., Mykkänen, H., & Poutanen, K. (2013). UPLC-QTOF/MS metabolic profiling unveils urinary changes in humans after a whole grain rye versus refined wheat bread intervention. *Molecular Nutrition and Food Research, 57*(3), 412-422.

Champavier, Y., Comte, G., Vercauteren, J., Allais, D. P., & Chulia, A. J. (1999). Norterpenoid and sesquiterpenoid glucosides from *Juniperus phoenicea* and *Galega officinalis*. *Phytochemistry, 50*(7), 1219-1223.

Cohen, S., Tzuri, G., Harel-Beja, R., Itkin, M., Portnoy, V., Sa'ar, U., Lev, S., Yeselson, L., Petrikov, M., Rogachev, I., Aharoni, A., Ophir, R., Tadmor, Y., Lewinsohn, E., Burger, Y., Katzir, N., & Schaffer, A. A. (2012). Co-mapping studies of QTLs for fruit acidity and candidate genes of organic acid metabolism and proton transport in sweet melon (*Cucumis melo* L.). *Theoretical and Applied Genetics, 125*(2), 343-353.

Dan, J., & Thakur, S. (1995). Constituent Acids of *Cucumis sativus* Fruit Cutin. *7, 2*, 381-388.

De Marino, S., Festa, C., Zollo, F., & Iorizzi, M. (2009). Phenolic glycosides from *Cucumis melo* var. inodorus seeds. *Phytochemistry Letters, 2*(3), 130-133.

Gómez-Romero, M., Segura-Carretero, A., & Fernández-Gutiérrez, A. (2010). Metabolite profiling and quantification of phenolic compounds in methanol extracts of tomato fruit. *Phytochemistry, 71*(16), 1848-1864.

Gonda, I., Bar, E., Portnoy, V., Lev, S., Burger, J., Schaffer, A. A., Tadmor, Y., Gepstein, S., Giovannoni, J. J., Katzir, N., & Lewinsohn, E. (2010). Branched-chain and aromatic amino acid catabolism into aroma volatiles in *Cucumis melo* L. fruit. *Journal of experimental botany, 61*(4), 1111-1123.

Huang, S.Y., Li, G.Q., Shi, J.G., Mo, S.Y., Wang, S.J., & Yang, Y.C. (2004). Chemical constituents of the rhizomes of *Coeloglossum viride* var. bracteatum. *Journal of Asian natural products research, 6*(1), 49-61.

Iha, A., Matsunami, K., Otsuka, H., Kawahata, M., Yamaguchi, K., & Takeda, Y. (2012). Three new aliphatic glycosides from the leaves of *Antidesma japonicum* Sieb. et Zucc. *Journal of Natural Medicines, 66*(4), 664-670.

Iswaldi, I., Gómez-Caravaca, A. M., Lozano-Sánchez, J., Arráez-Román, D., Segura-Carretero, A., & Fernández-Gutiérrez, A. (2013). Profiling of phenolic and other polar compounds in zucchini (*Cucurbita pepo* L.) by reverse-phase high-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry. *Food Research International, 50*(1), 77-84.

Jansen, R. J., Robinson, D. P., Stolzenberg-Solomon, R. Z., Bamlet, W. R., De Andrade, M., Oberg, A. L., Hammer, T. J., Rabe, K. G., Anderson, K. E., Olson, J. E., Sinha, R., & Petersen, G. M. (2011). Fruit and vegetable consumption is inversely associated with having pancreatic cancer. *Cancer Causes and Control, 22*(12), 1613-1625.

Kang, W. (2012). Quantitative determination of uridine in rabbit plasma and urine by liquid chromatography coupled to a tandem mass spectrometry. *Biomedical Chromatography, 26*(4), 541-544.

Kilic, A., Kollmannsberger, H., & Nitz, S. (2005). Glycosidically bound volatiles and flavor precursors in *Laurus nobilis* L. *Journal of Agricultural and Food Chemistry, 53*(6), 2231-2235.

Kourkoutas, D., Elmore, J. S., & Mottram, D. S. (2006). Comparison of the volatile compositions and flavour properties of cantaloupe, Galia and honeydew muskmelons. *Food Chemistry, 97*(1), 95-102.

Kuang, H.X., Xia, Y.G., Yang, B.Y., Liang, J., Zhang, Q.B., & Li, G.Y. (2009). A new caffeoyl phenylethanoid glycoside from the unripe fruits of *Forsythia suspensa*. *Chinese Journal of Natural Medicines, 7*(4), 278-282.

Kuroyanagi, M., Ishii, H., Kawahara, N., Sugimoto, H., Yamada, H., Okihara, K., & Shirota, O. (2008). Flavonoid glycosides and limonoids from *Citrus molasses*. *Journal of Natural Medicines, 62*(1), 107-111.

Lamikanra, O., Chen, J. C., Banks, D., & Hunter, P. A. (2000). Biochemical and microbial changes during the storage of minimally processed cantaloupe. *Journal of Agricultural and Food Chemistry, 48*(12), 5955-5961.

Lignou, S., Parker, J. K., Oruna-Concha, M. J., & Mottram, D. S. (2013). Flavour profiles of three novel acidic varieties of muskmelon (*Cucumis melo* L.). *Food Chemistry, 139*(1), 1152-1160.

Liu, L., Kakihara, F., & Kato, M. (2004). Characterization of six varieties of *Cucumis melo* L. based on morphological and physiological characters, including shelf-life of fruit. *Euphytica, 135*(3), 305-313.

Maietti, A., Tedeschi, P., Stagno, C., Bordiga, M., Travaglia, F., Locatelli, M., Arlorio, M., & Brandolini, V. (2012). Analytical Traceability of Melon (*Cucumis Melo* Var Reticulatus): Proximate Composition, Bioactive Compounds, and Antioxidant Capacity in Relation to Cultivar, Plant Physiology State, and Seasonal Variability. *Journal of Food Science, 77*(6), C646-C652.

Manohar, S. H., & Murthy, H. N. (2012). Estimation of phenotypic divergence in a collection of *Cucumis melo*, including shelf-life of fruit. *Scientia Horticulturae, 148*, 74-82.

Moco, S., Vervoort, J., Moco, S., Bino, R. J., De Vos, R. C. H., & Bino, R. (2007). Metabolomics technologies and metabolite identification. *TrAC - Trends in Analytical Chemistry, 26*(9), 855-866.

Moing, A., Aharoni, A., Biais, B., Rogachev, I., Meir, S., Brodsky, L., Allwood, J. W., Erban, A., Dunn, W. B., Kay, L., de Koning, S., de Vos, R. C. H., Jonker, H., Mumm,

R., Deborde, C., Maucourt, M., Bernillon, S., Gibon, Y., Hansen, T. H., Husted, S., Goodacre, R., Kopka, J., Schjoerring, J. K., Rolin, D., & Hall, R. D. (2011). Extensive metabolic cross-talk in melon fruit revealed by spatial and developmental combinatorial metabolomics. *New Phytologist, 190*(3), 683-696.

Obando-Ulloa, J. M., Ruiz, J., Monforte, A. J., & Fernández-Trujillo, J. P. (2010). Aroma profile of a collection of near-isogenic lines of melon (*Cucumis melo* L.). *Food Chemistry, 118*(3), 815-822.

Özcan, S., & Şenyuva, H. Z. (2006). Improved and simplified liquid chromatography/atmospheric pressure chemical ionization mass spectrometry method for the analysis of underivatized free amino acids in various foods. *Journal of Chromatography A, 1135*(2), 179-185.

Pang, X., Chen, D., Hu, X., Zhang, Y., & Wu, J. (2012). Verification of aroma profiles of jiashi muskmelon juice characterized by odor activity value and gas chromatography-Olfactometry/detection frequency analysis: Aroma reconstitution experiments and omission tests. *Journal of Agricultural and Food Chemistry, 60*(42), 10426-10432.

Pimenta Lange, M. J., Liebrandt, A., Arnold, L., Chmielewska, S., Felsberger, A., Freier, E., Heuer, M., Zur, D., & Lange, T. (2013). Functional characterization of gibberellin oxidases from cucumber, *Cucumis sativus* L. *Phytochemistry,* .

Poiroux-Gonord, F., Bidel, L. P. R., Fanciullino, A.L., Gautier, H., Lauri-Lopez, F., & Urban, L. (2010). Health benefits of vitamins and secondary metabolites of fruits and vegetables and prospects to increase their concentrations by agronomic approaches. *Journal of Agricultural and Food Chemistry, 58*(23), 12065-12082.

Seoane Spielgelberg, P., Rábade Rodríguez, M. T., & López Pérez, J. J. (2013). Anuario de estadística del Ministerio de Agricultura, Alimentación y Medio Ambiente. <http://www.magrama.gob.es/estadistica/pags/anuario/2012/AE_2012_Avance.pdf>.

Siciliano, T., De Tommasi, N., Morelli, I., & Braca, A. (2004). Study of flavonoids of *Sechium edule* (Jacq) Swartz (Cucurbitaceae) different edible organs by liquid chromatography photodiode array mass spectrometry. *Journal of Agricultural and Food Chemistry, 52*(21), 6510-6515.

Sidik, N. J., Hashim, S. N., Mohamad, Y. S., & Abdullah, S. (2012). Effects of natural and synthetic cytokinin hormone on shoot regeneration of rockmelon (*Cucumis melo*)

Glamour cv. by using nodal explants. *ISBELA 2012 - IEEE Symposium on Business, Engineering and Industrial Applications,* , 719-723.

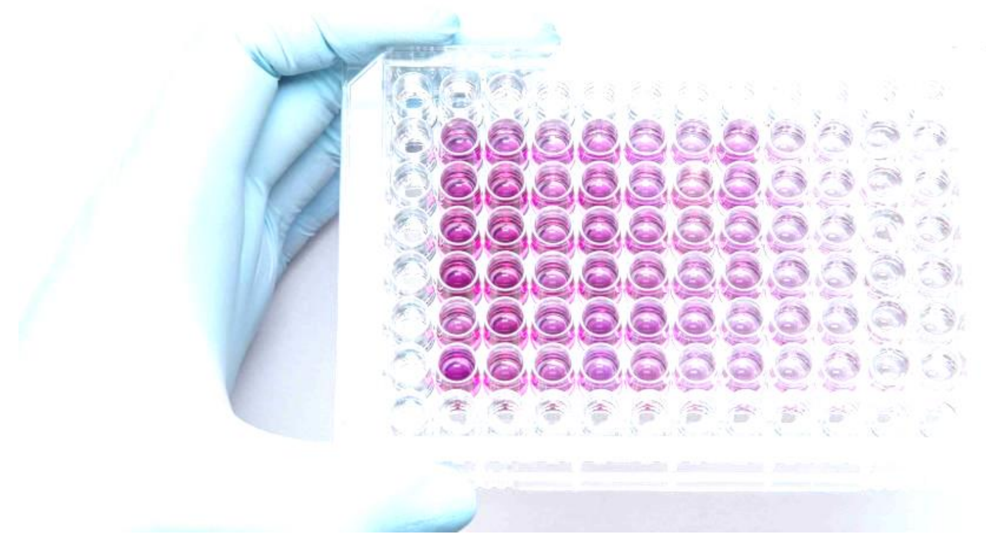
Taylor, V. F., March, R. E., Longerich, H. P., & Stadey, C. J. (2005). A mass spectrometric study of glucose, sucrose, and fructose using an inductively coupled plasma and electrospray ionization. *International Journal of Mass Spectrometry, 243*(1), 71-84.

Vallone, S., Sivertsen, H., Anthon, G. E., Barrett, D. M., Mitcham, E. J., Ebeler, S. E., & Zakharov, F. (2013). An integrated approach for flavour quality evaluation in muskmelon (*Cucumis melo* L. reticulatus group) during ripening. *Food Chemistry, 139*(1), 171-183.

Villanueva, M. J., Tenorio, M. D., Esteban, M. A., & Mendoza, M. C. (2004). Compositional changes during ripening of two cultivars of muskmelon fruits. *Food Chemistry, 87*(2), 179-185.

Vinholes, J., Grosso, C., Andrade, P. B., Gil-Izquierdo, A., Valentão, P., Pinho, P. G. D., & Ferreres, F. (2011). *In vitro* studies to assess the antidiabetic, anti-cholinesterase and antioxidant potential of *Spergularia rubra*. *Food Chemistry, 129*(2), 454-462.

Xiao, S., Zhao, S., Wang, C., Dong, Y., & Jiao, Z. (2010). Influences of grafting on aromatic compounds in muskmelon by solid phase microextraction with GC-MS. *Acta Horticulturae, 871*, 365-376.



Section II.

“Stability, *in vitro* and *in vivo* bioactivity evaluation of nutraceuticals and functional foods”

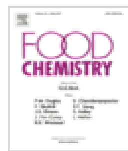
Chapter 6

Assessment of the stability of proanthocyanidins and other phenolic compounds in cranberry syrup after gamma-irradiation treatment and during storage

Food Chemistry, 2015, 174:392-399

Food Chemistry

Volume 174, 1 May 2015, Pages 392–399



Assessment of the stability of proanthocyanidins and other phenolic compounds in cranberry syrup after gamma-irradiation treatment and during storage

C. Rodríguez-Pérez[a, b], R. Quirantes-Piné[b], M. del M. Contreras[a, b], J. Uberos[c], A. Fernández-Gutiérrez[a, b], A. Segura-Carretero[a, b]

[a] Department of Analytical Chemistry, Faculty of Sciences, University of Granada, Avenida Fuentenueva s/n, 18071 Granada, Spain

[b] Research and Development Functional Food Centre (CIDAF), Health Science Technological Park, Avenida del Conocimiento s/n, Edificio BioRegión, 18017 Granada, Spain

[c] UGC Pediatría, Hospital Clínico San Cecilio Granada, 18012 Granada, Spain

Abstract

Shelf life of commercial cranberry syrup irradiated with gamma radiation at a rate of 5 kGy and stored for 6 months at 25ºC and 60% relative humidity (RH) and under accelerated stability conditions was investigated. High-performance liquid chromatography coupled to electrospray ionization quadrupole-time-of-flight mass spectrometry (HPLC-ESI-QTOF-MS) was used to characterize cranberry syrup. Afterwards, these compounds were quantified by HPLC-ESI-QTOF-MS and 4-dimethylaminocinnamaldehyde (DMAC) assay. A significant increase in the content of procyanidin B isomer 1 (from 4.4 μg/mL to 7.0 μg/mL) and procyanidin A2 (from 83 μg/mL to 93 μg/mL) was observed after irradiation and compared with the non-irradiated syrup. Procyanidin B isomers and prodelphinidin were stable at 25ºC during the first month of storage, whereas quercetin and some derivatives remained constant for 3 months of storage at this temperature. In short, after gamma-rradiation in dose of 5 kGy, most compounds were highly stable for a month at 25ºC.

Keywords: cranberry, DMAC, gamma-irradiation, HPLC, mass spectrometry, stability

❖ Introduction

American cranberry (*Vaccinium macrocarpon*) is a rich source of bioactive phenolic compounds with antiproliferative, antioxidant, anti-inflammatory, and antimicrobial activities (Es-Safi, Guyot & Ducrot, 2006). This berry has been traditionally used to treat and prevent urinary-tract infections in women and digestive-tract complaints. Today its anticancer properties have also been studied (Neto, 2011). Previous research suggests that some of these activities may be associated with its rich composition in flavonoids such as quercetin and proanthocyanidins (PACs) and its wealth of phenolic acids (Formica, 1995, Iswaldi *et al.*, 2012, Prior & Gu, 2005).

Proanthocyanidins (PACs) are oligomeric and polymeric end-products of the flavonoid-synthesis pathway, since they consist of sequences of flavanol monomeric units such as catechin, epicatechin, gallocatechin, epigallocatechin, afzelechin, and epiafzelechin, which are connected by C-C linkages (B-type) and sometimes also by C-O-C linkages (A-type), as classified by (He, Pan, Shi & Duan, 2008). PACs are noteworthy for their antioxidant activity, since their aromatic rings can effectively scavenge free radicals. Besides their antioxidant properties, these key compounds may present some pharmacological and medicinal properties, such as anti-carcinogenic, anti-inflammatory, and vasodilatory properties (Neto, 2011, Reed, 2002). Thus, considerable research has examined the therapeutic applications of these compounds (Vadodkar, Suman, Lakshmanaswamy & Damodaran, 2012), especially in regard to the prevention and reduction of urinary-tract infections.

To increase the shelf-life of food products, radiation is a well-established non-thermal physical mode for food preservation. This process is also known as cold-pasteurization because foods are kept near or at ambient temperature during processing (Alothman, Bhat & Karim, 2009). This technique has been introduced as an alternative to other preservation methods because it does not harm the environment, reduces the amount of weight loss during post-harvest, and leaves no residues on the fruits (Carocho, Barreira, Antonio, Bento, Kaluska & Ferreira, 2012). Currently, low-level irradiation of food and food-products has been approved by the Food and Drug

Administration (FDA) of the USA to reduce the incidence of illness associated with food-borne pathogens.

In this way, previous studies have evaluated the chemical changes of phenolic compounds in strawberries or its effect on the anthocyanins yield and shelf-life extension of grape pomace and pomegranate juices after gamma-irradiation (Alighourchi, Barzegar & Abbasi, 2008, Ayed, Yu & Lacroix, 1999, Breitfellner, Solar & Sontag, 2003). However, there is a paucity of information concerning the effect of irradiation methods and storage on the phytochemical composition from cranberry-derived products, and especially PACs.

The complexity of PACs in terms of molecular weight and linkage type makes it difficult to use a single quantification method. The 4-dimethylaminocinnamaldehyde (DMAC) spectrophotometric assay has become increasingly popular as a rapid technique to quantify the total amount of proanthocyanidins present in foods and beverages. The DMAC method was used to substantiate French health claims for the bacterial anti-adhesion activity of cranberry juice concentrate and juice-concentrate extract powder, cranberry-juice cocktail, and fresh frozen and pureed cranberry by the French food-safety authority “Agence Française de Sécurité Sanitaire des Aliments”, AFSSA (Prior *et al.*, 2010). In this way, DMAC is considered the best analysis available for a global quantitation of flavanols (flavan-3-ols), both simple and oligomeric ones (proanthocyanidins), but it does not offer information about the specific compounds present in foods (Boudesocque, Dorat, Pothier, Gueiffier & Enguehard-Gueiffier, 2013). For this reason it is necessary to combine this technique with other more selective ones. In this regard, high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) has become a powerful method due to its high resolution, the capacity to separate a wide range of polar compounds by means of polarity differences, as well as its specificity.

Thus, the aim of the present work is to evaluate the qualitative and quantitative changes in the phenolic composition of cranberry syrup after gamma-irradiation, as well as the stability of these compounds during 6 months of storage in the irradiated

cranberry syrup under different conditions: 25ºC and 60% relative humidity (RH) and under 40ºC and 75% RH, the latter for accelerated stability testing.

❖ Material and methods

Chemical

Standards of proanthocyanidin A2, *p*-coumaric acid, quercitrin, quercetin-3-*O*-glucoside, myricetin, (+)-catechin, and luteolin were from Extrasynthese (Genay, France). Formic acid and acetonitrile used for preparing mobile phases were from Sigma-Aldrich (Steinheim, Germany) and Lab-Scan (Gliwice, Sowinskiego, Poland), respectively. Ultrapure water with a resistivity value of 18.2 MΩ was obtained from Milli-Q system (Millipore, Bedford, MA, USA). Solvents were filtered before use with a Solvent Filtration Apparatus 58061 (Supelco, Bellefonte, PA, USA). HPLC grade methanol (99.9%) and HPLC grade acetone were purchased from Fisher Scientific (Loughborough, Leics, UK). Acetic acid of analytical grade was acquired from Sigma-Aldrich. Hydrochloric acid (37%) was from Panreac (Barcelona, Spain). HPLC grade ethanol (99%) was from Analar Normapul (Fontenay-sous-Bois, France) and 4-dimethylaminocinnamaldehyde (DMAC) was from Sigma-Aldrich.

Syrup sample preparation

A total of 15 g of a commercial extract of American cranberry *(Vaccinium macrocarpon)* were mixed with 500 mL of water to prepare a syrup. The syrup was separated into 0.5 mL aliquots to facilitate storage and the irradiation process.

Preparation of standards solution

Procyanidin A2 (purity ≥ 99%) was used as a standard in the DMAC assay and in HPLC-ESI-QTOF-MS quantification. For the plotting of the DMAC calibration curve, 1 mg was dissolved in ethanol to give a final concentration of 100 µg/mL and procyanidin A2 calibration curve was drawn ranging from 1 to 50 µg/mL. Also, for the HPLC-ESI-QTOF-MS quantification, standard calibration curves of procyanidin A2, *p*-coumaric acid, quercetin-3-*O*-glucoside, quercitrin, (+)-catechin and myricetin were

prepared using different concentrations in methanol. In addition, luteolin was dissolved in methanol at 1 mg/mL and used as an internal standard (I.S.) to compensate for the potential variations in the instrumental analysis.

Irradiation treatment

A cobalt-60 irradiator was used for the irradiation treatment. The absorbed dosage was 5 kGy. Aliquots were separated to facilitate the storage process.

Storage conditions

The samples were stored for 6 months in two different chambers with different temperature and humidity (chambers A and B). Chamber A had a temperature of 25ºC and 60% RH whereas chamber B was kept at 40ºC and 75% RH for the accelerated stability testing, according with the Committee for Human Medicinal Products (EMEA (European Medicines Agency), 2007).

Sample preparation

Syrup dilution for HPLC-ESI-QTOF-MS analysis

In 4 mL of methanol, 200 µL of syrup and 10 µL of the I.S. solution were dissolved, vortexed for 2 min in a G560E Vortex-Genie 2 (Scientific Industries, Bohemia, NY, USA), filtered with a polytetrafluoroethylene (PTFE) syringe filter (0.2 µm pore size), and injected directly into the HPLC system. Each sample was analyzed in triplicate.

PAC extraction for the DMAC assay

The extraction process was performed according to (Prior *et al.*, 2010), with some modifications. A total of 400 µL of syrup were dissolved in 5 mL of water. Bond Elut C_{18} cartridges (100 mg, 1 mL, Agilent Technologies, Palo Alto, CA, USA) were activated with 2 mL of methanol and washed with 2 mL of water. The cranberry syrup was added to each column and allowed to pass through the column by gravity. The column was washed twice with 1 mL of water and eluted twice with 1 mL of elution

solution consisting of ethanol:water (80:20, v/v) with 0.5% of acetic acid. The eluted extract (2 mL) was collected, diluted again in a proportion of 1:25 in ethanol, and vortexed to mix well prior to the analysis by DMAC assay. Each sample was analyzed in triplicate.

HPLC-ESI-QTOF-MS analysis

HPLC analyses were made with an Agilent 1200 series rapid-resolution LC system equipped with a binary pump, an autosampler, and a diode-array detector (DAD) following (Iswaldi *et al.*, 2012), and a Zorbax Eclipse Plus C_{18} analytical column (150 mm × 4.6 mm, 1.8 μm particle size) was used for separation. The mobile phases consisted of water:acetonitrile (90:10, v/v) with 1% formic acid (phase A) and acetonitrile (phase B) at a constant flow rate of 0.5 mL/min using the following multi-step gradient: 0-20 min, from 5% B to 20% B; 20-25 min, from 20% B to 40% B; 25-30 min, from 40% B to 5% B; and 30-35 min, isocratic of 5% B. The injection volume was 10 μL.

The HPLC system was coupled to a micrOTOF-Q II mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with ESI operating in negative mode. The flow from HPLC delivered into the MS detector was split using a flow splitter at 1:2 for stable electrospray ionization and reproducible results. The detection was made considering a mass-to-charge ratio of 50-1100 and using a capillary voltage of +4000 V, a dry gas temperature of 210ºC, a dry gas flow of 8.0 L/min, a nebulizer pressure of 2.0 bar, and spectra rate of 1 Hz. Moreover, automatic MS/MS experiments were performed using nitrogen as the collision gas and adjusting the collision-energy values as follows: *m/z* 100, 20 eV; *m/z* 500, 25 eV; *m/z* 1000, and 30 eV.

External instrument calibration was used to ensure the necessary mass accuracy to characterize compounds. The calibrant was sodium formate clusters consisting of 5 mM sodium hydroxide dissolved in water: 2-propanol, 1:1 (v/v), with 0.2% of formic acid. This calibrant was injected at the beginning of each run using a 74900-00-05

Cole Palmer syringe pump (Vernon Hills, IL, USA), which was equipped with a Hamilton syringe (Reno, NV, USA) and directly connected to the interface.

All procedures were controlled by DataAnalysis 4.0 software (Bruker Daltonik), which provided a list of possible elemental formulas by using the SmartFormula™ editor. The Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double-bond equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (sigma value) for increased confidence in the suggested molecular formula.

Quantification by HPLC-ESI-QTOF-MS and method validation

Quantification of the phenolic compounds was carried out by HPLC-ESI-QTOF-MS, using internal calibration with luteolin as the internal standard. The volume necessary to obtain a luteolin final concentration of 1 mg/mL was added to the standards and to the cranberry syrup samples. Extracted ion chromatograms of each compound were used to obtain the areas used in the quantification process.

The range of calibration of procyanidin A was from the limit of quantification (LOQ) to 100 ppm; p-coumaric acid was from LOQ to 250 ppm, and quercetin-3-*O*-glucoside, quercitrin, (+)-catechin and myricetin were from LOQ to 50 ppm, as showed in **Table 1**.

Limits of detection (LOD) and quantification (LOQ) of the method were estimated to be 3 and 10 times the signal-to-noise ratio, respectively. Intra-day instrumental precision was determined by injecting a cranberry syrup sample 3 consecutive times in one day. Inter-day instrumental precision was determined by repeating the inter-assay procedure over three consecutive days. The linearity was individually verified for each compound, with analytical curves made from five calibration points in triplicate.

Table 1. Intra and inter-day precision, limits of detection (LOD) and quantification (LOQ) and linearity data.

Analyte	RSD Intraday (n=3) (%)	RSD Interday (n=9) (%)	LOD (µg/mL)	LOQ (µg/mL)	Calibration range (µg/mL)	Calibration equations	r^2
Procyanidin A2	0.9	2.9	0.15	0.28	LOQ-100	y=0.0604 x+0.0025	0.997
***p*-coumaric acid**	1.2	3.8	0.20	0.67	LOQ-250	*y=0.0168 x +0.0576*	0.991
Quercetin-3-*O*-glucoside	1.5	4.1	0.05	0.17	LOQ-50	*y=0.0522 x +0.0027*	0.996
Quercitrin	2.1	4.7	0.06	0.18	LOQ-50	*y=0.05688 x +0.0728*	0.991
(+)-catechin	1.1	3.5	0.04	0.16	LOQ-50	*y=0.0397 x +0.0172*	0.991
Myricetin	0.2	3.1	0.05	0.65	LOQ-50	*y=0.1015 x +0.0393*	0.991

DMAC assay

DMAC is a colorimetric method that consists of the reaction between DMAC reagent and the C-8 position in the A-ring of the terminal unit of a proanthocyanidin (Wallace & Giusti, 2010). This reagent was found to be selective for flavanols, and also gives an intense reaction with monomers such as catechin or epicatechin. This assay was adapted from Prior *et al.* (Prior *et al.*, 2010). Briefly, procyanidin A2 standard (purity ≥ 99%) was dissolved in ethanol to give a final concentration of 100 µg/mL. The incubation chamber was pre-heated to 25ºC, and 70 µL of ethanol (blanks), standard or sample solutions were dispensed into wells of a 96-well plate. Then, 210 µL of DMAC solution, consisting of 0.1% (w/v) DMAC reagent in acidized ethanol (ethanol:water: HCl, 75:12.5:12.5) was added. The absorbance was measured at 640 nm using a microplate reader Synergy Mx Monochromator-Based Multi-Mode Micro plate reader, by Bio-Tek Instruments Inc. (Winooski, VT, USA). Procyanidin A calibration curve was built ranging from 1 to 50 µg/mL. All samples were analyzed in triplicate. The results were expressed as µg of procyanidin A2/mL of syrup.

Statistical analysis

Quantitative data are represented as mean ± standard deviation (SD). To evaluate the differences at a 95% confidence level ($p \leq 0.05$), a one-way analysis of variance (ANOVA) followed by Tukey's test was performed using Origin (version Origin Pro 8 SR0, Northampton, MA, USA).

❖ Results and discussion

Assessment of the HPLC-ESI-QTOF-MS method

Intraday and interday precision values were measured to evaluate the repeatability and reproducibility of the method and the relative standard deviations (RSDs) of the peaks area were determined. All the calibration curves presented good linearity, regression coefficients being higher than 0.99 (**Table 1**).

Compound characterization and quantification of the cranberry syrup

Compound characterization by HPLC-ESI-QTOF-MS and MS/MS

The base-peak chromatogram (BPC) of the non-irradiated cranberry syrup (control) obtained by HPLC-ESI-QTOF-MS analysis is shown in **Figure 1**. The phenolic compounds characterized are indicated with numbers according to their elution order. The characterization strategy was based on the accurate MS and MS/MS spectra of the compounds determined by QTOF mass analyzer and also taking account the data from literature. The identification of these compounds was also corroborated by comparing their retention times and MS/MS spectra with those of authentic standards whenever available. **Table 2** summarizes the MS data of the compounds characterized, including experimental and calculated *m/z* for the molecular ions, their error, and the main fragments determined by MS/MS. Among these compounds, 27 phenolic compounds were identified, including simple flavonols, catechin, and oligomers such as 3 isomers of procyanidin B, procyanidin A2 and a procyanidin A isomer, prodelphinidin, four isomers of procyanidin A trimer (*m/z* 863) and one procyanidin A pentamer, identified on the basis of its doubly charged ion at *m/z* 719 (Foo, Lu, Howell & Vorsa, 2000). Moreover, two isomers of myricetin and myricetin glycosylated derivatives, quercetin and quercetin glycosylated derivatives, and glycosylated derivatives of coumaric acid were detected as in Iswaldi *et al.* (Iswaldi *et al.*, 2012).

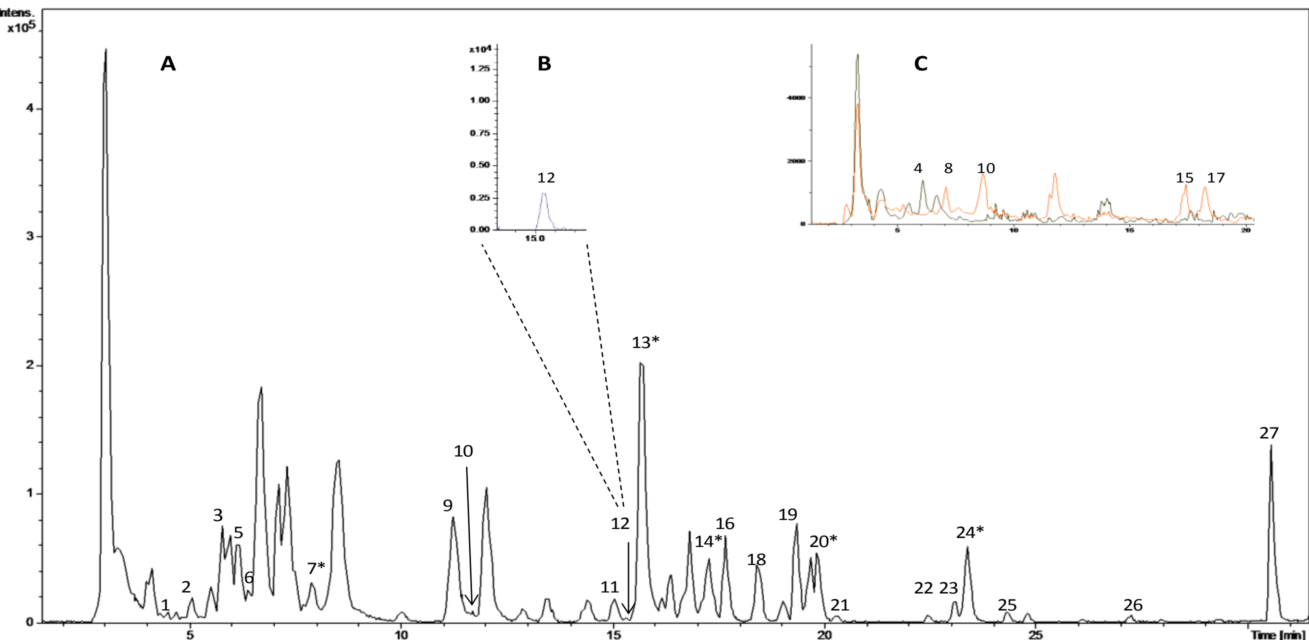


Figure 1. Base-peak chromatogram of non-irradiated cranberry syrup (control) obtained by HPLC-ESI-TOF-MS analysis using negative ion mode (A) and extracted ion chromatogram of peak 12 (procyanidin B isomer) (B) and procyanidin A trimers and pentamer (C). *Identification confirmed using commercial standards.

Table 2. Compounds characterized in non-irradiated cranberry syrup

Peak	RT (min)	m/z experimental	Molecular formula	Error (ppm)	Fragments	Compound
1	4.53	577.1375	$C_{30}H_{26}O_{12}$	4.1	289.0735 (100%); 125.0242 (88%); 4070728 (38%)	Procyanidin B isomer 1
2	5.29	575.1190	$C_{30}H_{24}O_{12}$	0.8	125.233 (100%); 289.0671 (44%); 285.0344 (31%)	Procyanidin A isomer
3	5.81	325.0918	$C_{15}H_{18}O_{8}$	3.4	119.0500 (100%); 163.0391 (40%)	Coumaroyl-hexose isomer 1
4	6.14	719.1503	$C_{75}H_{58}O_{30}$	2.9	289.0704 (100%); 125.0251 (63.7%); 151.0386 (45%); 287.0547 (42%); 325.0875 (36%)	Procyanidin A pentamer
5	6.21	325.0922	$C_{15}H_{18}O_{8}$	2.2	119.0286 (100%); 117.0340 (15%); 163.0433 (10%)	Coumaroyl-hexose isomer 2
6	6.41	577.1337	$C_{30}H_{26}O_{12}$	2.4	289.0708 (100%); 407.0788 (71%); 125.0233 (49%); 161.0253 (44%)	Procyanidin B isomer 2
7	8.36	289.0708	$C_{15}H_{14}O_{6}$	3.4	109.0268 (100%); 121.0307 (24%); 123.0399 (54%); 151.0387 (66%); 203.0696 (67%)	Catechin*
8	8.69	863.1849	$C_{45}H_{36}O_{18}$	2.3	289.0715 (100%); 287.0524 (42.8%); 285.0388 (35%); 575.1191 (16%)	Procyanidin A trimer isomer 1
9	11.17	479.0823	$C_{21}H_{20}O_{13}$	1.7	316.0216 (100%);317.0277 (22%); 179.0009 (10%)	Myricetin hexose
10	11.83	863.1814	$C_{45}H_{36}O_{18}$	4.5	575.1203 (100%); 423.0686 (74%); 289.0715 (59%); 285.0394 (56%)	Procyanidin A trimer isomer 2
11	15.04	449.0703	$C_{20}H_{18}O_{2}$	4.9	316.0216 (100%); 317.0277 (22%); 287.0161 (10%)	Myricetin pentose
12	15.33	577.1327	$C_{30}H_{25}O_{12}$	4.2	289.0683 (100%); 163.0384 (45%); 407.0814 (30%); 125.0220 (40%); 119.0542 (34%)	Procyanidin B isomer 3
13	15.65	463.0869	$C_{21}H_{20}O_{12}$	2.8	300.0265 (100%); 301.0325 (50%); 271.0234 (10%)	Quercetin 3-O-glucoside*
14	17.23	575.1179	$C_{30}H_{24}O_{12}$	2.8	285.0381 (100%); 289.0687 (70%); 125.0232 (32%)	Procyanidin A2*
15	17.40	863.1778	$C_{45}H_{36}O_{18}$	3.6	575.1218 (100%); 285.0387 (31%); 289.0674 (21%)	Procyanidin A trimer isomer 3
16	17.61	433.0762	$C_{20}H_{18}O_{11}$	3.3	300.0269 (100%); 301.0336 (50%); 271.0262 (7%)	Quercetin pentoside isomer 1
17	18.25	863.1826	$C_{45}H_{36}O_{18}$	1.9	575.1190 (100%); 285.0376 (21%); 289.0686 (19.5%)	Procyanidin A trimer isomer 4
18	18.37	433.0754	$C_{20}H_{18}O_{11}$	5.1	300.0273 (100%); 301.0332 (50%); 271.0255 (10%)	Quercetin pentoside isomer 2
19	19.33	433.0757	$C_{20}H_{18}O_{11}$	4.1	300.0267 (100%); 301.0331 (72%); 271.0269 (11%)	Quercetin pentoside isomer 3
20	19.85	447.0918	$C_{21}H_{20}O_{11}$	3.3	300.0262 (100%); 301.0324 (77%); 151.0030 (10%)	Quercetin rhamnoside (quercitrin)*

Table 2. *(Continued)*

Peak	RT (min)	m/z experimental	Molecular formula	Error (ppm)	Fragments	Compound
21	20.31	463.0874	$C_{21}H_{20}O_{12}$	1.7	301.0325 (100%)	Quercetin hexose
22	22.42	447.0914	$C_{21}H_{20}O_{11}$	4.2	314.0430 (100%); 315.0432 (26%); 300.1081 (20%)	Quercetin methyl ether-pentoside isomer 1
23	23.04	447.0906	$C_{21}H_{20}O_{11}$	6.1	314.0417 (100%); 315.0528 (23%); 243.0275 (10%)	Quercetin methyl ether-pentoside isomer 2
24	23.36	317.0289	$C_{15}H_{10}O_8$	4.3	151.0034 (100%); 137.0231 (68.4%); 179.9994 (10%); 165.0172 (7.5%)	Myricetin*
25	24.29	447.0905	$C_{21}H_{20}O_{11}$	6.3	314.0420 (100%); 315.0475 (33%); 285.0399 (17%); 243.0289 (10%)	Quercetin methyl ether-pentoside isomer 3
26	27.15	609.1236	$C_{30}H_{26}O_{14}$	2.3	NA	Prodelphinidin
27	30.41	301.0344	$C_{15}H_{10}O_7$	3.3	151.0019 (100%); 121.0296 (26%); 107.0137 (20%)	Quercetin

*Identification confirmed using commercial standards.

Quantification by HPLC-ESI-QTOF-MS and DMAC methods

The main phenolic compounds were quantified by the aforementioned HPLC-ESI-QTOF-MS method. In this way, myricetin, catechin, quercetin-3-*O*-glucoside, quercitrin, and procyanidin A2 were quantified by the calibration curves determined from their respective commercial standards. Since there are no available standards for all the characterized phenolic compounds, some compounds had their content estimated by using the analytical curves of compounds with similar chemical structures. In this way, proanthocyanidins were quantified using the procyandin A2 calibration curve; myricetin derivatives were quantified using the myricetin calibration curve; quercetin and its derivatives were quantified by using the quercetin-3-*O*-glucoside calibration curve; and coumaroyl glycosides isomers were quantified using the coumaric acid calibration curve. The quantitative results of the main compounds are displayed in **Figures 2**, **3**, and **4**, and the whole data are presented in **Table 3** and **Table 4**. The cranberry syrup used as control showed a rich composition in procyanidins, being procyanidin A2 the most abundant (83 µg/mL), which has been described as the most bioactive proanthocyanidin against urinary tract infections (Krueger, Reed, Feliciano & Howell, 2013), followed by procyanidin B isomer 2 (35 µg/mL) and isomer of procyanidin A (20 µg/mL). The main flavonols were quercetin (80 µg/mL) and its glucoside (320 µg/mL).

Table 3. Concentration of each phenolic compound in cranberry syrup irradiated and non-irradiated (control) and after irradiation during storage (1, 3 and 6 moths) in chamber A (25ºC/60% of RH) and expressed as mean ± SD (µg/mL, n=3)

	Chamber A				
Compound	**Control**	**0 IRRA**	**1 m**	**3 m**	**6 m**
Procyanidin B isomer 1	4.4±0.3^{b}	7.0±0.3^{a}	6.0±0.2^{c}	5.0±0.3^{b,d}	3.00±0.04^{e}
Procyanidin B isomer 2	35±3.0^{a}	37±1.0^{a}	36±1.0^{a}	30±0.4^{b}	14±0.6^{c}
Procyanidin B isomer 3	3.0±0.3^{a}	3.0±0.3^{a}	2.8±0.2^{a,b}	2.0±0.1^{b,c}	0.60±0.03^{d}
Procyanidin A isomer	20±2.0^{a}	20±0.4^{a}	14±1.0^{b}	14±0.1^{b,c}	10±0.5^{d}
Procyanidin A2*	83±4.0^{b}	93±5.0^{a}	86±2.0^{a,b}	70±3.0^{c}	40±1.0^{d}
Prodelphinidin	5.8±0.4^{a}	7.0±0.1^{a}	7.0±0.5^{a}	7.0±0.1^{a}	3.0±0.2^{b}
Myricetin hexose	70±7.0^{a}	72±2.0^{a}	70±3.0^{a}	60±4.0^{a}	50±3.0^{b}
Myricetin pentose	6.0±1.0^{a}	7.0±0.3^{a}	6.0±0.3^{a}	3.0±0.7^{b}	--
Myricetin*	20±2.0^{a}	20±1.0^{a}	14±0.6^{b}	10±0.4^{b,c}	4.0±0.7^{d}
Quercitrin*	30±5.0^{a}	33±1.0^{a}	30±2.0^{a}	30±2.0^{a}	20±0.6^{b}
Quercetin 3-*O*-Glucoside*	320±20.0^{a}	320±10.0^{a}	310±20.0^{a,b}	280±3.00^{b,c}	250±5.00^{d}
Quercetin hexose	4.0±0.2^{a}	4.0±0.3^{a}	3.0±0.6^{a}	3.0±0.2^{a}	--
Quercetin xylopiranoside isomer 1	50±4.0^{a}	60±2.0^{a}	60±7.0^{a}	60±0.1^{a}	40±2.0^{b}
Quercetin xylopiranoside isomer 2	40±5.0^{a}	40±2.0^{a}	40±3.0^{a}	40±1.0^{a}	30±1.0^{b}
Quercetin xylopiranoside isomer 3	70±7.0^{a}	72±5.0^{a}	68±5.0^{a}	68±5.0^{a}	40±2.0^{b}
Quercetin methyl ester pentoside isomer 1	4.0±0.3^{a}	4.0±0.1^{a}	4.0±0.4^{a,b}	3.0±0.3^{b,c}	2.0±0.1^{d}
Quercetin methyl ester pentoside isomer 2	15±1.0^{a}	15±0.3^{a}	14±0.7^{a}	14±0.5^{a}	10±0.2^{b}
Quercetin methyl ester pentoside isomer 3	7±1^{a,b}	7.00±0.04^{a}	6.0±0.2^{a,b}	6.0±0.1^{b}	3±0.3^{c}
Quercetin	80±9.0^{b}	100±2.00^{a}	70±7.0^{b,c}	80±0.1^{b,d}	50±2.0^{e}
Catechin*	65±4.0^{a}	50±1.0^{b}	40±2.0^{c}	40±1.0^{c,d}	26±1.0^{e}
Coumaroyl hexose isomer 1	56±1.0^{b}	90±4.0^{a}	40±1.0^{b,c}	100±5.00^{a,d}	80±8.0^{a,e}
Coumaroyl hexose isomer 2	90±3.0^{b}	110±15.0^{a}	150±10.0^{a,b}	130±5.00^{a,b}	60±10^{c}

Data are expressed as mean ± standard deviation. Mean values with different superscript letters are significantly different ($p < 0.05$).
*Identification confirmed using commercial standards.

Table 4. Concentration of each phenolic compound in cranberry syrup irradiated and non-irradiated (control) and after irradiation during storage (1, 3 and 6 moths) in chamber B (40ºC/75% of RH) and expressed as mean ± SD (µg/mL, n=3)

	Chamber B				
Compound	**Control**	**0 IRRA**	**1 m**	**3 m**	**6 m**
Procyanidin B isomer 1	4.4±0.3[b]	7.0±0.3[a]	6.0±0.4[b,c]	3.0±0.1[d]	--
Procyanidin B isomer 2	35±3.0[a]	37±1.0[a]	25±2.0[b]	10±0.4[c]	1.0±0.2[d]
Procyanidin B isomer 3	3.0±0.3[a]	3.0±0.3[a]	2.0±0.2[b]	2.00±0.05[b,c]	0.80±0.09[d]
Procyanidin A isomer 1	20±2.0[a]	20±0.4[a]	10±0.6[b]	6.0±0.2[c]	2.0±0.1[d]
Procyanidin A2*	83±4.0[b]	93±5.0[a]	60±1.0[c]	30±0.2[d]	10±1.0[e]
Prodelphynidin	5.8±0.4[a]	7.0±0.1[a,b]	7.0±0.7[a,b,c]	3.0±0.1[d]	1.0±0.1[e]
Myricetin hexose	70±7.0[a]	72±2.0[a]	60±10[a]	40±2.0[b]	20±3.0[c]
Myricetin pentose	6.0±1.0[a]	7.0±0.3[a]	1.0±0.4[b]	--	--
Myricetin*	20±2.0[a]	20±1.0[a]	10±1.0[b]	2.0±0.3[c]	--
Quercitrin*	30±5.0[a]	33±1.0[a]	20±0.1[a]	10±0.6[b]	1.0±0.1[c]
Quercetin 3-O-Glucoside*	320±20.0[a]	320±10.0[a]	270±10.0[a]	230±10.0[b]	130±6.00[c]
Quercetin hexose	4.0±0.2[a]	4.0±0.3[a]	3.0±0.3[b]	--	--
Quercetin xylopiranoside isomer 1	50±4.0[a]	60±2.0[a]	50±1.0[a]	30±0.5[b]	10±0.6[c]
Quercetin xylopiranoside isomer 2	40±5.0[a]	40±2.0[a]	40±3.0[a]	30±0.5[b]	10±0.1[c]
Quercetin xylopiranoside isomer 3	70±7.0[a]	72±5.0[a]	50±5.0[b]	20±1.0[c]	--
Quercetin methyl ester pentoside isomer 1	4.0±0.3[a]	4.0±0.1[a]	3.0±0.2[b]	2.0±0.2[c]	0.50±0.02[d]
Quercetin methyl ester pentoside isomer 2	15±1.0[a]	15±0.3[a]	10±1.0[a]	1.0±0.07[b]	3.0±0.1[c]
Quercetin methyl ester pentoside isomer 3	7±1[a]	7.00±0.04[a]	4.0±0.3[b]	0.60±0.02[c]	--
Quercetin	80±9.0[b]	100±2.00[a]	80±8.0[b,c]	70±0.3[b,c,d]	40±3.0[e]
Catechin*	65±4.0[a]	50±1.0[b]	40±4.0[c]	30±0.7[d]	--
Coumaroyl hexose isomer 1	56±1.0[b]	90±4.0[a]	100±10.0[a,c]	100±10.0[a,c,d]	80±8.0[a,c,d]
Coumaroyl hexose isomer 2	90±3.0[b]	110±15.0[a]	100±10.0[a,c]	30±4.0[d]	--

Data are expressed as mean ± standard deviation. Mean values with different superscript letters are significantly different ($p < 0.05$).
*Identification confirmed using commercial standards.

Currently, the cranberry industry is using DMAC as a standard method to measure the PACs content of products (Krueger, Reed, Feliciano & Howell, 2013). Thus, to complement the latter quantitative results of each compound determined individually, DMAC assay was also performed to determine the overall PACs concentration, which include high mass oligomeric ones. These results are available in **Table 5**. The total PACs was 4130 μg/mL in the non-irradiated syrup, which is higher than those results from the analysis by HPLC-QTOF-MS. It could be explained since one limitation of HPLC-MS methods is the difficulty to separate and detect higher polymeric proanthocyanidins, which may lead to underestimated quantitative results.

Table 5. Total PACs determined by DMAC assay

Time (months)	Mean ± SD (μg/mL)
0 Control	4130 ± 50[a]
0 Irradiated	3460 ± 80[b]
Chamber A	
1	2720 ± 40[c]
3	2150 ± 160[d]
6	1420 ± 30[e]
Chamber B	
1	1910 ± 60[f]
3	1130 ± 40[g]
6	450 ± 10[h]

Data are expressed as mean ± standard deviation (SD). Mean values with different superscript letters are significantly different ($p < 0.05$).

It is difficult to make a direct comparison between phenolic compounds found in this study and those reported by other authors because most articles refer to cranberry juices or fresh cranberries alone or in combination with other red fruits (Alighourchi, Barzegar & Abbasi, 2008, Boudesocque, Dorat, Pothier, Gueiffier & Enguehard-Gueiffier, 2013, Grace, Massey, Mbeunkui, Yousef & Lila, 2012, Sánchez-Patán, Bartolomé, Martín-Alvarez, Anderson, Howell & Monagas, 2012). In particular, PACs are usually characterized and quantified in previous studies by the

degree of polymerization, but not individually due to their structural heterogeneity and difficult analytical separation (Pappas & Schaich, 2009).

Resistance of phenolic compounds to gamma-irradiation and stability during storage

Assessment by HPLC-QTOF-MS

The main compounds were quantified by HPLC-ESI-QTOF-MS in syrup samples before gamma-irradiation, immediately after applying gamma irradiation and after 1, 3, and 6 months of storage of the irradiated syrup at 25ºC and 60% RH (chamber A) and 40ºC and 75% RH (chamber B). Interestingly, after the gamma-irradiation treatment, the qualitative composition of the syrup was similar to the non-irradiated one, showing poor quantitative differences. In any case, a significant increase ($p < 0.05$) in the content of procyanidin B isomer 1 (from 4.4 µg/mL to 7.0 µg/mL) and procyanidin A2 (from 83 µg/mL to 93 µg/mL) was observed after irradiation and compared with the non-irradiated syrup (control). The increase in procyanidin A2 could be associated with the degradation of procyanidin A trimers or pentamer, which were only observed in non-irradiated syrup. In this regard, other authors have reported greater anthocyanins content after irradiation at 5-6 kGy in strawberries (Ayed, Yu & Lacroix, 1999) and other vegetable foods (Dixit, Bhatnagar, Kumar, Chawla, Fakhruddin & Bhatnagar, 2012), but the formation pathway is not clear.

Figure 2 displays the stability of the concentration of the flavanols, including PACs and the monomer catechin, after gamma-irradiation and during storage under the two conditions studied. In general, the most stable PACs were procyanidin B isomers 2 and 3, and prodelphinidin while the other isomer of procyanidin B and the isomer of procyanidin A showed higher susceptibility, e.g. isomers 2 and 3, remained stable in at 25ºC/60% RH (chamber A) until the 1st and the 3rd month of storage, respectively. Similarly, (Chang, Zuo, Chow & Ho, 2006) also demonstrated the instability of B-type proanthocyanidins at room temperature after 6 months of storage, but remarkably our results show that this trend depends on the structure of procyanidin B. Not

surprisingly, compared with the initial time, the greatest losses were found when the syrup was stored under accelerated stability conditions, (40ºC/75% RH), in chamber B. In this case, in which all PACs showed significant changes ($p < 0.05$) in their concentration during storage except prodelphinidin, which remained constant under these conditions up to the first month.

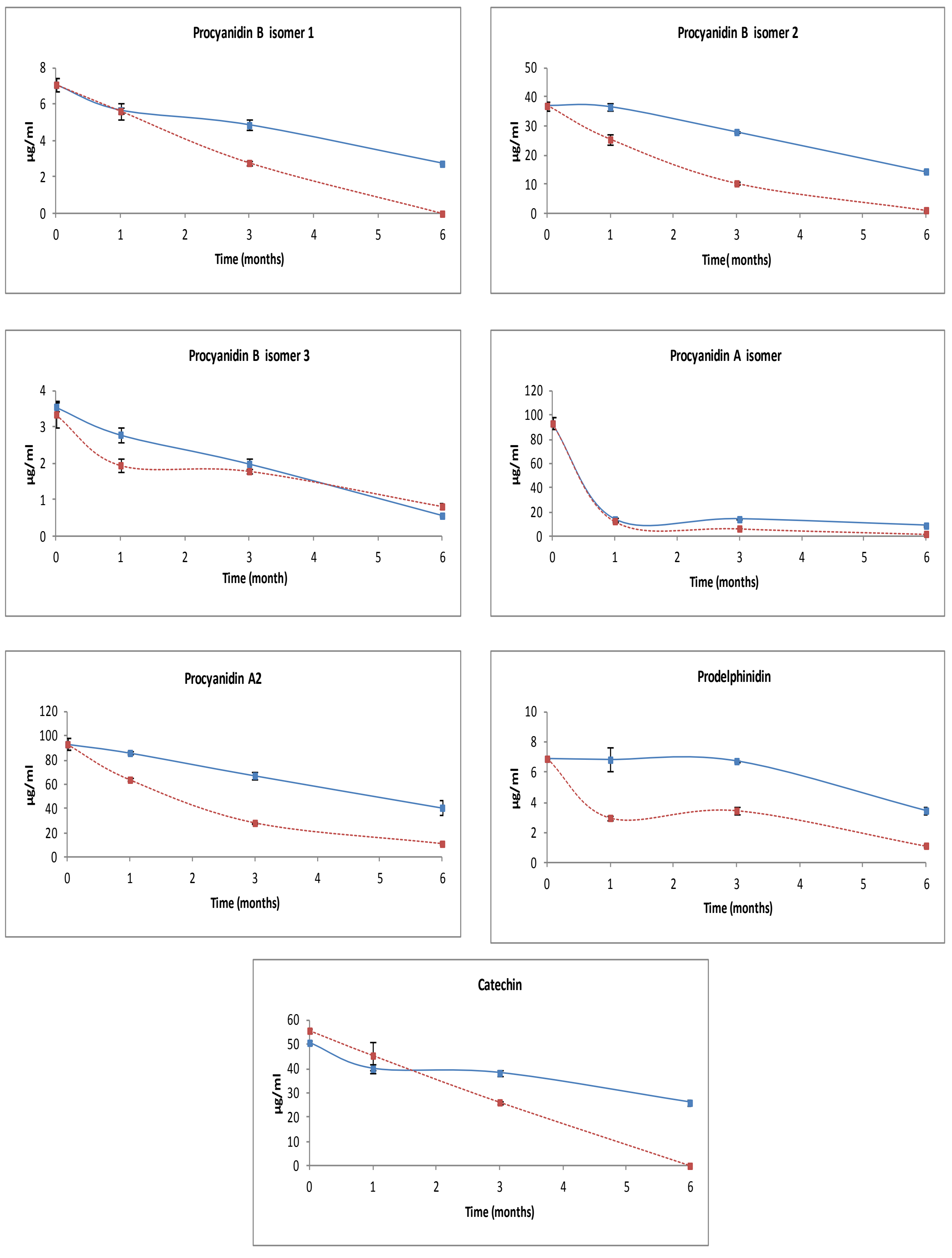


Figure 2. Stability of PACs and catechin from gamma-irradiated cranberry syrup stored for 6 months at 25ºC/60% RH, chamber A (plain line), and at 40ºC/75% RH, chamber B (discontinuous line).

In the case of the flavanol catechin, its content significantly decreased (from 65 µg/mL to 50 µg/mL), around 23%, after gamma-irradiation and was less stable than other simple flavonoids. The same effect was observed by (Breitfellner, Solar & Sontag, 2003) in strawberries in which the catechin concentration decreased as the irradiation dosage increased; specifically, at dosage of 5 kGy, the loss was over 25%. As found for PACs, the loss of this compound in chamber A was more gradual than in chamber B (**Figure 2**), where it was more accused.

Flavonols were highly resistant to gamma-irradiation, and, as for flavanols, were less stable in chamber B conditions. As shown in **Figure 3**, quercetin was the most stable compound over time under different storage conditions. The total loss in chamber A, at 6 months of storage, was approximately 35%, whereas in chamber B the loss was around 50%. Regarding myricetin, quercetin and its derivatives, there were no significant differences ($p < 0.05$) between irradiated samples and non-irradiated controls at the starting time. Quercetin-3-*O*-glucoside was the main flavonol in cranberry syrup control (320 µg/mL) and remained constant until 1 month of storage in both chambers A and B. Breiffellner *et al.* also reported that quercetin-3-*O*-glucoside in strawberry remained unchanged up to a dosage of 6 kGy (Breitfellner, Solar & Sontag, 2003). The rest of the studied flavonols (myricetin hexose, quercitrin, quercetin xylopiranoside isomers, and methyl quercetin pentoside isomer 2) remained constant until the 3rd month of storage in chamber A, as showed in **Figures 3** and **4**. In view of these results, most of the cranberry's phenolic compounds were quite stable for one month of storage, but the combination of gamma-irradiation with other treatments (refrigeration, freezing, etc.) may improve the shelf-life of this syrup.

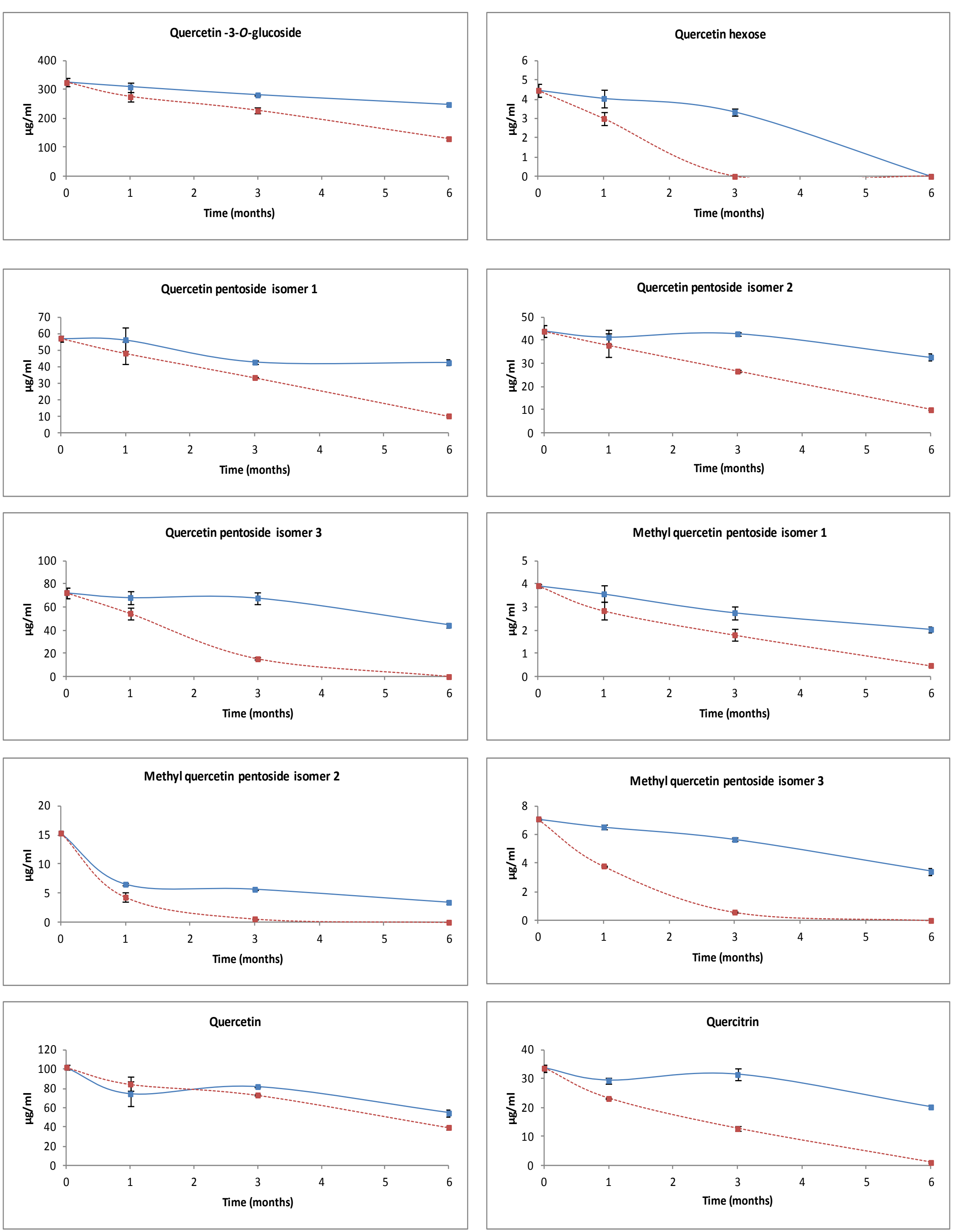


Figure 3. Stability of quercetin and quercetin derivatives from gamma-irradiated cranberry syrup stored for 6 months at 25ºC/60% RH, chamber A (plain line), and at 40ºC/75% RH, chamber B (discontinuous line).

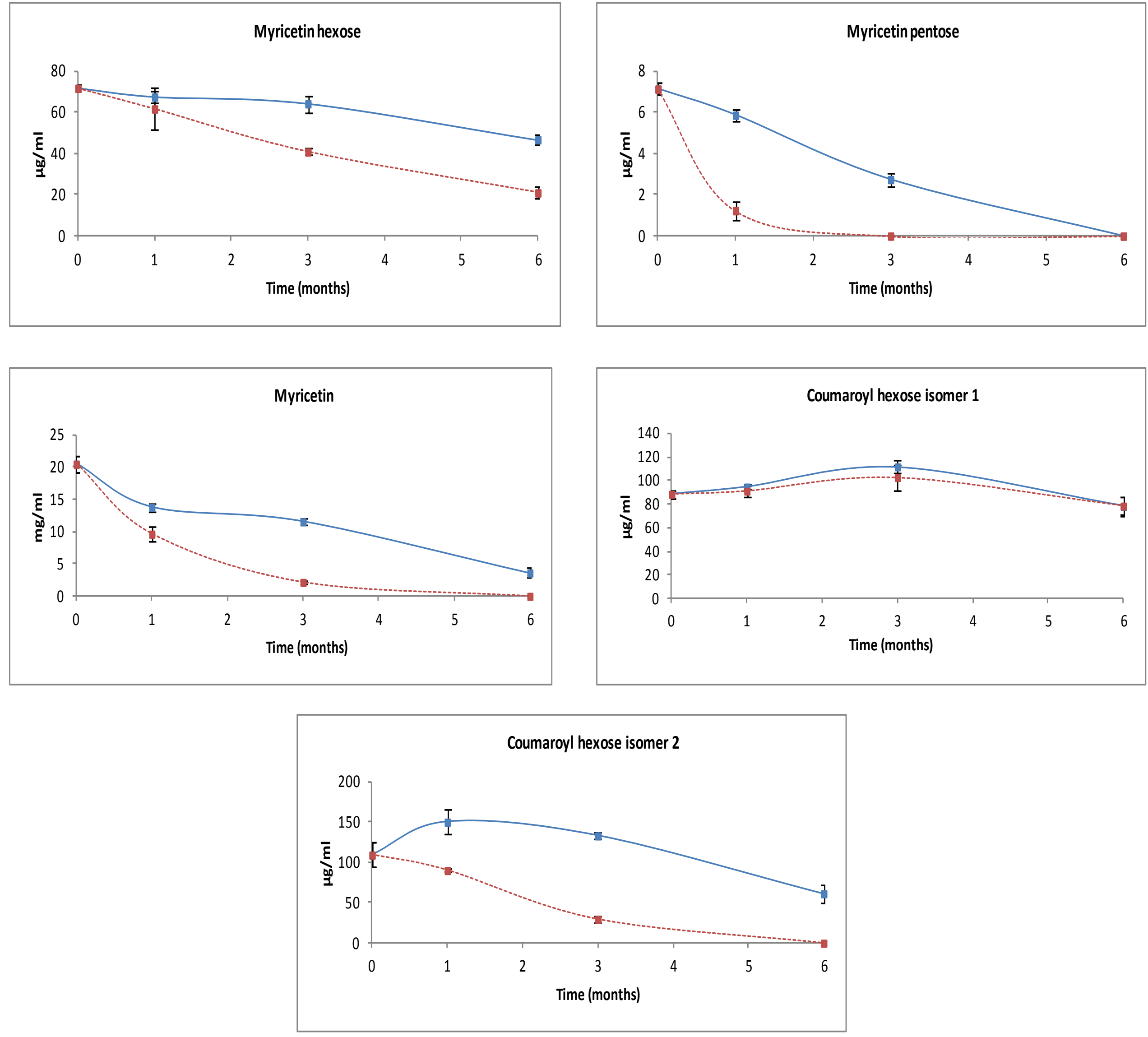


Figure 4. Stability of myricetin, myricetin derivatives, and coumaroyl derivatives from gamma-irradiated cranberry syrup stored for 6 months at 25ºC/60% RH, chamber A (plain line), and at 40ºC/75% RH, chamber B (discontinuous line).

Assessment by DMAC assay

The results from the DMAC assay showed that the total concentration of procyanidins decreased with the time in both chambers, PACs being present in less concentration over time in chamber B (**Table 1**). Thus, non-irradiated syrup (control) had a concentration of 4130 ± 50 µg/mL, which is significantly higher than that found in gamma-irradiated syrup (3460 ± 80 µg/mL). However, this only meant a loss of only about 16%.

On the other hand, the concentration of PACs in samples stored at 25ºC/60%RH (chamber A) significantly differed between the different months of storage, with a total loss over 60% after 6 months. The loss of PACs in samples stored at 40ºC/75%RH in chamber B was higher after 6 months, the total loss of PACs being around 80%. These results from the DMAC assay agreed with results from HPLC-ESI-QTOF-MS quantification. Overall, using the latter method and compared with the control samples, the total loss of PACs was over 65% in chamber A whereas in the chamber B the total loss was over 92%. Although the degradation pathway was not investigated in the present study, they could be related to their oxidation, hydrolysis or isomerization (Chang, Zuo, Chow & Ho, 2006).

Although, DMAC assay is consider to be the best analysis available to perform an overall quantification of flavanols (Boudesocque, Dorat, Pothier, Gueiffier & Enguehard-Gueiffier, 2013), it is important to complement these results with the analysis of each individual compound, since the behavior depend on the chemical structure, even stereochemistry, such as our results found for procyanidin B isomers.

❖ Conclusion

The cranberry syrup studied was rich in flavonoids, mainly procyanidins and flavonols such as quercetin, myricetin, and their derivatives. The compounds were highly resistant to gamma-irradiation (dose of 5 kGy) and after one month of storage at room temperature. The combination of HPLC-ESI-QTOF-MS and DMAC assay

demonstrated to be powerful techniques to quantify flavanols and other phenolic compounds. Our results showed that both methods should be used in a complementary way in order to gain more exhaustive information about the phenolic composition during processing and storage, particularly flavanols. However, further studies under different storage conditions are necessary to optimize preservation.

Acknowledgements

This work was supported by the project AGL2011-29857-C03-02 (Spanish Ministry of Science and Innovation), as well as P09-CTS-4564, P10-FQM-6563 and P11-CTS-7625 (Andalusian Regional Government Council of Innovation and Science) and PI070274 (Carlos III Institute of Health for Clinical Research, Madrid, Spain). The authors are grateful to the Spanish Ministry of Economy and Competitiveness (MINECO) for a FPU fellowship AP2010-1551 (C. Rodríguez-Pérez), a "Personal técnico de apoyo" Grant PTA2012-6956-E (R. Quirantes-Piné) and a Juan de la Cierva contract (M. del M. Contreras).

References

Alighourchi, H., Barzegar, M., & Abbasi, S. (2008). Effect of gamma irradiation on the stability of anthocyanins and shelf-life of various pomegranate juices. *Food Chemistry*, 110(4), 1036-1040.

Alothman, M., Bhat, R., & Karim, A. A. (2009). Effects of radiation processing on phytochemicals and antioxidants in plant produce. *Trends in Food Science and Technology*, 20(5), 201-212.

Ayed, N., Yu, H., & Lacroix, M. (1999). Improvement of anthocyanin yield and shelf-life extension of grape pomace by gamma irradiation. *Food Research International*, 32(8), 539-543.

Boudesocque, L., Dorat, J., Pothier, J., Gueiffier, A., & Enguehard-Gueiffier, C. (2013). High performance thin layer chromatography-densitometry: A step further for quality control of cranberry extracts. *Food Chemistry*, 139(1-4), 866-871.

Breitfellner, F., Solar, S., & Sontag, G. (2003). Radiation induced chemical changes of phenolic compounds in strawberries. *Radiation Physics and Chemistry*, 67(3-4), 497-499.

Carocho, M., Barreira, J. C. M., Antonio, A. L., Bento, A., Kaluska, I., & Ferreira, I. C. F. R. (2012). Effects of electron-beam radiation on nutritional parameters of Portuguese chestnuts (*Castanea sativa* Mill.). *Journal of Agricultural and Food Chemistry*, 60(31), 7754-7760.

Chang, Q., Zuo, Z., Chow, M. S. S., & Ho, W. K. K. (2006). Effect of storage temperature on phenolics stability in hawthorn (*Crataegus pinnatifida* var. major) fruits and a hawthorn drink. *Food Chemistry*, 98(3), 426-430.

Dixit, A. K., Bhatnagar, D., Kumar, V., Chawla, D., Fakhruddin, K., & Bhatnagar, D. (2012). Antioxidant potential and radioprotective effect of soy isoflavone against gamma irradiation induced oxidative stress. *Journal of Functional Foods*, 4(1), 197-206.

EMEA (European Medicines Agency) (2007). Committee for Human Medicinal Products. http://www.ema.europa.eu/ema/, 2007.

Es-Safi, N., Guyot, S., & Ducrot, P. (2006). NMR, ESI/MS, and MALDI-TOF/MS analysis of pear juice polymeric proanthocyanidins with potent free radical scavenging activity. *Journal of Agricultural and Food Chemistry*, 54(19), 6969-6977.

Foo, L. Y., Lu, Y., Howell, A. B., & Vorsa, N. (2000). The structure of cranberry proanthocyanidins which inhibit adherence of uropathogenic P-fimbriated *Escherichia coli in vitro*. *Phytochemistry*, 54(2), 173-181.

Formica, J. V. (1995). Review of the biology of quercetin and related bioflavonoids. *Food and Chemical Toxicology*, 33(12), 1061-1080.

Grace, M. H., Massey, A. R., Mbeunkui, F., Yousef, G. G., & Lila, M. A. (2012). Comparison of health-relevant flavonoids in commonly consumed cranberry products. *Journal of Food Science*, 77(8), H176-H183.

He, F., Pan, Q., Shi, Y., & Duan, C. (2008). Biosynthesis and genetic regulation of proanthocyanidins in plants. *Molecules*, 13(10), 2674-2703.

Iswaldi, I., Gómez-Caravaca, A. M., Arráez-Román, D., Uberos, J., Lardón, M., Segura-Carretero, A., & Fernández-Gutiérrez, A. (2012). Characterization by high-

performance liquid chromatography with diode-array detection coupled to time-of-flight mass spectrometry of the phenolic fraction in a cranberry syrup used to prevent urinary tract diseases, together with a study of its antibacterial activity. *Journal of pharmaceutical and biomedical analysis*, 58(1), 34-41.

Krueger, C. G., Reed, J. D., Feliciano, R. P., & Howell, A. B. (2013). Quantifying and characterizing proanthocyanidins in cranberries in relation to urinary tract health. *Analytical and Bioanalytical Chemistry*, 405(13), 4385-4395.

Neto, C. C. (2011). Cranberries: Ripe for more cancer research? *Journal of the science of food and agriculture*, 91(13), 2303-2307.

Pappas, E., & Schaich, K. M. (2009). Phytochemicals of cranberries and cranberry products: Characterization, potential health effects, and processing stability. *Critical reviews in food science and nutrition*, 49(9), 741-781.

Prior, R. L., Fan, E., Ji, H., Howell, A., Nio, C., Paynef, M. J., & Reed, J. (2010). Multi-laboratory validation of a standard method for quantifying proanthocyanidins in cranberry powders. *Journal of the science of food and agriculture*, 90(9), 1473-1478.

Prior, R. L., & Gu, L. (2005). Occurrence and biological significance of proanthocyanidins in the American diet. *Phytochemistry*, 66(18 SPEC. ISS.), 2264-2280.

Reed, J. (2002). Cranberry flavonoids, atherosclerosis and cardiovascular health. *Critical reviews in food science and nutrition*, 42(3 Suppl), 301-316.

Sánchez-Patán, F., Bartolomé, B., Martín-Alvarez, P. J., Anderson, M., Howell, A., & Monagas, M. (2012). Comprehensive assessment of the quality of commercial cranberry products. Phenolic characterization and *in vitro* bioactivity. *Journal of Agricultural and Food Chemistry*, 60(13), 3396-3408.

Vadodkar, A. S., Suman, S., Lakshmanaswamy, R., & Damodaran, C. (2012). Chemoprevention of breast cancer by dietary compounds. *Anti-Cancer Agents in Medicinal Chemistry*, 12(10), 1185-1202.

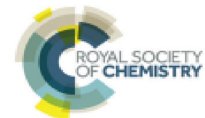
Wallace, T. C., & Giusti, M. M. (2010). Evaluation of parameters that affect the 4-dimethylaminocinnamaldehyde assay for flavanols and proanthocyanidins. *Journal of Food Science*, 75(7), C619-C625.

Chapter 7

Antibacterial activity of isolated phenolic compounds from cranberry (*Vaccinium macrocarpon*) against *Escherichia coli*

Food and Function, 2016, 7(3):1564-1573

Food & Function



PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/c5fo01441g

Antibacterial activity of isolated phenolic compounds from cranberry (*Vaccinium macrocarpon*) against *Escherichia coli*†

Celia Rodríguez-Pérez,[a,b] Rosa Quirantes-Piné,*[b] José Uberos,[c] Cecilia Jiménez-Sánchez,[a,b] Alejandro Peña[d] and Antonio Segura-Carretero[a,b]

[a] *Department of Analytical Chemistry, Faculty of Sciences, University of Granada, Avenida Fuentenueva s/n, 18071 Granada, Spain*

[b] *Research and Development Functional Food Center (CIDAF), Health Science Technological Park, Avenida del Conocimiento 37, Edificio BioRegión, 18016 Granada, Spain*

[c] *Unit of Clinical Pediatrics, San Cecilio University Hospital, 18012 Granada, Spain*

[d] *Microbiology Department, San Cecilio University Hospital, Instituto Biosanitario de Investigación (IBIG), Granada, Spain*

Abstract

Phenolic compounds from a cranberry extract were isolated in order to assess their contribution to the antibacterial activity against urophatogenic strains of *Escherichia coli* (UPEC). With this purpose, a total of 25 fractions from a cranberry extract were isolated using semipreparative high performance liquid chromatography (HPLC) and characterized based on the results obtained by reversed-phase HPLC coupled to mass spectrometry detection. Then, the effect on UPEC surface hydrophobicity and biofilm formation of the cranberry extract as well as the purest fractions (a total of 13) was tested. As expected, the whole extract presented a powerful antibacterial activity against UPEC while the selected fractions presented different behavior. Myricetin and quercitrin significantly decreased ($p < 0.05$) *E. coli* biofilm formation compared with the control, while dihydroferulic acid glucuronide, procyanidin A dimer, quercetin glucoside, myricetin and prodelphinidin B led to a significant decrease on the surface hydrophobicity compared with the control. The results suggest that apart from

proanthocyanidins, other compounds, mainly flavonoids, can act against *E. coli* biofilm formation and also modify UPEC surface hydrophobicity *in vitro*, one of the first steps of adhesion.

Keywords: cranberry, semipreparative-HPLC, phenolic compounds, adherence, biofilm, surface hydrophobicity, *Escherichia coli*.

❖ Introduction

Cranberries (*Vaccinium macrocarpon*) are popularly consumed as part of the human diet both fresh and processed forms. Additionally, their derived extracts are also used, mainly as part of some botanical dietary supplements forms due to their renowned human health benefits[1]. Cranberry has proved to be an excellent source of bioactive compounds such as flavonoids (procyanidins, flavonols), and phenolic acids derivatives[2]. Thanks to these health-promoting compounds, cranberry and cranberry-based products consumption has been correlated with recurrent urinary tract infections (UTIs) prophylaxis[3,4]. UTI has been defined as the presence of significant number of pathogenic bacteria or organisms in the urinary system and it is considered the most common type of infection in the body, which affects women in a greater extent than men[5]. *Escherichia coli* (*E. coli*) is the main responsible bacterial species for the appearance of this infection, and causes more than 80 percent of all acquired UTIs in the community[6]. Concretely, the ability of urophatogenic *Escherichia coli* (UPEC) to form biofilm has been strongly associated with recurrent UTIs[7, 8] and there have been proven that surface hydrophobicity is conductive to adhesion to surfaces and to penetration of host tissues[9] since bacteria have developed many different ways to use hydrophobic effect in order to adhere to substrata, such as previously described by Doyle *et al*[10]. The importance of biofilms in public health is related to the decreased susceptibility to antimicrobial agents that biofilm-associated microorganisms exhibit. This is the case of *E. coli* which has shown to be increasingly resistant to some of the antibiotics currently used in the treatment of UTIs[11,12]. In addition, the public interest in herbal medicines and natural products is still growing. For this reason, researchers have concluded the re-evaluation of first and second-line therapies for the treatment of UTIs becomes to be pivotal[13]. Consequently, the antimicrobial effect of cranberry products and their phenolic compounds have been widely studied, especially to develop new healthy food ingredients, functional foods, nutraceuticals, and pharmaceuticals[14]. The most accepted theory about the mechanism of action of cranberry compounds for the promotion of urinary tract health is based on the effects

of fructose and PACs in inhibiting the adherence of type 1 and P fimbriae of *E. coli* to the uroepithelial cell receptors[15,16]. Without adhesion, the bacteria cannot infect the mucosal surface. Despite a large number of studies highlighted that there are synergisms between different compounds present in cranberry extracts[17-19], other authors such as Hisano *et al.* concluded that the use of the whole cranberry for UTIs prevention was not scientifically supported, and for that reason, it is pointed out the necessity of research focused on bioactive compounds from cranberry instead of the entire fruit[3]. However, the isolation of simultaneous compounds from cranberry extracts is an arduous task due to its complexity. Reversed-phase semipreparative high performance liquid chromatography (semipreparative-HPLC) has been increasingly used once possesses an interesting target separation ability, great efficiency and high recovery[20], and therefore can be a valuable tool to solve the aforementioned difficulty.

In this sense, the aims of the present research were to fractionate phenolic compounds from a cranberry extract by semipreparative-HPLC and to give new insights into their contribution to the antibacterial effect by testing the *in vitro* effect of the entire extract and the isolated fractions against *E. coli* surface hydrophobicity and biofilm formation.

❖ Results and discussion

Isolation of phenolic compounds from cranberry extracts by semipreparative-HPLC and characterization of fractions by HPLC-ESI-MS.

Natural extracts usually consist of hundreds of compounds, and the isolation of particular components presents unique problems because the methods used to isolate them are based mainly on their polarity. The similarity of some polyphenolic structures makes that compounds elute at similar retention times, making difficult their separation. For that reason, only few studies have focused on the chromatographic methods for the isolation of multiple compounds simultaneously. In this regard, semipreparative-HPLC is a robust, versatile, and usually rapid technique by which compounds can be purified from complex mixtures[21].

In the current research, the analytical HPLC method previously developed for the characterization of phenolic compounds from cranberry extracts[19] was scaled-up to semipreparative-HPLC scale. Different gradients were tested to enhance the separation of the compounds (data not shown), selecting as optimum the method described in "experimental" section. **Figure 1** shows the UV chromatogram of the cranberry extract under study acquired with the proposed method, where the fractions collected are indicated according to their elution order.

The isolated fractions were subsequently analyzed by HPLC-ESI-QTOF-MS in negative ionization mode. Characterization strategy was carried out by generation of the candidate molecular formula with a mass accuracy limit of 5 ppm, considering their MS spectra determined by quadrupole time-of-flight mass spectrometer (QTOF–MS), and also comparing with those of authentic standards whenever available and data from the literature. Databases such as SciFinder Scholar (http://scifinder.cas.org), MassBank (http://massbank.jp), and METLIN Metabolite Database (http://metlin.scripps.edu) were consulted in order to acquire chemical structure information.

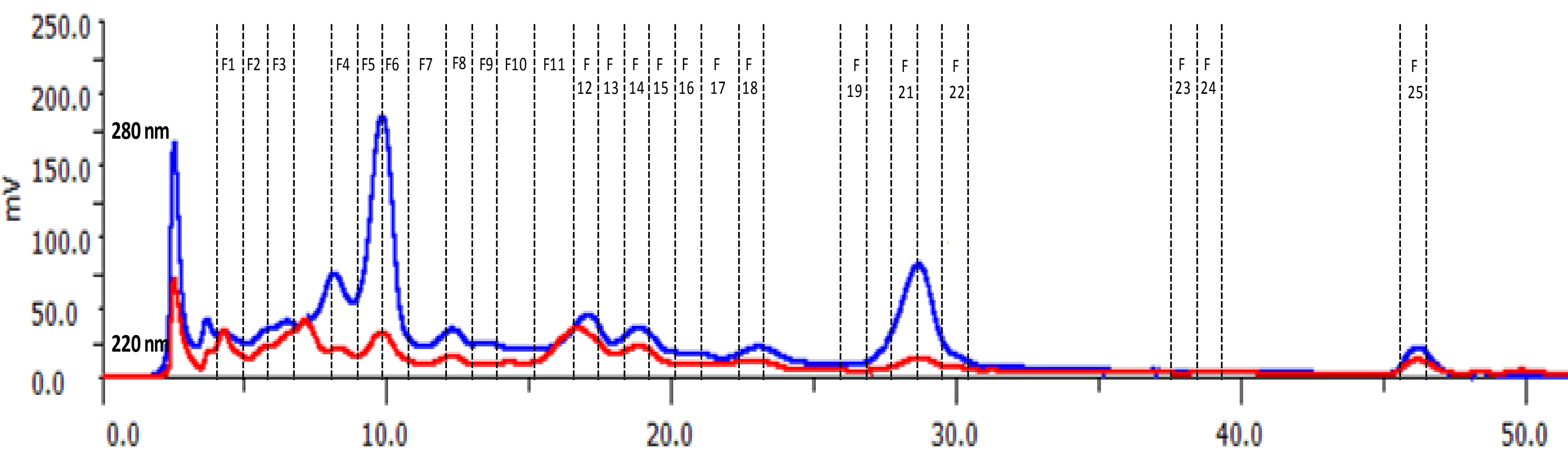


Figure 1. Semipreparative-HPLC-UV chromatograms of cranberry extract indicating the collected fractions.

280 nm CF 1 CF 2 CF 3 CF 4 CF 5 CF 6 CF 7 CF 8 CF 9 CF 10 CF 11 CF 12 CF 13

220 nm

Despite the scarcity of literature on the fractionation of cranberry using semipreparative-HPLC makes difficult to contrast our optimized method with others, and the results could not be comparable, the optimized method allowed obtaining 25 fractions from the cranberry extract (**Table 1**), which were composed predominantly by procyanidins (PACs) and flavonols. Even though the difficulty in separating and purifying PACs has been previously highlighted[22], the current method allowed isolating some of them, including A-type procyanidin dimmers, an A-type procyanidin trimer (cinnamtannin B1) and a gallocatechin dimer (prodelphinidin). PACs are the most typical compounds characterized in cranberry, noteworthy for their antioxidant activity, although they may also present other pharmacological and medicinal properties such as anti-carcinogenic, anti-inflammatory, and vasodilator[23]. Isolated cranberry flavonols included quercetin derivatives which have been previously demonstrated to have both *in vivo* and *in vitro* antioxidant, anti-inflammatory, anticancer, and antidiabetic activities[24]. In addition, four myricetin derivatives were characterized. These compounds are also common dietary flavonoids which have demonstrated antioxidant, cytoprotective, antiviral, antimicrobial, anticancer and antiplatelet activities[25]. Apart from these compounds, one hydroxicinnamic acid derivative (dihydroferulic acid glucuronide) was isolated.

Among these 25 eluted fractions, 13 were chosen in order to test their antibacterial activity against *E. coli,* namely F: 6, 8, 9, 11, 13–16, 18, 19, 21, 23, and 25. These fractions were selected on the basis of their purity, due to they showed a purer composition than the rest, presenting up to two target phenolic compounds. HPLC-ESI-QTOF-MS chromatograms from these nearly pure fractions are displayed in **Figure 2**. Semipreparative-HPLC allowed getting 1.1 mg of F6, F8, and F18; 0.9 mg of F9 and F15; 1.7 mg of F11; 1.5 mg of F13; 1 mg of F14; 0.7 mg of F16 and F21; 0.6 mg of F19 and F25; and 0.5 mg of F23. Different concentrations tested are depicted in **Table 2.** The use of different concentrations of each fraction was established in order to simulate their contribution in the whole extract.

Table 1. Retention time and mass spectral data of the compounds characterized in the fractions from cranberry extract by HPLC-ESI-MS in negative mode.

Proposed compound	Retention time (min)	Molecular Formula	Calculated m/z ($[M-H]^-$)	Fractions
Quinic acid	5.212	$C_7H_{12}O_6$	191.0561	1,2
Kaempferol arabinoside	5.527	$C_{20}H_{18}O_{10}$	417.0827	1
Procyanidin B	5.736	$C_{30}H_{26}O_{12}$	577.1351	3
Caffeic acid glucoside	6.588	$C_{15}H_{18}O_9$	341.0878	1
Cinnamtannin B1 isomer 1	7.13	$C_{45}H_{36}O_{18}$	863.1829	1,4
Myricetin arabinoside	7.421	$C_{20}H_{18}O_{12}$	449.0725	5
Catechin *	7.765	$C_{15}H_{14}O_6$	289.0718	4
Procyanidin C1	9.689	$C_{45}H_{38}O_{18}$	865.1985	4
Myricetin glucoside isomer 1	9.065	$C_{21}H_{20}O_{13}$	479.0831	6
Myricetin glucoside isomer 2	9.123	$C_{21}H_{20}O_{13}$	479.0831	7
Dihydroferulic acid glucuronide	9.183	$C_{16}H_{20}O_{10}$	371.0984	4,8
Procyanidin A dimer isomer 1	10.611	$C_{30}H_{24}O_{12}$	575.1195	9
Quercetin glucoside isomer 1	12.155	$C_{21}H_{20}O_{12}$	463.0882	12
Quercetin glucoside isomer 2	12.191	$C_{21}H_{20}O_{12}$	463.0882	11
Procyanidin A dimer isomer 2	12.973	$C_{30}H_{24}O_{12}$	575.1195	12,13
Quercetin-3-O-glucoside *	14.775	$C_{21}H_{20}O_{12}$	463.0882	10
Cinnamtannin B1 isomer 2	15.019	$C_{45}H_{36}O_{18}$	863.1829	14
Quercetin glucoside isomer 3	15.095	$C_{21}H_{20}O_{12}$	463.0882	10
Quercetin arabinoside isomer 1	15.202	$C_{20}H_{18}O_{11}$	433.0776	14, 16
Quercitrin isomer 1	15.663	$C_{21}H_{20}O_{11}$	447.0933	17,18
Quercetin arabinoside isomer 2	16.013	$C_{20}H_{18}O_{11}$	433.0776	15
Myricetin *	20.229	$C_{15}H_{10}O_8$	317.0303	21
Quercitrin *	20.847	$C_{21}H_{20}O_{11}$	447.0933	19,20,21
Quercitrin isomer 2	21.668	$C_{21}H_{20}O_{11}$	447.0933	22
Prodelphinidin B	24.246	$C_{30}H_{26}O_{14}$	609.125	23,24
Quercetin	26.56	$C_{15}H_{10}O_7$	301.0354	25

*Compounds identified with standard.

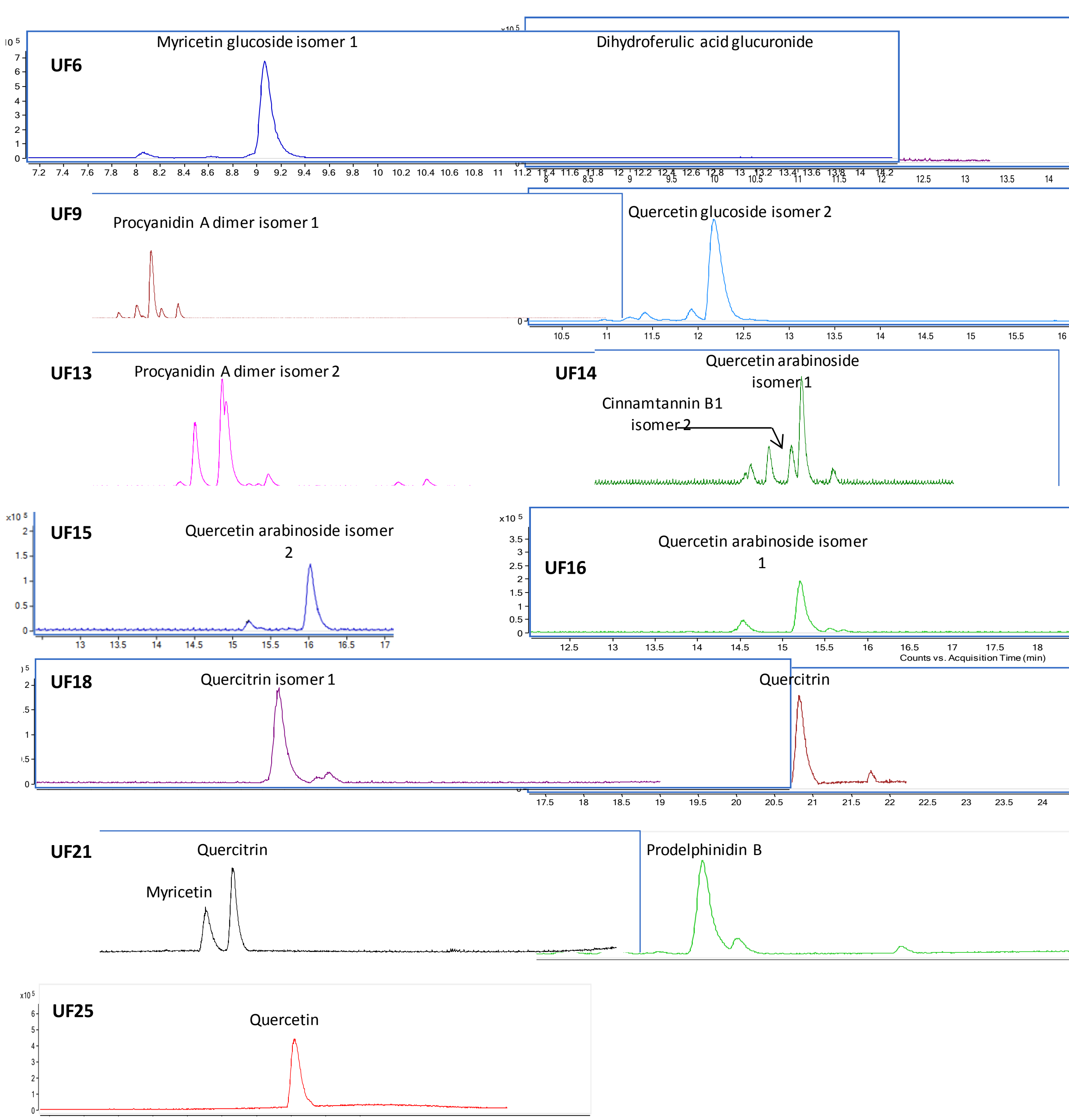


Figure 2. HPLC-MS chromatograms of the isolated fractions from cranberry extract.

Table 2. Concentration tested in bioactivity assays.

Fractions tested	Dilution A ($\mu g\ mL^{-1}$)	Dilution B ($\mu g\ mL^{-1}$)
F6	550	275
F8	550	275
F9	450	225
F11	850	425
F13	750	375
F14	500	250
F15	450	225
F16	350	175
F18	550	275
F19	300	150
F21	350	175
F23	250	125
F25	300	150

Antibacterial activity

Although some authors reported that cranberry does not have any effect against Gram-negative bacteria pathogens such as *E. coli*[26], most of the research converges on the fact that berries, and especially cranberry and cranberry-based products, have both *in vitro* and *in vivo* antibacterial activity[14,16,19,22,27,28]. As aforementioned, the most accepted mechanism of action of cranberry focuses primarily on its ability to prevent bacterial binding to host cell surface membrane[29], one of the initial steps in the infection process. This process is initially mediated by the electrostatic charge (characterized by determining its zeta potential) and consequently surface hydrophobicity of microorganisms followed by other factors such as formation of fimbriae and specific adhesins[30]. Thus, surface physicochemical parameters such as electrostatic charge are then fundamentally important with regard to influencing overall polarity in order to maintain the degree of bacterial surface hydrophobicity necessary for the bacterial adhesion. Subsequently, adhesion of bacteria to host surfaces is finally a key element in the formation of biofilms that constitutes a protected mode of growth that allows bacteria to survive in hostile environment[31]. For that reason, the effect of the previously isolated fractions as well as the whole extract

on biofilm formation and surface hydrophobicity of fourteen UPECs has been tested as a way to evaluate the individual contribution of every compound to the antibacterial activity.

Figures 3 and **4** show the mean and standard deviation (SD) of biofilm formation and surface hydrophobicity for *E. coli* after incubation with each isolated fraction and with the cranberry extract, respectively, at two different assayed concentrations. **Table 3** summarizes the Wilcoxon matched-pairs signed-ranks analysis for the biofilm formation and surface hydrophobicity of the isolated fractions and the whole extract.

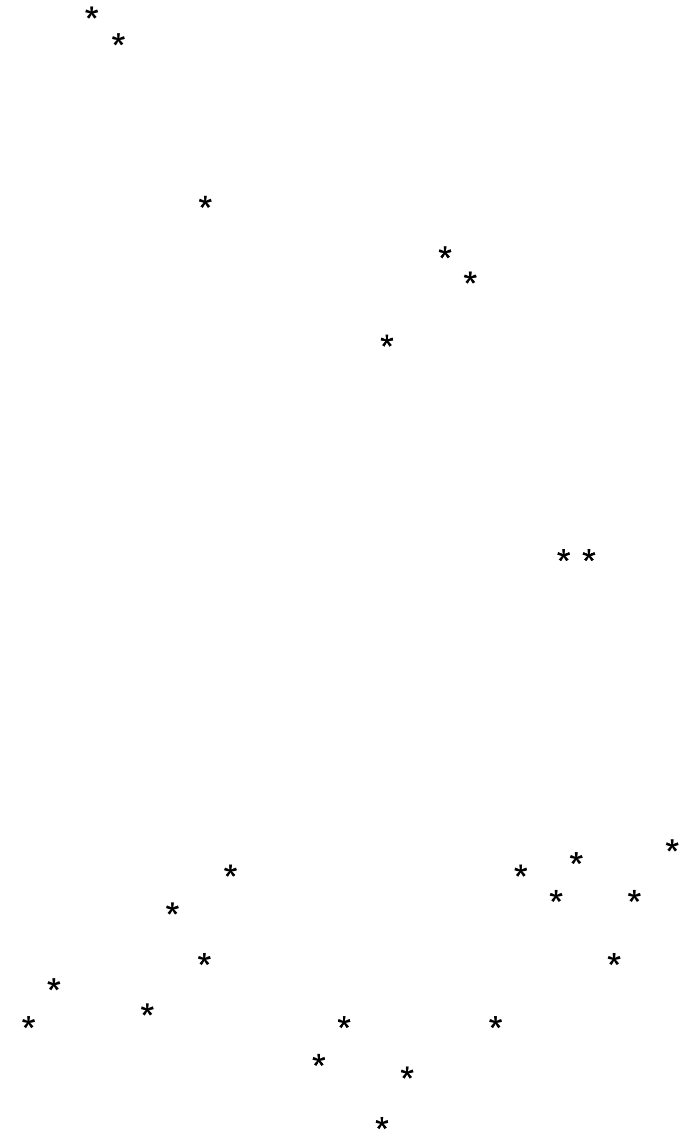


Figure 3. (a) Mean and standard deviation in biofilm formation after incubating *E. coli* strains with each selected fraction; (b) Mean and standard deviations of surface hydrophobicity after incubating *E. coli* strains with each selected fraction. *Significant differences between control group and tested fraction ($p < 0.05$).

Figure 4. (a) Mean and standard deviation in biofilm formation after incubating *E. coli* strains with cranberry extract; (b) Mean and standard deviations of surface hydrophobicity after incubating *E. coli* strains with cranberry extract. *Significant differences between control group and tested extract ($p < 0.05$).

Table 3. Surface hydrophobicity and adherence after incubating *E. coli* with each cranberry fraction compared with control. Dil A, dilution at 1 mg mL^{-1}; Dil B, dilution at 0.5 mg mL^{-1}. *Significant differences between control group and tested extract ($p < 0.05$).

Hydrophobicity	z	Asymptotic significance	Adherence	z	Asymptotic significance
Extract Dil A	-3.063	0.002*	**Extract Dil A**	- 3.296	0.001*
Extract Dil B	-3.065	0.002*	**Extract Dil B**	- 3.296	0.001*
F6 Dil A	-1.913	0.056	**F6 Dil A**	-0.795	0.427
F6 Dil B	-0.577	0.564	**F6 Dil B**	-0.852	0.394
F8 Dil A	-3.083	0.002*	**F8 Dil A**	-0.795	0.427
F8 Dil B	-3.081	0.002*	**F8 Dil B**	-1.931	0.053
F9 Dil A	-0.122	0.903	**F9 Dil A**	-3.408	0.001*
F9 Dil B	-1.294	0.196	**F9 Dil B**	-3.296	0.001*
F11 Dil A	-3.117	0.002*	**F11 Dil A**	-0.659	0.510
F11 Dil B	-2.988	0.003*	**F11 Dil B**	-0.471	0.638
F13 Dil A	-3.113	0.002*	**F13 Dil A**	-3.124	0.002*
F13 Dil B	-2.671	0.008*	**F13 Dil B**	-1.704	0.088
F14 Dil A	-1.256	0.209	**F14 Dil A**	-1.590	0.112
F14 Dil B	-0.723	0.470	**F14 Dil B**	-1.533	0.125
F15 Dil A	-3.074	0.002*	**F15 Dil A**	-0.738	0.460
F15 Dil B	-3.074	0.002*	**F15 Dil B**	-0.454	0.650
F16 Dil A	-3.315	0.001*	**F16 Dil A**	-2.556	0.011*
F16 Dil B	-3.188	0.001*	**F16 Dil B**	-1.533	0.125
F18 Dil A	-2.456	0.014*	**F18 Dil A**	-2.668	0.008*
F18 Dil B	-0.586	0.558	**F18 Dil B**	-2.731	0.006*
F19 Dil A	-3.237	0.001*	**F19 Dil A**	-0.284	0.776
F19 Dil B	-2.989	0.003*	**F19 Dil B**	-0.966	0.334
F21 Dil A	-2.849	0.004*	**F21 Dil A**	-2.840	0.005*
F21 Dil B	-2.673	0.008*	**F21 Dil B**	-3.067	0.002*
F23 Dil A	-2.833	0.005*	**F23 Dil A**	0.00	1.00
F23 Dil B	-2.631	0.009*	**F23 Dil B**	-1.420	0.156

Table 3. *(Continued)*

Hydrophobicity	z	Asymptotic significance	Adherence	z	Asymptotic significance
F25 Dil A	-2.449	0.014*	**F25 Dil A**	-0.454	0.650
F25 Dil B	-0.791	0.429	**F25 Dil B**	-0.284	0.776

After testing the selected fractions, two concentrations of F9 made up of procyanidin type-A dimer, showed a statistically significant increase in biofilm formation compared with the control (**Figure 3**). Other research has also described an increase of biofilm formation in four of the 20 *E. coli* strains tested after consuming cranberry juice[7] and a reduction of biofilm formation only in one of them. However, F9 did not significantly change surface hydrophobicity. On the other hand, F13 (made up of other isomer of procyanidin type-A dimer) at the highest concentration (dilution A) caused an increase in biofilm formation while both concentrations tested significantly decreased surface hydrophobicity. In any case, it should be pointed out that the hydrophobicity of bacteria can vary even within the same strain depending on the mode and stage of growth[32]. Despite the study of PACs in *E. coli* has been widely described, controversial results are still reported in literature. Foo *et al*. also found a weak activity of procyanidin A2 against the inhibition of adherence of *E. coli*[33]. In another study, PACs as a group of compounds inhibited the growth of *E. coli* CM 871, with no inhibition of *E. coli* 50[17]. Foo *et a.l* also proved the anti-adherent effect of procyanidin trimers[33]. However, no statistical differences were found between F14 (made up of cinnamtannin B1 and quercetin arabinoside) and the control in both assays tested. Prodelphinidin B (F23) also influenced the antibacterial effect against *E. coli* by decreasing the bacteria surface hydrophobicity. Prodephinidins with pyrogallol groups, which have similar structures to procyanidins except for their hydroxyphenyl group, have reported to have stronger antibacterial activity than procyanidins with the catechol groups[34]. However, the different results obtained from different isolated PACs, reinforce the theory proposed by Schmidt *et al.* who concluded that it was

likely that a mixture of several high molecular weight PACs were responsible for the anti-proliferation and anti-adhesion activity.

Regarding isolated flavonols, fraction formed by myricetin and quercitrin (F21) was the most active fraction against the *E. coli* biofilm formation and also influenced the decrease in *E. coli* surface hydrophobicity. Bacterial hydrophobicity has been proved to be largely influenced by the residues and structures on the surface of the cell[32]. In this way, recent research has pointed out that phytochemicals such as flavonoids can modify bacterial membrane surface hydrophobicity[35] probably based on their ability to complex with extracellular and soluble proteins as well as with bacterial cell walls. Concretely, three mechanisms of action of flavonoids have been proposed: inhibition of nucleic acid synthesis, cytoplasmic membrane damage and inhibition of energy metabolism[36]. Although the anti-adherent effect of myricetin remains controversial, some authors have found that 0.5 mg mL^{-1} of myricetin strongly inhibited the growth of *E. coli*[17]. Only few studies have been carried out in order to assess the flavonoids structure-antibacterial activity relationship. In this sense, some authors concluded that the hydroxylation at position 5 on the A ring and at position 3 on the C ring improves the antibacterial activity of flavones decreasing membrane fluidity[37,38]. These previous results could explain the antibacterial effects that the combination of quercitrin and myricetin (F21) showed in both assays. Cowan *et al.* reported that more lipophilic flavonoids may disrupt microbial membranes[26]. Furthermore, Wojnicz, *et al*. affirmed that flavonoids such as quercetin, reduced biofilm synthesis because they can suppress autoinducer-2 activity, which is responsible for cell-to-cell communication[39]. In particular other authors have described the existence of antibacterial activity of quercetin against *E. coli*[6]. Contrary to these previous findings, F25, formed by pure quercetin, a molecule that has a lipophilic character despite the presence of five hydroxyl groups in its structure, not only did not show statistical differences in UPEC biofilm formation at two tested concentrations, but also significantly increased the UPEC surface hydrophobicity compared with control at the highest concentration tested (dilution A). Some authors affirmed, in base

of their results, that the degree of hydroxylation might affect the antimicrobial activity of phenolic compounds, indicating that the more polar flavonoids, the more antibacterial effect[17]. In the current study, this theory could be applicable when comparing F25 (quercetin) and F21 (quercitrin and myricetin). The addition of one more hydroxyl group on the aromatic ring of myricetin compared with quercetin may be responsible for its antimicrobial activity. Other research attributes its antimicrobial mechanism against Gram-negative to a reaction with DNA or inhibition of protein synthesis bacteria[40,41]. An early theory based on that hydrophobic effect may be the primary driving force for the adhesion of most pathogens was also proposed[42]. However, taking into account the abovementioned case of quercetin, no relation was observed between *E. coli* surface hydrophobicity and biofilm formation rates.

Despite the great general interest in glycosylated flavonoids due to their diverse bioactivity, research focused on their antibacterial properties is still at the developmental stage. None of the tested concentrations of F6 (myricetin glucoside) showed any activity against biofilm formation nor modifying surface hydrophobicity. Some authors have pointed out that the glycosylation of flavonoids leads to a loss of activity against some Gram-negative bacteria[43]. In addition, early studies concluded that quercetin monosaccharide derivatives showed weak activity against *E. coli* [44]. Following with these compounds, other plant extracts such as white garlic extract, which contains a high concentration of quercetin-4-*O*-glucoside and quercetin-3,4-*O*-diglucoside, had a large inhibiting activity on the growth of *E. coli*, among other Gram-negative bacteria[6]. The current results show that quercetin derivatives do not always produce the same antibacterial effect. On one hand, fractions 18 and 19, made up of quercitrin isomer and quercitrin (quercetin-3- rhamnoside) respectively, showed different antibacterial activity. While incubation with F18 caused a statistically significant increment of UPEC biofilm formation compared with the control and did not present significant differences on surface hydrophobicity, F19 (quercitrin) did not show statistical differences in biofilm formation rates but produced a significant reduction on surface hydrophobicity. Taking into account that F19 was tested at lower

concentrations than F18, as depicted in **Table 2**, this fact suggests that the position of sugar moieties influences the antibacterial activity of flavonoids. Previous studies reported that among quercetin glycosides tested, quercetin-3-rhamnoside exhibited the strongest antibacterial activity against Gram-negative bacteria whereas other quercetin glycosides showed weak or no activity against the same Gram-negative bacteria[45]. On the other hand, F15 and F16, made up of quercetin arabinoside isomers, showed similar trends in significant surface hydrophobicity reduction even testing different concentrations (**Table 3**) while only F16 at 300 µg mL^{-1} (dilution A) significantly increased the biofilm formation rate.

In addition, both tested concentrations of fraction F8, made up of mainly dihydroferulic acid glucuronide, also showed a reduction in the hydrophobicity of *E. coli*. In this regard, Borges *et al*. found that ferulic acid had antimicrobial activity against *E. coli* by irreversible changes in membrane properties through hydrophobicity changes that caused local rupture or pore formation in the cell membranes causing the loss of essential intracellular constituents[46]. Despite Borges *et al.* also concluded in other study that ferulic acid reduced mass of biofilm formed by Gram-negative bacteria[47], dihydroferulic acid glucuronide did not show statistically differences compared with the control.

If we look at the whole extract, the data revealed statistical differences with respect to control in both, biofilm formation and surface hydrophobicity, after incubating UPEC strains with the cranberry extract independent of the concentrations tested (**Figure 4**). This finding suggests that even at low dosage, cranberry extract presents antibacterial activity *in vitro*. As pointed out along the text, the hydrophobic properties of microbial surfaces are conducive to adhesion and, thus, to penetration of host tissues. Taking into account the capacity of UPEC to form biofilms, it could be expected a positive relationship between hydrophobicity and biofilm formation. However, the nonparametric Kendall's rank correlation disclosed that there was no trend between surface hydrophobicity and adherence ($W=0.236$; $p=0.019$) of UPEC tested after the incubation with cranberry extract. These results could be attributed to

the different behavior of each strain. In fact, despite most of UPEC strains are *in vitro* positive for biofilm production[48], it has been previously reported that even the same strain can respond very differently to biofilm formation depending on the environmental factors, among others[49]. Thus, the fact that complete extracts showed stronger inhibitions in surface hydrophobicity and biofilm formation compared with isolated fractions reinforces the theory that the antimicrobial activity of cranberry extracts is a synergistic effect of various phenolic compounds, many of which are probably still unidentified.

❖ Conclusions

In conclusion, the present work showed that semipreparative-HPLC proved to be a powerful tool for the fractionation of phenolic compounds from complex matrices like cranberry extracts. The results suggested that apart from PACs, other compounds, mainly flavonoids, can act against uropathogenic *E. coli* biofilm formation and also modifying UPEC surface hydrophobicity *in vitro*, one of the first steps of adhesion. Additionally, a synergism between compounds could affect the antibacterial effects of the studied extracts. However, further studies *in vivo* are necessary to confirm their antibacterial activity.

❖ Experimental

General Experimental Procedures

Formic acid and acetonitrile used for preparing mobile phases were from Sigma-Aldrich (Steinheim, Germany) and Fisher Scientific (Loughborough, Leics, UK), respectively. Ultrapure water with a resistivity value of 18.2 MΩ was obtained from Milli-Q system (Millipore, Bedford, MA, USA). HPLC grade methanol (99.9%) was purchased from Fisher Scientific (Loughborough, Leics, UK). For microbiological determinations, tryptic soy broth (TSB) (Fluka), phosphate buffered saline pH-7.4 (PBS), ammonium phosphate; acetic acid, methanol, and Hucker's cristal violet were supplied from Sigma-Aldrich (Steinheim, Germany).

Sample preparation

A commercial extract in capsules of American cranberry consisted on concentrated cranberry juice was used to carry out this study (Urell® Pharmatoka, Rueil Malmaison, France). The content of five capsules (200 mg each) was mixed and 5 mg of the cranberry extract were weighted and dissolved in 5 ml of a (50:50, v/v) methanol/water mixture to obtain a final concentration of 1 mg mL^{-1}. Then, the solutions were vortexed for 2 min, sonicated for 10 min, and centrifuged at 984 × g. Finally, the supernatants were filtered through 0.2 µm regenerated cellulose syringe filters. The extraction procedure was carried out in triplicate.

For isolation of phenolic compounds from cranberry extract, solution stock at 50 mg mL^{-1} was prepared by dissolving the appropriate amount of cranberry extract in (50:50, v/v) methanol/water mixture, and the aforementioned procedure was followed.

To develop the antimicrobial assays, two solutions of the extract were prepared at 1 mg mL^{-1} (dilution A) and 0.5 mg mL^{-1} (dilution B) in phosphate buffered saline (PBS), pH 7.4.

Isolation of compounds by semipreparative-HPLC

Fractionation was conducted at room temperature using a Gilson semipreparative HPLC system (Gilson Inc., Middleton, WI, USA) equipped with a binary pump (model 331/332), automated liquid handling solutions (model GX-271), and UV-Vis detector (model UV-Vis 156). To separate the target compounds, an Ascentis C_{18} column (10 µm, 250 × 212 mm) was used. The mobile phases consisted of 1% formic acid in water-acetonitrile (90:10, v/v) (phase A) and acetonitrile (phase B). The following optimized multi-step linear gradient was developed: 0 min, 5% B; 10 min, 9.5% B; 35 min, 17.5% B; 50 min, 25% B; 55 min, 100% B; 57 min, 5% B; 62 min, 0% B. The initial conditions were held for 10 min. The injection volume was 1 mL. The flow rate used was 15 mL min^{-1}. The separated compounds were monitored with UV-Vis (220–280 nm). The fraction-collection step consisted of UV-based purification, determining the elution time window for collecting each fraction. Finally,

a total of 25 fractions were collected, and the solvent was evaporated under vacuum. The residue of each fraction was weighted and dissolved a) in methanol to obtain a final concentration of 100 ppm to analyze them by HPLC-ESI-MS, and b) in 2 mL of PBS to carry out the antibacterial assays.

Characterization of the fractions by HPLC-ESI-MS

Analyses were carried out by an Agilent 1200 series rapid resolution (Santa Clara, CA, USA) equipped with a binary pump, a vacuum degasser, an autosampler, a thermostated column compartment, and a diode array detector (DAD). Compounds were separated at room temperature using a Zorbax Eclipse Plus C_{18} column (1.8 µm, 150 × 4.6 mm) (Agilent Technologies, Palo Alto, CA, USA) according to the method proposed by Iswaldi *et al.*[19].

The compounds detection was carried out using a QTOF mass spectrometer (Agilent 6540) equipped with Jet Stream dual electrospray ionization (ESI) interface operating in negative ionization mode. To maintain mass accuracy during the run time, continuous infusion of a reference mass solution containing ions m/z 112.985587 (trifluroacetate anion) and 1033.988109 (trifluroacetic adduct of hexakis (1H, 1H, 3H-tetrafluoropropoxy)phosphazine or HP-921) was used. Data acquisition in profile mode was governed *via* MassHunter Workstation Software (Agilent Technologies). Data analysis was performed on MassHunter Qualitative Analysis Version B.06.00 (Agilent Technologies).

Bacteria and cultures

A mixture of fourteen strains of uropathogenic *E. coli* (UPEC) were used, ten obtained from patients with acute pyelonephritis (471, 787, 753, 472, 595, 760, 695, 697, 629, and 795), together with four strains obtained from the Spanish Type Culture Collection (CECT): CECT 424 (F- thr- leu- lacY mtl- thi- ara gal ton 2 malA xyl, resistant to phages T1, T2, and T6.), CECT 4076 (Serovar. O157:H7, originally isolated from haemorrhagic colitis), CECT 417 (SupE44 (am). mutant tRNA), and CECT 743 (Serovar. O142 K86B:H6, isolated from children with diarrhea).

Biofilm formation and surface hydrophobicity

To determine the adherence and subsequent biofilm formation of tested mixture of UPEC, a tube test proposed by Stepanovic *et al.*[50] was performed. Briefly, the mixture of uropathogenic strains were subcultured at 37ºC for 24 h in glass tubes with 2.5 mL of tryptic soy broth (TSB). Then, 0.5 mL of the aforementioned culture and 50 µL of the cranberry extract and each selected fraction at two different concentrations displayed in **Table 2** were placed into Eppendorf tubes. An Eppendorf tube without inoculums containing the same amount of TSB was used as a negative control, while 0.5 mL of the bacterial suspension in an Eppendorf tube together with 50 µL of phosphate buffer saline (PBS) was used as a positive control. After incubating for 24 h, the content of each tube was aspirated carefully and washed three times with 1 mL of PBS. Tubes were air dried and 200 µL of 99% methanol were added as a fixative. After 15 min, the excess of methanol was removed and the tubes were air dried. Then, 200 µL of the colorant Hucker's cristal violet solution (2% dye content) were added, and after 5 min the tubes were submerged in distillate water to take out the surplus. After air drying, biofilm was dissolved in each tube with 1 mL 33% acetic acid. Once the absorbance was measured at 570 nm using Boehringer–Mannheim photometer-4010 model (Boehringer GmbH, Mannheim, Germany), results were calculated according to Eq. (1), where OD is the optical density of the strains incubated with the cranberry extract or with each phenolic fraction and ODc is the optical density from the strains after incubating with the same volume of PBS.

(1) Δ biofilm = OD/ODc

In order to determine the surface hydrophobicity, the ammonium sulphate aggregation test, described by Lindahl *et al.*[51], was carried out. In brief, a mixture of strains was performed in 2 mL of TSB medium. The culture was washed three times with PBS and centrifuged at 562 × g for 10 minutes. Bacteria were resuspended into 0.002 mol L^{-1} sodium phosphate (OD1 at 540 nm). Then, 10 µL of the cranberry extract and each selected fraction at two different concentrations displayed in **Table 2** were incubated at room temperature for 30 min in a rotary shaker (Heidolph Reax,

ConThermo GmH & Co. KG, Germany) with 100 μL of the bacterial suspension of the selected strains, in PBS. Several solutions of ammonium sulphate at osmolarities ranged from 0.2 to 4 mol L^{-1} in sodium phosphate 0.002 mol L^{-1} were prepared. Then, 10 μL of bacterial suspension with the same volume of ammonium sulphate were added on a slide. The lowest concentration of ammonium sulphate which produced visible aggregation after 30 seconds gentle manual rotation at room temperature was written down. Aggregation with 4 mol L^{-1} solution was interpreted as 0% hydrophobicity, while aggregation with 0.2 mol L^{-1} was interpreted as 95% hydrophobicity. The results obtained, expressed as % hydrophobicity, were calculated according to Eq. (2) where ΔH is the ratio of the hydrophobicity of the strains incubated with the whole extract or with each phenolic fraction and ΔHc are the hydrophobicity of the strains after incubation with an equal volume of PBS.

(2) $\% \text{ hydrophobicity} = \Delta H / \Delta Hc * 100$

Statistical analysis

Data of bioactivity are expressed as mean ± standard deviation. Significant differences in the adherence and surface hydrophobicity of *E. coli* pre and post-incubated with the extract or phenolic fractions were determined using the Wilcoxon matched pairs signed rank test by IBM SPSS Statistics (Chicago, IL, USA). Differences between means were considered to be significant when the p value was below 0.05. In addition, Kendall's correlation coefficients of inter-variable concordance were calculated.

Conflicts of interest

The authors declare no competing financial interest.

Acknowledgements

This work was supported by the projects AGL2011-29857-C03- 02, P09-CTS-4564, P10-FQM-6563, and P11-CTS-7625 (Andalusian Regional Government Council of Innovation and Science) and PI070274 (Carlos III Institute of Health for Clinical

Research, Madrid, Spain). The authors are grateful to the Spanish Ministry of Economy and Competitiveness (MINECO) for a FPU fellowship AP2010-1551 (Spanish Ministry of Science and Innovation) (C. Rodríguez-Pérez), and a grant "Personal técnico de apoyo" PTA2012-6956-E (R. Quirantes-Piné).

References

1 N.P. Seeram, L.S. Adams, Y. Zhang, R. Lee, D. Sand, H.S. Scheuller, and D. Heber, *J Agric Food Chem*, 2006, **54**, 9329-9339.

2 E. Pappas and K.M. Schaich, *Crit Rev Food Sci Nutr*, 2009, **49**, 741-781.

3 M. Hisano, H. Bruschini, A.C. Nicodemo and M. Srougi, *Clinics*, 2012, **67**, 661-667.

4 K.L. Kaspar, A.B. Howell and C. Khoo, *Food Funct*, 2015, **6**, 1212-1217.

5 B. Foxman, *Am J Med*, 2002, **113**, 5-13.

6 E. Coppo and A. Marchese, *Curr Pharm Biotechnol*, 2014, **15**, 380-390.

7 T. Tapiainen, H. Jauhiainen, L. Jaakola, J. Salo, J. Sevander, I. Ikäheimo, A.M. Pirttila, A. Hohtola and M. Uhari, *Eur J Clin Microbiol Infect Dis*, 2012, **31**, 655-662.

8 A.L. Flores-Mireles, J.N. Walker, M. Caparon and S.J. Hultgren, *Nat Rev Microbiol,* 2015, **13**, 269-284.

9 A. Krasowska and K. Sigler, *Front Cell Infect Microbiol* 2014, **4**, 1-7.

10 R.J. Doyle, *Microb Infect*, 2000, **2**, 391-400.

11 M.E.T. Mcmurdo, I. Argo, G. Phillips, F. Daly and P. Davey, *J Antimicrob Chemother,* 2009, **63**, 389-395.

12 J. Uberos, M. Nogueras-Ocana, V. Fernandez-Puentes, R. Rodriguez-Belmonte, E. Narbona-López, A. Molina-Carballo and A. Muñoz-Hoyos, Open Access *J Clin Trials*, 2012, **4**, 31-38.

13 G.G. Zhanel, T.L. Hisanaga, N.M. Laing, M.R. DeCorby, K.A. Nichol, L.P. Palatnik,J, Johnson, A. Noreddin, G.K. Harding, L.E. Nicolle and D.J. Hoban, *Int J Antimicrob Agents,* 2006, **27**, 468-475.

14 R. Puupponen-Pimiä R, L. Nohynek, H. Alakomi and K. Oksman-Caldentey, *Appl Microbiol Biotechnol,* 2005, **67**, 8-18.

15 I. Ofek, D.L. Hasty and N. Sharon, *FEMS Immunol Med Microbiol,* 2003, **38**, 181-191.

16 A.B. Howell, H. Botto, C. Combescure, A. Blanc-Potard, L. Gausa, T. Matsumoto T, P. Tenke, A. Sotto and J.P. Lavigne, *BMC Infec Dis,* 2010, **10**, 94-105.

17 R. Puupponen-Pimiä, L. Nohynek, C. Meier, M. Kähkönen, M. Heinonen, A. Hopia and K.M. Oksman-Caldentey, *J Appl Microbiol,* 2001, **90**, 494-507.

18 K.L. Laplante, S.A. Sarkisian, S. Woodmansee, D.C. Rowley and N.P. Seeram, *Phytotherapy Research*, 2012, **26**, 1371-1374.

19 I. Iswaldi, A.M. Gómez-Caravaca, D. Arráez-Román, J. Uberos, M. Lardón, A. Segura-Carretero and A. Fernández-Gutiérrez, *J Pharm Biomed Anal,* 2012, **58**, 34-41.

20 T. Chen, Y. Liu, D. Zou, C. Chen , J. You, G. Zhou, J. Sun and Y. Li, *J Sep Sci,* 2014, **37**, 165-170.

21 Z. Latif and S.D. Sarker, *Methods Mol Biol*, 2012, **864**, 255-274.

22 B.M. Schmidt, A.B. Howell, B. McEniry, C.T. Knight, D. Seigler, J.W. Erdman Jr. and M.A. Lila, *J Agric Food Chem*, 2004, **52**, 6433-6442.

23 C. Rodríguez-Pérez, R. Quirantes-Piné, M.D.M. Contreras, J. Uberos, A. Fernández-Gutiérrez and A. Segura-Carretero, *Food Chem*, 2015, **174**, 392-399.

24 K. Kawabata, R. Mukai and A. Ishisaka, *Food Funct*, 2015, **6**, 1399-1417.

25 K.P. Devi, T. Rajavel, S. Habtemariam, S.F. Nabavi and S.M. Nabavi, *Life Sci*, 2015, **142**, 19-25.

26 M.M. Cowan, *Clin Microbiol Rev*, 1999, **12**, 564-582.

27 J. Lavigne, G. Bourg, C. Combescure, H. Botto and A. Sotto, *Clin Microbiol Infec,* 2008, **14**, 350-355.

28 J. Uberos, R. Rodríguez-Belmonte, C. Rodríguez-Pérez, M. Molina-Oya, E. Blanca-Jover, E. Narbona-López and A. Muñoz-Hoyos, *J Funct Foods*, **18**, 608-616.

29 R. Jepson and J. Craig, G. Williams, *JAMA*, 2013, **310**, 1395-1396.

30 K. Otto, J. Norbeck, T. Larsson, K. Karlsson and M. Hermansson, *J Bacteriol*, 2001, **183**, 2445-2453.

31 D. Ribet and P. Cossart, *Microb Infect*, 2015, **17**, 173-183.

32 R.M. Goulter, I.R. Gentle and G.A. Dykes, *Lett Appl Microbiol*, 2009, **49**, 1-7.

33 L.Y. Foo, Y. Lu, A.B. Howell and N. Vorsa, *J Nat Prod*, 2000, **63**, 1225-1228.

34 T. Taguri, T. Tanaka and I. Kouno, *Biol Pharm Bull*, 2006, **29**, 2226-2235.

35 J. Monte, A. Abreu, A. Borges, L. Chaves Simões and M. Simões, *Pathogens,* 2014, **3**, 473-498.

36 T.P.T. Cushnie and A.J. Lamb, 2005, **26**, 343-356.

37 T. Wu, M. He, X. Zang, Y. Zhou, T. Qiu, S. Pan and X. Xu, *Biochim Biophys Acta (BBA),* 2013, **1828**, 2751-2756.

38 K. Šmejkal, S. Chudík, P. Kloucek, R. Marek, J. Cvacka, M. Urbanová, O. Julínek, L. Kokoska, T. Slapetová, P. Holubová, A. Zima and M. Dvorská, *J Nat Prod*, 2008, **71**, 706-709.

39 D. Wojnicz, Z. Sycz, S. Walkowski, J. Gabrielska, W. Aleksandra, K. Alicja, S.L. Anna and A.B. Hendrich, *Phytomedicine,* 2012, **19**, 506-514.

40 E. Czinner , A. Kéry, K. Hagymási, A. Blázovics, A. Lugasi, E. Szõke and E. Lemberkovics, *Eur J Drug Metab Pharmacokinet,* 1999, **24**, 309-313.

41 R. Lin, Y. Chin and M. Lee, *Phytother Res*, 2005, **19**, 612-617.

42 W.C. Duncan-Hewitt, In: Doyle, R.J. & Rosenberg M., editor. Microbial Cell Surface Hydrophobicity Washington, D.C.: ASM Publications; 1990. pp 39-73.

43 H. Xu and S.F. Lee, *Phytotherapy Research*, 2001, **15**, 39-43.

44 F. Bernard, S. Sablé, B. Cameron, J. Provost, J. Desnottes, J. Crouzet and F. Blanche, *Antimicrob Agents Chemother*, 1997, **41**, 992-998.

45 K. Waage S and A. Hedin P, *Phytochemistry*, 1985, **24**, 243-245.

46 A. Borges, C. Ferreira, M.J. Saavedra and M. Simões, *Microbial Drug Resistance,* 2013, **19**, 256-265.

47 A. Borges, M.J. Saavedra and M. Simões, *Biofouling*, 2012, **28**, 755-767.

48 U.B. Maheswari, S. Palvai, P.R. Anuradha and N. Kammili, *Indian J Urol*, 2013, **29**, 77-281.

49 A. Reisner, K.A. Krogfelt, B.M. Klein, E.L. Zechner and S. Molin, *J Bacteriol*, 2006, **188**, 3572-3581.

50 S. Stepanovic, D. Vukovic, I. Dakic, B. Savic and M. Švabic-Vlahovic, *J Microbiol Methods*, 2000, **40**, 175-179.

51 M. Lindahl, A. Faris, T. Wadström and S. Hjertén, *BBA - General Subjects* 1981, **677**, 471-476.

Chapter 8

Docosahexaenoic acid attenuates cardiovascular risk factors via a decline in proprotein convertase subtilisin/kexin type 9 (PCSK9) plasma levels

Lipids, 2016, 51(1):73-83

Lipids (2016) 51:75–83
DOI 10.1007/s11745-015-4099-4

ORIGINAL ARTICLE

Docosahexaenoic Acid Attenuates Cardiovascular Risk Factors via a Decline in Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) Plasma Levels

Celia Rodríguez-Pérez[1,2] · Vanu Ramkumar Ramprasath[3,4] · Shuaihua Pu[3,4] · Ali Sabra[4] · Rosa Quirantes-Piné[2] · Antonio Segura-Carretero[1,2] · Peter JH Jones[3,4,5]

[1] *Department of Analytical Chemistry, Faculty of Sciences, University of Granada, Avenida Fuentenueva s/n, 18071 Granada, Spain*
[2] *Research and Development Functional Food Centre (CIDAF), Health Science Technological Park, Avenida del Conocimiento, 37 Edificio BioRegión, 18016 Granada, Spain*
[3] *Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, MB, Canada*
[4] *Richardson Centre for Functional Foods and Nutraceuticals, Winnipeg, MB, Canada*
[5] *Department of Food Sciences, University of Manitoba, Winnipeg, MB, Canada*

Abstract

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a circulating protein that regulates cholesterol metabolism by promoting LDL receptor degradation in the liver and has recently been proposed as a therapeutic target in the management of hyperlipidaemia. We investigated the impact of dietary fat on the metabolism of sterols and on plasma PCSK9 concentrations to explore likely clinical usefulness. In a *post hoc* analysis of a double-blinded randomised crossover controlled feeding trial [The Canola Oil Multicenter Intervention Trial (COMIT)], volunteers (n= 54) with at least one condition related to metabolic syndrome consumed diets with one of the following treatment oils in beverages: 1) conventional canola oil (Canola); 2) canola oil rich in docosahexanoic acid (DHA) (CanolaDHA); and 3) high-oleic acid canola oil (CanolaOleic). The enrichment in oleic acid resulted in lower plasma cholesterol concentration compared with diets enriched in DHA. Contrarily, DHA-enriched oil significantly decreased plasma PCSK9 and triacylglycerols levels, but increased circulating levels of sterols. The variations in lathosterol, sitosterol and campesterol indicate that plasma PCSK9 levels are sensitive to changes in cholesterol synthesis and/or absorption. There was a significant correlation between plasma PCSK9 levels

and plasma triacylglicerol and apolipoprotein B (apoB) levels, which was not affected by dietary fat. Therefore, our results suggest that the impact of dietary fats should not be discarded as complementary treatment in the management of patients with hyperlipidaemia. These findings should be considered in the analysis of ongoing studies and may represent a cautionary note in the treatment of patients with cardiovascular risk.

Keywords: PCSK9, fatty acids, docosahexaenoic acid, sterols, hyperlipidaemia.

❖ Introduction

Coronary mortality remains the most common cause of early death in the Western world[1]. Accordingly, the European Guidelines on cardiovascular disease prevention in clinical practice affirm that more than 50% of the reduction in cardiovascular disease (CVD) mortality relates to changes in risk factors, and 40% to improved treatments[2]. In this regard, the quality of dietary fats is important in preventing the development and progression of chronic diseases such as CVD. These European Guidelines report that increased plasma total and low-density lipoprotein (LDL) cholesterol are among the main risk factors for CVD[2]. Because of this, proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibition has become a therapeutic target in order to control LDL-C levels in plasma as alternative drugs of statin classes[3,4].

Abifadel *et al.* first reported an association between autosomal dominant hypercholesterolaemia (ADH) and PCSK9[5]. Since its discovery, PCSK9 has been proposed as the third gene related to autosomal dominant familial hypercholesterolaemia, after LDL receptor (LDLR) and apoB-100[5]. The principal mechanism of PCSK9 in increasing plasma cholesterol is based on promoting LDLR degradation in liver, the primary conduit for the removal of LDL from the circulation, leading to an increase in blood LDL-C levels[6], and as such is considered a novel target in cholesterol-lowering therapy. In fact, the Copenhagen Heart Study showed that loss of function of PCSK9 is associated with an 11–15% reduction in the LDL-C level, which resulted in a 6–46% risk reduction in coronary heart disease events[7]. LDLR and PCSK9 are transcriptionally regulated by sterol response element-binding proteins (SREBPs), a family of transcription factors involved in the regulation of lipid homeostasis, including the activation of genes required for cholesterol biosynthesis[8].

Although relatively modest, diet-related reductions in hepatic PCSK9 secretion could be amplified over the longer term via the reciprocal relationships in the LDLR-PCSK9-LDL axis[9]. Other study has demonstrated that PCSK9 expression is dependent on nutritional regulation, decreasing upon fasting and increasing after re-

feeding with a high carbohydrate diet in rodents[10]. The relationship between PCSK9 and sterols is being investigated. Some research demonstrated that PCSK9 expression follows a diurnal rhythm accompanied by parallel fluctuations in the lathosterol-to-cholesterol ratio[11]. In addition, it has been suggested that plasma PCSK9 concentration reflect hepatic SREBP2 activity due to fasting induces changes in both, PCSK9 and markers of cholesterol synthesis in humans and these parameters downstream effects of SREBP2[12]. However, only a few studies have described a dietary modulation of PCSK9 in humans. One of them showed that the Mediterranean Diet, in the absence of weight loss, reduced plasma PCSK9 in men (n=19) with metabolic syndrome[13], while a randomised controlled trial demonstrated that n-6 polyunsaturated fatty acids (PUFA) lower PCSK9 plasma levels[14].

Despite many randomised controlled trials showing a reduction in CV risk-factors with decreases or modifications in dietary fat intake[15,16], little is known about whether divergent dietary intakes with varying fatty acid compositions directly impact circulating PCSK9 levels. Therefore, the objective of the present study was to investigate the impact of dietary fat – including conventional canola oil, high-oleic acid canola oil enriched with DHA canola oil, and high-oleic canola oil – on the metabolism of sterols and on plasma PCSK9 concentrations to explore likely clinical usefulness.

❖ Materials and Methods

Trial design and study subject

The Canola Oil Multicenter Intervention Trial (COMIT) was a double-blind, randomised, crossover trial conducted at the University of Manitoba, Laval University and the Pensylvania State University. This study was carried out according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subject were approved by the Biomedical Ethics Board at the University of Manitoba, Comité sectoriel d'éthique de la recherche en sciences de la santé de l'Université Laval, and the Institutional Review Board at the Pennsylvania State University. The trial was registered at clinicaltrials.gov under the registration

number NCT01351012. Written informed consent was obtained after an initial interview with each participant. The interview included a full description of the study and a discussion of the compliance issues and study expectations. The COMIT design, procedure and clinical protocol have been described in detail elsewhere[17].

Briefly, participants with at least one of the metabolic syndrome risk factors based on the International Diabetes Federation definition[17] were recruited. As a sub-study of the COMIT, 54 participants (16 males and 38 females) aged 18 years or older at RCFFN were selected for the present work based on the sample availability.

Study diets

Importance of canola oil

Traditionally, the terms canola oil and rapeseed oil has been interchangeably used. However, in spite that both oils belong to the Brassicaceae family, by definition, canola has specific cut-off levels of erucic acid (< 2%) and glucosinolates (< 30 umol/g) for both human and animal consumption[18]. Canola oil is the third largest source of vegetable oil in the world, according with the United States Department of Agriculture (USDA)[19]. The importance of its consumption reside in its composition in low saturated fat and high monounsaturated fatty acids (MUFA) and PUFA concentrations which improve lipid profiles and reduction of CVD risk[18]. In fact, in the USA, the Food Drug Administration (FDA) approved a health claim regarding the association between canola oil consumption and reduction of risk of coronary heart disease[20].

Diet design

Three out of five COMIT interventions were chosen in order to estimate the impacts of oleic acid and DHA on PCSK9 plasma levels. Study diets were prepared in a metabolic kitchen facility where kitchen staff and clinical coordinators were blinded to the treatments. Three isocaloric meals were cooked each day and two snacks were provided per day for each subject in a 7-day rotating menu cycle. Subjects consumed

at least one of three daily meals under supervision. The other meals were prepared and packed for each subject to be taken away. Weekend meals and shakes were distributed to the participant's place of residence or were delivered to the feeding site on Fridays. The study intervention oils were delivered in a shake-style beverage, divided into two equal doses to be consumed at breakfast and supper each day[(17,21)]. The majority of the oil treatments were consumed under the supervision of a clinical coordinator to ensure optimal compliance. Subjects were also instructed to consume only the prepared meals and not to consume alcohol or caffeinated beverages.

Diets were provided at caloric levels that matched the individual volunteer's daily requirements, which were calculated by using the Mifflin-St Jeor equation[(22)]. The nutrient content of the diet was analysed using FOOD PROCESSOR (Food Processor, Salem, OR) to ensure the adequacy of macro- and micronutrients. **Table 1** summarises the specific composition of oils with different fatty acids [(21)]. Briefly, subjects consumed a fixed composition of a precisely controlled basal, weight-maintaining diet (35% energy from fat, 50% carbohydrate, and 15% protein) supplemented with the following treatment oils: 1) Canola (59% OA, 20% LA, and 10% ALA); 2) CanolaDHA (63% OA and 6% DHA); 3) CanolaOleic (72% OA and 15% LA). The quantities of oil provided were 60, 48 or 36 g oil per day, based on the calculated energy requirements of the participants 3000, 2400 or 1800 Kcal respectively. Oils were weighed to the nearest gram and added to the shakes. During the study period, body weight was monitored so that the baseline weight did not fluctuate by more than 1 kg and if subjects gained or lost weight energy adjustments were made.

Baseline characteristics of the participants are summarised in **Table 2**.

Table 1. Fatty acid and sterol profile of the treatments oils[1].

	Canola	CanolaDHA	CanolaOleic
Fatty acids (%)			
16:0, palmitic acid	4.1	5.3	3.7
18:0, stearic acid	1.8	1.7	1.8
18:1, oleic acid	58.6	63.2	71.5
18:2, linoleic acid	19.5	12.7	14.7
18:3, α-linolenic acid	9.8	2.0	2.3
20:4, arachidonic acid	0	0.1	0
20:5, EPA	0	0.2	0
22:5, DPA	0	2.4	0
22:6, DHA	0	5.8	0
Total SFA	7.3	9.0	6.9
Total MUFA	60.3	64.7	73.2
Total PUFA	29.3	23.1	17.0
Sterols (µmol/L)			
Brassicasterol	91	95	109.9
Lathosterol	0.0	0.8	0.6
Stigmasterol	1.9	26.2	2.7
Sitosterol	390.6	343.1	381.4
Lanosterol	0.0	3.4	0.0
Campesterol	257.7	233.4	253

[1]The amount of treatment oil was adjusted to contribute 18% of energy for each calorie level. Canola, conventional canola oil (Richardson Oilseed Ltd); CanolaDHA, high–oleic acid canola oil (Richardson Oilseed Ltd) with DHA (Martek Biosciences Corporation) and CanolaOleic, high–oleic acid canola oil.

Table 2. Baseline characteristics in 54 COMIT participants from RCFFN

Sex (*n*)	
Men	**16**
Women	**38**
BMI (kg/m2)	29.68 ± 5.03
Age (y)	43.87 ± 15.81
TC (mmol/L)	5.39 ± 1.13
HDL-C (mmol/L)	1.25 ± 0.28
LDL-C (mmol/L)	3.41 ± 1.01
Triacylglycerols (mmol/L)	1.61 ± 0.92
Glucose (mmol/L)	5.43 ± 1.65
Body weight (kg)	80.69 ± 15.93
Waist (cm)	
Women	90.64 ± 11.92
Men	103.37 ± 13.76
SBP (mm Hg)	122.44 ± 20.33
DBP (mm Hg)	81.24 ± 12.41

Adapted from Jones PJ *et al.*[29]. DBP, diastolic blood pressure; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; RCFFN, Richardson Centre for Functional Foods and Nutraceuticals, Winnipeg, Manitoba, Canada; SBP, systolic blood pressure; TC, total cholesterol.

Sample collection

Twelve-hour fasting blood samples were collected on days 29 and 30 (endpoint) of each phase. Endpoint values were determined as the mean values from days 29 and 30. After centrifugation of blood, plasma and serum samples were separated and stored at - 80°C until further analysis.

Biochemical analysis

Sterols and serum lipids analysis and quantification

The extraction methodology was performed as described by Mackay *et al.*[23] with some modifications regarding derivatization. 5α-Cholestane (100 μL; 1 mg/mL) was added to 500 μL of each plasma sample as the internal standard. Alkaline hydrolysis was performed using methanolic KOH to convert sterol fatty acid esters to free sterols prior to solvent extraction.

Total cholesterol and non-cholesterol sterols were characterised and measured using an Agilent 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA, USA), equipped with a flame ionization detector (FID) and an on-column injector. A SAC-5 capillary column (30 m x 0.25 mm x 0.25 μm, Supelco Inc., Bellefonte, PA) was used. Peak identification was accomplished by comparing the retention times with those of a standard mixture of pure sterols. Since 5α-cholestane has been reported to have a similar FID response to sterols, quantification of compounds was performed using 5α-cholestane as the internal standard.

Serum lipids were analysed using a Roche Cobas 6000 c501 System (Roche Diagnostics, Indianapolis, IN) and standardized with the Centers for Disease Control and Prevention (CDC) Lipid Standardization Program for external quality control. LDL cholesterol concentrations were calculated using the Friedewald formula. The concentrations of apolipoprotein A-1 and B (apoA and apoB respectively) were measured by nephelometry (BN Prospec, Siemens).

PCSK9 measurement

Plasma PCSK9 concentrations were measured using a commercial high-sensitivity, quantitative sandwich enzyme immunoassay technique (Quantikine ELISA, R&D Systems Europe Ltd, Sweden). Plasma PCSK9 measurement was carried out following meticulously the manufacturer's protocol. Briefly, plasma samples were 20-fold diluted in dilution buffer. 50 μL of standards and plasma samples were loaded into the 96-well microplate that was coated with a monoclonal antibody specific for human PCSK9 and it was incubated for 2 h at room temperature. Then, a total of 4 washes with wash buffer were done and when the microplate was totally dried, 200 μL of PCSK9 conjugate were added to each well follow by an another 2h-incubation. In order to remove any unbound antibody-enzyme reagent, other 4 washes were carried out. Once dry, 200 μL of a substrate solution which contains stabilized hydrogen peroxide and tetramethylbenzidine were added to achieve a coloured development. After 30 min, 50 μL of stop solution were added into each well and the optical density was measured at 450 nm using a Varian 50 MPR Microplate Reader and Cary 50 Bio UV-Visible Spectrophotometer (Varian). PCSK9 levels after each of the three dietary interventions were tested in duplicate. In order to minimise the analytical variations all the measurement were carried out by the same researcher. The intra-assay analytical variation (CV) varied from 0.05% to 12.61%. Linearity of all 0.625 to 40 ng/mL calibration curves were excellent ($R^2 > 0.99$).

Statistical Analysis

Results are presented as mean ± SD. Data were analysed using the PROC MIXED procedure for subjects over treatment periods in Statistical Analysis Software (SAS) (version 9.2; SAS Institute, Inc., Cary, NC, USA) with treatment, age and sex used as fixed effects, repeated over treatment periods. Tukey-adjusted *P* values were used to examine differences between treatments. The Pearson's correlation coefficient was applied for quantitative variables. Statistical significance is displayed at a 95% confidence level ($P \leq 0.05$).

❖ Results

Plasma sterol levels

Plasma sterol levels after the three dietary treatments are summarised in **Table 3**. Consumption of the CanolaDHA diet resulted in a significantly higher ($p=0.0104$) total cholesterol level compared with the CanolaOleic, but not with Canola diet, whereas there was no significant differences observed between Canola and CanolaOleic treatments (**Table 3**). Sterols including desmosterol, lathosterol, campesterol, β-sitosterol and cholestanol were identified in the plasma samples of all participants. No differences were observed in desmosterol levels between diets. The plasma lathosterol level was significantly higher ($p < 0.0001$) in the CanolaDHA group compared with the other two interventions. Highest plasma concentrations of campesterol and cholestanol were observed after the CanolaDHA treatment compared with the CanolaOleic treatment ($p=0.0063$ and $p=0.0010$, respectively). In addition, β-sitosterol levels were higher after the CanolaDHA intervention compared with the Canola ($p=0.0252$) and CanolaOleic ($p=0.0015$) treatments.

Table 3. Between-treatments comparisons of plasma plant sterols, cholesterol, and plant sterols-to-cholesterol ratios

	Canola	CanolaDHA	CanolaOleic
TC (mmol/L)	4.55 ± 0.12[a,b]	4.65 ± 0.12[a]	4.37 ± 0.12[b]
Desmosterol (μmol/L)	3.77 ± 0.47	3.91 ± 0.47	3.64 ± 0.47
Lathosterol (μmol/L)	6.64 ± 0.32[b]	8.63 ± 0.33[a]	6.31 ± 0.33[b]
Campesterol (μmol/L)	12.58 ± 0.68[a,b]	13.09 ± 0.67[a]	11.94 ± 0.67[b]
Sitosterol (μmol/L)	5.00 ± 0.38[b]	5.57 ± 0.38[a]	4.79 ± 0.38[b]
Cholestanol (μmol/L)	6.52 ± 0.35[a,b]	6.81 ± 0.35[a]	6.34 ± 0.35[b]
Total PS (μmol/L)*	17.54 ± 1.02[a]	18.68 ± 1.02[a]	16.76 ± 1.02[a,b]
Desmosterol-to-cholesterol ratio	0.83 ± 0.11	0.84 ± 0.11	0.81 ± 0.11
Lathosterol-to-cholesterol ratio	1.45 ± 0.07[b]	1.81 ± 0.07[a]	1.38 ± 0.07[b]
Campesterol-to-cholesterol ratio	2.84 ± 0.13[a]	2.88 ± 0.13[a]	2.73 ± 0.13[a,b]
Sitosterol-to-cholesterol ratio	1.12 ± 0.08[b]	1.23 ± 0.08[a]	1.11 ± 0.08[b]
Cholestanol-to-cholesterol ratio	1.45 ± 0.07	1.47 ± 0.07	1.43 ± 0.07

All values are least-squares means ± SD; n=54. Mean values with different superscript letters are significantly different between treatments; $p < 0.05$. TC, total cholesterol (mmol/L); Total PS, total plan sterols (μmol/L); *Total plant sterols (campesterol + sitosterol).

Plasma PCSK9 levels

Plasma PCSK9 concentrations at the endpoints are presented in Table 4. Consumption of the CanolaDHA diet resulted in the significantly lowest plasma PCSK9 concentration (212.53 ± 10.2 ng/mL) compared with Canola (238.54 ± 10.3 ng/mL) and CanolaOleic (234.18 ± 10.2 ng/mL) treatments (p=0.0042 and p=0.0179, respectively). Plasma obtained from subjects during consumption of the Canola and CanolaOleic diets was not significantly different in terms of plasma PCSK9 concentrations.

Table 4. Between-treatment comparison of plasma lipidic profile and PCSK9

	Canola	CanolaDHA	CanolaOleic
PCSK9 (ng/mL)	238.54 ± 10.29 [a]	212.53 ± 10.20 [b]	234.18 ± 10.23 [a,c]
apo A-1 (mmol/L)	1.48 ± 0.20 [a]	1.54 ± 0.24 [a]	1.50 ± 0.21 [a]
apo B (mmol/L)	0.96 ± 0.26 [a]	1.01 ± 0.27 [a]	0.96 ± 0.25 [a]
HDL-C (mmol/L)	1.25 ± 0.29 [b]	1.38 ± 0.36 [a]	1.25 ± 0.29 [b]
LDL-C (mmol/L)	3.02 ± 0.90 [b]	3.22 ± 0.99 [a]	2.94 ± 0.82 [b]
TAG (mmol/L)	1.64 ± 0.99 [a]	1.30 ±0.70 [b]	1.68 ± 1 [a]

All values are least-squares means ± SD; n=54. apo, apolipoprotein; Canola, conventional canola oil; CanolaDHA, high-oleic acid canola oil (Richardson oilseed Ldt) with DHA (Market Biosciences Corporation); HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; TAG, Triglycerides. Values with different letters are significant at $p < 0.05$.

Serum lipids and apolipoprotein levels

Serum lipid profiles, as well as apolipoprotein and PCSK9 concentrations, across the three oil interventions are presented in Table 4. LDL-C and HDL-C levels were higher after the CanolaDHA treatment (3.22 ± 0.99 and 1.38 ± 0.36 mmol/L, respectively) versus the other two treatments, whereas the LDL-C plasma concentration showed the lowest concentration after CanolaOleic intervention. Plasma apolipoprotein A-1 and B concentrations were similar across the three treatments. Moreover, mean plasma TAG concentrations were lower after consumption of the

CanolaDHA diet compared with the Canola and CanolaOleic (1.30 ± 0.70 mmol/L respectively) interventions.

Correlations between plasma PCSK9 and metabolic parameters

Table 5 summarises the correlations between plasma PCSK9 and metabolic parameters across the three diet interventions. Briefly, PCSK9 was significantly correlated with TC, TAG, apoB and cholesterol synthesis markers (desmosterol and lathosterol). However, a weak correlation was found between PCSK9 and cholesterol absorption markers (campesterol, β-sitosterol and cholestanol). Finally, there was no significant correlation between PCSK9 and HDL-C.

Comparisons between the most relevant correlations of the 3 diets are displayed in **Figure 1**. The most remarkable finding was that CanolaOleic presented the strongest correlation between PCSK9 and TC (r=0.483, p=0.000) compared with the other two interventions, while CanolaDHA led to the strongest correlation between PCSK9 and apoB (r=0.883; $p < 0.0001$).

Table 5. Correlations between circulating PCSK9 and metabolic parameters at the end of each experimental phase (n=54)

	All treatments		Canola		CanolaDHA		CanolaOleic	
Correlation with PCSK9	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**
TC (mmol/L)	0.300	0.000	0.279	0.041	0.348	0.009	0.483	0.000
HDL-C (mmol/L)	-0.045	0.565	-0.108	0.439	0.005	0.972	0.023	0.867
LDL-C (mmol/L)	0.202	0.010	0.172	0.214	0.229	0.096	0.265	0.055
TAG (mmol/L)	0.449	<.0001	0.484	0.000	0.356	0.008	0.464	0.000
apoA (mmol/L)	0.224	0.004	0.222	0.106	-0.139	0.317	0.287	0.037
apoB (mmol/L)	0.348	<.0001	0.366	0.007	0.883	<.0001	0.439	0.001
Desmosterol (µmol/L)*	0.322	<.0001	0.340	0.011	0.298	0.028	0.365	0.007
Lathosterol (µmol/L)*	0.261	0.000	0.309	0.023	0.353	0.009	0.304	0.027
Campesterol (µmol/L)**	-0.152	0.06	-0.130	0.347	-0.179	0.197	-0.127	0.365
β-sitosterol (µmol/L)**	-0.181	0.021	-0.102	0.464	-0.249	0.069	-0.129	0.355
Cholestanol (µmol/L)**	0.074	0.352	0.113	0.415	-0.049	0.719	0.188	0.178

Pearson's correlations between plasma PCSK9 levels and metabolic parameters in volunteers with at least one condition of metabolic syndrome (n=54) after different diet treatments. * Cholesterol synthesis markers; ** Cholesterol absorption markers.

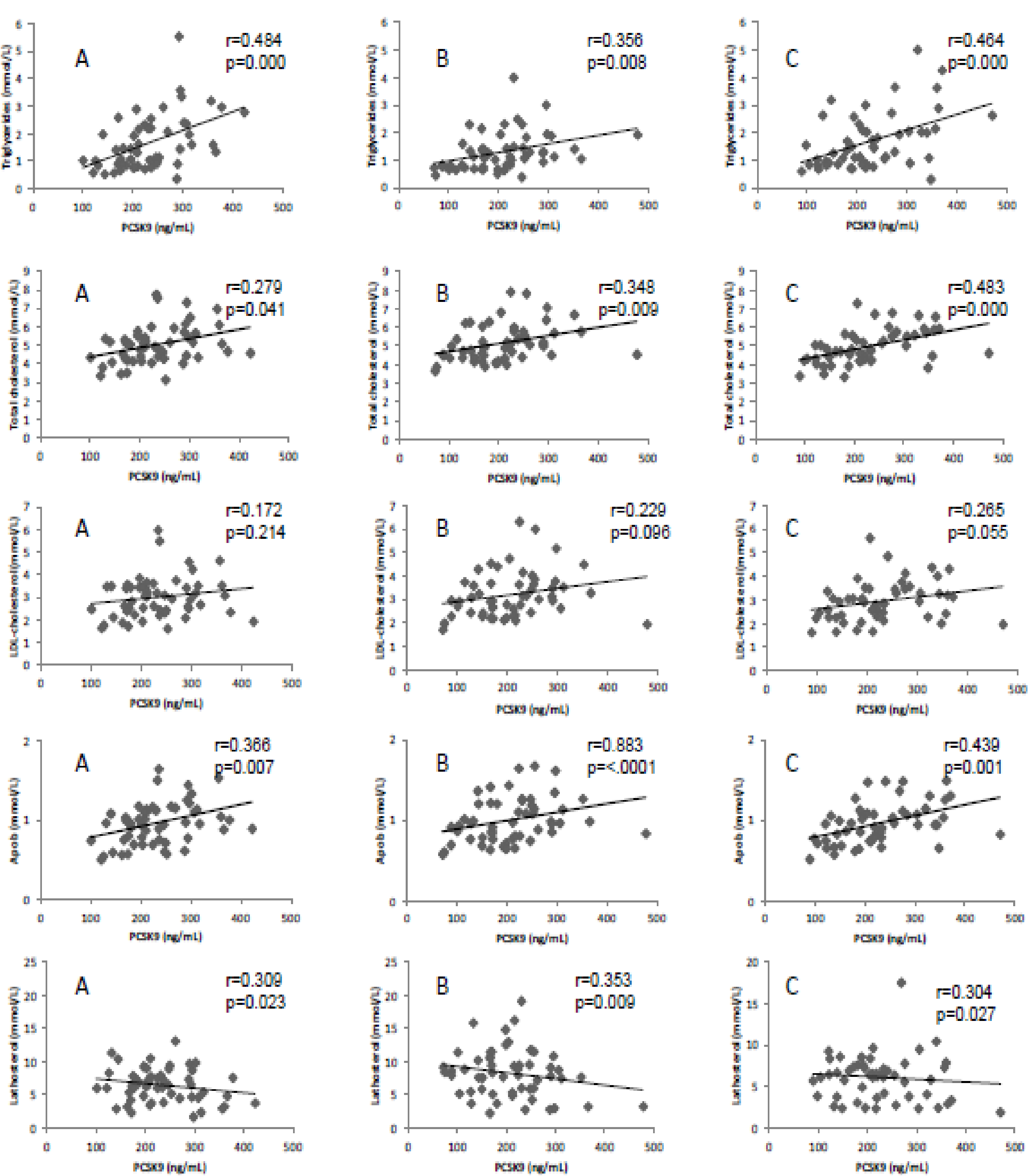


Figure 1. Comparative correlations between plasma PCSK9 and metabolic parameters across the tree diets. A. Conventional Canola oil; B. High oleic canola oil enriched with DHA; C. High oleic canola oil.

❖ Discussion

This *post-hoc* analysis from the COMIT trial provides the first line of the evidence that sterol metabolism and plasma PCSK9 concentration could be influenced by consumption of different dietary fats. In fact, the current study is the first to correlate the effect of the blend of fat intake from different canola oils and plant sterols and PCSK9 plasma concentrations.

Our results showed that plasma levels of PCSK9 were positively correlated with cholesterol synthesis markers (lathosterol and desmosterol) across all three diets, corroborating that plasma PCSK9 levels are sensitive to changes in cholesterol synthesis. Recently, investigators have presented study findings in a positive relationship between the diurnal variations of plasma PCSK9 and the lathosterol-to-cholesterol ratio(24). Additionally, a strong correlation between PCSK9 and lathosterol in humans, suggesting that hepatic PCSK9 and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase are regulated by the same mechanism, SREBP-2, has been previously reported(25).

One of the most remarkable finding in the current research was that CanolaDHA led to the highest plasma TC while simultaneously presenting the lowest plasma PCSK9. It is commonly believed that PCSK9 binds to hepatic low-density lipoprotein receptors (LDLRs), thus preventing LDLR recycling to the cell surface after endocytosis, resulting in a reduction in LDLRs on the surface of hepatocytes, decreased cellular uptake of LDL-C, and higher plasma LDL-C levels(6). The relationship between PCSK9/TC has been previously investigated in 198 normolipidaemic subjects resulting in positive correlation of plasma PCSK9 with TC in men, but not in women(26).

Data from several researches have suggested that PUFA such as DHA could help in the reduction of plasma PCSK9 concentration inhibiting its actuation(27,28). However, the mechanism underlying the interplay between different fatty acids and PCSK9 is not well-established. With respect to the reduction of circulating PCSK9 after consuming PUFA, some authors showed this mechanism is in line with the

notion that PUFA increases hepatic cholesterol, resulting in decreased activity of SREBP-2, and subsequently reduced serum concentrations of PCSK9[(14)]. Nevertheless, PCSK9 expression is not only influenced by SREBP-2. Recently, it has been demonstrated that SREBP-1c can also regulate PCSK9 via the same response element as SREBP-2 in the promoter of PCSK9[(10)]. Accumulating evidence supports that peroxisome proliferator-activated receptor γ (PPARγ) can be activated by ligands such as DHA, among others factors[(29,30)]. In other studies it has been shown that while PPARα suppresses PCSK9 and activates furin cleavage of PCSK9, PPARγ induces PCSK9 transcription but reduces PCSK9 secretion[(31)]. For that reason, another mechanism that should not be discarded is the fact that the reduction of PCSK9 plasma concentration after CanolaDHA treatment could be owing to the down-regulation of SREBP-1c signalling by DHA[(32)].

The connection between PCSK9 and different plasma lipid parameters is being extensively investigated. Our current results show that plasma PCSK9 was positively correlated with apoB ($p < 0.001$) in all three oil interventions (**Figure 1**). These results are consistent with previous clinical trials that reported a relationship between the apoB and PCSK9[(33,34)]. However, the association between these parameters is still controversial and remains unclear.

Some researches show that elevations in PCSK9 increase cholesterol, TAG and apoB levels in blood in different mouse models, indicating that PCSK9 prevents the intracellular degradation of apoB and increases very low-density lipoprotein (VLDL) secretion, which provides an additional mechanism whereby PCSK9 could increase circulating cholesterol and TAG[(33)]. This fact is supported in part by our results, in which the dietary interventions Canola and CanolaOleic showed the highest PCSK9 plasma levels and led to the highest TAG plasma concentrations.

Furthermore, recent analyses, including data from The Dallas Heart Study carried out with 3138 subjects, showed positive and significantly correlated levels of PCSK9 with the plasma levels of LDL-C, HDL-C and triacylglycerols[(34)]. In agreement with these results, we found significant correlations between plasma

PCSK9 and plasma TAG and LDL-C ($p < 0.05$) in all three oil interventions, but no correlations were found between PCSK9 and HDL-C within this study.

There is a lack of information regarding the impact of the diet and particularly dietary fats in PCSK9 plasma concentrations. However, the potential of the diet in helping to prevent hyperlipidaemia and other CV-risk factors should not be forgotten, especially in those treated with monoclonal antibodies to inhibit PCSK9.

Strengths and limitations

Strengths of this trial include the controlled feeding (participants were provided all meals, with one meal a day eaten under supervision for the majority of days) and the crossover design. However, the scarcity of previous information about the effect of fatty acids on PCSK9 made it difficult to compare our results with previous data in the same field. In addition, our study provides the first approach between the effects of the oils studied in PCSK9 plasma concentration. Limitations of the present study were the lack of a control Western diet and the relatively high oil supplementation.

❖ Conclusions

The current study presents a new approach in understanding how different dietary fats could influence several factors related to CVD. The suppression of plasma PCSK9 concentrations with DHA-enriched high–oleic acid canola oil treatment may be of importance, which could lead to further investigations of mechanism of actions of DHA on PCSK9. To the best of our knowledge, it is the first time such a response has been observed in humans. Our findings support that DHA-enriched high–oleic acid canola oil treatments could attenuates CVD risk factors via a decline in PCSK9 plasma levels. Further long-term studies are necessary to determine the clinical importance of the blends of the studied oils on PCSK9.

Acknowledgements

We gratefully acknowledge Khatima Khalloufi and Mohammad Abdullah from the Richardson Centre for Functional Foods and Nutraceuticals for her technical laboratory support and his help with the ELISA tests, respectively. We would like to express our gratitude to Jorge Joven, Director of Laboratoris Clínics i de la Unitat de Recerca Biomèdica (URB-CRB), for his kind advice and suggestions regarding the manuscript organisation. The authors are also grateful to the Spanish Ministry of Economy and Competitiveness (MINECO) for a FPU fellowship AP2010-1551 (C. Rodríguez-Pérez), a "Personal técnico de apoyo" grant PTA2012-6956-E (R. Quirantes-Piné) and to CEIBiotic's predoctoral mobility grant (C. Rodríguez-Pérez).

Financial Support

Supported by Grants from Canola Council of Canada, Flax Council of Canada, Agriculture and Agri-Food Canada, Dow AgroSciences and Western Grains Research Foundation and Canada Research Chairs Program collectively provided funding for this trial through the Growing Forward program of Agriculture and Agri-Food Canada. Also supported by NIH Grant HL 49373 and National Center for Advancing Translational Sciences, National Institutes of Health, through Grant UL1TR000127. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Conflict of Interest

Peter Jones reported receiving grants from Advanced Foods and Materials Network (AFMNet), Danone, Enzymotec, Unilever, the Canadian Institutes of Health Research (CIHR) and Canada Research Chair Endowment (CRCE) of the Federal Government of Canada. Dr. Jones also serves as President of Nutritional Fundamentals for Health Inc, which markets plant sterols among other nutraceuticals.

The other authors did not report any conflicts of financial interest.

Authorship

PJJ is responsible for the integrity and the accuracy of the data and had full access to the complete data set in the study. Study concept and design: PJJ, VRR and SP. Acquisition of data: PJJ, VRR and SP, Analysis and interpretation of data: CRP, AS, PJJ, VRR and SP. Drafting of the manuscript CRP, AS, PJJ, VRR and SP. Critical revision of the manuscript for important intellectual content and final approval of the manuscript: CRP, RQP, ASC, PJJ, VRR and SP. Obtained funding: PJJ. Study supervision: PJJ, VRR and SP. All authors reviewed the manuscript. All authors read and approved the final manuscript.

References

1. Ellegård LH, Andersson SW, Normén AL, *et al.* (2007) Dietary plant sterols and cholesterol metabolism. Nutr Rev 65:39-45.

2. Cífková R, Vaverková H, Filipovský J *et al.* (2014) Summary of the European Guidelines on cardiovascular disease prevention in clinical practice prepared by the Czech Society of Cardiology. Cor Vasa 56:169-189.

3. Davidson MH. (2013) Emerging low-density lipoprotein therapies: Targeting PCSK9 for low-density lipoprotein reduction. J Clin Lipidol 7:S11-S15.

4. Dadu RT, Ballantyne CM. (2014) Lipid lowering with PCSK9 inhibitors. Nat Rev Cardiol 10:563-575.

5. Abifadel M, Varret M, Rabès JP, *et al.* (2003) Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. Nat Genet 34:154-156.

6. Lose JM, Dorsch MP, Bleske BE. (2013) Evaluation of proprotein convertase Subtilisin/Kexin Type 9: Focus on potential clinical and therapeutic implications for low-density lipoprotein cholesterol lowering. Pharmacotherapy 33:447-460.

7. Benn M, Nordestgaard BG, Grande P, *et al.* (2010) PCSK9 R46L, Low-density lipoprotein cholesterol levels, and risk of ischemic heart disease. 3 Independent Studies and Meta-Analyses. J A. Coll Cardiol 55:2833-2842.

8. Browning JD, Horton JD (2010) Fasting reduces plasma proprotein convertase, subtilisin/kexin type 9 and cholesterol biosynthesis in humans. J Lipid Res 51:3359-3363.

9. Lagace TA. (2014) PCSK9 and LDLR degradation: regulatory mechanisms in circulation and in cells. Curr Opin Lipidol 25(5):387–393.

10. Costet P, Cariou B, Lambert G *et al.* (2006) Hepatic PCSK9 expression is regulated by nutritional status via insulin and sterol regulatory element-binding protein 1c. J. Biol. Chem 281 (10):6211-6218.

11. Cariou B, Langhi C, Le Bras M *et al.* (2013) Plasma PCSK9 concentrations during an oral fat load and after short term high-fat, high-fat high-protein and high-fructose diets. Nutr Metab 10:4.

12. Brouwers MCGJ, Konrad RJ, van Himbergen TM *et al.* (2013). Plasma proprotein convertase subtilisin kexin type 9 levels are related to markers of cholesterol synthesis in familial combined hyperlipidemia. Nutr Metab Cardiovasc Dis 23 (11):1115-1121.

13. Richard C, Couture P, Desroches S *et al.* (2011) Effect of the Mediterranean diet with and without weight loss on cardiovascular risk factors in men with the metabolic syndrome. Nutr Metab Cardiovasc Dis 21 (9):628-635.

14. Bjermo H, Iggman D, Kullberg J, *et al.* (2012) Effects of n-6 PUFAs compared with SFAs on liver fat, lipoproteins, and inflammation in abdominal obesity: A randomized controlled trial. Am J Clin Nutr 95:1003-1012.

15. Hooper L, Summerbell CD, Higgins JPT, *et al.* (2001) Dietary fat intake and prevention of cardiovascular disease: Systematic review. Br Med J 322:757-763.

16. Swain JF, McCarron PB, Hamilton EF, *et al.* (2008) Characteristics of the diet patterns tested in the optimal macronutrient intake trial to prevent heart disease (OmniHeart): Options for a heart-healthy diet. J Am Diet Assoc 108:257-265.

17. Senanayake VK, Pu S, Jenkins DA, *et al.* (2014) Plasma fatty acid changes following consumption of dietary oils containing n-3, n-6, and n-9 fatty acids at different proportions: Preliminary findings of the Canola Oil Multicenter Intervention Trial (COMIT). Trials 15:136-147.

18. Lin L, Allemekinders H, Dansby A *et al.* (2013). Evidence of health benefits of canola oil. Nutr Rev 71(6): 370–385

19. FDA (Food and Drug Administration) (2006). Unsaturated fatty acids from canola oil and reduced risk of coronary heart disease docket No. 2006Q-0091. http://www.fda.gov/Food/IngredientsPackagingLabeling/LabelingNutrition/ucm072958.htm. Accessed 21 October 2015.

20. USDA (United States Department of Agriculture) (2012). Canola. http://www.ers.usda.gov/topics/crops/soybeans-oil-crops/canola.aspx#Consumption. Accessed 21 October 2015.

21. Jones PJ, Senanayake VK, Pu S, *et al.* (2014) DHA-enriched high-oleic acid canola oil improves lipid profile and lowers predicted cardiovascular disease risk in the canola oil multicenter randomized controlled trial. Am. J Clin Nutr 100:88-97.

22. Mifflin MD, St Jeor ST, Hill LA, *et al.* (1990) A new predictive equation for resting energy expenditure in healthy individuals. Am J Clin Nutr 51:241-247.

23. Mackay DS, Jones PJH, Myrie SB *et al.* (2014) Methodological considerations for the harmonization of non-cholesterol sterol bio-analysis. J Chromatogr B 957:116-122.

24. Cariou B, Le May C, Costet P. (2011) Clinical aspects of PCSK9. Atherosclerosis 216:258-265.

25. Persson L, Cao G, Ståhle L *et al.* (2010) Circulating proprotein convertase subtilisin kexin type 9 has a diurnal rhythm synchronous with cholesterol synthesis and is reduced by fasting in humans. Arterioscler Thromb Vasc Biol 30:2666-2672.

26. Mayne J, Raymond A, Chaplin A *et al.* (2007) Plasma PCSK9 levels correlate with cholesterol in men but not in women. Biochem Biophys Res Commun 361:451-456.

27. Milne JC, Jirousek MR, Vu CB, (2013) Methods of lowering proprotein convertase subtilisin/kexin type 9 (PCSK9). *WO2013177536 A2.* http://www.google.com/patents/WO2013177536A3?cl=en. (accessed December 2014).

28. Graversen CB, Lundbye-Christensen S, Thomsen B *et al.* (2015) Marine n-3 polyunsaturated fatty acids lower plasma proprotein convertase subtilisin kexin type 9 levels in pre- and postmenopausal women: A randomised study. Vascul Pharmacol. *Article in press,* doi:10.1016/j.vph.2015.07.001.

29. Li H, Ruan XZ, Powis SH *et al.* (2005) EPA and DHA reduce LPS-induced inflammation responses in HK-2 cells: evidence for a PPAR-gamma-dependent mechanism. Kidney Int 67 (3):867-874.

30. Grygiel-Gorniak B. (2014) Peroxisome proliferator-activated receptors and their ligands: nutritional and clinical implications – a review. Nutrition Journal 13 (1):17.

31. Schulz R, Schlüter KD, Laufs U. (2015) Molecular and cellular function of the proprotein convertase subtilisin/kexin type 9 (PCSK9). Basic Res Cardiol 110 (2):19.

32. Jump, DB. (2008) N-3 polyunsaturated fatty acid regulation of hepatic gene transcription. Curr Opin Lipidol 19 (3):242-247.

33. Sun H, Samarghandi A, Zhang N, *et al.* (2012) Proprotein convertase subtilisin/kexin type 9 interacts with apolipoprotein B and prevents its intracellular degradation, irrespective of the low-density lipoprotein receptor. Arterioscler Thromb Vasc Biol 32:1585-1595.

34. Lakoski SG, Lagace TA, Cohen JC, *et al.* (2009) Genetic and metabolic determinants of plasma PCSK9 levels. J Clin Endocrinol Metab 94:2537-2543

Conclusions/Conclusiones

❖ CONCLUSIONS

1. The ultrasound-assisted extraction using ethanol:water 50% was the optimal extraction system for the recovery of phenolic compounds from *Moringa oleifera* leaves. The use of HPLC-ESI-QTOF-MS enabled the tentative characterization of 59 phenolic compounds (mainly flavonoids) from the leaves of *Moringa oleifera*, 30 of them have not been previously described in this matrix, demonstrating its potential as source of bioactive compounds.

2. The optimal conditions which allowed the higher extraction yield, total phenolic content, and total flavonoid content as well as the best antioxidant activity under microwave-assisted extraction were an extraction time of 20 min, 42% of ethanol and 158ºC, while the optimal extraction parameters for pressurized liquid extraction were extraction time of 20 min, 35% of ethanol and 128ºC. After the HPLC-ESI-QTOF-MS analytical characterization under the optimal extraction conditions, it was confirmed that MAE extracts were richer in flavonoids whereas PLE extracts were richer in more polar compounds, those with more hydroxyl groups, and temperature sensitive compounds such as glucosinolates.

3. The optimization of the downstream green extraction systems, namely SFE, GXLs, and PHWE proved to be a powerful tool that enabled the extraction of diverse fractions rich in different families of bioactive compounds with various polarities from *Moringa oleifera* leaves. A volatile fraction rich in fatty acids, alcohols, ketones, alkanes, and esters, a flavonoid-rich fraction and fractions rich in phenolic acids were produced. Although the fractions extracted by SFE and GXLs presented a similar composition in volatile compounds, the latest was more suitable for extracting ketones and esters compared to SFE. The fraction resulting from PHWE presented a higher content of phenolics than did the GXL fraction, while the latter was richer in flavonoids.

4. An HPLC-ESI-QTOF-MS method was optimized for the qualitative and quantitative characterization of *Pistacia lentiscus* leaves. The optimal parameters established for a higher resolution, less time of analysis, and the best peak width were mobile phases consisting of methanol and 0.25% of acetic acid in water, a flow rate of 0.8 mL/min, an injection volume of 10 µL, and a final analysis time of 45 min. The optimized analytical method revealed that the leaves of *Pistacia lentiscus* are a good source of bioactive compounds, mainly flavonoids.

5. The polar fraction of the Spanish melon varieties Galia, Cantaloupe, and Piel de Sapo was characterized in depth for the first time. This fraction was rich in amino acids and derivatives, phenolic acids, and derivatives, and organic acids, among other compounds such as lignans and flavonoids. The principal-component analysis (PCA) gave a clear differentiation between the 3 varieties through 12 compounds which could be identified.

6. Proanthocyanidins and flavanols studied in a cranberry syrup proved highly resistant to gamma irradiation and they were stable up to 1 month of storage at room temperature. Among these, quercetin and some of its derivatives were the most stable compounds, remaining unaltered for 3 months of storage at room temperature.

7. The use of semi-preparative HPLC proved to be a powerful technique for the fractionation of complex matrices such as the extract of commercial cranberry capsules. Furthermore, it was demonstrated that, apart from proanthocyanidins, other phenolic compounds have *in vitro* antibacterial activity against *Escherichia coli* adherence and biofilm formation. The entire extract presented a clear antimicrobial effect by means of diminishing the adherence and biofilm formation of the mixture of 14 strains of uropathogenic *E. coli*, suggesting a synergic effect between the compounds from the matrix under study.

8. A total of 54 plasma samples were taken during a clinical trial in which volunteers consumed different types of canola oils: conventional canola oil, high oleic acid canola oil and docosahexaenoic-acid-enriched canola oil. These plasma samples were analyzed by GC-FID, and PCSK9 plasma proprotein was quantified by ELISA assay. The results showed that plasma levels of PCSK9 were positively correlated with cholesterol synthesis markers (lathosterol and desmosterol) across all three diets, corroborating that plasma PCSK9 levels are sensitive to changes in cholesterol synthesis. In addition, the results demonstrated for the first time that the intake of DHA-enriched oil diminish the PCSK9 plasma concentration by almost 10%, suggesting that the intake of some types of dietary fat could act as a complementary treatment in the management of patients with hyperlipidemia.

❖ CONCLUSIONES

1. La extracción asistida por ultrasonidos utilizando como disolvente una mezcla de etanol y agua al 50 % resultó ser el sistema de extracción óptimo de compuestos fenólicos totales de las hojas de *Moringa oleifera*. El uso de HPLC-ESI-QTOF-MS permitió la caracterización tentativa de 59 compuestos fenólicos (mayoritariamente flavonoides) en la hoja de *Moringa oleifera,* 30 de los cuales no habían sido descritos previamente en esta matriz, demostrando su potencialidad como fuente de compuestos bioactivos.

2. En la extracción asistida por microondas de hojas de *Moringa oleifera*, las condiciones óptimas que permitieron obtener el mayor rendimiento, mayor contenido en compuestos fenólicos y flavonoides totales, así como la mejor capacidad antioxidante fueron 20 minutos de extracción, 42% de etanol y 158°C, mientras que los parámetros óptimos de extracción mediante fluidos presurizados fueron 20 minutos de extracción, 35% de etanol y 128°C. Tras la caracterización analítica mediante HPLC-ESI-QTOF-MS de los extractos obtenidos en las condiciones optimizadas para cada técnica, se comprobó que los extractos obtenidos por MAE fueron más ricos en flavonoides y sus derivados glicosados como kaempferol y quercetina, mientras que los extractos obtenidos por PLE fueron más ricos en compuestos más polares, con más grupos hidroxilo, así como en compuestos termolábiles como los glucosinolatos.

3. La optimización de los sistemas de extracción limpios, SFE, GXLs y PHWE, llevados a cabo hasta agotar la muestra de hoja de *Moringa oleifera* mostraron ser una potente herramienta la cual permitió la obtención de diversas fracciones ricas en diferentes familias de compuestos bioactivos con diferentes polaridades. De esta forma se obtuvieron tres fracciones: una fracción volátil rica en ácidos grasos, alcoholes, cetonas, alcanos y ésteres, otra fracción rica en flavonoides y la última fracción rica en ácidos fenólicos. Las fracciones

extraídas mediante SFE y GXLs presentaron una composición similar en compuestos volátiles, siendo la última más favorable para extraer compuestos como cetonas y alcoholes. La fracción obtenida mediante PHWE presentó un contenido mayor de compuestos fenólicos que la fracción GXLs, mientras que el contenido total de flavonoides fue superior en esta última.

4. Se optimizó un método de HPLC-ESI-QTOF-MS para la caracterización cualitativa y cuantitativa de las hojas de *Pistacia lentiscus*. Los parámetros óptimos establecidos para la obtención de una mayor resolución, menor tiempo de análisis y una mejor forma de pico fueron el uso de fases móviles consistentes en metanol y agua con 0.25% de ácido acético, flujo de 0.8 mL/min, volumen de inyección de 10 μL y un tiempo de análisis de 45 minutos. El método analítico optimizado reveló que las hojas de *Pistacia lentiscus* son una importante fuente en compuestos bioactivos, mayoritariamente flavonoides.

5. Se caracterizó por primera vez la fracción polar de las variedades españolas de melón Galia, Cantaluz y Piel de Sapo, las cuales fueron ricas en aminoácidos y derivados, ácidos fenólicos y derivados, así como ácidos orgánicos, entre otros compuestos como lignanos o flavonoides. El análisis de componentes principales (PCA) permitió una clara diferenciación entre las 3 variedades, siendo 12 los compuestos diferenciadores que pudieron ser identificados.

6. Las proantocianidinas y flavanoles estudiados procedentes de un jarabe de arándano rojo americano fueron bastante resistentes a la radiación gamma y se mantuvieron estables a temperatura ambiente hasta 1 mes de almacenamiento. De entre ellos, la quercetina y algunos de sus derivados fueron de los compuestos más estables, permaneciendo sin alterarse hasta 3 meses de almacenamiento a temperatura ambiente.

7. El uso de cromatografía semipreparativa se presentó como una potente técnica para el fraccionamiento de matrices complejas como es el caso del extracto procedente de cápsulas a base arándano rojo americano. Además, se demostró

que otros compuestos fenólicos aparte de las proantocianidinas tienen efecto antimicrobiano *in vitro* en cuanto a la adherencia y la formación de biofilm de *Escherichia coli* se refiere. El extracto procedente de cápsulas a base de arándano rojo americano presentó un claro efecto antimicrobiano, disminuyendo la adherencia y la formación de biofilm *in vitro* frente a 14 cepas uropatógenas de *E. coli*, sugiriendo un efecto sinérgico entre lo compuestos presentes en la matriz de estudio.

8. 54 muestras de plasma sanguíneo procedentes de un ensayo clínico en el que los voluntarios consumieron diferentes tipos de aceites de canola: aceite de canola convencional, aceite de canola enriquecido en ácido oleico y aceite de canola alto oleico enriquecido en omega 3 (DHA), fueron analizadas mediante GC-FID y se cuantificó la proproteína PCSK9 mediante ELISA. Los resultaros mostraron que los niveles plasmáticos de PCSK9 estuvieron positivamente correlacionados con los marcadores de la síntesis de colesterol (latosterol y desmosterol) en las tres intervenciones dietéticas, corroborando que los niveles plasmáticos de PCSK9 son sensibles a cambios en la síntesis del colesterol. Los resultados obtenidos demostraron por primera vez que el consumo de aceite enriquecido en DHA disminuye cerca de un 10% la concentración de la proproteína PCSK9, sugiriendo que el consumo de determinados tipos de grasa en la dieta podría actuar como tratamiento complementario para tratar a pacientes con hiperlipidemia.